RESEARCH REPORT

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Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity

Part 1. Biologic Responses in Rats and Mice to Subchronic Inhalation of Diesel Exhaust from U.S. 2007-Compliant Engines: Report on 1-, 3-, and 12-Month Exposures in the ACES Bioassay
Jacob D. McDonald et al.

Part 2. Assessment of Genotoxicity After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay
Jeffrey C. Bemis et al.

Part 3. Assessment of Genotoxicity and Oxidative Stress After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay
Lance M. Hallberg et al.

Part 4. Effects of Subchronic Diesel Engine Emissions Exposure on Plasma Markers in Rodents: Report on 1- and 3-Month Exposures in the ACES Bioassay
Daniel J. Conklin and Maiying Kong

Includes a Commentary by the Institute's ACES Review Panel

Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity

with a Commentary by the HEI ACES Review Panel

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI's research and analyses to public and private decision makers.

HEI typically receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. Specific components of the ACES program were funded by the U.S. Department of Energy and the Federal Highway Administration. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research. For the ACES studies, a special ACES Review Panel — comprising Health Review Committee members and outside experts — fulfilled this role.

All project results and accompanying comments by the Health Review Committee (or, in this case, the ACES Review Panel) are widely disseminated through HEI's Web site (*www.healtheffects.org*), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 166, *Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity*, presents four studies funded by the Health Effects Institute. This report contains the following main elements:

The Preface, prepared by staff at HEI, is a brief summary of HEI's rationale for undertaking the ACES program and the selection of the investigators and development of the protocol for the ACES health studies described in this report, as well as an overview of Phases 1, 2, and 3A of the ACES program.

The Investigators' Reports for the core animal bioassay and three ancillary studies describe the scientific background, aims, methods, results after 1 and 3 months of exposure, and interpretations and conclusions of the studies. The report on the core bioassay by McDonald and colleagues also includes some results from 12 months of exposure.

The Commentary on the four studies was prepared by members of the HEI ACES Review Panel (see below) with the assistance of HEI staff. The Commentary provides scientific background to the studies, comments on the methods used and strengths and limitations of the observations, discusses conclusions that can be drawn, and puts the studies into a broad scientific and regulatory perspective.

The four reports in this publication have gone through HEI's rigorous review process. Thus, each investigator team submitted a draft final report. These draft reports were evaluated by the HEI ACES Review Panel — an independent panel of distinguished scientists who had no involvement in selecting or overseeing these studies and included some members of the HEI Review Committee. Comments from the Panel were sent to the investigators, who revised their reports as they considered appropriate. The final report was again evaluated by the Panel, which prepared the Commentary based on the contents of the final reports.

The final results from the animal bioassay and the ancillary studies will be published at the end of the program, when the animals have been exposed to diesel exhaust for up to 30 months. The final results and commentary are expected to be published in 2014.

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PREFACE

HEI's Advanced Collaborative Emissions Study: Phase 3B

INTRODUCTION

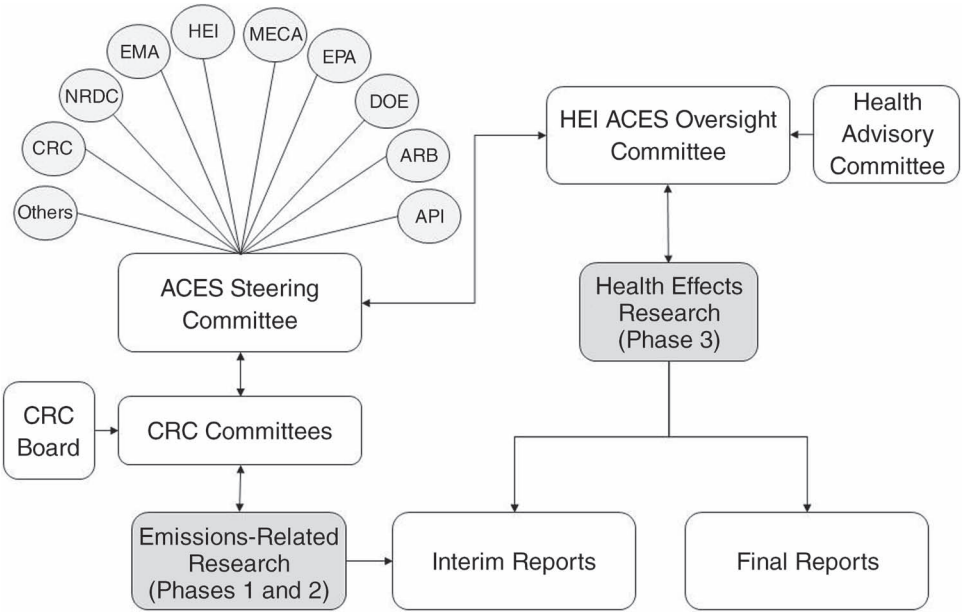
Because of health concerns related to exposure to diesel exhaust (DE*) emissions, the U.S. Environmental Protection Agency (U.S. EPA) and the California Air Resources Board (CARB) in 2001 adopted stringent new standards for diesel fuel and for heavy-duty diesel engine emissions. In 2007, engines were required to meet a new standard for particulate matter (PM) and, in 2010, to conform to a new standard for nitrogen oxides (NO_x). In response, industry developed a combination of advanced-technology compression-ignition engines, exhaust control systems, and reformulated fuels to meet these stringent standards, which were expected to result in substantially reduced emissions of other exhaust constituents as well. The U.S. EPA and CARB projected that the targeted emissions reductions of about 90% (compared with emissions from pre-2007 heavy-duty diesel engine systems) would have substantial public health benefits.

To characterize the emissions and assess the safety of these new, advanced heavy-duty engine systems and fuels, the Health Effects Institute and the Coordinating Research Council (CRC; a nonprofit organization that directs engineering and environmental studies on the interaction between automotive or other mobility equipment and petroleum products) developed the Advanced Collaborative Emissions Study (ACES), a cooperative, multiparty effort that was conceived in 2005 and consisted of three phases:

- **Phase 1:** Extensive emissions characterization of four production-ready heavy heavy-duty diesel (HHDD; i.e., gross vehicle weight higher than 33,000 lb) engines equipped with control systems designed to meet the 2007 standards for reduced PM. This phase was conducted at the Southwest Research Institute (SwRI) in 2007 and 2008 and was the basis for selecting one HHDD engine/control system for health testing in Phase 3.
- **Phase 2:** Extensive emissions characterization of a group of diesel engine and control systems intended for production that met the more stringent 2010 standards (including more advanced NO_x controls). This phase is being conducted at SwRI in 2012.
- **Phase 3:** Health effects assessment in rodents using one selected 2007-compliant heavy-duty diesel engine system. This phase started in 2008 with the installation of a specially designed emissions-generation and animal exposure facility at the Lovelace Respiratory Research Institute (LRRI) and is being conducted in two parts. Phase 3A included setting up the engine, characterizing the engine performance and emissions to make sure it was operating as intended, and generating and characterizing the exposure atmospheres in the animal inhalation chambers at three dilution levels. Phase 3B includes a 90-day inhalation study in mice and a chronic inhalation study in rats with health measurements at several time periods, as described later in this Preface.

The Figure in this Preface provides an overview of the ACES entities and stakeholders. The CRC initiated and oversaw the emissions characterizations in Phases 1 and 2 of ACES under its committee structure for such projects. The design and implementation of the health effects research in Phase 3 and overall reporting of results for ACES are the responsibility of HEI and are overseen by the HEI ACES Oversight Committee (a subset of the HEI Research Committee, augmented by independent experts from several disciplines), with advice from a Health Advisory Committee of ACES stakeholder experts. The overall effort has been guided by an ACES Steering Committee consisting of representatives of HEI and CRC, along with the U.S. Department of

*A list of abbreviations and other terms appears at the end of the Preface.



ACES flowchart including the ACES entities and stakeholders. API = American Petroleum Institute; ARB = Air Resources Board (California); CRC = Coordinating Research Council; DOE = U.S. Department of Energy; EMA = Truck & Engine Manufacturers Association; EPA = U.S. Environmental Protection Agency; HEI = Health Effects Institute; MECA = Manufacturers of Emission Controls Association; NRDC = Natural Resources Defense Council.

Energy, U.S. EPA, engine manufacturers, the petroleum industry, CARB, emission control manufacturers, the National Resources Defense Council, and others. At the inception of ACES, the first step taken jointly by the CRC and HEI committees was the development of a detailed project plan that formed the basis for the subsequent requests for applications (RFAs) issued by CRC and HEI.

This Preface accompanies a Research Report comprising investigators' reports resulting from studies funded by HEI to conduct work in Phase 3B. The sections that follow provide some background on the process leading to the selection of the investigators for Phase 3, an overview of the studies funded under Phase 3B, and a description of the development of a protocol for the chronic bioassay, as well as background on emissions and animal exposure characterization conducted in Phase 3A. More detailed information about Phase 3A and the engine selection process can be found in HEI Communication 17 (Mauderly and McDonald 2012). Results from Phase 1, the emissions characterization of four 2007-compliant engines, have been published elsewhere (Khalek et al. 2009; 2011).

The investigators' reports in this Research Report describe the results from 1- and 3-month exposures in rats and mice, as well as some key results at 12 months in rats in the core study. A second series of investigators' reports, to be published in 2014, will describe the results from exposures up to 30 months in rats.

SELECTION OF PHASE 3 INVESTIGATORS

In 2006, HEI issued request for proposals (RFP) 06-1, *Exposure Facility and Conduct of a Chronic Bioassay,* in tandem with RFA 06-2, *Additional Health Effects Measurements During the Chronic Bioassay and Short-Term Study*. RFP 06-1 solicited proposals from multidisciplinary teams to design and implement an engine facility with an engine dynamometer and a dilution system, to characterize the engine exhaust and animal chamber exposure, and to conduct a chronic inhalation bioassay in rats and mice. The bioassay would include standard assessments of general health status, tumor incidence, and histopathologic changes in all major organs, similar to the standard rodent bioassay conducted by the National Toxicology Program (NTP) to evaluate potential carcinogenicity of chemical compounds (NTP 2011). In addition, the bioassay was intended to provide information on chronic toxicity

(through histopathologic analyses of multiple organs at interim necropsies and at the end of the study) and on in vivo genotoxicity, inflammation, and other noncancer health endpoints that have been associated with exposure to DE and that are not part of the standard NTP bioassays (NTP 2011).

A team led by Drs. Joseph Mauderly and Jacob McDonald at LRRI submitted a proposal for RFP 06-1 in July 2006. The proposal was reviewed by the HEI ACES Oversight Committee, which recommended the LRRI team for funding to conduct the core inhalation studies in Phase 3 (Table). After a review of the project costs and the key health studies needed to assess any health effects of these new technologies, the HEI ACES Oversight Committee decided to conduct the bioassay only in rats; mice were to be used only to study the effects of short-term exposures.

One important element of the ACES plan was to make maximum, cost-effective use of the exposures being conducted in the Phase 3 bioassay using mice and rats. Thus, RFA 06-2 solicited applications to measure additional endpoints, such as vascular markers of inflammation and damage, genotoxicity, respiratory infections, and immune responses, that were not

Summary of Studies Conducted in Phase 3B of the ACES Program

Request Number and Title / Primary Investigator(s) (Institution)	Study Title	Main Health Endpoints in Rats and Mice
RFP 06-1 Exposure Facility and Conduct of a Chronic Inhalation Bioassay		
Joseph L. Mauderly and Jacob D. McDonald (Lovelace Respiratory Research Institute, Albuquerque, N.M.)	Development of a diesel exhaust exposure facility and conduct of a chronic inhalation bioassay in rats and mice	Mortality and morbidity, body weight, clinical observations (including hematology and serum chemistry), gross pathology of major organs Lung: Cytotoxicity, inflammation, oxidative stress, cytokines; cell proliferation; pulmonary function
RFA 06-2 Additional Health Effects Measurements During the Chronic Bioassay and Short-Term Study		
Jeffrey C. Bemis (Litron Laboratories, Rochester, N.Y.)	Genotoxicity of inhaled diesel exhaust: Examination of rodent blood for micronucleus formation	Micronucleated reticulocytes and normochromatic erythrocytes in serum
Daniel J. Conklin (University of Louisville, Louisville, Ky.)	Effects of diesel emissions on vascular inflammation and thrombosis	Inflammatory cytokines and chemokines; lipids; acute phase reactants; markers of thrombosis, vascular inflammation, and oxidative stress in serum and aorta Immunoglobulins in serum (added at the request of HEI)
Lance M. Hallberg (University of Texas Medical Branch, Galveston, Tex.) and Jeffrey Wickliffe (Tulane University Health Sciences Center, New Orleans, La.)	Assessment of the genotoxicity of diesel exhaust/diesel exhaust particulates from improved diesel engines	Oxidative DNA damage in serum; DNA damage in lung; lipid peroxidation in brain
Qinghua Sun (Ohio State University, Columbus, Ohio)	Diesel exhaust exposure and cardiovascular dysfunction: ROS mechanism	ROS (reactive oxygen species) generation and vascular inflammation in cardiac muscle, aortic rings, mesenteric arterioles, and cremasteric muscle
John Veranth (University of Utah, Salt Lake City, Utah)	Lung cell gene transcription responses to diesel exhaust	DNA damage, oxidative stress, and apoptosis (cell death) in lung tissue Signaling pathways related to DNA damage using RNA from lung tissue

included in the core health effects studies funded under RFP 06-1. Through a competitive selection process, five studies were approved for funding by an expert panel consisting of members of the HEI ACES Oversight Committee and additional outside experts (Table). Three of the funded studies measured endpoints of genotoxicity and mutation, and two studies measured markers of vascular damage and inflammation. HEI did not receive any applications on immunotoxicity or respiratory infection. Because the HEI ACES Oversight Committee thought measures of immunotoxicity would be important to incorporate, such measures were included in the ancillary study by Conklin and colleagues. The ancillary studies led by John Veranth and Qinghua Sun were terminated after they were initiated because of technical issues.

PROTOCOL DEVELOPMENT

A detailed project plan was developed for the design of all three phases of the ACES program. For Phase 3, the project plan included recommendations about the rodent strains, exposure concentrations and duration, and endpoints to be covered. However, the specific details were to be worked out and agreed upon with the selected team of investigators. Several important recommendations made by the HEI ACES Oversight Committee, and approved by the HEI Research Committee, are described in this Preface.

Selecting a Rat Strain

Criteria considered in selecting a rat strain were longevity, whether a strain had been used previously in chronic inhalation studies (particularly of DE), whether there existed a historical database of cancer incidence (based on control groups from chronic studies using inhalation or other methods of administering toxic compounds), and the maximum body weight reached by males (because it affects housing in inhalation chambers with limited space available; male rats typically gain more weight than female rats). A full discussion of these criteria is provided in Appendix G in McDonald et al. (available on the HEI Web site at *www.healtheffects.org*).

When RFP 06-1 was issued, two rat strains were being considered for ACES: F344 and Wistar WU. Historically, F344 had been the strain of choice for chronic NTP bioassays and had been used in many of the chronic bioassays of DE in the United States, whereas the Wistar strain had been used in chronic bioassays in Europe (including of DE). During 2005 and 2006, however, the NTP was reconsidering its use of the F344 strain because it had a high spontaneous incidence of leukemia, which could reduce the statistical power for distinguishing exposure-associated lung lesions and shorten the life span of the animals (King-Herbert and Thayer 2006). In late 2006 (i.e., after RFP 06-1 was issued), the NTP decided to use the Wistar Han strain for chronic bioassays (King-Herbert et al. 2010). This decision subsequently guided the discussions by the HEI ACES Oversight Committee that led to recommending the Wistar Han strain for the ACES program as well.

However, the NTP changed its recommendation again in 2010 — when the ACES chronic bioassay was about to start — in favor of using the Sprague Dawley rat strain (King-Herbert et al. 2010). This decision was based in part on the observation that Wistar Han rats produced fewer offspring than were required to successfully conduct developmental and reproductive toxicology studies. Because the chronic bioassay had not yet started, the HEI ACES Oversight Committee revisited the rat strain decision but decided to stay with the original recommendation of the Wistar Han strain because ACES did not include developmental or reproductive outcomes. The HEI ACES Oversight Committee also considered questions raised by CARB regarding the Wistar strain, because a portion of the animals may have a mutation in the Ah receptor gene that could affect their responsiveness to exposure to polycyclic aromatic hydrocarbons. The Committee again weighed this issue against all factors that influenced the rat strain selection and decided not to change its decision. More information on this issue is provided in the HEI ACES Review Panel Commentary accompanying the investigators' reports by McDonald, Bemis, Hallberg, and Conklin and their colleagues.

Selecting a Mouse Strain

Similar criteria (i.e., longevity, background tumor incidence, and use in previous inhalation studies) were used to identify a suitable mouse strain for the ACES program. RFP 06-1 stated that three mouse strains could be considered for ACES: C57BL/6, CD-1, and B6C3F_1. B6C3F_1 mice (a cross between female C57BL/6 and

male C3H mice) are used in the standard NTP bioassay (King-Herbert et al. 2010), but have not been used in previous DE bioassays and have a relatively high spontaneous liver tumor incidence. Previous DE bioassays have used a variety of mouse strains, including NMRI, CD-1, C57BL/6N, SENCAR, and Strain A. Because the C57BL/6 strain has a relatively low incidence of spontaneous lung tumors and has been used in previous DE bioassays, RFP 06-1 indicated that there may be advantages to using that strain. When proposing a particular strain, applicants were asked to consider differences in the sensitivity of strains in their response to exposure to chemicals via inhalation. Based on longevity and a lower incidence of lung tumors than other strains under consideration, the C57BL/6 strain was proposed by the LRRI team and approved by the HEI ACES Oversight Committee.

Selecting Time Points for Evaluating Health Endpoints

To standardize the evaluations among the six ACES studies and to accommodate cost considerations, it was agreed that all studies should evaluate blood and tissue samples collected in rats after 1, 3, 12, and 24 months of exposure and in mice after 1 and 3 months of exposure.

RFP 06-1 indicated that about half the number of animals (180 of 288 rats and 180 of 360 mice; equal numbers of males and females) per exposure level would be assigned to the terminal necropsy at 24 or 30 months (see the subsection "Duration of Chronic Exposures in Rats"). The RFP stated that the remaining rats and mice would be available for intermediate necropsies at 1, 3, 6, 12, and 18 months, plus 24 months if animals were kept on the study for 30 months. These time points were proposed to capture changes at short-, intermediate-, and long-term exposure durations. Applicants to RFA 06-2 were asked to indicate, justify, and estimate costs for a number of time points that they thought would be optimal for measuring their proposed endpoints. Final decisions about the times of measurements and numbers of animals were made during protocol development.

During the budget negotiations with the LRRI team, the HEI ACES Oversight Committee and ACES stakeholders discussed options to lower the cost of the study by reducing the scope of the chronic bioassay. One major reduction was to limit the chronic bioassay to rats only, in part because there were more data available on DE exposures in rats than in mice and because rats are considered slightly more relevant than mice when extrapolating results to humans. Consequently, the duration of the mouse exposures was reduced to 90 days, a typical time span for short-term exposures. In addition, the number of intermediate necropsies was reduced.

Collaboration and Logistics

LRRI investigators facilitated the collaboration with the additional investigators funded under RFA 06-2, by conducting all animal exposures and necropsies and by devising a scheme for sharing tissues among the five ancillary studies. Because of limited blood volumes available from individual mice, they exposed additional mice to accommodate all requests for blood and tissue samples. All samples were collected and distributed by LRRI personnel according to procedures specified by the investigators of the ancillary studies and approved by the HEI ACES Oversight Committee. In addition, unused blood and tissue samples are being banked for future analyses if the need arises.

Duration of Chronic Exposures in Rats

Inhalation exposures of rats are being conducted 16 hours per day, 5 days a week for 30 months (through 2012). Based on survival rates at 23 months of exposure, a decision was made to continue until 30 months of exposure before euthanizing the rats, which provides a longer time window to examine whether tumor formation occurs.

GENERATION AND CHARACTERIZATION OF THE EXPOSURE ATMOSPHERE

The LRRI team and collaborators spent considerable effort in optimizing engine operation, testing the emissions to ensure comparability with Phase 1 results, determining the appropriate dilution factors, maintaining a constant temperature in the inhalation chambers in spite of introducing hot exhaust, and making sure the whole system — comprising engine, dilution, and animal inhalation facilities — operated reliably and consistently. The results of these important efforts are presented in detail in HEI Communication 17

(Mauderly and McDonald 2012). A brief summary of the major decisions is presented here.

Exposure Atmosphere Generation

Because the chosen HHDD engine was considerably larger than any engine previously used at LRRI (which were light-duty engines), LRRI constructed a special facility for housing the dynamometer and the engine and for delivering the exhaust to the animal exposure chambers. A protocol for commissioning the dynamometer and characterizing the engine exhaust and the exhaust in the exposure chambers was developed by the LRRI team and approved by the HEI ACES Oversight Committee after receiving input from the Health Advisory Committee (see Appendices B and C of Communication 17, available at *www.healtheffects.org*). A decision as to which test cycle to use was made in Phase 1.

16-Hour Test Cycle

During Phase 1, a specially designed 16-hour test cycle was developed by a team at West Virginia University for use during animal exposures. The cycle includes four 4-hour parts consisting of Federal Test Procedure segments mixed with segments of the CARB HHDD engine-5 (CARB 5-Modes) driving cycles. It was designed to be representative of modern truck usage and included a broad range of engine loads and speeds reflecting both urban and rural (highway) driving. Details about the development of the cycle can be found in an article by Clark and colleagues (2007). The 16-hour cycle also allowed for collection of useful information on emissions during particle filter regeneration, which does not occur during the shorter test cycles.

Selection of Main and Backup Engines

After the emissions characterization of the four engines in May 2008 during Phase 1, HEI randomly selected engine B for the health studies, as described in the *Final Plan for Engine Selection* provided in Appendix A of Communication 17 (Mauderly and McDonald 2012; available on the HEI Web site at *www.healtheffects.org*). A duplicate engine (referred to as *engine B′*) of the same model and make as the engine selected was obtained as a backup in the event of a breakdown of the primary engine for which repairs were estimated to last more than one or two days. Engine B′ underwent a set of emissions tests similar to those for engine B. There were some differences between the engines (see Tables 10–15 of HEI Communication 17 [Mauderly and McDonald 2012]). However, the differences were small — given the low level of emissions from both engines — and could be due to inter-engine variability and the fact that engine B′ was produced about a year after engine B and had updated engine and emissions controls. At the time Phase 3B was about to start, engine B′ had a larger share of the market for this model (more than two-thirds of the 2007-compliant engine market) than the original engine B, and its market share was expected to increase over time. Therefore, after consultation with the engine manufacturer, the HEI ACES Oversight Committee decided that engine B′ should be used as the main engine for the animal exposures at LRRI, with the original engine B serving as the backup engine.

Exhaust Dilutions

Four chamber exposure levels were targeted for the animal bioassay: low, mid, and high DE dilutions, and clean air. In previous DE animal exposure studies, dilution levels were based on particle mass concentrations. However, this approach was no longer viable because of the low particle concentrations in the exhaust of the 2007-compliant diesel engine and control systems. The HEI ACES Oversight Committee therefore decided, after discussion with the investigators and the ACES stakeholders, to set dilutions based on predetermined NO_2 concentrations. This decision was made because NO_2 is the pollutant with the highest concentration in the exhaust of the 2007-compliant engines and because noncancer health effects have been observed with exposure to NO_2 and with exposure to whole DE (particle and gaseous components) from older engines in previous animal inhalation studies. The NO_2 concentrations selected and the rationale for their selection are as follows:

1. **The highest concentration of NO_2 would be 4.2 ppm.** This concentration was derived from a prior study of chronic NO_2 exposures (Mauderly et al. 1989) in which animals were exposed to NO_2 at 9.5 ppm for 7 hours per day for 6 months. This concentration would serve as the maximum tolerated dose. The equivalent

concentration for a 16-hour exposure duration is 4.15 ppm. However, given that actual concentrations vary during the 16-hour cycle and may end up slightly below or above the target, the HEI ACES Oversight Committee recommended that concentrations should not go below 4.0 ppm. At this concentration, it was deemed possible to control the exposure chamber temperature to within the specified range.

2. **The lowest concentration would be 0.1 ppm**, or as close as possible to that concentration, in order to provide a likely no-observed-adverse-effect level. This concentration approaches the U.S. EPA ambient NO_2 air quality standard of 0.053 ppm. Concentrations in this range would be expected to be quite variable because of the high dilution ratio.
3. **The intermediate concentration would be 0.8 ppm.** Based on the highest and lowest concentrations, the HEI ACES Oversight Committee recommended targeting 0.8 ppm (but not exceeding it) and going no lower than 0.7 ppm.

Because of the overall low exhaust emissions, an additional issue considered in setting the highest chamber concentration was the chamber temperature. The acceptable range of temperatures with the animals in the chamber was set at 75° ± 3°F. A cooler was installed in the chamber to ensure that temperatures did not rise too high during the exposures.

SUMMARY

The multiparty ACES project was initiated to evaluate 2007- and 2010-compliant HHDD engine and control technologies. Phase 3 of ACES includes exposure atmosphere generation and characterization before the start of the animal exposures (Phase 3A, described in HEI Communication 17 [Mauderly and McDonald 2012]) and the evaluation of biologic responses associated with exposure to whole DE for up to 3 months for all studies in rats and mice and up to 12 months for the core study in rats (Phase 3B, described in this Research Report) from one randomly selected 2007-compliant diesel engine. A subsequent Research Report will describe the health effects of chronic inhalation in rats exposed for up to 30 months.

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ABBREVIATIONS AND OTHER TERMS

ACES	Advanced Collaborative Emissions Study
API	American Petroleum Institute
CARB	California Air Resources Board
CRC	Coordinating Research Council
DE	diesel exhaust
DOE	U.S. Department of Energy
EMA	Truck & Engine Manufacturers Association
HHDD	heavy heavy-duty diesel
LRRI	Lovelace Respiratory Research Institute
MECA	Manufacturers of Emission Controls Association
NO_x	nitrogen oxides
NRDC	Natural Resources Defense Council
NTP	National Toxicology Program
PM	particulate matter
RFA	request for applications
RFP	request for proposals
SwRI	Southwest Research Institute
U.S. EPA	U.S. Environmental Protection Agency

HEI STATEMENT
Synopsis of Research Report 166, Parts 1–4

Effects of Subchronic Exposure of Rats and Mice to Inhaled 2007-Compliant Diesel Exhaust

INTRODUCTION

This Statement summarizes HEI's independent evaluation, conducted by a specially convened Review Panel, of four studies conducted as a single phase (Phase 3B) of the Advanced Collaborative Emissions Study (ACES) program. The ACES Phase 3B studies investigated the health effects of subchronic exposures of mice and rats to diesel exhaust emissions from a heavy-duty diesel engine system compliant with 2007 regulations. The studies were led by Drs. Jacob D. McDonald of the Lovelace Respiratory Research Institute, Albuquerque, New Mexico, Jeffrey C. Bemis of Litron Laboratories, Rochester, New York, Lance M. Hallberg of the University of Texas Medical Branch, Galveston, Texas, and Daniel J. Conklin of the University of Louisville, Louisville, Kentucky.

BACKGROUND

In light of concerns identified over many decades about the potential health effects of diesel emissions, the U.S. Environmental Protection Agency and the California Air Resources Board adopted stringent new standards for diesel exhaust emissions and fuel for light- and heavy-duty highway diesel engines. Light-duty engines were required to meet a new standard for particulate matter by 2006, and heavy-duty engines by 2007. A tighter standard for nitrogen oxides (primarily nitric oxide [NO] and nitrogen dioxide [NO_2]) came into effect in 2010. The regulatory agencies also mandated that sulfur in fuel be reduced substantially. To address these regulations and standards, motor vehicle and engine manufacturers introduced new technologies. These developments were expected to result in substantial reductions in emissions from diesel engines.

To characterize the exhaust emissions from heavy-duty diesel engines that met the new standards and to assess the possible adverse health effects of exposure to these emissions, HEI, working in collaboration with the Coordinating Research Council, a nonprofit organization with expertise in emissions characterization, launched the multiphase Advanced Collaborative Emissions Study (ACES). Phases 1, 2, and 3A focused on emissions characterization.

Phase 3B of ACES was designed to evaluate health outcomes in animals exposed to diesel exhaust from a 2007-compliant engine for up to 30 months. Through competitive processes, HEI funded several investigator teams: a core study, led by McDonald (who became principal investigator after the retirement of the original principal investigator, Dr. Joe L. Mauderly), and ancillary studies to evaluate endpoints not assessed in the core study. The overall hypothesis for the ACES health study was that emissions from the 2007-compliant engine would not cause an increase in tumor formation or substantial toxic health effects in rats and mice, although some biologic effects might occur.

This Statement summarizes results reported from the core study and the ancillary studies led by Bemis and Hallberg, which assessed genotoxic endpoints in the exposed animals, and by Conklin, which assessed inflammatory and thrombotic endpoints. The investigator teams' reports were reviewed by the specially convened ACES Review Panel, comprising members of HEI's Health Review Committee and outside experts.

APPROACH

McDonald and colleagues generated exhaust from a 2007-compliant heavy heavy-duty diesel engine

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Drs. Jacob D. McDonald of the Lovelace Respiratory Research Institute, Albuquerque, New Mexico, Jeffrey C. Bemis of Litron Laboratories, Rochester, New York, and Lance M. Hallberg of the University of Texas Medical Branch, Galveston, Texas, and their colleagues, and Daniel J. Conklin and Maiying Kong of the University of Louisville, Louisville, Kentucky. Research Report 166 contains both the detailed Investigators' Reports and a Commentary on the study prepared by the Institute's ACES Review Panel.

(defined as >33,000 lb; hereafter called *heavy-duty*) equipped with emission controls. The engine was fueled with ultra-low-sulfur diesel fuel meeting current on-road specifications and was operated with a dynamometer.

Male and female Wistar Han rats and male and female C57BL/6 mice were exposed to one of three dilutions of whole diesel exhaust — 4.2 (high), 0.8 (mid), or 0.1 (low) ppm NO_2 — or to filtered air as a control. Exposure levels were set based on NO_2 because earlier phases of ACES had established that levels of NO_2 were much higher than levels of particulate matter in the emissions. In addition, the highest NO_2 exposure level was chosen to provide a comparison with the same cumulative exposure to NO_2 (the product of concentration and exposure duration) that was used in prior HEI-funded long-term inhalation studies in rats conducted by Mauderly and colleagues, in which some responses were detected in the lungs (HEI Research Reports 8 [1987] and 30 [1989]).

Exposures were conducted for 16 hours per day from approximately 1600 to 0800 hours for 5 days per week. The emissions were characterized both before they reached the animal exposure chambers and inside the exposure chambers; in this way, the investigators could assess how the presence of the animals affected the composition of the emissions. For this study, groups of male and female rats were euthanized after 1, 3, and 12 months of exposure, and male and female mice were euthanized after 1 and 3 months. Investigators at Lovelace Respiratory Research Institute harvested blood and tissues for their analyses (10 animals of each sex per exposure group) and also sent aliquots of blood and appropriate tissue samples from 5 to 10 animals of each sex per exposure group to the ACES Phase 3B ancillary studies investigators.

McDonald and colleagues examined a vast array of biologic endpoints: histopathologic (multiple tissues, including the airways), hematologic (several cell types, plus coagulation), serum chemistry (including triglyceride and protein components), lung lavage (including numbers of cells and levels of multiple cytokines and markers of oxidative stress), and pulmonary function.

For the assessments of genotoxicity, Bemis and colleagues measured the number of micronuclei detected in peripheral blood reticulocytes, which are immature red blood cells. Micronuclei can form as a result of a break in deoxyribonucleic acid (DNA) or from the disruption of chromosome segregation during division. Hallberg and colleagues assessed several markers of oxidative damage to cell components, which is believed to be involved in the induction of carcinogenesis. To detect damage to DNA, the Hallberg team used a Comet assay on lung cells and measured 8-hydroxy-deoxyguanosine levels in blood. As a measure of damage to lipids, they assessed levels of thiobarbituric acid reactive substances in brain tissue. Conklin and Kong measured multiple plasma markers of inflammation and thrombosis, including levels of lipids and lipoproteins involved in cholesterol transport and function. They also measured levels of multiple immunoglobulin (Ig) classes.

All four studies evaluated these endpoints in rats and mice after 1 and 3 months of exposure (with the exception of pulmonary function in mice, which was not evaluated at either time point). McDonald and colleagues also present histopathologic and respiratory function data from the 12-month exposure.

RESULTS AND CONCLUSIONS

In its independent review of the four reports, the HEI ACES Review Panel concluded that McDonald and colleagues' core study was wide ranging and well executed. In addition, the studies by Bemis and colleagues and Hallberg and colleagues were generally well implemented and assessed accepted genotoxic endpoints that are not normally part of chronic inhalation bioassays. Conklin and Kong's study was also wide ranging in its attempt to measure multiple plasma markers (approximately 30) associated with inflammation and thrombosis to identify possible cardiovascular markers of diesel exhaust exposure.

The panel highlighted several strong points in the McDonald study. The study is the first to conduct a careful and comprehensive evaluation of the subchronic effects in rodents of inhalation of diesel exhaust from a heavy-duty 2007-compliant engine at a range of levels. Even while applying a unique and strenuous 16-hour engine operating cycle, McDonald and colleagues successfully maintained the continuous operation for more than 12 months of a facility in which engine exhaust was generated and transported to rodent exposure chambers.

In their extensive analysis of the physical and chemical composition of the emissions, McDonald and colleagues found that the most abundant pollutants were carbon dioxide, carbon monoxide (CO),

NO, and NO_2, whereas concentrations of particulate matter, sulfur dioxide, and semivolatile and volatile organic species were very low. These findings confirm that the components of emissions from the 2007-compliant engine differ strikingly from those of older engines, in which particulate matter concentrations are much higher. The multiple standardized toxicity endpoints evaluated in this study — including histology, serum chemistry, and respiratory function — were appropriate for evaluating the hypothesis. The panel agreed with McDonald and colleagues that there were no changes in health endpoints for the majority of biologic tests conducted in rats and mice. When results were compared at 1 and 3 months across the species, the few changes observed were reported more often in rats than mice and almost exclusively with exposure to high-level diesel exhaust.

Mild histologic changes associated with diesel exhaust exposure were detected in the respiratory tract of rats. In the lung, changes were detected after 3 months of exposure to high-level diesel exhaust and had progressed at the 12-month exposure time point (in that changes were more widespread within the lung and found in more animals). Nonetheless, the histologic changes were still mild as defined by the investigators' scoring system (i.e., a score of 1 on a 4-point scale). The investigators noted a mild thickening of the central acinus (the junction of the conducting airways and the gas exchange region of the lung). In addition, the nose and turbinate of a very small number of rats showed scattered changes after 3 months of exposure, and these generally mild changes were detected in a few more male and female rats at 12 months at all diesel exhaust exposure levels. The panel agreed with the investigators' suggestion that the histologic changes in the lung in the current study that were observed after exposure to diesel exhaust emitted by a 2007-compliant engine are consistent with effects observed in Mauderly and colleagues' earlier studies of long-term exposure to NO_2. However, the effects of other gaseous components of diesel exhaust cannot be ruled out.

Some small changes in respiratory function were noted at 3 months in rats, but of these, only a decrement in diffusing capacity of CO (DL_{CO}, a measure of the lung's ability to transport gas into and out of the blood) may have persisted at 12 months. A decrease in DL_{CO} suggests the possibility of effects on pulmonary gas transfer or pulmonary circulation, which would be consistent with the observed histologic changes in the gas exchange region of the lung. In rats, some small changes in biochemical endpoints, particularly related to oxidative stress pathways, were also noted in lung lavage fluid and lung tissue at 1 and 3 months. (The results of the 12-month biochemical assessments will be included in the final report from the investigators). Overall though, these changes were small, and there was a lack of coherence among the endpoints; that is, the endpoints that might have been expected to change in concert — because they share a common pathway — did not do so. These discrepancies among endpoints in the same pathway may reflect the different sensitivities of the individual assays used to measure changes, or they may be just anomalous observations.

The panel noted that the study design did not include a side-by-side comparison with an older pre-2007 model-year engine. While recognizing that such a "positive control" could not be included as it would have substantially increased the complexity and cost of the study and would have posed enormous logistical challenges, the panel thought that such a side-by-side comparison could have enhanced the study. The panel also identified some other limitations to McDonald and colleagues' study — some biochemical assays lacked positive controls (to determine that each was sensitive enough to detect changes). In addition, rather than using a standard three-way analysis of variance on the entire data set, in some statistical approaches the investigators combined data from both sexes and used a trend analysis. The panel also thought that more precise quantitative histopathologic information (such as morphometric readings in the lung) would have enhanced the study.

The panel concluded that the ancillary studies assessed generally well-accepted markers of both genotoxicity — micronuclei formation in reticulocytes (in the report by Bemis et al.) and DNA damage and lipid peroxidation (in the report by Hallberg et al.) — and systemic inflammation and thrombosis (in the report by Conklin and Kong) and that they were valuable extensions to the ACES core study. The panel agreed with the investigators that no genotoxic effects could be detected that were associated with exposure for up to 3 months to any level of diesel exhaust from the 2007-compliant engine. The small group size (only 5 animals of each sex in each exposure group) and the assessment of genotoxic endpoints that, although well validated, are relatively short term (lasting one month or less) slightly reduced confidence in the utility of these

negative findings. Most of the thrombotic and inflammatory endpoints measured in plasma in Conklin and Kong's study were not affected by exposure to diesel exhaust, but some scattered changes — including changes in rat levels of cholesterol and high density lipoprotein — were detected. The pathophysiologic significance of these scattered changes was uncertain, however, because they were seen at only one of the two exposure time points in each sex, and the direction of the change (positive or negative) differed at the different exposure time points. Similarly, inconsistent and scattered changes in levels of IgE were seen, but these changes did not support the interpretation that new-technology diesel emissions acted as an adjuvant — an enhancer of specific immune responses — for the allergic response, as has been suggested for the effects of diesel exhaust particles in some prior animal and human studies.

Overall, these results indicate that rats exposed to one of three levels of diesel exhaust from a 2007-compliant engine for up to 12 months, for 16 hours per day, 5 days a week, with use of a strenuous operating cycle that was more realistic than cycles utilized in previous studies, showed few biologic effects related to diesel exhaust exposure. Even fewer exposure-related biologic effects were found in mice exposed for 3 months to diesel exhaust. In rats, the effects that were observed were limited to the respiratory tract and were mild, and the changes in lungs were consistent with previous findings after long-term exposure to NO_2 — a major component of the exposure atmosphere. No exposure-related genotoxic effects were found in rats or mice after 3 months of exposure, and few, if any, cardiovascular effects were detected that were sustained or detectable after 1 or 3 months of exposure. Rats will continue to be exposed for up to 30 months. At the end of the study, all the ACES investigators will submit reports that will be reviewed by the ACES Review Panel. The publication of these reports and accompanying commentary, anticipated in 2014, will provide an extensive overview of the effects of long-term exposure to diesel exhaust emitted by a 2007-compliant engine.

Part 1. Biologic Responses in Rats and Mice to Subchronic Inhalation of Diesel Exhaust from U.S. 2007-Compliant Engines: Report on 1-, 3-, and 12-Month Exposures in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 1. Biologic Responses in Rats and Mice to Subchronic Inhalation of Diesel Exhaust from U.S. 2007-Compliant Engines: Report on 1-, 3-, and 12-Month Exposures in the ACES Bioassay

Jacob D. McDonald, Melanie Doyle-Eisele, Andrew Gigliotti, Rodney A. Miller, Steve Seilkop, Joe L. Mauderly, JeanClare Seagrave, Judith Chow, and Barbara Zielinska

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ABSTRACT

The Health Effects Institute and its partners conceived and funded a program to characterize the emissions from heavy-duty diesel engines compliant with the 2007 and 2010 on-road emissions standards in the United States and to evaluate indicators of lung toxicity in rats and mice exposed repeatedly to diesel exhaust (DE*) from 2007-compliant engines. The preliminary hypothesis of this Advanced Collaborative Emissions Study (ACES) was that 2007-compliant on-road diesel emissions ". . . 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 . . . although some biological effects may occur." This hypothesis is being tested at the Lovelace Respiratory Research Institute (LRRI) by exposing rats by chronic inhalation as a carcinogenicity bioassay, measuring indicators of pulmonary toxicity in rats after 1, 3, 12, and 24–30 months of exposure (final time point depends on the survival of animals), and measuring similar indicators of pulmonary toxicity in mice after 1 and 3 months of exposure. This report provides results of exposures through 3 months in rats and mice.

Emissions from a 2007-compliant, 500-horsepower-class engine and aftertreatment system operated on a variable-duty cycle were used to generate the animal inhalation test atmospheres. Four treatment groups were exposed to one of three concentrations (dilutions) of exhaust combined with crankcase emissions, or to clean air as a negative control. Dilutions of exhaust were set to yield average integrated concentrations of 4.2, 0.8, and 0.1 ppm nitrogen dioxide (NO_2). Exposure atmospheres were analyzed by daily measurements of key components and periodic detailed physical-chemical characterizations. Exposures were conducted 16 hr/dy (overnight), 5 dy/wk. Rats were evaluated for hematology, serum chemistry, bronchoalveolar lavage (BAL), lung cell proliferation, and histopathology after 1 month of exposure, and the same indicators plus pulmonary function after 3 months. Mice were evaluated for BAL, lung cell proliferation, and respiratory tract histopathology after 1 month of exposure, and the same indicators plus hematology and serum chemistry after 3 months. Samples from both species were collected for ancillary studies performed by investigators who were not at LRRI and were funded separately.

Exposures were accomplished as planned, with average integrated exposure concentrations within 20% of the target dilutions. The major components were the gaseous inorganic compounds, nitrogen monoxide (NO), NO_2, and carbon monoxide (CO). Minor components included low concentrations of diesel particulate matter (DPM) and volatile and semivolatile organic compounds (VOCs and SVOCs). There were no exposure-related differences in mortality or clinically evident morbidity. Among the more

This Investigators' Report is one part of Health Effects Institute Research Report 166, which also includes a Commentary by the HEI ACES Review Panel and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Jacob D. McDonald, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108.

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* A list of abbreviations and other terms appears at the end of the Investigators' Report.

than 100 biologic response variables evaluated, the majority showed no significant difference from control as a result of exposure to DE. There was evidence of early lung changes in the rats, accompanied by a number of statistically significant increases in inflammatory and oxidative stress indicators, and some evidence of subtle changes in pulmonary function. In general, statistically significant effects were observed only at the highest exposure level. The mice did not have the same responses as the rats, but did have small but statistically significant increases in lavage neutrophils and the cytokine IL-6 at 1 month (but not at 3 months). These findings suggest that the rats were more sensitive than mice to the subchronic exposures.

INTRODUCTION

Mobile source emissions are a significant source of exposure of people to air pollution. There has been longstanding interest in the health effects of DE, largely because of its historically high concentrations of particulate matter (PM). The presence of trace amounts of known carcinogens and mutagens in DE, and especially on DPM, raised concern for potential cancer risk from occupational and environmental exposures. Numerous epidemiologic studies of occupational groups having known or presumed high exposure to DE have demonstrated an increased risk for lung cancer, although uncertainties in estimating exposure made ascribing effects to DE and estimating unit risks correspondingly uncertain.

Several near-lifetime (chronic) inhalation studies of DE in rodents during the 1980s evaluated the potential for DE to cause cancer and noncancer biologic responses. The studies clearly showed that extreme exposures of rats (as described in Mauderly et al. 1987b), caused exposure-related increases in lung neoplasms. Similar exposures of mice and Syrian hamsters caused noncancer disease, but little or no increase in lung tumors. Further study revealed that the lung tumor response of rats could be largely ascribed to a chemically nonspecific, species-specific response to the loading of the lung with poorly soluble particles (Nikula et al. 1995; Mauderly and McCunney 1996). However, the rat response is frequently cited as supporting evidence for a cancer hazard from DE. Exposure–response results from the program were used to identify the threshold nature of the rat lung tumor response (Valberg and Crouch 1999; Mauderly and Garshick 2009) and to set a reference concentration for human noncancer effects on the part of the U.S. Environmental Protection Agency (EPA 2002).

Emissions from on-road diesel engines have been markedly reduced in the United States and other developed nations in response to progressively stringent fuel-quality and emissions standards. The 2007 standards for diesel fuel composition and tailpipe emissions of DPM and nitrogen oxides (NO_x) represent a major benchmark in this progression in the U.S. Emissions from 2007-compliant on-road diesel engines are markedly lower than those used in the earlier rodent inhalation studies, and the relative ratios of different physical–chemical components have also changed. It is reasonable to determine whether repeated exposure to DE from 2007-compliant engines causes effects in rodents similar to those caused by emissions from older engines.

HEI and its partners conceived and funded a program aimed at characterizing the emissions of heavy-duty diesel engines compliant with U.S. 2007 and 2010 on-road emissions standards and at evaluating indicators of lung toxicity in rats and mice exposed repeatedly to 2007-compliant DE. The research program, called the Advanced Collaborative Emissions Study (ACES), funded the work reported here under a contract to evaluate biologic responses of rats and mice to repeated inhalation exposure to combined tailpipe exhaust and crankcase emissions from a heavy-duty diesel engine and fuel meeting U.S. 2007 on-road emission standards. No comprehensive toxicologic studies have been done using DE from this generation of engines. This information on health hazards in rodents will facilitate updated views of the potential health risks from human exposures. This report provides the results of exposures of rats and mice for 1 and 3 months (4 and 13 weeks). Exposures of rats are continuing; exposures of mice concluded at 3 months. The results from similar evaluations of rats after 12 months of exposure and from the chronic-inhalation carcinogenicity bioassay are summarized briefly in Appendix F of this report. Results after up to 30 months of exposure will be communicated in a later report.

SPECIFIC AIMS

The aim of the subchronic portion of this study was to compare certain biologic responses in rats and mice exposed for 1 or 3 months to DE from a heavy-duty engine and fuel compliant with U.S. 2007 fuel and on-road emission standards. Biologic response indicators for this portion of the study focused primarily on standardized toxicity endpoints such as clinical chemistry, clinical pathology, and pathologic and biochemical toxicity indicators of inflammation and oxidative stress. Pulmonary function was assessed in the rats at 3 months, but not in the mice. Animals were exposed 16 hr/dy, 5 dy/wk to match the exposure protocol of a previous HEI-funded study comparing lung carcinogenicity in rats after chronic exposure to historical DE and carbon black (Mauderly et al. 1994; Nikula et al. 1995).

METHODS AND STUDY DESIGN

EXPERIMENTAL DESIGN

The animal groups used in this study are described in Table 1. HsdRccHan:Wist (Wistar Han) rats (10/sex/group) and C57BL/6 (10/sex/group) mice were exposed by inhalation 16 hr/dy, 5 dy/wk to one of three dilutions of whole DE from a 2007-compliant system (encompassing engine, fuel, and aftertreatment) or to clean air as a control. Rats will be exposed for 24 months, with the option to extend exposures to 30 months if survival permits. Some of the rats have been and will be euthanized after 1, 3, 12, and 24 months of exposure for interim evaluations. These evaluations include pulmonary function, necropsy and organ weights, BAL, hematology, serum chemistry, lung cell proliferation, and histopathology of lesions and standard organ sections. Mice were similarly exposed for up to 3 months, with one-half euthanized after 1 month and the remaining half after 3 months of exposure. The respiratory tracts of the mice were analyzed by histopathology at the 3-month time point. Tissue and fluid samples were also collected from euthanized animals for the evaluation of additional responses in ancillary studies by investigators external to the core LRRI team (see the other Investigators' Reports in this volume).

ANIMALS AND MAINTENANCE

Wistar Han rats (Harlan Laboratories) and C57BL/6 mice (Charles River Laboratories) were received at 6 weeks of age and quarantined in inhalation exposure chambers for a period of no less than 14 days before being assigned to groups. Animals were randomly assigned to treatment groups after being released from quarantine. They were housed individually in stainless-steel wire mesh cages within stainless-steel 1- or 2-m^3 whole-body inhalation exposure chambers (H1000 and H2000, respectively; Hazleton Lab Products) throughout the quarantine/conditioning and exposure periods. Animal chambers were maintained daily by cleaning of excreta trays and visually inspecting the animals. Ad libitum water availability was verified daily by bleeding out the water lines. Chambers were washed and sanitized weekly, and the automatic watering system was also sanitized and checked at that time. The cage units within each chamber were rotated one position each week, so that each cage unit was rotated through all positions within its chamber as the study progressed.

In order to verify the specific pathogen-free status of the rodents in the study, blood for serological analysis was obtained from each group of animals purchased before their entry into the exposure chambers (after release from quarantine). Five unexposed sentinel mice and rats per sex were sampled for serology at the 3-month (final) necropsy time. Serum separated from the cell fraction of each blood sample was frozen and shipped to BioReliance (Rockville, MD) for analysis of the presence of antibodies to common rodent pathogens. The BioReliance 80-221 panel was used for rats and includes H-1 parvovirus, Kilham rat virus, cilia-associated respiratory (CAR) bacillus, pneumonia virus of rats, rat corona virus/Sialodacryoadenitis virus, and Sendai virus. The 80-220 panel was used for mice and includes enteric diarrhea of infant mice, Theiler's encephalomyelitis virus, mouse hepatitis virus, mouse minute virus, *Mycoplasma pulmonis*, CAR bacillus, Pneumonia virus of mice, and Sendai. No serology abnormalities were observed.

Exposure chamber flow rates were adjusted to maintain a minimum of 12 air changes per hour. Chambers were

Table 1. Study Animals[a]

Treatment Group	Purpose	Species/Strain	Age of Animals at Study Start	Total Number of Animals/ Group/Sex
Rats				
1-Month sacrifice	Pulmonary toxicity	Harlan Wistar HsdRccHan:Wist	Young adult (6 weeks)	10
3-Month sacrifice	Pulmonary toxicity	Harlan Wistar HsdRccHan:Wist	Young adult (6 weeks)	10[a]
Mice				
1-Month sacrifice	Pulmonary toxicity	C57BL/6 mice	Young adult (6 weeks)	10[b]
3-Month sacrifice	Pulmonary toxicity	C57BL/6 mice	Young adult (6 weeks)	10

[a] n = 20/group for respiratory function measurement (males/females combined).
[b] Histopathology and serum chemistry not conducted at 1-month mouse sacrifice.

held at 2 to 5 cm of negative water pressure with respect to the room. Animals were housed in separate compartments within the chamber. Daily average temperatures were maintained within a target range of 18 to 26°C. This temperature fluctuated depending on the temperature of the room and would typically increase during operation. In general, the highest exposure level had the highest temperature, which was typically 1.5 to 2 degrees higher than the lower exposure levels and control. Relative humidity was monitored, but not controlled or alarmed. A 12-hour daylight cycle from approximately 0600 to 1800 hours was used. All animals were supplied with tap water, and 2016C Harlan Global Certified Rodent Chow was available ad libitum.

EXPOSURES AND MEASUREMENT OF EXPOSURE ATMOSPHERES

A 2007-compliant engine ("Engine B′", selected from four candidate engines in Phase 1 of the ACES program; see Khalek et al. 2009) and its stock aftertreatment system was mated to a Dyne Systems, 550-hp, 660-A, alternating-current dynamometer and controlled remotely using a Dyne Systems, Inter-Loc V, Digital Multi-Loop Controller interfaced to a personal computer. The engine test cycle was controlled with the Dyne Systems Cell Assistant for Windows software. The engine was operated on a 16-hour duty cycle developed specifically for the ACES program (Clark et al. 2007). Diesel fuel meeting current on-road specifications was delivered to LRRI from a local commercial source (Chevron-branded D-2 [legal for on-road use], Ever-Ready Oil Co., Albuquerque, NM). The engine and associated systems were maintained as recommended by the engine manufacturer. Crankcase lubricating oil was changed every 250 hours. The oil was a proprietary blend approved by HEI and the Coordinating Research Council (CRC), provided by Lubrizol Corp., and also used in Phase 1 of the ACES Program. The manufacturer-recommended oil filter was changed with each change of lubrication oil. A more detailed description of the exposure system is provided in Appendix B.

Exhaust was passed through a stock aftertreatment system before injection into a 35.6 cm internal diameter dilution tunnel. The crankcase ventilation effluent joined the exhaust stream downstream of the aftertreatment system. The exhaust was diluted with filtered air under turbulent conditions at the point of injection. The dilution tunnel supply-air flow was approximately 3000 cubic feet per minute. The primary dilution tunnel was a constant–pressure tunnel, rather than constant volume. When exhaust flow increased, the increased pressure caused the dilution air to be dumped into a bypass leg in the test cell. At a distance of 5.5 m from the injection point (in the exposure room), a portion of the diluted exhaust was drawn through an in-line extraction probe. The exhaust mixture was withdrawn from the primary dilution plenum through individual probes and transit lines for each exposure chamber. Each exposure chamber had its own extraction probe and dilution system. Subsequent to this extraction, the exhaust was diluted with filtered, compressed air provided through a rotary dilution/dilution bypass system. Diluting flows were adjusted as needed to reach the final dilution and concentration targets. The residence time of DE in the dilution tunnel and transit lines was less than 5 seconds. After the exhaust reached the exposure chamber, the residence time was approximately 4 minutes. All dilution and transit lines were constructed of stainless steel, and were of near equal lengths for each of the exposure levels. Exhaust was diluted to achieve pre-selected target exposure concentrations of 4.2, 0.8, and 0.1 ppm NO_2. This required dilution ratios of approximately 25:1, 115:1, and 840:1. The dilution rate in the primary dilution tunnel was approximately 5:1.

Exposures of the mice preceded those of rats; thus, average concentrations of some components were slightly different for the two species. Exposures were conducted 16 hr/dy, 5 dy/wk from approximately 1600 to 0800 hours Sunday through Thursday. Because the animals were to be exposed at least 1 day immediately before necropsy, the Sunday–Thursday schedule allowed necropsies during the regular Monday–Friday work week. Exposures were conducted for 16 hours plus the time to reach 90% of the target atmosphere (T_{90}). The system was operated without continuous operator presence between 1800 and 0800 hours, although surveillance personnel were always available and checked the system periodically during the night. Several control-alarm systems were in place to automatically shut down the engine and notify personnel in case of malfunction. The system was programmed to automatically terminate the engine cycle and switch the exposure chambers to clean dilution air at 0800 hours.

Exposure atmosphere measurements were collected throughout the 16-hour exposure period. Concentrations of NO and NO_2 were measured directly from each exposure level throughout each exposure day. NO_x were also measured from the primary dilution tunnel to enable measurements of the secondary dilution rate (the ratio of tunnel concentration and chamber concentration). CO, carbon dioxide (CO_2), non-methane hydrocarbons, particle mass by a Dekati Mass Monitor (DMM) and black carbon by a Photoacoustic Soot Spectrometer (PASS) were measured daily from DE at the high-exposure level. During periodic intensive characterizations, these measurements were taken at the other exposure levels, and on those days the measurements were not made at the high level. Particle

size was measured as part of the DMM analysis. A more detailed measurement of particle size was conducted once per week at each exposure level by the Fast Mobility Particle Sizer (TSI, St. Paul, MN). A periodic measurement of size in the chamber was conducted with an Aerodynamic Particle Sizer (TSI, St Paul, MN). Particle mass concentration by gravimetric analysis of Teflon-membrane filters at the inlet of the chamber and inside the exposure chamber was measured once a week at each exposure level. More detailed atmospheric monitoring was conducted in April 2010 and September 2010, as described in Appendix B.

EVALUATION OF HEALTH EFFECTS

The biologic response indicators are summarized in Table 2. Animals were observed twice daily for morbidity and mortality, and were weighed once per month. In addition to pathologic and biochemical toxicity, rats were

Table 2. Biologic Response Indicators (with Units) Used to Indicate Effects of Inhaled DE

Hematology
Red blood cell count (10^6/μL)
Hemoglobin (g/dL)
Hematocrit (%)
Mean corpuscular volume (fL)
Mean corpuscular hemoglobin concentration (g/dL)
Mean corpuscular hemoglobin (pg)
Platelet count (10^3/μL)
Percentage of reticulocytes
White blood cell count and absolute differential (10^3/μL)
White blood cell count (10^3/μL)
Neutrophils (10^3/μL)
Lymphocytes (10^3/μL)
Monocytes (10^3/μL)
Eosinophils (10^3/μL)
Basophils (10^3/μL)
Large unstained cells (10^3/μL)
Coagulation:
  Partial thromboplastin time (sec)
  Prothrombin time (sec)

Serum Chemistry
Alanine aminotransferase (alanine transaminase)-serum (IU/L)
Albumin (g/dL)
Aspartate aminotransferase (aspartate transaminase)-serum (IU/L)
Bilirubin (total) (mg/dL)
Blood urea nitrogen (mg/dL)
Calcium (mg/dL)
Chloride (serum) (mmol/L)
Cholesterol (total) (mg/dL)
Creatinine (serum) (mg/dL)
Glucose (mg/dL)
Gamma glutamyltransferase (IU/L)
Alkaline phosphatase (IU/L)
Phosphates (mg/dL)
Potassium (serum) (mmol/L)
Protein (total) (g/dL)
Sodium (serum) (mmol/L)
Triglycerides (mg/dL)
Calculated variables and ratios:
  Albumin/globulin
  Blood urea nitrogen/creatinine
Globulin (g/dL)

Lung Lavage
Lactate dehydrogenase activity (IU/L)
Protein (g/dL)
Albumin (μg/mL)
Hemoglobin (mg/mL)
Alkaline phosphatase (IU/L)
Total cell counts/differentials (10^5)
Total antioxidant capacity (μM)
Sodium (serum) (mmol/L)
Triglycerides (mg/dL)
Lung tissue
IL-1β (pg/mg)
TNF-α (pg/mg)
MIP-2 (pg/mg)
KC (pg/mg)
IL-6 (pg/mg)
Oxidized/reduced glutathione (nmol/mg)
Heme oxygenase-1 (ng/mg)
8-Hydroxy-guanosine (ng/g)
Cell proliferation (data not shown)

Pulmonary Function (Rats Only)
Minute volume/body weight (mL/min/kg)
Total lung capacity (mL)
Total lung capacity/kg (mL/Kg)
Dynamic lung compliance (mL/cm H_2O)
Quasistatic chord compliance (mL/cm H_2O)
CO diffusing capacity/alveolar volume (mL/min/mm Hg)
Forced expiratory flow (mL/sec)
Mean mid expiratory flow (mL/sec)

Other Clinical Observations
Mortality
Body weight (g)
Organ weights (g)
Tissue histopathology

assessed for pulmonary function at 3 months. The animals were euthanized with an overdose of a pentobarbital-based euthanasia solution (Euthasol). Body and organ weights, blood, bronchoalveolar lavage fluid (BALF), and frozen tissue specimens were collected in addition to fixed tissue specimens. The lungs were weighed, and the right lung lobes lavaged with phosphate-buffered saline (PBS). Right lung lobes were then frozen individually, and the left lung instilled with neutral-buffered formalin (NBF) at 25 cm of hydrostatic pressure before immersion fixation. All major organ systems were examined and fixed in NBF.

On animals designated for clinical pathology analyses (hematology, serum chemistry, coagulation, and BALF cell count, cell differential, and chemistry), blood was collected at necropsy via open cardiac puncture and immediately placed into potassium ethylenediaminetetraacetate anticoagulant tubes for hematology analysis, sodium citrate anticoagulant tubes for coagulation analyses, and/or a gel serum separator tube for serum chemistry analyses. Because of blood volume limitations in mice, full hematology and serum chemistry panels were not performed. BAL was performed on the right lung only, preserving the left lung exclusively for histopathology. All clinical pathology analyses were performed by trained, certified (American Society for Clinical Pathology) medical technologists. Hematology analysis was performed using an automated analyzer (Advia120 Hematology System, Siemens Medical Solutions Diagnostics, Tarrytown, NY). Serum and BAL supernatant (rats only because of volume limitations in mice) chemistry analyses were conducted using a Hitachi Modular Analytics Clinical Chemistry System (Roche Diagnostics, Indianapolis, IN). An Amax Destiny Plus coagulation analyzer (Trinity Biotech, Jamestown, NY) was used for coagulation analyses (prothrombin time and activated partial thromboplastin time). BAL cell counts were performed manually using a Neubauer hemacytometer, and modified Wright-Giemsa stained cytospin preparations were used to conduct manual differential counts on BAL specimens.

Tissues (as detailed in the protocol; see Appendix H on the HEI Web site at *www.healtheffects.org*) for histologic examination were processed routinely, embedded in paraffin, sectioned at approximately 4 µm, placed on glass slides, and stained with hematoxylin and eosin for microscopic examination. Full sets of tissues were evaluated in controls and high-exposure animals, and one section of the left lung lobe on all remaining animals was evaluated. In accordance with standard practice in toxicologic pathology as recommended by the Society of Toxicologic Pathology (Crissman et al. 2004), initial examinations were conducted by the pathologist with the full knowledge of the exposure groups. All lesions were graded subjectively by the designated pathologist, and diagnoses received a severity grade on a 0-4 scale. For example, for the lung, indicators of inflammation, cytotoxicity, and parenchymal changes (alveolar wall thickening, fibrosis, and pneumocyte hyperplasia) were evaluated for each animal. Frequent communication between the pathologists reviewing the mouse and rat studies, as well as limited image review of selected slides using the Internet, ensured diagnostic consistency and lesion agreement. After the initial histopathology evaluation, the treatment-related changes in the lung that were observed at 3 months in the rats exposed to high-levels of DE and the absence of similar changes at 3 months in the high-exposure mice were verified by blinded evaluation by the study pathologist and colleagues. Finally a histopathology peer review of the studies was performed by HEI commissioned pathologists Drs. Ernest E. McConnell and Ronald Herbert (see Appendix A).

For biochemical toxicity, lung tissue was homogenized in a buffer consisting of 1× Dulbecco's phosphate-buffered saline (DPBS), 0.5% Triton X-100 and a proteinase cocktail including 1mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride (serine proteinase inhibitor), 15 µMN trans-epoxysuccinyl-L-leucine-4-guanidinobutylamide (E-64, cysteine proteinase inhibitor), 20 µM pepstatin A (aspartic acid proteinase inhibitor) and 5 mM 1,10-phenanthroline (metalloproteinase inhibitor). The tissues were weighed and combined with 5 volumes of the lysis buffer and then homogenized on ice using a Tissumizer. The homogenates were centrifuged, and the supernatant was frozen until use. The protein content was measured using a bicinchoninic acid reagent kit (Pierce Chemical Co.) using bovine serum albumin as the standard. Hemoglobin in BALF was assayed using Eagle Diagnostics hemoglobin reagent set (cat. no. 6200-1) according to the procedures provided by the supplier. The assay is based on measurement of cyanmethemoglobin after the reaction of the hemoglobin with ferricyanide, which converts oxyhemoglobin to methemoglobin, and with cyanide, converting the methemoglobin to cyanmethemoglobin. The reactions were carried out in microtiter plates, and the optical density at 540 nm was read on a spectrophotometric plate reader using human hemoglobin (Sigma Chemical Co.) as a standard.

BALF albumin was measured using enzyme-linked immunosorbant assays (ELISA): Immunology Consultants Laboratory kit E-25AL for rat BALF diluted 1:1000 in PBS, and Bethyl Laboratories E99-134 for mice, BALF diluted 1:1000 in PBS, according to the procedures provided by the kit suppliers. Glutathione was analyzed in lung tissue and BALF. BALF samples were deproteinized with 5% sulfosalicylic acid (SSA) as soon as possible after collection and centrifuged in a microfuge at full speed (14,000*g*) for 1 minute. The supernatants were flash-frozen in liquid nitrogen and stored at –80°C until analysis. Portions of the

lungs (lavaged) were flash-frozen in liquid nitrogen. The frozen lung tissues were weighed, and 20 volumes of 5% SSA was added before homogenization using a Tissumizer; the samples were maintained on ice during the homogenization. The homogenates were centrifuged at 2000*g* for 5 minutes at 4°C. The supernatant was used for the glutathione assay, and the pellet was solubilized in DPBS (pH adjusted to be 7.0–7.5 after addition to the SSA pellets) for protein determination using the Coomassie assay. Glutathione measurements were performed according to the Anderson modification (Anderson 1985) of the Tietze enzymatic recycling method (Tietze 1969). Results for the lung tissue are normalized to the total protein.

Cytokines were analyzed in lung tissue homogenates, prepared as just described. Mouse (KC, IL-1β, IL-6, TNF-α, and MIP-2) and rat (KC, IL-1β, IL-6, and TNF-α) cytokines were measured using a Luminex bead-based multiplex immunoassay (Millipore Milliplex). Rat CINC-3 (MIP-2) was assayed with an ELISA kit (R&D Systems, cat. no. RCN300). Results were normalized to the protein content of the lysates. Lung tissue homogenates were analyzed for HO-1 measured using ELISA. For mice, the ELISA was constructed using an ImmunoSet antibody pair (Enzo Life Sciences, cat. no. ADI-960-071) according to the procedures provided by the supplier. For rats, a commercially available ELISA (Enzo Life Sciences, cat. no. ADI-EKS-810A) was used. The rat HO-1 standard was used for the mouse ELISA as well. For mouse BALF, lactate dehydrogenase was measured using a microtiter plate method based on the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) in the presence of pyruvate. Alkaline phosphatase was measured using a microtiter plate method based on hydrolysis of para-Nitrophenylphosphate (March et al. 2002). Total protein was measured using a Coomassie Blue reagent (Pierce Chemical Co.) using bovine serum albumin as the standard. Lavage Trolox equivalent antioxidant capacity (TEAC) was measured as previously described (Troost et al. 2003). Briefly, buffer consisting of 150 mM sodium phosphate pH 7.4 and 150 mM sodium chloride was bubbled with nitrogen. A mixture of freshly prepared 0.23 mM 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid/2.3 mM 2,2′-azobis(2-amidinopropane) was prepared in the buffer and heated to 70°C for 15 minutes. A stock solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Santa Cruz Biotechnology, Santa Cruz, CA) was prepared in ethanol and diluted in nitrogen-sparged DPBS to a range of concentrations from 10 to 100 µM and used as a standard. Samples or standard were combined with the Trolox solution, incubated for 5 minutes at room temperature, and then read in a spectrophotometric plate reader (Molecular Devices VersaMax) at 734 nm.

For determination of 8-Oxo-2′-deooxyguanosine (8-Oxo-dG), DNA was first isolated from rat lung tissue using a Qiagen (Valencia, CA) AllPrep DNA/RNA/protein isolation kit. The isolated DNA was then hydrolyzed in a 37°C hot water bath with deoxyribonuclease for 10 minutes followed by the addition of phosphor-diesterase and alkaline phosphatase for 1 hour. Throughout the extraction procedure the DNA was protected from hydrolyzation using tetramethylpiperidine-1-oxide (TEMPO, Sigma-Aldrich, St. Louis, MO). The samples were then cleaned up using a Millipore YM-10 microcentrifuge filter. The 8-Oxo-dG was then isolated from the hydrolyzed DNA using an Agilent 1100 HPLC coupled to a fraction collector. Liquid chromatography/mass spectrometry analysis was performed on fractions containing 8-Oxo-dG using an Applied Biosystems API-4000 instrument (Foster City, CA) employing an electrospray ionization source operated in positive multiple reaction monitoring mode. Parent/daughter ion pairs of 284/168 Da for 8-Oxo-dG and 289/173 Da for the internal standard ($^{15}N_5$-8-Oxo-dG) were used for analysis.

Mucous cell metaplasia was analyzed in lung tissue sections prepared from the paraffin-embedded, fixed, left lung, described earlier in the "Evaluation of Health Effects" section. Antigen was recovered by heating the tissue for 2 minutes under pressure in a commercial pressure cooker (Presto, 6 qt; National Presto Industries) in 10 mM sodium citrate buffer with 0.05% Tween (pH 6.0), a polysorbate surfactant. Endogenous peroxidases were quenched by treatment with 3% hydrogen peroxide in methanol for 30 minutes, and the sections were blocked by incubation in 10% goat serum in Tris-buffered saline for 1 hour. The primary antibody (monoclonal anti-Ki67, Abcam) was diluted to 0.05 µg/mL in the same buffer with 10% goat serum, and the slides were incubated with this solution overnight at 4°C in a humidified chamber. After the slides were washed, the secondary antibody (biotinylated goat anti-mouse) was diluted (1:400) in the same buffer with 10% normal goat serum and incubated with the samples for 1 hour at room temperature. Finally, the antigen was visualized using the avidin-biotin-peroxidase complex (1:50 in PBS, Vectastain Elite ABC kit, Vector Laboratories), and the slide was incubated for 5 minutes with diaminobenzidine as the substrate. Sections were counterstained with hematoxylin.

Each experimental series included a negative control in which an isotype-matched (IgG1) nonspecific antibody was substituted for the primary antibody. A tracheobronchial or mesenteric lymph node collected from a control animal during each necropsy served as a positive control and was processed identically. Slide images were collected using virtual microscopy (NanoZoomer Digital Pathology, Hamamatsu Photonics). Images were then imported into

Visiopharm (Visiopharm, Medicon Valley, Denmark) software using the MicroImager module. Sections were defined, and sampling regions were collected for 10% of each section's area. Two representative regions were selected per slide. The percentage of cells testing positive for Ki-67 was determined for the parenchymal tissue within the sampling area. Ki-67 labeling indices were expressed as the percentage of cells with positive nuclear staining.

Selected pulmonary function parameters were measured on 10 male and 10 female rats per treatment group after 3 months of exposure using well-established methods (Harkema et al. 1982; Mauderly 1995). Rats were anesthetized with isofluorane, intubated per os with semi-rigid, thin-walled endotracheal catheters (modified 12- or 14-gauge intravenous catheters, Cathlon, Jelco) (Mauderly 1977) and liquid-filled esophageal catheters, and placed prone in a 9.2 L heated flow plethysmograph. The plethysmograph was fitted with plastic spacers to reduce dead volume to 2.6 L at this measurement time, while allowing for future sex- and age-related differences in body size. Flows were determined by measuring plethysmograph-to-room differences in pressure (Validyne MP-45, ± 2 cm H_2O) across a resistance element consisting of five 400-mesh stainless-steel screens fixed rigidly over a 1.27-cm hole at the rear of the plethysmograph. The resistance element was sized for an estimated maximum peak flow of 200 mL/sec, using an established formula (Mauderly 1995). Transpulmonary pressure (P_{tp}) was measured as the difference between esophageal and airway pressure (Motorola MPX11DP). Flow, volume, and P_{tp} signals were acquired via preamplifiers (Validyne) and a PC-based programmable system (Acknowledge V.3.7.2, Biopac Systems). Body temperature was measured using a rectal thermometer.

The test sequence consisted of three steps involving single-breath inflations and deflations by syringes or standardized positive and negative airway pressures during brief hyperventilation-induced apnea. The lung diffusing capacity for carbon monoxide (DL_{CO}) was measured first by the traditional single-breath method (Ogilvie et al. 1957). The injected volume required to inflate the lung to a P_{tp} of +20 cm H_2O was determined during apnea using an air-filled syringe. A dual syringe–stopcock assembly was flushed with a test gas mixture containing 0.3% CO and 0.5% neon (Ne) in air. Apnea was reinduced, the test gas was injected into the lung, most of the gas was withdrawn after 6 seconds, and a sample of gas from the lung was collected in a small side syringe. The CO and Ne concentrations in the collected sample were measured by gas chromatography (Model 111, Carle). The differences in gas concentrations, inflation time, barometric pressure, and body and room temperatures were entered into a standardized program to calculate DL_{CO}, DL_{CO} per kg body weight, and DL_{CO} normalized by the inflated alveolar volume estimated by Ne dilution.

The relation between lung pressure and volume was measured during a quasistatic inflation–deflation cycle. During apnea, the lung was connected to a ± pressure reservoir–solenoid system via an airway in order to minimize dead space and abrupt changes in internal diameter. The lung was inflated to a P_{tp} of 30 cm H_2O (defined as total lung capacity) and deflated until flow stopped at standardized flow rates of 5 and 3 mL/sec. The exhaled volume was measured as the slow vital capacity (SVC), and quasistatic lung compliance (Cqs) was measured over the descending chord at a P_{tp} of between plus or minus 10 and 0 cm H_2O.

A slow inhalation and forced exhalation was performed as described above, but without limitation of expiratory flow. Flow was driven by a vacuum reservoir maintained at a P_{tp} of –50 cm H_2O and connected to the airway via a solenoid valve having a 9.5 mm orifice (V52DA3012, Skinner) and an airway having a minimum internal diameter of 2.5 mm. The forced expiratory maneuver was performed (with intervening recovery of spontaneous respiration) at least twice, and a third time if the first two flow-time curves were noticeably different or if the pressure or volume traces were not acceptable. Measured and calculated variables included forced vital capacity (FVC), forced expired volume in 0.1 second ($FEV_{0.1}$, as mL and percentage of FVC), peak expiratory flow rate (PEFR), forced expiratory flows at 75%, 50%, 25%, and 10% of FVC (FEF_{75}, FEF_{50}, FEF_{25}, FEF_{10}), mean mid-expiratory flow (MMEF, the average flow between 75% and 25% FVC), and all flows normalized by FVC.

After the test series, anesthesia was discontinued, the rats were allowed to recover, and the catheters were removed at the first sign of voluntary movement. The rats were returned to housing in bedded plastic cages with food and water until necropsy the following day.

STATISTICAL METHODS AND DATA ANALYSIS

Both one-way and two-way analyses of variance (ANOVA) were used to evaluate exposure-related effects at each of the exposure time points (1 and 3 months). One-way ANOVA was used to assess diesel exposure effects for each sex. Two-way ANOVA models contained terms for exposure group (control, low-, mid-, and high-level DE exposures), sex, and sex × exposure group interaction effects. A three-way ANOVA was also conducted to evaluate interactions, and the results of that separate analysis are reported in Appendix I (available only on the Web at *www.healtheffects.org*). The comparability of sex-based differences in DE exposure–related response was assessed with

the sex × exposure group interaction term in the ANOVA. Where there was no substantial evidence of sex-based differences in exposure-related response ($P > 0.05$), the effects were assessed across sexes using a two-way ANOVA with no interaction term. If the ANOVA (one-way or two-way) indicated a significant ($P < 0.05$) difference among exposure group responses, Dunnett's multiple comparison procedure (Dunnett 1955; 1980) was performed to compare DE-exposed group means with those of controls. The linear term of the ANOVA (under the assumption of equal exposure spacing, using orthogonal coding of −3, −1, 1, 3) was used to assess evidence of exposure-related trends in response across the control and exposed groups. Endpoints that had 10 or fewer distinct values (e.g., cellular count data) were analyzed with an analogous ANOVA and trend analysis approach for categorical data based on weighted least squares (Grizzle et al. 1969). *P* values for multiple comparisons of DE exposure groups against the control group were adjusted using the Bonferroni correction method.

There was significant heteroscedasticity for several endpoints, with exposure group variances increasing in magnitude with their associated sample means. Logarithmic transformation of these data was employed before analysis to satisfy the homogeneity of variance assumptions underlying the ANOVA regression model. For heteroscedastic endpoints that had values of zero (where the logarithmic transformation was undefined), an offset constant was added to values before logarithmic transformation (Rocke and Durbin 2003).

The data are summarized as mean values plus or minus standard errors of the mean (mean ± SEM). In analyses across sexes, the reported mean values are the averages of the means by sex in the four exposure groups, and the reported standard errors are based on pooled sample variance estimates, after adjustment for sex differences (as opposed to variances calculated across all animals without regard for differences between sexes). All *P* values are two-sided, and statistical significance was assessed at $P = 0.05$ and $P = 0.01$. Statistical calculations were performed using the SAS software system, version 9.2. Examples of the statistical approach for selected endpoints are provided in Appendix C.

QUALITY ASSURANCE

This research was conducted in a manner that is consistent with many of the standards developed for Good Laboratory Practices (GLP), although full compliance with GLP requirements was not a requirement of the protocol. Quality control (QC) consisted of the conduct of all work according to approved protocols and standard operation procedures, the inclusion of verified QC standards for the calibration of the certification of system performance, and third-party verification of all data before submission to the statistician for analysis. These QC processes applied to all aspects of the study, including the test and evaluation of engine performance, the verification of fuel and oil composition, the receipt and husbandry of animals, the analysis of test atmospheres, the evaluation of clinical signs or pulmonary function, and the evaluation of changes in tissue or other biologic responses in animals. All animal activities, including receipt, husbandry, clinical observations and necropsy/tissue collection, and histopathology/clinical pathology were tracked through a validated software system (Provantis, Instem).

For each of the analytical tools, calibration or "span" checks were conducted each time of use. There was a wide array of biologic assays employed for this study. The sensitivity and range of the assays varied, and may have not been optimal for the ranges required for this study. They were, however, internally consistent with the standards of operation, and matched well against historical control data. Most of the assays did not have "positive controls" to verify their ability to detect a change if there were to be a biologic effect, as this is not standard practice at LRRI for the assays that were employed.

In addition to QC, the LRRI Quality Assurance Unit independently monitored the quality of operations and scientific data. The Quality Assurance Unit duties for this protocol included evaluating study-start parameters, auditing exposure system functions, auditing study-related animal care functions, auditing the processes of scientific data collection, and determining if data were recorded and stored according to LRRI Policy 602. The data were stored in a secure electronic database.

RESULTS

EXPOSURE ATMOSPHERES

Exposure atmospheres were analyzed in detail, as described in Appendix B. Tables 3 and 4 provide the mean of the atmospheric components that were monitored routinely, for mice and rats, respectively. NO_2 concentrations, which were used as the dilution indicator, were within 20% of target at all levels. Real-time particle mass and particle size distribution were also monitored routinely, and representative illustrations of both are shown in Figures 1 and 2, respectively. The results shown in Figures 1 and 2 were from samples from the breathing zone of the rodents in the high-level exposure chamber. Figure 3 summarizes the composition of the atmospheres (at the rodent

Table 3. Mean Concentrations of Major Constituents (minus CO_2 and H_2O) of Exposure Atmospheres for Mice

	Mouse Exposure			
	High Mean ± SD	Mid Mean ± SD	Low Mean ± SD	Control Mean ± SD
Gases				
NO_2 (ppm)	4.3 ± 0.7	0.8 ± 0.3	0.10 ± 0.041	0.000 ± 0.007
NO (ppm)	5.1 ± 0.8	0.9 ± 0.4	0.1 ± 0.049	0.001 ± 0.001
NO_x (ppm)	9.3 ± 1.3	1.7 ± 0.7	0.2 ± 0.086	0.001 ± 0.008
CO (ppm)[a]	7.1 ± 1.1	NA	NA	NA
NMHC (ppm)[a]	0.3 ± 0.3	NA	NA	NA
SO_2 (ppb)[a]	25.80 ± 8.0	NA	NA	NA
PM ($\mu g/m^3$)				
DPM: chamber inlet (filter)	9 ± 4	3 ± 1	2 ± 2	NA
PM: chamber (filter)	35 ± 19	38 ± 86	34 ± 26	32 ± 27

[a] CO, NMHC, and SO_2 measured daily only at high-exposure level (NA indicates not available).

Table 4. Mean Concentrations of Major Constituents (minus CO_2 and H_2O) of Exposure Atmospheres for Rats

	Rat Exposure			
	High Mean ± SD	Mid Mean ± SD	Low Mean ± SD	Control Mean ± SD
Gases				
NO_2 (ppm)	3.6 ± 1.2	0.95 ± 0.57	0.11 ± 0.12	0.003 ± 0.013
NO (ppm)	5.1 ± 1.6	1.47 ± 0.82	0.15 ± 0.17	0.002 ± 0.004
NO_x (ppm)	8.6 ± 2.6	2.41 ± 1.34	0.26 ± 0.28	0.004 ± 0.015
CO (ppm)[a]	10.5 ± 5.1	NA	NA	NA
NMHC (ppm)[a]	0.3 ± 0.5	NA	NA	NA
SO_2 (ppb)[a]	20.61 ± 6.7	NA	NA	NA
PM ($\mu g/m^3$)				
DPM: Chamber inlet (filter)	13 ± 5.7	4 ± 4	2 ± 6	NA
PM: Chamber (filter)	32 ± 21	30 ± 24	27 ± 31	37 ± 48

[a] CO, NMHC, and SO_2 measured daily only at high-exposure level (NA indicates not available).

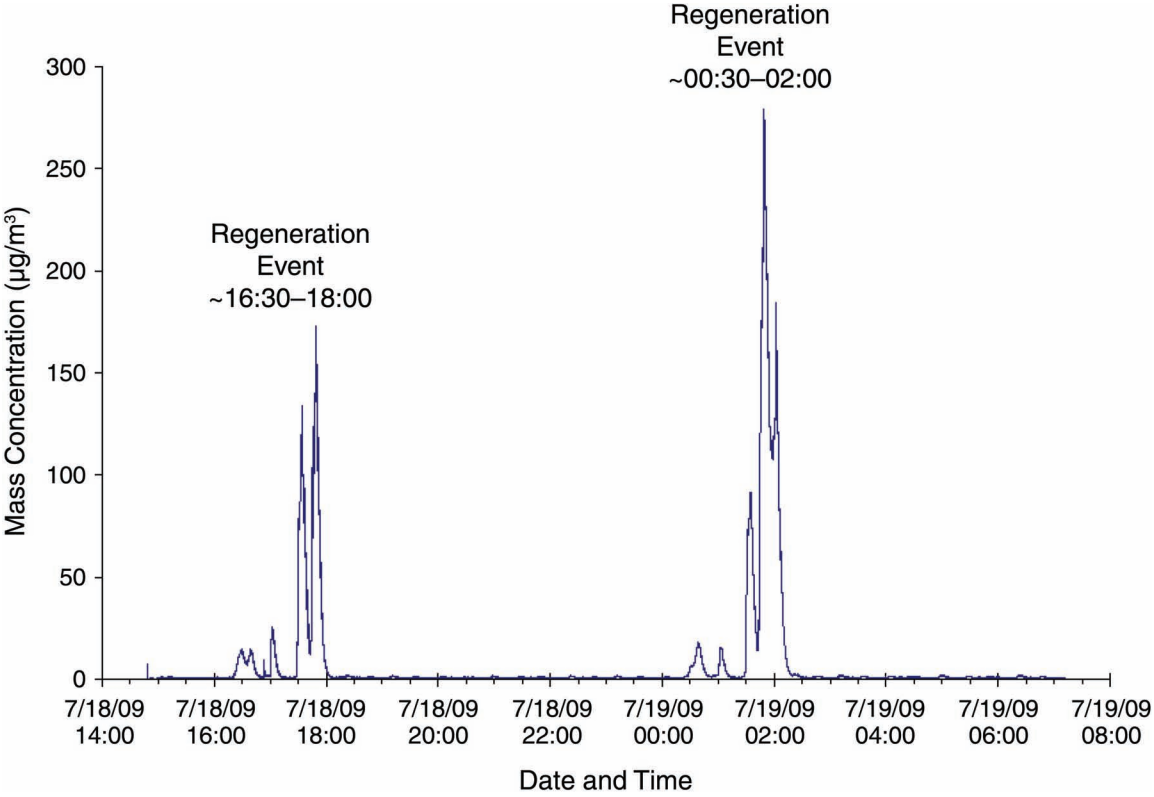

Figure 1. Real-time particle mass concentration during a 16-hour ACES engine cycle. Note that particle mass was observed only during periods where the trap was undergoing a regeneration.

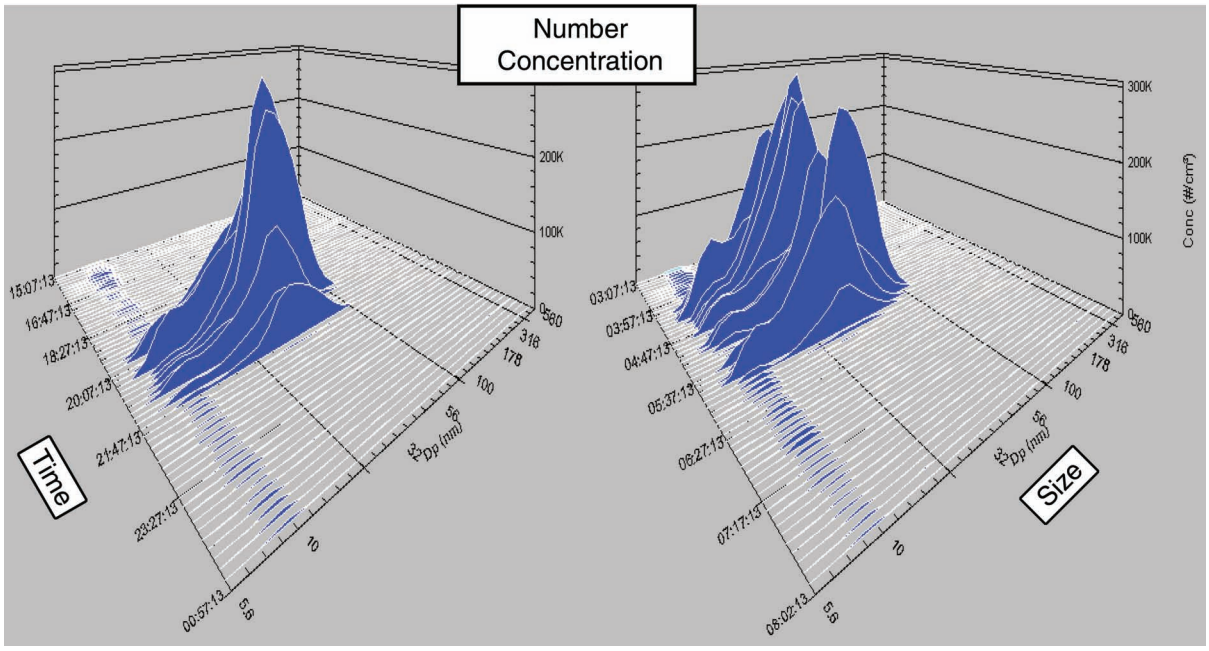

Figure 2. Real-time particle number–based size distribution during a 16-hour ACES engine cycle. Particle counts were observed only during trap regeneration.

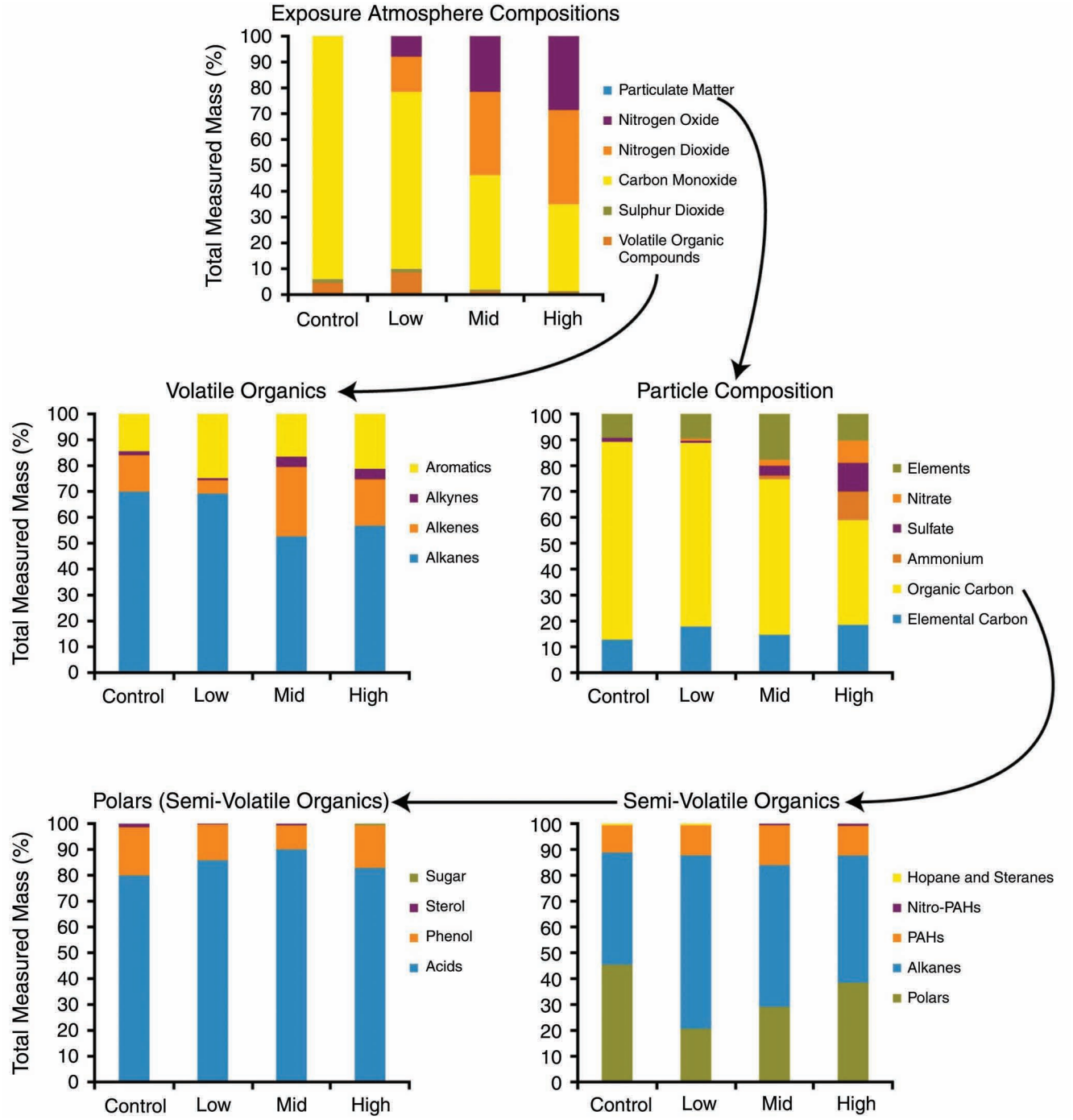

Figure 3. Composition of exposure atmospheres (from both mouse and rat chambers) expressed as a percentage of the total mass fraction for each grouping. Gaseous inorganics accounted for most of the DE atmospheres.

breathing zone) as the percentage of total measured mass for each chemical class. The composition details and the standard deviations for the measurements of the atmospheres shown in Figure 3 are provided in Appendix B.

The PM mass concentrations were measured at the inlet to the chamber and in the chamber to allow the contribution from animals to be distinguished from the DE contribution. As expected, the concentration in the chamber was much higher than that in the chamber inlet, showing that the major portion of PM mass was due to contributions from animals in the chamber. As a result, particle mass concentration within the chambers was not closely dilution-dependent. In contrast, there was a dilution-dependent difference in mass at the inlet. In addition, there was a dilution-dependent difference in particle number count, with the highest particle number observed in the high-level chamber. The particle number–based size distribution had a median size of approximately 20 nm, and the particle mass size distribution had a median size of 40 nm at the mid and high levels. The low and control levels had much larger sizes, but the mass concentrations were very low.

As Figure 3 indicates, NO, NO_2 and CO accounted for most of the mass of the measured components of the exposure atmospheres. The contribution of NO and NO_2 as a fraction of total mass increased proportionally with exposure level, as expected. In general the VOCs accounted for a small proportion of the mass, but because of the low concentrations of CO and NO_x, the VOCs accounted for up to 10% of the mass at the low and control exposure levels. It is noteworthy that this result indicates the animals contributed a significant portion of the measured VOCs. Sulfur dioxide was low at all exposure levels. PM was also low in concentration, as expected. The composition of the PM resulted from a combination of DPM and PM derived from animals in the chamber. The animal contribution undoubtedly included both dander and fine food dust. In addition, some PM likely formed in the chamber as a result of reactions between exhaust gases and ammonia, as previously shown (e.g., McDonald et al. 2004a). As a result, the PM composition was slightly different at each of the exposure levels. This was shown by a change in the proportional amount of inorganic PM that occurred through these reactions in the chamber. The proportions of organic carbon also varied at different levels. Because the sum of organic carbon and additional species exceeded the total measured mass, the organic carbon measurements are considered to be biased by a sampling artifact (organic vapor adsorption). Furthermore, some of the organic carbon measured in the chamber may have come from the animals, including dander and SVOC emissions from respiration. The high-exposure level had the largest contribution of PM that was derived from the engine (see Table B.2). At that level, carbon accounted for about 50% of the mass, and the remainder was a combination of the inorganic ions and elements. The elements were primarily composed of zinc, manganese, copper, and iron. Potassium and calcium were also present. The metal concentrations were small and not substantially different from background in most cases (see Table B.2 in Appendix B).

The VOC concentrations in general showed dilution proportionality, with the highest concentration at the high-exposure level when measuring total mass. However, concentrations at the lower exposure levels were not significantly higher than the control background with the exception of a higher concentration at the low exposure level during the April sampling period. The VOCs that could be attributed to DE were primarily the alkanes (straight chain), alkenes, and aromatics. The carbonyls (which are included in Table B.2 of Appendix B, but not in Figure 3 of the main report) showed no concentration proportionality with dilution, and in fact were lower in concentration at the higher exposure levels. While animals do contribute to a background carbonyls concentration, the dilution-dependent decrease suggested that reactions occurred with NO_x at the time samples were being taken. Further investigation into the chromatograms (data not shown) from the carbonyl analysis suggested that, indeed, the NO_x–carbonyl reactions occurred at the high level. A cobalt oxide denuder was used to attempt to remove NO_x before sampling. During the investigation, it was discovered that the denuder was not effectively removing the NO_x during the sample collections. It was found that a heat and oxygen treatment after sampling each day would regenerate the denuder to enable it to remove more than 95% of the NO_x. For subsequent sampling, the NO_x denuder regeneration procedure was implemented.

Among the measured SVOCs, the alkanes, polycyclic aromatic hydrocarbons (PAHs), and polars had approximately equivalent contributions. Hopanes and steranes, as well as nitro-PAHs, were substantially lower, as expected. The very low concentrations of hopanes and steranes indicated a negligible contribution from oil. The polar organics were primarily aliphatic and aromatic acids, which are likely from the oxidation of the parent compounds present in the fuel, and phenolic compounds.

BIOLOGIC RESPONSE IN THE WISTAR HAN RAT

There were no significant exposure-related findings in mortality or clinical signs in the rat (see Appendix E for a summary of results). With the exception of histopathology (discussed later in this section), biologic response data that showed statistically significant differences are summarized in Table 5. This effect did not persist at later time

Table 5. Findings in Lung Tissue and BALF in Wistar Han Rats After 4 and 13 Weeks of Exposure to a Clean Air Control or DE

Lung Tissue and BALF / Time Point / Group	Male		Female		Both	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
µTP BALF						
13 Weeks						
Control	10	6.0 ± 1.0	10	7.8 ± 0.6	20	6.9 ± 0.6
Low	10	5.8 ± 0.7	10	6.7 ± 0.5	20	6.3 ± 0.4
Mid	10	5.0 ± 1.0	10	6.8 ± 0.8	20	5.9 ± 0.6
High	10	10.8 ± 1.8[a]	10	11.2 ± 1.5	20	11.0 ± 1.2[b]
Trend[c]		0.030 (+)		0.072 (+)		0.004 (+)
Albumin BALF (µg/ml)						
13 Weeks						
Control	9	43 ± 4	10	64 ± 5	19	54 ± 3
Low	10	41 ± 3	10	53 ± 5	20	47 ± 3
Mid	10	38 ± 4	10	50 ± 5	20	44 ± 3
High	10	79 ± 15[b]	10	81 ± 10	20	80 ± 9[b]
Trend[c]		0.007 (+)		0.319		0.007 (+)
Cytokine IL-1β Lung (pg/mg protein)						
4 Weeks						
Control	10	90 ± 20	10	49 ± 6	20	69 ± 11
Low	10	65 ± 7	10	47 ± 7	20	56 ± 5
Mid	10	53 ± 5	9	55 ± 7	19	54 ± 4
High	10	61 ± 4	10	58 ± 9	20	59 ± 5
Trend[c]		0.209		0.337		0.904
13 Weeks						
Control	10	56 ± 6	10	38 ± 2	20	47 ± 3
Low	10	52 ± 5	10	48 ± 6	20	50 ± 4
Mid	10	59 ± 6	10	41 ± 4	20	50 ± 4
High	10	50 ± 3	10	65 ± 10[b]	20	57 ± 5
Trend[c]		0.695		0.011 (+)		0.104
Cytokine IL-6 Lung (pg/ml)						
4 Weeks						
Control	10	20.8 ± 2.9	10	21.4 ± 0.7	20	21.1 ± 1.5
Low	10	22.6 ± 2.0	10	23.3 ± 2.0	20	23.0 ± 1.4
Mid	10	20.9 ± 1.8	9	23.5 ± 1.5	19	22.2 ± 1.2
High	10	24.3 ± 2.4	10	24.5 ± 1.4	20	24.4 ± 1.4
Trend[c]		0.407		0.151		0.136
13 Weeks						
Control	10	14.3 ± 2.6	10	11.5 ± 2.4	20	12.9 ± 1.7
Low	10	15.4 ± 3.2	10	16.8 ± 3.6	20	16.1 ± 2.4
Mid	10	17.5 ± 3.1	10	11.8 ± 1.7	20	14.7 ± 1.8
High	10	15.7 ± 2.5	10	31.7 ± 6.7[a]	20	23.7 ± 3.6
Trend[c]		0.288		0.036 (+)		0.038 (+)

(*Table continues on next page*)

[a] Significance versus control; $P < 0.05$.

[b] Significance versus control; $P < 0.01$.

[c] Trend directions reported for $P < 0.1$.

Table 5 (*Continued*). Findings in Lung Tissue and BALF in Wistar Han Rats After 4 and 13 Weeks of Exposure to a Clean Air Control or DE

Lung Tissue and BALF / Time Point / Group	Male		Female		Both	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
GSH Lung (nmol/mg protein)						
4 Weeks						
Control	10	0.031 ± 0.004	10	0.024 ± 0.002	20	0.027 ± 0.002
Low	10	0.033 ± 0.004	10	0.028 ± 0.004	20	0.031 ± 0.003
Mid	10	0.029 ± 0.004	9	0.030 ± 0.004	19	0.029 ± 0.003
High	10	0.036 ± 0.005	10	0.045 ± 0.006[a]	20	0.040 ± 0.004[a]
Trend[c]		0.560		0.009 (+)		0.023 (+)
13 Weeks						
Control	10	0.021 ± 0.004	10	0.016 ± 0.005	20	0.019 ± 0.003
Low	10	0.023 ± 0.003	10	0.020 ± 0.005	20	0.021 ± 0.003
Mid	10	0.024 ± 0.004	10	0.022 ± 0.004	20	0.023 ± 0.003
High	10	0.031 ± 0.007	10	0.026 ± 0.003	20	0.028 ± 0.004
Trend[c]		0.188		0.109		0.036 (+)
HO-1 Lung (ng/mg protein)						
4 Weeks						
Control	10	1.4 ± 0.1	10	1.4 ± 0.1	20	1.4 ± 0.1
Low	10	1.8 ± 0.2	10	1.5 ± 0.1	20	1.6 ± 0.1
Mid	10	1.5 ± 0.1	9	1.6 ± 0.1	19	1.6 ± 0.1
High	10	1.7 ± 0.1	10	1.7 ± 0.2	20	1.7 ± 0.1
Trend[c]		0.139		0.131		0.034 (+)
13 Weeks						
Control	10	1.2 ± 0.1	10	1.1 ± 0.1	20	1.1 ± 0.1
Low	10	1.3 ± 0.1	10	1.2 ± 0.1	20	1.3 ± 0.1
Mid	10	1.4 ± 0.1	10	1.4 ± 0.1	20	1.4 ± 0.1[a]
High	10	1.7 ± 0.2[b]	10	1.7 ± 0.1[b]	20	1.7 ± 0.1[b]
Trend[c]		0.002 (+)		<0.001 (+)		<0.001 (+)
Total Glutathione Lung (nmol/mg protein)						
4 Weeks						
Control	10	0.036 ± 0.004	10	0.028 ± 0.002	20	0.032 ± 0.002
Low	10	0.037 ± 0.005	10	0.035 ± 0.004	20	0.036 ± 0.003
Mid	10	0.032 ± 0.005	9	0.034 ± 0.004	19	0.033 ± 0.003
High	10	0.039 ± 0.005	10	0.049 ± 0.007[a]	20	0.044 ± 0.004
Trend[c]		0.945		0.025 (+)		0.098 (+)
13 Weeks						
Control	10	0.038 ± 0.004	10	0.033 ± 0.004	20	0.035 ± 0.003
Low	10	0.042 ± 0.004	10	0.036 ± 0.005	20	0.039 ± 0.003
Mid	10	0.044 ± 0.007	10	0.045 ± 0.004	20	0.045 ± 0.004
High	10	0.051 ± 0.010	10	0.044 ± 0.004	20	0.048 ± 0.005
Trend[c]		0.165		0.033 (+)		0.017 (+)

(*Table continues on next page*)

[a] Significance versus control; $P < 0.05$.

[b] Significance versus control; $P < 0.01$.

[c] Trend directions reported for $P < 0.1$.

Table 5 (*Continued*). Findings in Lung Tissue and BALF in Wistar Han Rats After 4 and 13 Weeks of Exposure to a Clean Air Control or DE

Lung Tissue and BALF / Time Point / Group	Male		Female		Both	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
TEAC BALF (μM)						
4 Weeks						
Control	10	39.7 ± 4.1	10	33.8 ± 3.1	20	36.8 ± 2.5
Low	10	31.9 ± 1.5	10	23.5 ± 1.0[b]	20	27.7 ± 0.9[b]
Mid	10	34.7 ± 3.2	10	23.8 ± 1.1[b]	20	29.3 ± 1.7[a]
High	10	36.0 ± 3.3	10	28.1 ± 1.4	20	32.0 ± 1.8
Trend[c]		0.617		0.141		0.192
13 Weeks						
Control	10	40.2 ± 3.2	10	43.6 ± 3.5	20	41.9 ± 2.4
Low	10	30.9 ± 2.2[a]	10	29.4 ± 1.8[b]	20	30.1 ± 1.4[b]
Mid	10	29.2 ± 2.1[b]	10	31.3 ± 1.8[b]	20	30.3 ± 1.4[b]
High	10	29.4 ± 1.3[a]	10	31.4 ± 1.9[b]	20	30.4 ± 1.1[b]
Trend[c]		0.004 (−)		0.007 (−)		<0.001 (−)

[a] Significance versus control; $P < 0.05$.
[b] Significance versus control; $P < 0.01$.
[c] Trend directions reported for $P < 0.1$.

points. Males had a significantly higher weight at the mid exposure level at 3 months. At necropsy, there were scattered gross findings at both 1 and 3 months. These findings, which were typically lesions on internal organs or dermal abrasions, were not considered exposure related, as the pattern of findings was distributed across treatment groups. There were no statistically significant differences in organ weights at 1 month (Table E.3). The lung-to-body weight ratio of the males exposed to high levels of DE at 3 months was significantly increased compared with controls (Table E.4), and there was a trend with exposure across levels. Absolute heart weights were significantly decreased in females at the high-exposure level and in males at all DE exposure levels. Heart-to-brain weight ratios were significantly decreased in males at the low and high-exposure levels.

There were no significant differences in hematology, serum chemistry or coagulation indicators in the rats. However, there were several statistically significantly higher values for clinical pathology and biochemical toxicity indicators, primarily at the high-exposure level. Micro–total protein (μTP) in BALF was significantly increased at 3 months in males and in combined sexes at the high-exposure level. The increase in protein appeared to be driven by increases in albumin where again, strong increases in the high-exposure group males and combined sexes were observed (Tables 5 and E.6). Female rats at 3 months also showed a statistically significant increase in IL-6 and IL-1β at the highest level, and a similar trend was observed in the combined sexes for IL-6. One of the statistically strongest effects observed in the biochemical parameters was a decrease in TEAC in BALF (Figure 4). The TEAC assay measures the ability of the BALF to prevent oxidation of a chromophore by a chemically generated free radical. The results are compared with a dilution series (standard curve) of Trolox, a water soluble analog of vitamin E. Statistically significant effects were observed at the low and mid levels in the females and combined sexes at 1 month, and in both sexes at all levels at 3 months (Table 5). At 3 months, a significant increase in the oxidative stress indicator heme-oxygenase-1 (HO-1) was observed in lung tissue at the highest exposure level in both sexes (Figure 5 and Table 5). Interestingly, a trend toward higher total lung tissue glutathione was observed in females and the combined sexes at both 1 and 3 months, although there was no effect on oxidized glutathione. This suggested an oxidative stress-induced upregulation of synthetic and reducing enzymes for this key endogenous antioxidant. Additional assays of oxidative stress, such as changes in glutathione, were not observed.

Assessments of pulmonary function at 3 months revealed statistically significant effects on alveolar-capillary

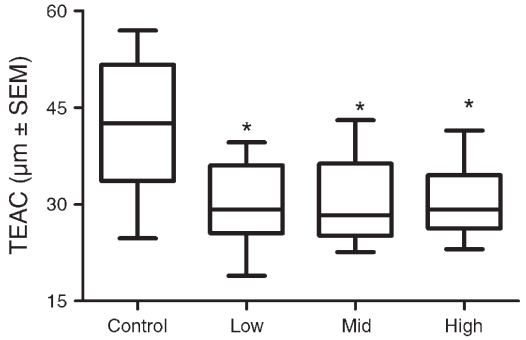

Figure 4. TEAC response in combined sexes of rats exposed for 3 months. Box plots provide the median and interquartile ranges along with the maximum and minimum values. Data were normalized by overall sex response mean. A two-way ANOVA (sex and exposure as factors with no interaction) of the logarithmically transformed data confirmed the strong evidence of exposure effects across sexes, with highly statistically significant ($P < 0.01$) differences between controls and all diesel exposure group means.

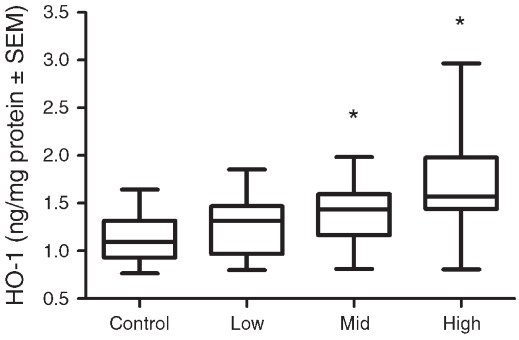

Figure 5. HO-1 response in combined sexes of rats exposed for 3 months. Box plots provide the median and interquartile ranges along with the maximum and minimum values. The mid ($P < 0.05$) and high ($P < 0.01$) groups showed statistical significance against control means by two-way ANOVA. Positive trends were statistically significant ($P < 0.001$).

gas exchange (or diffusing capacity in the lung for CO, DL_{CO}), the forced expiratory variables of mean mid expiratory flow (MMEF) as a trend in females, and FVC when sexes were combined (Table 6). These effects were primarily observed as trends, and only when the sexes were combined to enable a decrease in the overall standard error for the statistical analysis. DL_{CO} was slightly reduced in both males and females. Although the trend was progressive in males, it was not statistically significant. The trend reached statistical significance for combined sexes, but DL_{CO} at the high exposure level did not differ significantly from control (Figure 6). Normalization of DL_{CO} by body weight or lung volume rendered the trend insignificant, but the normalized data still suggested that DL_{CO} may have been slightly affected by exposure in a manner unrelated to lung size (Table E.10).

Histologic analysis revealed a number of exposure-related findings. Tables 7 and 8 summarize the incidence and severity of selected findings in the lungs and nasal regions, respectively. Statistical significance of the severity score findings against control is noted. There were no exposure-related findings in extra-respiratory tract organs (data not shown). Lesions were observed at 3 months in the lungs of both male and female rats exposed at the high level. These lesions were not evident among the lower level groups or controls. Figure 7 provides illustrative photomicrographs of the lung findings. The lung lesions were primarily characterized by an increased number and prominence of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli that were in close proximity to the terminal bronchial and alveolar ducts. The distribution was rather uniform and focused at the central acinus. The hyperplasia observed was not accompanied by an increase in mucous cell metaplasia. In addition to the epithelial proliferation, there was a subtle accumulation of pulmonary alveolar macrophages, with a tendency to be concentrated in and centered on periacinar areas. A lesser infiltrate of scattered mixed inflammatory cells was present in those areas as well. In some animals, there was also a minimal increase in fibrous connective tissue along and around alveolar ducts and the junctions of the alveolar ducts with alveolar walls in periacinar areas.

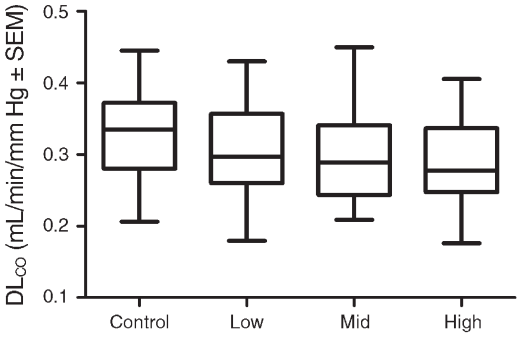

Figure 6. DL_{CO} (mL/min/mm Hg) response in combined sexes of rats exposed for 3 months. Box plots provide the median and interquartile ranges along with the maximum and minimum values. Data were normalized by overall sex response mean. Negative trends were statistically significant ($P = 0.05$). Individual ANOVAs for each of the sexes gave neither statistically significant evidence of differences in mean values among experimental groups (males, $P = 0.55$; females, $P = 0.44$) nor compelling evidence of exposure-related trends (males, $P = 0.16$; females, $P = 0.19$).

Table 6. Pulmonary Function Findings in Wistar Han Rats After 13 Weeks of Exposure (See Also Table E.10)[a]*

Respiratory Function	Male		Female		Both	
	n	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE
FVC						
Control	10	17.4 ± 0.8	10	12.8 ± 0.5	20	15.1 ± 0.4
Low	10	16.2 ± 0.4	10	12.5 ± 0.5	20	14.4 ± 0.3
Mid	10	16.6 ± 0.3	10	12.8 ± 0.3	20	14.7 ± 0.2
High	10	16.1 ± 0.6	10	11.8 ± 0.3	20	13.9 ± 0.3[b]
Trend[c]		0.172		0.125		0.042 (−)
PEFR						
Control	10	82 ± 2	10	75 ± 2	20	78 ± 1
Low	10	81 ± 2	10	67 ± 4	20	74 ± 2
Mid	10	81 ± 2	10	71 ± 3	20	76 ± 2
High	10	80 ± 2	10	65 ± 1[b]	20	73 ± 1
Trend[c]		0.55		0.039 (−)		0.045 (−)
FEF_{25}						
Control	10	44 ± 3	10	37 ± 2	20	41 ± 2
Low	10	47 ± 3	10	33 ± 3	20	40 ± 2
Mid	10	42 ± 3	10	36 ± 3	20	39 ± 2
High	10	42 ± 3	10	28 ± 2[b]	20	35 ± 2
Trend[c]		0.458		0.030 (−)		0.045 (−)
MMEF						
Control	10	60 ± 2	10	54 ± 1	20	57 ± 1
Low	10	61 ± 2	10	48 ± 4	20	55 ± 2
Mid	10	58 ± 1	10	52 ± 2	20	55 ± 1
High	10	58 ± 2	10	45 ± 1[b]	20	52 ± 1
Trend[c]		0.329		0.054 (−)		0.035 (−)
DL_{CO}						
Control	10	0.36 ± 0.02	10	0.28 ± 0.02	20	0.32 ± 0.01
Low	10	0.35 ± 0.01	10	0.26 ± 0.02	20	0.30 ± 0.01
Mid	10	0.34 ± 0.02	10	0.25 ± 0.01	20	0.30 ± 0.01
High	10	0.33 ± 0.02	10	0.25 ± 0.01	20	0.29 ± 0.01
Trend[c]		0.158		0.189		0.050 (−)

[a] All parameters were normalized.

[b] Significance versus control; $P < 0.05$.

[c] Trend directions reported for $P < 0.1$.

* See Table 2 for units.

Table 7. Incidence of Histologic Findings in Lungs of Male and Female Wistar Han Rats After 4 or 13 Weeks of DE Exposure

	Incidence[a] of Lesions with Severity Score > 0[b] (*P* value)[c]		
Finding/Group	Males	Females	Both
Accumulation, macrophage (13 wk)			
Control	0/10 (0.012)	0/10 (0.012)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	3/10 (0.105)	3/10 (0.105)	6/20 (0.010)
Fibrosis, interstitial (13 wk)			
Control	0/10 (0.002)	0/10 (0.058)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	4/10 (0.043)	2/10 (0.237)	6/20 (0.010)
Histiocytosis, alveolar, focal (13 wk)			
Control	2/10 (0.384)	2/10 (0.131)	4/20 (0.137)
Low	4/10 (0.314)	0/10 (0.237)	4/20 (0.653)
Mid	4/10 (0.314)	5/10 (0.175)	9/20 (0.088)
High	3/10 (0.500)	3/10 (0.500)	6/20 (0.358)
Hyperplasia, epithelium, periacinar (13 wk)			
Control	0/10 (<0.001)	0/10 (<0.001)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	10/10 (<0.001)	9/10 (<0.001)	19/20 (<0.001)
Infiltrate, mixed cell (13 wk)			
Control	4/10 (0.500N)	5/10 (0.200)	9/20 (0.310)
Low	3/10 (0.500)	4/10 (0.500)	7/20 (0.374)
Mid	5/10 (0.500)	8/10 (0.175)	13/20 (0.170)
High	3/10 (0.500)	6/10 (0.500)	9/20 (0.624)
Histiocytosis, alveolar, focal (4 wk)			
Control	3/10 (0.332)	4/10 (0.117N)	7/20 (0.338N)
Low	3/10 (0.686)	5/10 (0.500)	8/20 (0.500)
Mid	4/10 (0.500)	2/10 (0.314)	6/20 (0.500)
High	4/10 (0.500)	2/10 (0.314)	6/20 (0.500)
Infiltrate, mixed cell (4 wk)			
Control	5/10 (0.254N)	1/10 (0.308)	6/20 (0.445N)
Low	0/10 (0.016)	0/10 (0.500)	0/20 (0.010)
Mid	1/10 (0.070)	0/10 (0.500)	1/20 (0.046)
High	3/10 (0.325)	2/10 (0.500)	5/20 (0.500)
Trachea inflammation (13 wk)			
Control	1/10 (0.628)	0/10 (1.000)	1/10 (0.628)
Low	0/10 (0.500)	0/10 (1.000)	0/10 (0.500)
Mid	0/10 (0.500)	0/10 (1.000)	0/10 (0.500)
High	1/10 (0.763)	0/10 (1.000)	1/10 (0.763)

[a] Incidences reported where there was more than one lesion-bearing animal across genders.

[b] Lesion-bearing animals/total number of animals necropsied. Unless otherwise indicated, reported incidence reflects number of animals with a severity score of 1.

[c] *P* values for treated groups reflect Fisher two-sided exact tests against control incidence. *P* value listed for control group is for Cochran-Armitage one-sided trend test; N denotes a decreasing trend across exposures.

Table 8. Incidence of Histologic Findings in Noses of Male and Female Wistar Han Rats After 4 or 13 Weeks of DE Exposure

	Incidence[a] of Lesions with Severity Score > 0[b] (*P* Value)[c]		
Finding / Group	Males	Females	Both
Turbinate 1			
Metaplasia, squamous, lateral (13 wk)			
Control	1/10 (0.340)[d]	0/10 (0.308)	1/20 (0.207)[d]
Low	1/10 (0.763)	1/10 (0.500)	2/20 (0.500)
Mid	1/10 (0.763)	1/10 (0.500)	2/20 (0.500)
High	2/10 (0.500)[d]	1/10 (0.500)	3/20 (0.302)[d]
Metaplasia, squamous, lateral (4 wk)			
Control	0/10 (0.004)	2/10 (0.340)	2/20 (0.011)
Low	0/10 (1.000)	0/10 (0.237)	0/20 (0.244)
Mid	1/10 (0.500)	0/10 (0.237)	1/20 (0.500)
High	4/10 (0.043)[d]	3/10 (0.500)	7/20 (0.064)[d]
Turbinate 2			
Degeneration, olfactory epithelial (4 wk)			
Control	0/10 (0.250)	0/10 (0.372)	0/20 (0.153)
Low	0/10 (1.000)	1/10 (0.500)	1/20 (0.500)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	1/10 (0.500)[d]	1/10 (0.500)[d]	2/20 (0.244)[e]
Metaplasia, squamous, olfactory (4 wk)			
Control	0/10 (0.500)	0/10 (0.500)	0/20 (0.627)
Low	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
Mid	0/10 (1.000)	1/10 (0.500)	1/20 (0.500)
High	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Turbinate 3			
Degeneration, olfactory epithelial (4 wk)			
Control	0/10 (0.250)	0/10 (0.500)	0/20 (0.187)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	1/10 (0.500)	1/20 (0.500)
High	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
Metaplasia, squamous, olfactory (4 wk)			
Control	0/10 (1.000)	1/10 (0.372N)	1/10 (0.372N)
Low	0/10 (1.000)	0/10 (0.500)	0/10 (0.500)
Mid	0/10 (1.000)	1/10 (0.763)[d]	1/10 (0.763)[d]
High	0/10 (1.000)	0/10 (0.500)	0/10 (0.500)

[a] Incidences reported where there was more than one lesion-bearing animal across genders.

[b] Lesion-bearing animals/total number of animals necropsied. Unless otherwise indicated, reported incidence reflects number of animals with a severity score of 1.

[c] *P* values for treated groups reflect Fisher two-sided exact tests against control incidence. *P* value listed for control group is for Cochran-Armitage one-sided trend test; N denotes a decreasing trend across exposures.

[d] One animal had a severity score of 2.

[e] Both animals had severity score of 2.

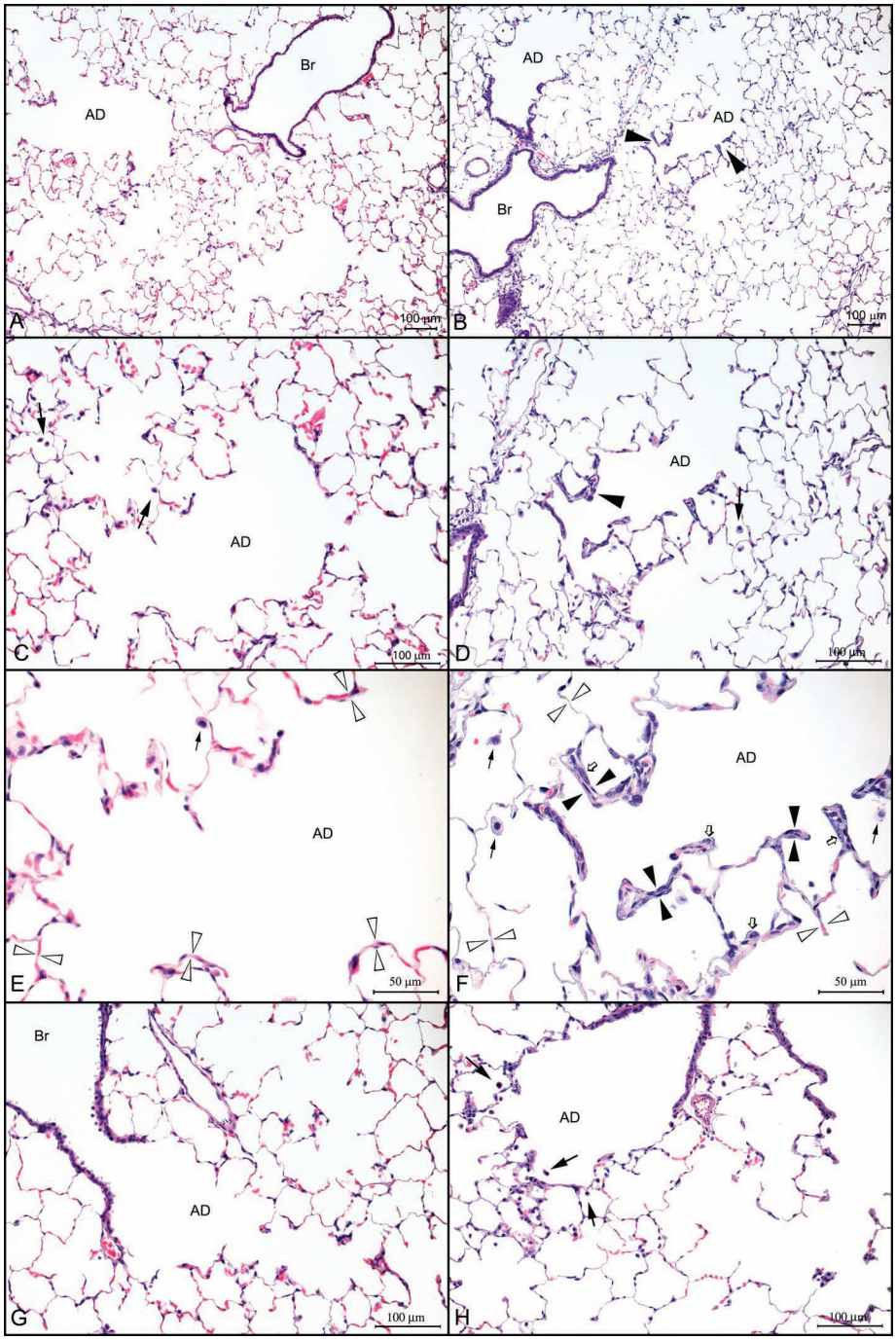

Figure 7. Photomicrographs of lungs from male or female Wistar Han rats after 13 weeks of exposure to either clean air (control) or the highest concentration (high) of 2007-compliant DE. These photomicrographs illustrate typical findings, where the lungs in the control group were unremarkable, and the high group male and female lungs showed periacinar epithelial hyperplasia: **(A)** *Control male*: relatively low magnification of unremarkable lung; **(B)** *High group male*: relatively low magnification demonstrating periacinar epithelial hyperplasia. Note apparent thickening of alveolar duct (AD) septae (arrowheads) resulting primarily from increased numbers and prominence of basophilic epithelial cells; **(C)** *Control male*: medium magnification of same unremarkable lung as in panel A; **(D)** *High group male*: medium magnification of same lung in panel B. Epithelial cell changes (hyperplasia, basophilia) are apparent (arrowhead), and there may be a slight accumulation of alveolar macrophages (arrow) within affected areas; **(E)** *Control male*: high magnification of same unremarkable lung as in panel C. Note unremarkable alveolar septae (between open arrowheads) and rare alveolar macrophages (arrows); **(F)** *High group male*: high magnification of same lung in panel D to illustrate specific changes. Note thickened alveolar septae (arrowheads) lining much of alveolar duct, with unremarkable alveolar septae (open arrowheads) in surrounding areas. Prominent, basophilic epithelial cells (short open arrows) are primary contributors to the septal thickening. Alveolar macrophages (arrows) may accumulate to a minor degree within affected areas; **(G)** *Control female*: unremarkable lung at medium magnification (as panel C); **(H)** *High group female*: lung at medium magnification demonstrating changes similar to high group male (see panel D). (A and B: original magnification ×100; C and D: original magnification ×200; E and F: original magnification ×400; and G and H: original magnification ×200).

Table 9. Statistically Significant Findings in C57BL6/N Mice After 4 and 13 Weeks of Exposure to a Clean Air Control or DE

Endpoint / Time Point / Group	Males		Females		Both	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Albumin (mg/ml)						
13 Weeks						
Control	10	177 ± 14	8	134 ± 16	18	156 ± 11
Low	7	175 ± 12	10	154 ± 10	17	164 ± 8
Mid	10	168 ± 12	9	161 ± 9	19	165 ± 7
High	9	295 ± 50[a]	9	142 ± 10	18	218 ± 26
Trend[c]		0.008 (+)		0.317		0.022 (+)
Cytokine IL-6 lung (ng/mg protein)						
4 Weeks						
Control	10	3.4 ± 0.6	10	3.3 ± 0.6	20	3.3 ± 0.4
Low	10	3.8 ± 1.1	9	4.8 ± 1.1	19	4.3 ± 0.8
Mid	10	2.6 ± 0.5	10	3.1 ± 0.4	20	2.9 ± 0.3
High	10	11.5 ± 3.8[a]	10	5.5 ± 1.3	20	8.5 ± 2.0[b]
Trend[c]		0.035 (+)		0.093 (+)		0.007 (+)
13 Weeks						
Control	10	43.0 ± 13.1	10	9.8 ± 0.7	20	26.4 ± 6.6
Low	10	36.7 ± 3.2	10	11.5 ± 1.6	20	24.1 ± 1.8
Mid	10	36.1 ± 6.3	10	8.6 ± 0.8	20	22.4 ± 3.2
High	10	45.4 ± 15.9	10	18.6 ± 6.2	20	32.0 ± 8.5
Trend[c]		0.913		0.090 (+)		0.261

(*Table continues on next page*)

[a] Significance versus control; $P < 0.05$.
[b] Significance versus control; $P < 0.01$.
[c] Trend directions reported for $P < 0.1$.

In addition to the findings in the lung, there was a small change in the turbinate areas in the noses of male rats that may or may not have been related to exposure. Minimal squamous metaplasia was seen in the transitional epithelium of the lateral wall of the most anterior nose section. Some incidence in control animals suggests that the lesions may also occur in the absence of exposure. This type of lesion is often observed as spontaneous.

BIOLOGIC RESPONSE IN MICE

There were no significant exposure-related differences in mortality, clinical signs, or histopathology in the mice (see Appendix D for a summary of the results). Biologic responses showing statistically significant differences are summarized in Table 9.

The lung-to-body weight ratio in females exposed to high levels of DE after 1 month was significantly increased compared with controls (Table D.3). The significantly decreased body weight of these mice contributed to the apparent ratio increase, but the absolute lung weights also showed a trend to increase with exposure. Neither the higher lung-to-body weight ratio nor the reduced body weight was apparent at 3 months (Table D.4). There was no exposure-related difference in organ weights at 1 month. The lung-to-body-weight ratio in males exposed to high levels of DE was significantly increased compared with controls, and showed a trend to increase with exposure. Absolute heart weights were significantly decreased in females at the high-exposure level and in males at all DE exposure levels. Heart-to-brain weight ratios were significantly

Table 9 (*Continued*). Statistically Significant Findings in C57BL6/N Mice After 4 and 13 Weeks of Exposure to a Clean Air Control or DE

Endpoint / Time Point / Group	Males		Females		Both	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Macrophages differential cell count (%)						
13 Weeks						
Control	10	99.70 ± 0.300	10	99.60 ± 0.221	20	99.65 ± 0.186
Low	9	100.0 ± 0.000	10	99.40 ± 0.267	19	99.70 ± 0.141
Mid	10	98.90 ± 0.547	10	99.50 ± 0.269	20	99.20 ± 0.305
High	10	99.10 ± 0.526	10	98.80 ± 0.512	20	98.95 ± 0.367
Trend[c]		0.107		0.158		0.031 (−)
LDH (pg/ml)						
13 Weeks						
Control	10	375 ± 48	10	346 ± 43	20	361 ± 32
Low	10	177 ± 50	10	275 ± 69	20	226 ± 43
Mid	10	505 ± 116	10	494 ± 65	20	499 ± 67
High	10	440 ± 51	10	432 ± 51	20	436 ± 36
Trend[c]		0.114		0.074 (+)		0.017 (+)
PMN absolute count (%)						
13 Weeks						
Control	10	0.004 ± 0.004	10	0.000 ± 0.000	20	0.002 ± 0.002
Low	9	0.000 ± 0.000	10	0.001 ± 0.001	19	0.000 ± 0.000
Mid	10	0.002 ± 0.002	10	0.003 ± 0.003	20	0.002 ± 0.002
High	10	0.005 ± 0.003	10	0.014 ± 0.008	20	0.009 ± 0.004
Trend[c]		0.844		0.051 (+)		0.015 (+)
PMN differential cell count						
13 Weeks						
Control	10	0.200 ± 0.200	10	0.000 ± 0.000	20	0.100 ± 0.100
Low	9	0.000 ± 0.000	10	0.100 ± 0.100	19	0.050 ± 0.053
Mid	10	0.300 ± 0.300	10	0.200 ± 0.200	20	0.250 ± 0.180
High	10	0.500 ± 0.307	10	0.900 ± 0.458	20	0.700 ± 0.276[a]
Trend[c]		0.268		0.034 (+)		0.004 (+)

[a] Significance versus control; $P < 0.05$.
[b] Significance versus control; $P < 0.01$.
[c] Trend directions reported for $P < 0.1$.

decreased in males at the low and high-exposure groups. Group differences in other organ weights were generally unremarkable; other differences achieving statistical significance but considered unlikely to be clinically meaningful are noted in Table 9.

Scattered gross lesions were observed at necropsy at both 1 and 3 months. These findings, which were typically lesions on internal organs or dermal abrasions, were not considered exposure-related because the pattern was distributed across treatment groups. The primary gross finding was alopecia without grossly evident dermatitis (consistent with barbering, common in this mouse strain). Common "background" lesions in mice of preputial gland enlargement (typical of preputial adenitis) and uterine dilatation (typical of mucometra) also occurred. All gross findings were subjectively examined for evidence of an exposure–response pattern of occurrence, which could dictate further investigation (i.e., a histologic exam).

Preputial gland enlargement in males was somewhat more prominent in exposed groups than in controls, but was not progressive with exposure level (control = 0, low = 3, medium = 2, high = 2). Because this is a relatively common background lesion in male mice, it was not examined histologically or statistically.

There were no significant group differences in hematology, serum chemistry or coagulation indicators. However, there were several statistically significant increases in clinical pathology and biochemical toxicity indicators. At 3 months, lavage neutrophils were significantly increased in the high-level females and in the combined sexes, with a strong exposure–response trend. These mice also showed increases in IL-6, a pro-inflammatory cytokine, but in this case the effect was observed only at 1 month. At that time, there was a statistically significant increase in the high level males and combined sexes, and a weak trend (P = 0.09) with exposure in the females. The weak trend persisted in the females at 3 months, but not in males. Male mice at 1 month but not females at 1 month or either sex at 3 months had increased total glutathione in lung tissue at the highest exposure level (Table D.7). No effects on glutathione were observed in the lavage fluid (Table D.6).

Histologic analysis of mouse lungs revealed only minor, scattered findings that consisted primarily of minimal perivascular mixed inflammatory infiltrates. These findings were routinely minimal in severity and were indistinguishable at the light microscopic level in character and severity between control and exposed animals at either 1 or 3 months.

SUMMARY OF BIOLOGIC FINDINGS

Over 100 biologic response variables were assessed. Most indicators demonstrated no statistically significant differences from control as a result of exposure. Rats showed statistically significant responses in most of the indicators that correlate with oxidative stress in the lung, and also had increases in lung protein and cytokines that correlate with a mild inflammatory response. The mice showed fewer responses than the rats; specifically, the mice showed increases in neutrophil inflammation (PMN) and an increase in the cytokine IL-6. There were mild histopathologic changes in the noses and lungs of rats, but not in mice. The lesions were mild in severity, and primarily characterized by an increased number and prominence of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli in close proximity to the terminal bronchial and alveolar ducts. These were not accompanied by an increase in mucous cell metaplasia in this high-exposure group. Rats also showed slight but statistically significant changes in pulmonary function, which included lower values for DL_{CO}, FVC, and MMEF.

DISCUSSION AND CONCLUSIONS

This study is the first to evaluate potential biologic responses to inhaled DE generated from a 2007-compliant system. The study was designed with three exposure levels and a clean air control, and at the time points reported here, responses were evaluated in both mice and rats. The study was intentionally designed to potentially cause statistically significant exposure-related effects at the most extreme (highest) exposure level, and to potentially demonstrate no statistically significant effects at one or both of the two lower exposure levels. It was anticipated that any biologic effects may be related to the concentration of NO_2 in the chambers, which was thus used as the target component for setting and controlling the DE dilutions. The highest exposure level of NO_2 (4.2 ppm target) was selected to approximate the weekly time-integrated exposure to NO_2 in a previous HEI-funded chronic inhalation study of rats exposed to NO_2 alone (Mauderly et al. 1990). That study evaluated some biologic endpoints similar to those used in the present study, but used a different strain of rats and evaluated responses only at later time points. Although NO_2 was the primary dilution indicator in the present study, it is important to acknowledge that the exposure material was a complex mixture containing many components. While some reflections are made later in this discussion on the plausibility that NO_2 caused the effects observed in the present study, it is premature to assume that NO_2 was partly or indeed solely responsible for the observed responses. The present study design does not allow discrimination among the contributions of the different exposure components to the observed effects.

None of the observed biologic responses led to clinically observable morbidity among either the mice or rats. We acknowledge that the large number of responses measured in this study combined with a criterion for statistical significance of $P \geq 0.05$ could result in a small number of statistically significant differences from control attributable to chance. Statistically significant differences that might be suspected to have occurred by chance were identified by a lack of evidence of an exposure–response trend, lack of corroboration by occurrence in both sexes, lack of changes in biologically related parameters, or occurrences in responses in which minimal differences were large in relation to mean values (e.g., values for rare granulocytes such as basophils may change severalfold when only a few additional cells are seen). As exposures of the remaining rats continue, some changes that are presently judged likely to have little relevance to exposure may indeed prove to have been harbingers of persistent or progressive effects, and some may not. The histopathology and pulmonary function data from the rats at 12 months are described in Appendix F.

The histologic evidence of lung changes in the rats, combined with the significant increases in several inflammatory and oxidative stress indicators and a slight change in pulmonary function, confirmed biologic response at 13 weeks. These findings were primarily observed at the highest exposure level. The mice demonstrated fewer biologic responses and did not have the lung tissue injury that was observed in the rats. The mice did show small increases in lavage neutrophils, which were statistically significant only when the sexes were combined, and in the cytokine IL-6, but this did not persist to 3 months.

In general, the statistically significant differences from control were accompanied by statistically significant trends with exposure concentration. This evidence strongly suggests that the significant differences reflected true exposure effects, rather than being chance findings. In some instances, biologic responses were not corroborated in other assessments. For example, in the assays for indicators of oxidative stress, HO-1 and TEAC levels changed in rats, but there was no corresponding change in glutathione levels, which would be expected for oxidative injury. Although the reason for this inconsistency is not known, it may be linked to a mismatch between the sensitivity of the assays and the relatively low magnitude of oxidative injury.

Another finding in rats was that hyperplasia was observed in the absence of cell proliferation. However, the observed hyperplasia was confined to a limited area within the central acinus, and the cell proliferation assay may not have been optimized to detect these subtle differences, which were observed in only a limited portion of the airways. It is plausible that the increases in pulmonary inflammation and oxidative stress were related to the hyperplasia observed in the central acinus of the deep lung, and it is possible that the reduced forced flow rates of female rats were also related to the tissue changes. Moreover, it is plausible that these tissue responses at the alveolar duct-alveolar level could have led to a slight impairment of pulmonary function, as reflected by a subtle decrease in gas exchange and forced flow rates. If these postulations prove correct, deterioration of the tissue and functional effects might be expected at later measurement times.

Overall, the pulmonary function results in rats suggested that 13 weeks of exposure may have slightly reduced alveolar-capillary gas exchange (measured as DL_{CO}), the mobile lung volume (SVC and FVC), lung compliance in the region of tidal breathing (Cqs), and maximal expiratory airflow (PEFR, MMEF, FEF_{50}, FEF_{25}, and FEF_{10}). The magnitudes of most effects were very small. Sex-specific statistically significant trends occurred only among females (n = 10/group) (PEFR, MMEF, and FEF_{25}), and additional trends (in DL_{CO}, FVC, FEF_{50}, and FEF_{10}) reached statistical significance only for combined sexes (n = 20). The increased animal number led to statistical significance because of the corresponding decrease in standard error among the groups. The justification for the approach of analyzing the pooled sexes for these endpoints is discussed further in Appendix C ("Examples of Statistical Approach").

Most of the mass emitted in DE from modern diesel technology consists of CO_2 and H_2O. The remaining mass consists primarily of other inorganic gases such as CO, NO, and NO_2. DPM and VOCs account for only a very small fraction of the exhaust compared with those gases. Thus, the exposure atmospheres used in this and earlier DE studies (e.g., McDonald et al. 2004a) are primarily gaseous inorganics with small amounts of PM and VOCs. The primary differences between the exposure atmosphere in the present study and those of earlier DE studies using pre–2007-compliant technologies are the concentration and composition of DPM relative to other pollutants. The other notable distinction is the higher concentration of NO_2 compared with the other gases. The aftertreatment technology used in the present study oxidizes NO to NO_2, resulting in proportions of NO_2 that account for approximately 40 to 50% of the NO_x, while previous technologies produced exhaust in which typically 10% of the NO_x was NO_2 (e.g., Cheng et al. 1984; McDonald et al. 2004a).

Both DPM and NO_2 have historically been primary targets of concern for DE-related health hazards although, certainly, other components could potentially also exert toxicity. The DPM and NO_2 concentrations at the highest level in the present study averaged 9 $\mu g/m^3$ (chamber inlet) and 4.3 ppm (6766 $\mu g/m^3$), respectively, during the mouse exposure and 13 $\mu g/m^3$ and 3.6 ppm (5665 $\mu g/m^3$), respectively, during the rat exposure (Tables 3 and 4), or NO_2/PM mass ratios of 752:1 and 436:1, respectively. Although many experimental exposures of animal and human subjects to DE have been published, most have used exposure atmospheres that are not closely comparable to those of the present study, and especially not with respect to the NO_2/PM mass ratio. The remote relevance of exposure materials in published DE studies to contemporary diesel technology was recently reviewed (Mauderly 2010). At that time (summer of 2010), the most contemporary engine system used in a published study was 19 years old and did not include aftertreatment technology to reduce PM emissions (Barath et al. 2010). The same group subsequently used the same engine (1991 Volvo TD40 GJE 4.0L) operating on a variable-duty cycle, burning contemporary European fuel (5–7 ppm sulfur, 2–6% PAH), and equipped with or without a continuously regenerating PM trap (DPF-CRT, Johnson Matthey, Royston, U.K.) to evaluate the effects of the trap on vascular responses in human

subjects (Lucking et al. 2011). They reported that exposure to DE without the trap (PM = 320 μg/m^3, NO_2 = 0.7 ppm, NO_x = 6.4 ppm) significantly reduced the response in humans of forearm blood flow to vasodilators and significantly increased ex vivo thrombus formation as seen in the previous study, but that both effects were ameliorated and the results were not significantly different from control using the DPF-CRT trap (PM = 7 μg/m^3, NO_2 = 3.4 ppm, NO_x = 5.5 ppm). The study by Lucking and colleagues was the first to employ an NO_2/PM mass ratio (approximately 760:1) somewhat similar to that of the present study (although the present study did not evaluate either acute responses or vascular responses), and it demonstrated a marked reduction of acute biologic responses. An earlier study reported by McDonald and colleagues (2004b) had demonstrated that the use of low-sulfur fuel and a PM trap reduced lung inflammation, oxidant stress, and susceptibility to lung viral infection in DE-exposed mice, compared with mice exposed to DE using then-contemporary higher-sulfur fuel and no PM trap. However, although the trap reduced PM mass from 236 to 7 μg/m^3, a concentration similar to that in the present study, the NO_2 concentrations were not reported.

It is challenging to compare the present results from rats and mice after 3 months of exposure to results from historical DE studies, because of differences in experimental design as well as differences in exposure material. Most of the historical DE rodent studies were focused on long-term carcinogenicity and did not evaluate responses as early as after 3 months of exposure, although some began interim necropsies at 6 months. Perhaps the study closest in design to the present study was the study funded by HEI and the Department of Energy of rats exposed for 24 months to compare the carcinogenicity of DE and carbon black at similar PM concentrations (Mauderly et al. 1994; Nikula et al. 1995). In that study, F344 rats were exposed 16 hr/dy, 5 dy/wk to exhaust from 1988 GM LH6 6.2L engines burning then-contemporary D-2 certification fuel (300+ ppm sulfur), operating on a variable-duty cycle without exhaust aftertreatment. As was typical of historical DE, NO_2 constituted 10 to 20% of NO_x. The low and high-exposure levels contained DPM (PM in exposure chambers minus PM in control chambers) at 2390 and 6280 μg/m^3 and NO_2 at 0.73 and 3.78 ppm, for NO_2/DPM mass ratios of 0.48:1 and 0.95:1, respectively — strikingly different from the ratios in the present study. Lung tumors after long-term exposure were the focus of the 1994 study, but limited observations were made on rats euthanized after 3, 6, 12, and 18 months of exposure. At 3 months, body weight was reduced, and lung weight was increased relative to controls in an exposure-related manner, but the effects were not statistically significant. Mild alveolar macrophage and alveolar epithelial hyperplasia, with incidence and severity related to exposure, was observed at 3 months. BAL was performed at later intervals, but not at 3 months. Continued exposure resulted in significant, exposure-related accumulation of DPM in the lungs, accompanied by chronic active lung inflammation and progressive lung pathology, including tumorigenesis. Although some effects observed at 3 months in the earlier and present studies were similar, a striking difference was the observation of much greater accumulation of DPM in macrophages in the earlier study.

A preliminary study of F344 rats and CD-1 mice exposed repeatedly to DE for up to 12 weeks (Mauderly et al. 1980) was conducted at LRRI before initiation of the chronic carcinogenicity bioassays of rats and mice reported later (Mauderly et al. 1987b; 1996). In the preliminary study, animals were exposed beginning at age 12 weeks for 7 hr/dy, 5 dy/wk to diluted exhaust from 1980 5.7L GM engines operating on a variable-duty cycle and burning then-contemporary D-2 certification fuel (300+ ppm sulfur) without aftertreatment technology, or to clean air as control. Three dilutions contained DPM, at 4675, 950, and 240 μg/m^3. At the highest exposure level, the NO_2 concentration was reported as 0.05 ppm, which yielded an NO_2/PM mass ratio of only 0.17:1. NO_2 data were not reported for the lower levels. Animals were euthanized at times ranging from 1 to 12 weeks for evaluations of respiratory histopathology, BALF, tracheal mucociliary clearance of radiolabeled albumin, clearance of inhaled *Pseudomonas*, antibody-forming cells in lung-associated lymph nodes, and pulmonary function. At 12 weeks, neither body nor lung weight was increased. There was an exposure-related accumulation of DPM-laden macrophages in alveoli, extending into the interstitium and lung-associated lymph nodes at the highest exposure level. Alveolar epithelium was relatively unaffected. There was an exposure-related increase in macrophages and glutathione reductase in BALF (most BALF variables in the present study were not measured in this preliminary study). Tracheal clearance of radiolabeled albumin and lung clearance of *Pseudomonas* were slightly slowed. There were no statistically significant differences in pulmonary function, which included the variables measured in the present study. Thus, the most striking effects after 12 weeks of exposure were an accumulation of DPM in the lung and lymph nodes, and a subtle slowing of particle clearance from the lung and trachea.

To the extent that the evaluations of responses were comparable after 13 (in the present study) and 12 weeks (in the preliminary study) of exposure, the present results differ from those of the earlier study in demonstrating less

accumulation of DPM, greater alveolar epithelial hyperplasia, and some evidence of impact on pulmonary function. Of course, the total exposure time differed substantially between the studies; 1040 hours in the present study compared with 420 hours in the earlier study. In a study contemporaneous with the 1980 study by Mauderly and colleagues, Heinrich and colleagues (1986) also observed a slowing of the clearance of tracer PM from the lungs of rats after 3 months of exposure 19 hr/dy, 5 dy/wk to DE at a PM mass concentration of 4240 $\mu g/m^3$ and an NO_2 concentration of 1.5 ppm (NO_2/PM mass ratio approximately 0.6:1).

Because of the difference in experimental design, these comparisons are not particularly informative regarding either the differences in biologic effects after 3 months of exposure or the nature or magnitude of effects that might be produced by continued exposure in the present study. The results of the comparisons are consistent with the premise that chronic effects associated with the accumulation of DPM in the lung in historical studies should be less in the present study. Both neoplastic and nonneoplastic effects in the historical studies have largely been ascribed to the accumulation of large amounts of DPM, both as a cause of progressive lung hyperplasia and fibrosis and as a potential initiator and promoter of carcinogenesis. It is reasonable to speculate that the markedly lower, and nearly negligible, exposures to DPM in the present study may preclude DPM-caused effects. The potential, as shown in the results of this study, for progressive lung remodeling associated with exposure to NO_2 is less certain, but certainly plausible — especially at the highest exposure level.

Two previous HEI-funded studies of rats exposed repeatedly to NO_2 alone offer the closest comparison to the NO_2 exposure rate of the present study. In the first, Mauderly and colleagues (1987a) exposed 6-month-old male F344 rats 7 hr/dy, 5 dy/wk for 6 months to NO_2 at 9.5 ppm as part of a project to compare the effects of DE and NO_2 in adult and developing rat lungs. The weekly cumulative exposure rate in that study was 332.5 ppm · hour, which was nearly identical to the design rate of 336 ppm · hour (4.2 ppm × 80 hr/wk) of the present study and only somewhat higher than the actual rate of 288 ppm · hour (3.6 ppm × 80 hr/wk) for the first 3 months of the present rat study. After 6 months of exposure in the older study, lung weight was increased 7% compared with controls, but the increase was not statistically significant. The histological appearance of the lungs was reported to be unremarkable, although cell proliferation was not assessed and the appearance of epithelium in the centriacinar region was not specifically noted. Inflammatory cells were not increased in BALF, but other variables reflected cytotoxicity and oxidative stress. There were no significant differences in pulmonary function from control values. In summary, the structural and functional effects of 6 months of exposure of male F344 rats to NO_2 at a rate somewhat higher than in the present study may have been similar to the effects in the male Wistar rats exposed to high levels of DE in the present study. Considering the differences in rat strain, study design, and endpoints, the comparison might be interpreted to suggest that the effects in the present study could have been caused largely by NO_2.

In the second HEI-funded NO_2 rat study (Mauderly et al. 1989; 1990), normal and emphysematous young adult male F344 rats were exposed to NO_2 to determine the influence of pre-existing emphysema on NO_2 effects. Those rats were also exposed 7 hr/dy, 5 dy/wk to 9.5 ppm NO_2 (332.5 ppm · hour/wk), but the exposures continued for 24 months. There was no interim evaluation at a time comparable to the present study, but the results suggest effects that might occur after 24 months in the present study if the effects were due solely to NO_2. Body weight and mortality were unaffected, and no clinical morbidity was observed throughout the exposure. At 24 months, the lung-to-body weight ratio was increased 25% compared with controls as a result of small changes in both variables. Morphometric measures of mean linear intercept and internal surface area gave no evidence of emphysematous changes from NO_2 alone. There was mild hyperplasia of epithelium in terminal bronchioles and "bronchiolization" (a change in the normal flat epithelium, rendering it cuboidal and similar to cells lining the terminal bronchioles) of proximal alveoli, accompanied by occasional foci of inflammatory cells in the same region. Overall, the inflammatory response was minimal. Responses in BALF components reflected modest cytotoxicity and oxidative stress, and total lung collagen was increased. Clearance of inhaled radiolabeled tracer particles was not impaired. Two-way ANOVA identified statistically significant effects of both NO_2 and emphysema on pulmonary function, but the effects were modest in magnitude. Lung volumes at standard inflation pressures and lung compliance were increased, and forced expiratory flows were slightly decreased, but CO diffusing capacity was not reduced. These findings might suggest that the structural and functional changes observed at 3 months in the present study might progress only modestly through 24 months, and that the NO_2 exposure would not impair clearance of the small amount of DPM deposited in the lung.

Other studies have shown that high levels of NO_2 can cause significant biochemical and pathologic changes in a concentration range of 5 to 20 ppm (e.g., Pickrell et al. 1981; Gregory et al. 1983). Gregory and colleagues exposed

Fischer 344 rats at 1 to 5 ppm for 6 to 7 hr/dy, 5 dy/wk for up to 6 months. At the high exposure level, which is similar to the NO_2 concentration in this study, there were no detectable effects after 0.4 weeks of exposure, but after 1.7 weeks of exposure, there were significant elevations in biochemical parameters in both lavage and lung tissue. There were no changes in pathology after 1.7 or 2.7 weeks of exposure, but 15 weeks of exposure led to subpleural alveolar macrophage accumulation and airway enlargement. It is notable that these findings do not parallel the findings observed in the DE-exposed animals described here, potentially due to differences in the time points for evaluation or the rat species evaluated. In other studies that may be used for comparison, Wistar Han rats were exposed to 15 ppm NO_2 for 20 weeks (Freeman et al. 1969) or 16 ppm for 7 months (Juhos et al. 1980). However, the changes described in the previous report suggest emphysematous responses, while the current study shows hyperplasia responses that are projected to lead to bronchiolization.

When completed, this phase of the ACES program will provide an updated assessment from animal data of the potential health hazards, and particularly the carcinogenic hazard, of repeated, chronic inhalation of near-contemporary (U.S. 2007-compliant) DE. It will especially be of interest to determine the contrast between the final results and those of historical animal exposures to DE that have helped shape regulatory interpretations of hazard (U.S. EPA 2002). Because the ACES protocol did not include groups exposed to "old" emissions, the relative effects of new and old emissions must necessarily be inferred by comparison of the ACES study results to results of previous studies using similar methods. These comparisons will be further facilitated by results obtained at later time points in the present exposure study, because of the better match to evaluation times in the older studies.

The findings reported here demonstrate that lung inflammation, lung remodeling, and possible effects on pulmonary function occurred early in the chronic exposure. Most of these effects were observed only at the high-exposure level, and the results show that the rats were more sensitive than the mice. The rats were selected for the lifetime study both because they were expected to be the more sensitive species and because the previous hazard evaluations of diesel have focused on responses in the rat. It is tempting to ascribe the effects observed through 3 months of exposure to NO_2, but this cannot be confirmed by the study design, there were no previous studies that evaluated effects of NO_2 in this strain of rats at the same time points, and the NO_2 was delivered as a component of a complex mixture. Regardless of the causal component(s), it will be of great interest to determine whether, and to what extent, the biochemical, histological, and functional effects progress with continued exposure.

12-MONTH RESULTS IN RATS

Although not a focus of this report, results of the histopathology and pulmonary function assessments in rats at 12 months are provided in Appendix F. The histologic findings at 12 months showed an increase in the extent of the tissue changes in the respiratory tract, but the severity of the changes remained small. As with the 3-month findings, the lung lesions were characterized by an increased number and prominence of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli that were in close proximity to the terminal bronchial and alveolar ducts. The distribution was rather uniform and focused at the central acinus, and the animals exposed for 12 months showed the tissue changes reaching further into the central acinar region compared with the 3-month animals. Furthermore, the incidence of fibrotic tissue at 12 months was found across all animals at the high exposure level, compared with only scattered observations of this finding at 3 months. In addition to the epithelial proliferation, there was a subtle accumulation of pulmonary alveolar macrophages, coupled to slight bronchiolization in some animals. The pulmonary function changes observed at 3 months in the rats persisted only for DL_{CO} at 12 months. The changes in DL_{CO} remained small, and did not progress compared with the 3-month observations. In addition, the 12-month results, like the 3-month findings, were statistically significant only when the sexes were combined and also normalized for body weight. Overall, the lack of observed findings or progression in the pulmonary function parameters suggests that the biologic significance of these observations is small.

SUMMARY

This report provides the results of inhalation exposures of mice and rats for 4 and 13 weeks to combined tailpipe exhaust and crankcase emissions from a heavy-duty diesel engine system and fuel meeting U.S. 2007 on-road emission standards. The three exposure dilutions were based on target concentrations of NO_2 that alone might be anticipated to elicit biologic responses at the highest level. The exposure atmosphere consisted of low concentrations of PM that had minimal soot content, small amounts of VOCs and SVOCs, and high concentrations of other gases such as CO, NO, and NO_2.

After 3 months of exposure, the rats showed statistically significant, exposure-level–related responses in indices that correlate with oxidative stress in the lung, and also

had statistically significant increases in lung protein and cytokines that correlate with a mild inflammatory response. The mice showed fewer responses than the rats; the mouse responses were small increases in neutrophil inflammation and an increase in cytokines. Mild lung pathology was observed in the noses and lungs of rats, but not mice. The lesions were primarily characterized by an increased number and prominence of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli in close proximity to the terminal bronchial and alveolar ducts. These responses were not accompanied by increased mucous cell metaplasia. Rats also had mild, but statistically significant, effects on pulmonary function, including reduced forced expiratory flows and alveolar-capillary gas exchange. As described in Appendix F, the pulmonary function findings did not all persist at 12 months. Only DL_{CO} trends, which were statistically significant only when both sexes were included in the analysis and also normalized for body weight, were observed at both time points.

To date, the exposures have been accomplished without major disruption, and the exposed animals have not manifested exposure-related mortality or morbidity. The exposures of rats are continuing, and the measurements conducted at 3 and 12 months will be repeated at 24 months.

IMPLICATIONS OF FINDINGS

This is the first study to evaluate the biologic responses of mice and rats to repeated, chronic inhalation of emissions from a fuel, engine, and aftertreatment system compliant with U.S. 2007 on-road standards. The engine and aftertreatment systems have operated reliably with minimal attention to maintenance, and the exposures have been accomplished as planned. The primary importance of these early interim results is their demonstration that (1) several plausibly coherent responses indicate early, exposure-related, subclinical impacts on lung inflammation and deep lung tissue of both sexes of rats; (2) statistically significant effects were mostly limited to the highest exposure level, and no effects were observed at the lowest level; and (3) mice were less responsive than rats to 3 months of exposure. The present findings provide targets of interest with regard to their potential for progression with continued exposure. It was not expected that lung tumors would be an outcome of these subacute exposures; the potential for tumorigenesis awaits much longer-term exposure study for observation. Although the study design does not allow for identification of the causal components of the exposure atmosphere, the responses observed to date, complemented by comparisons to previous studies, suggest that they could plausibly have been driven primarily by NO_2. That this might be true is of interest because NO_2 emissions are now considerably lower under the current U.S. 2010 on-road emission standards.

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APPENDIX A. Pathology Evaluation and Peer Review for ACES Phase 3B

PROCEDURE AND SUMMARY OF FINDINGS

Described here are the steps taken to assure that the histopathology in Phase 3B of the Advanced Collaborative Emissions Study (ACES) is of the highest quality.

The principal pathologists responsible for evaluating the histopathology were Dr. Rodney Miller of Experimental Pathology Laboratories (EPL), Research Triangle Park, N.C., for the rat exposures, and Dr. Andrew Gigliotti of the Lovelace Respiratory Research Institute (LRRI), Albuquerque, N.M., for the mouse exposures. The tissue slides from rats were delivered to EPL from LRRI for histopathologic evaluation by Dr. Miller. The tissue slides from mice for histopathologic evaluation were read at LRRI by Dr. Gigliotti, and the slides were subsequently sent to EPL for peer review, as described below. This appendix describes the pathology evaluation and review of the 1- and 3-month exposures in rats and mice and the 12-month exposures in rats.

Initial Evaluation by the Principal Pathologist

The tissue evaluation process and sequence are consistent with that used by contractors to the National Toxicology Program (NTP) for light microscopic tissue evaluation of interim sacrifices. Following this well-established and accepted procedure in rodent toxicology studies (Morton et al. 2010), both rat and mouse slides were evaluated starting with the control animals (to familiarize the evaluator with background changes) of one sex and then followed by that sex's high-exposure, mid-exposure, and low-exposure groups. The other sex was then evaluated in the same group sequence.

The data (diagnoses and the severity grades of lesions) for the 1- and 3-month rat necropsies were recorded on a data sheet for each individual rat and subsequently entered into Provantis software. A list of organs and tissues that were evaluated is provided in the protocol (Appendix H, available on the HEI Web site at *www.healtheffects.org*). Diagnoses were graded on a relative scale of 1 to 4 (where *minimal* [1] was considered difficult to observe, *mild* [2] quite easy to find, *moderate* [3] quite extensive, and *marked* [4] involving most of an organ). Histopathologic findings from the 1- and 3-month mouse necropsies were directly entered into the Provantis system at the time of examination.

Verification of Findings by the Study Pathology Team

Verification of the pathologic findings occurred in the following ways. After the initial reading of the 1-month rat necropsies, in which no lesions related to exposure were observed, the slides were blinded by Dr. Miller. He re-examined the slides and verified that the rats in the high-exposure group could not be separated from the rats in the control group. After the initial reading of the 3-month rat necropsies, Dr. Miller mixed all lung slides from rats in the control and high-exposure groups and read them blinded. He was able to separate control animals from animals in the high-exposure group without knowledge of the treatment, thus verifying that there were treatment-related changes in the centriacinar areas of the lung. Lung slides from the low- and mid-exposure groups could not be distinguished from the control group when examined blindly.

Additionally, a set of blinded lung slides from rats in the control and high-exposure groups (3-month necropsy)

were given to another EPL pathologist, and that pathologist could also successfully separate control and exposed lungs. Dr. Gigliotti (LRRI) reviewed some rat lung slides and concurred with the treatment-related changes seen in the high-exposure group from the 3-month necropsy.

Verification of Findings by Independent Peer Reviewers

Finally, exposure-related effects observed microscopically in the lungs of rats (both sexes) in the high-exposure group from the 3-month necropsy were reviewed blindly by two peer-review pathologists, Dr. Ernest E. McConnell (ToxPath, Inc.), a member of the HEI ACES Oversight Committee, and Dr. Ronald Herbert (NTP, National Institute for Environmental Health Sciences, Research Triangle Park, N.C.) during a visit to EPL in March 2011, and the lesions were again verified (see below). They confirmed that there were no treatment-related lesions in the lungs of the 1-month necropsied rats. They also examined the mouse lung slides from the 1-month and 3-month necropsies in a blind fashion and could not identify a treatment-related effect. This independent peer review thus verified the findings of Dr. Gigliotti: that there were no treatment-related lesions in mice. Drs. McConnell, Gigliotti, and Miller again reviewed the mouse lung slides at LRRI in May 2011 and again agreed that no lesions related to exposure could be observed in the lungs of mice in the 3-month high-exposure group. More details are provided in the section "March 2011 Independent Peer Review."

In December 2011, a similar review by Drs. McConnell and Herbert was conducted of lung and nasal turbinate slides of rats exposed for 12 months. They confirmed that both male and female rats in the high-exposure group showed exposure-related effects. The lesions in the lung were for the most part still of minimal severity and had not progressed significantly from the lung lesions observed after 3 months of exposure. More details are provided in the section "December 2011 Independent Peer Review."

March 2011 Independent Peer Review The following are the process and results of a pathology peer review of the ACES Phase 3B study conducted by Drs. McConnell and Herbert. Dr. Miller was present to answer questions. Dr. Annemoon van Erp (from HEI, the sponsor of the study) observed the review and answered questions about the study design and conduct. The peer review was conducted at EPL, Research Triangle Park, N.C., on March 14, 2011.

Objective The objective of the peer review of this diesel exhaust study was to provide an independent opinion of the histopathologic findings reported by Dr. Miller (EPL), the study pathologist responsible for evaluating pathology in exposed rats and Dr. Gigliotti (LRRI), the study pathologist responsible for reviewing pathology in exposed mice. The animals for review were selected by Dr. McConnell in conjunction with Dr. Miller before the review.

Rationale for Slide Selection The slides for review were selected based on the reported findings of the study pathologists. Briefly, the only treatment-related lesions reported were in the lungs of rats in the high-exposure group, especially in the males. The reported lesions (all of minimal severity) of most interest consisted of alveolar epithelial hyperplasia of the proximal alveolar duct, macrophage infiltration of the alveolar space, and interstitial fibrosis.

Approach to Slide Review Based on the reported findings, it was decided to initially examine the lung slides from all (10) male rats in the high-exposure group. Because of the lesions observed in these rats, it was decided to examine the lungs from all 10 male rats from the mid-exposure group. Subsequently, five randomly-selected lung slides were examined from females in the high-exposure group. The reason for examining fewer females was because there were few differences of opinion between the original pathologist and the peer-review pathologists in the review of the males in the high-exposure group. It was apparent after reviewing the males that he was reporting the lesions accurately. The lungs from three randomly selected males and two females in the control group were first examined as a reference point. Two rats that had died prematurely were also examined; both were males in the high-exposure group. The death of these rats was not related to the exposure.

While no treatment-related lesions were reported in the mice, it was decided to examine the lungs from five randomly selected male and female mice from the high-exposure group based on the findings in the rats and the route of exposure (inhalation) to verify the lack of lesions. Again, the lungs from three male and female mice in the control group were first examined and used as a reference point.

All slides were examined without knowledge of the diagnoses of the study pathologists. After Drs. McConnell and Herbert completed the examination of a given slide, their findings were compared with the reported findings of the study pathologists, Drs. Miller and Gigliotti.

Results and Conclusions The reviewing pathologists were in almost universal agreement with the diagnoses of lung lesions made by the study's designated pathologists. Therefore, the reviewing pathologists concluded that it is appropriate to use the study pathologists' findings in the Investigators' Report tables to interpret the study to date (1- and 3-month exposures).

December 2011 Independent Peer Review The following are the process and results of a pathology peer review of the ACES Phase 3B study conducted by Drs. McConnell and Herbert. Dr. Miller was present to answer questions. Dr. van Erp observed the review and answered questions about the study design and conduct. The peer review was conducted at the NTP, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, using a multiheaded microscope on December 12, 2011.

Objective The objective of the peer review of this diesel exhaust study was to provide an independent opinion of the histopathologic findings reported by Dr. Miller, the study pathologist responsible for evaluating pathology in exposed rats. The animals for review were selected by Dr. McConnell in conjunction with Dr. Miller before the review.

Rationale for Slide Selection The slides for review were selected based on the reported findings of the study pathologist. Briefly, the only treatment-related lesions reported were in the lung, turbinates, and larynx of rats in the high-exposure group. The reported lesions (mainly of minimal severity) of most interest were the pulmonary lesions, consisting of alveolar epithelial hyperplasia of the proximal alveolar duct, macrophage infiltration of the alveolar space, bronchiolization, and interstitial fibrosis.

Approach to Slide Review Based on the reported findings, it was decided to initially examine the lung slides from two male and two female control rats to establish "background" changes that are found in a 14-month-old rat. Next, two male rats in the high-exposure group were examined to identify the types and severity of lesions observed and reported by the study pathologist. This was followed by a blind examination (slides selected by Dr. van Erp) of the other eight males in both the control and high-exposure groups to see if the exposed and untreated animals could be differentiated from each other. After this, five male rats from the mid-exposure group were examined to evaluate whether an exposure–response relation could be observed. No lesions were observed in this group. Subsequently, five randomly selected lung slides were examined from females in the high and mid-exposure groups. The reason for examining fewer females was because there were few differences of opinion between the original pathologist and the peer-review pathologists in the review of the males in the high-exposure group.

Finally, several sections of larynx and turbinates from both males and females in the high-exposure group were reviewed to confirm the presence of various types of lesions in these tissues. Again, as in the lung, the lesions in these tissues were of minimal to mild severity.

Results and Conclusions The reviewing pathologists were in almost universal agreement with the diagnoses in the lung, turbinates, and larynx made by the study pathologist, Dr. Miller. Therefore, the reviewing pathologists concluded that it is appropriate to use the study pathologist's findings in the Investigators' Report tables to interpret the study to date (12-month exposures).

Overall, the lesions in the lung were for the most part still of minimal severity and had not progressed significantly from the observations after 3 months of exposure, albeit there was evidence of metaplastic change of the alveolar epithelium at the level of the proximal alveolar duct/alveoli (termed "centriacinar"). This change is often referred to as "bronchiolization" because the normal flat epithelium has changed to a ciliated cuboidal shape, similar to cells lining the terminal bronchioles. There was not a great deal of difference in severity between males and females.

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APPENDIX B. ACES Engine and Exposure Facility

GENERATION OF TEST ATMOSPHERES

Fuel Identity, Source, and Storage

Diesel fuel meeting current on-road specifications was delivered to LRRI by tanker truck from a local commercial source (Chevron No. 2, ultra-low sulfur diesel [legal for on-road use], Ever-Ready Oil Co., Albuquerque, NM) and stored in an above-ground storage tank. Fuel was transferred on-site directly into the 5000-gallon 8 × 14 ft Fireguard storage tank (SN 19911; Brown-Minneapolis Tank Co., Albuquerque, NM).

Fuel Handling

Fuel from the storage tank was delivered to an intermediate day-tank in the test cell. This process occurs automatically when the Dyne Systems (Jackson, WI) programmable logic controller (PLC) receives a fill signal from the fuel level sensor (Flowline, Los Alamitos, CA). The

pump was filled once a full level signal was received. The temperature of fuel in the day tank was maintained at 70°F by an electric heater within the tank, which was gravity fed through a fuel-conditioning delivery system to the engine. The excess fuel was returned from the engine to the fuel-conditioning delivery system, where it was filtered and cooled by a heat exchanger to a temperature of 104°F before being returned to the engine. Engine fuel filters were changed on a schedule set by the engine manufacturer.

Engine Operation

Schematics of the engine test cell and laboratory are provided in Figures B.1 and B.2. The engine was mated to a Dyne Sytems, 550-hp, 660-amp, alternating-current dynamometer and controlled remotely using a Dyne Systems Inter-Loc V Digital Multi-Loop Controller interfaced to a personal computer running Dyne Systems Cell Assistant for Windows software. The 16-hour ACES test cycle was controlled by the Cell Assistant software. Each daily exposure began with a room-temperature "cold" start.

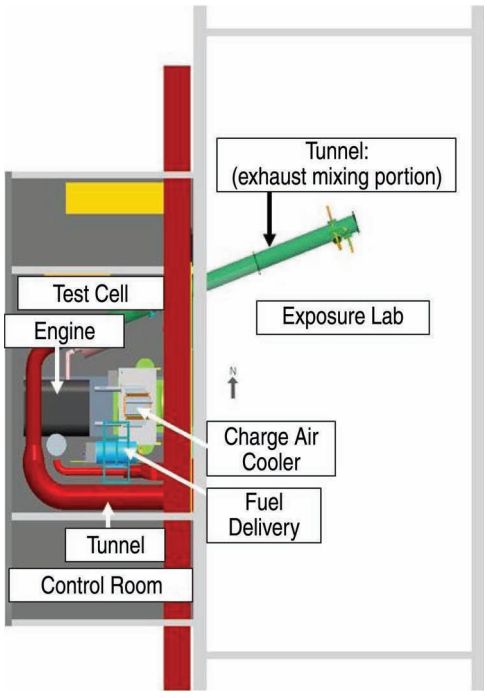

Figure B.1. Overhead view of the engine laboratory and dilution tunnel. The tunnel portions shown in red are upstream from the engine exhaust mixing. The exhaust mixing portion is green, and the extraction point for the exposure chamber distribution system is at the end of the dilution tunnel (the remainder is routed to a waste stack). The charge air cooler is shown in grey, the engine in black, the fuel delivery system in blue, and the dynamometer in light green.

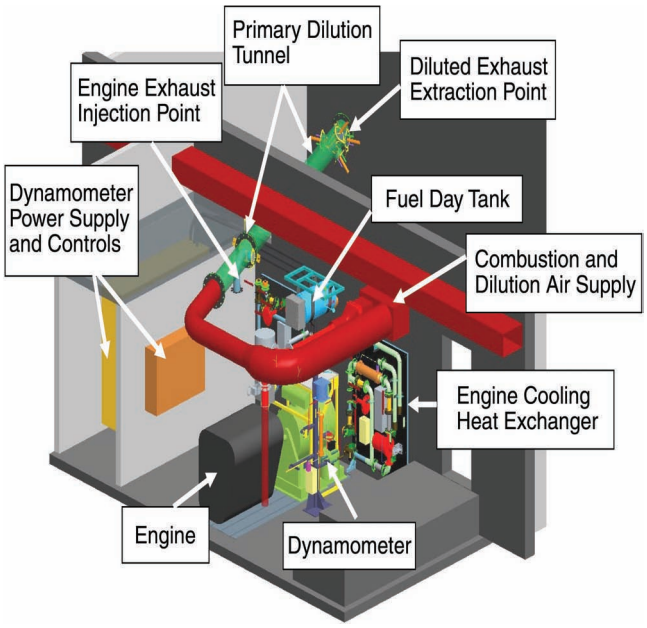

Figure B.2. Front quarter view of engine laboratory and dilution tunnel. The tunnel portions shown in red are upstream of the engine exhaust mixing. The exhaust mixing section and the extraction point for the exposure chamber distribution system are shown in green, the engine in black, the fuel delivery system in blue, and the dynamometer in light green.

Aftertreatment regeneration events were allowed to occur as controlled by the engine's electronic control unit. Engine cooling (jacket water temperature) was controlled by an ethylene glycol closed-loop heat exchanger that is supplied by the chilled water supply. The jacket water cooling system was maintained at a set point of 190°F using feedback from the Dyne Systems PLC.

The engine intake air (charge air) supply was conditioned to a set point of 77°F, filtered and delivered to the turbo charger. The charge air was then compressed by the turbo charger and cooled as it passed through the intercooler. The intercooler was cooled through a closed-loop heat exchanger to maintain 77°F using feedback from the Dyne Systems PLC. Lubricating oil was changed on a schedule recommended by the engine manufacturer. The crankcase lubricating oil was a proprietary blend approved by HEI and the Coordinating Research Council (CRC) and provided by Lubrizol. Aliquots of used oil were obtained at each oil change for potential analysis. The oil filter (Power Guard, serial number 23530573) was changed with the change in lubrication oil.

Dilution System

Exhaust was passed through a stock aftertreatment system before injection into a 35.6 cm internal diameter

dilution tunnel. The crankcase ventilation effluent rejoined the exhaust stream downstream of the particle trap. The exhaust was diluted with filtered air under turbulent conditions at the point of injection. The dilution tunnel supply-air flow was approximately 3000 cubic feet per minute. The primary dilution tunnel was a constant–pressure tunnel, rather than constant-volume. When exhaust flow increased, the increased pressure caused the dilution air to bypass the dilution tunnel into the test cell. At a distance of 5.5 m from the injection point (in the exposure room), a portion of the diluted exhaust was drawn through an in-line extraction probe. The exhaust mixture was withdrawn from the primary dilution plenum through individual probes and transit lines for each exposure chamber (Figures B.3 and B.4). The transit time from exhaust to exposure chamber was less than 5 seconds. Once in the exposure chamber, the residence time was approximately 4 minutes. Each exposure chamber had its own extraction probe and dilution system. Subsequent to this extraction, the exhaust was diluted by filtered, compressed air provided through a rotary dilution/dilution bypass system. Diluting flows were adjusted as needed to reach the final dilution and concentration targets. All dilution and transit lines were constructed of stainless steel, and were of near equal lengths for each of the exposure levels. Exhaust was diluted to achieve target exposure concentrations of 4.2, 0.8 and 0.1 ppm NO_2. This required dilution ratios of 25:1, 115:1, and 840:1. The dilution rate in the tunnel was approximately 5:1.

System Operation

Exposures were conducted 16 hr/dy, 5 dy/wk from approximately 1600 to 0800 hours, Sunday through Thursday. Because the animals were to be exposed at least 1 day immediately before necropsy, the Sunday–Thursday schedule

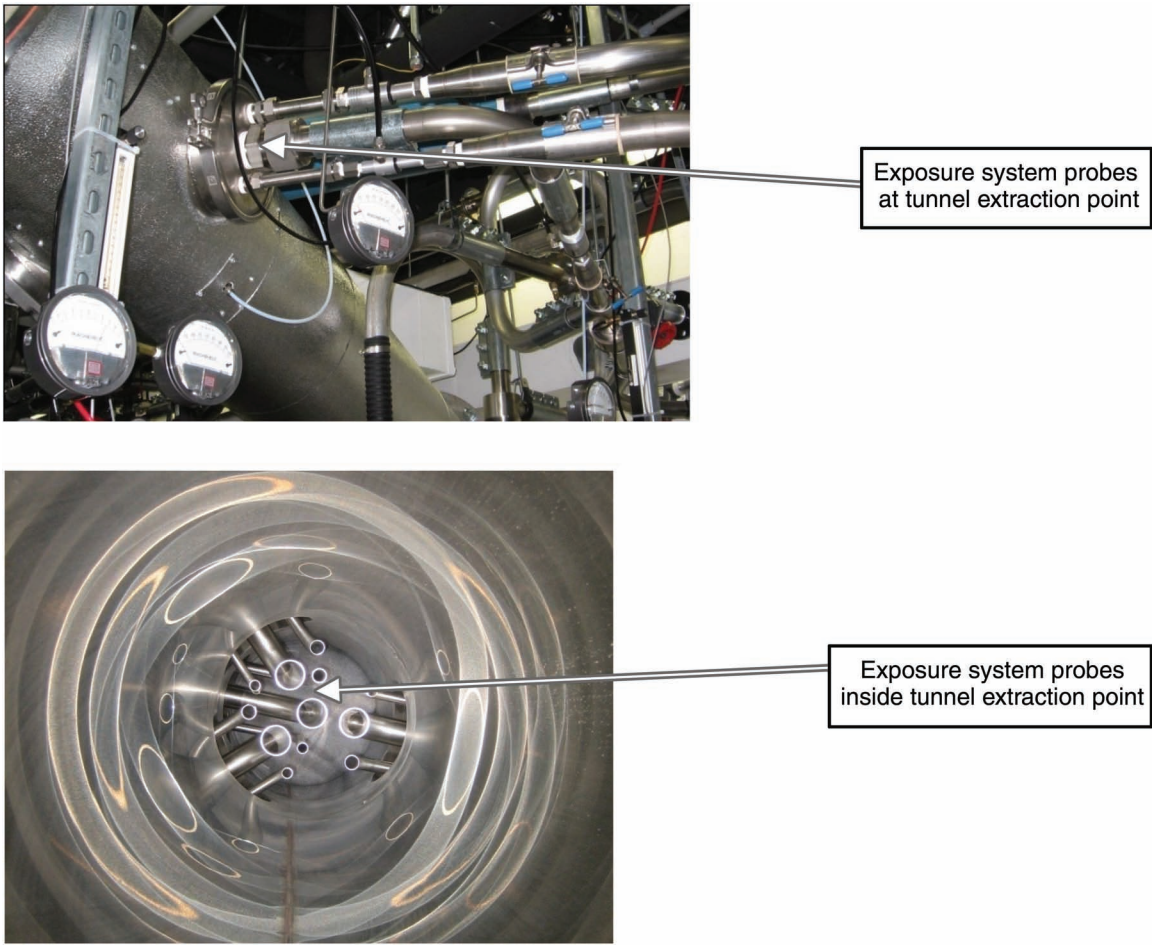

Figure B.3. Photographs of sample probes on the outside and inside of the dilution tunnel at the exposure system extraction point.

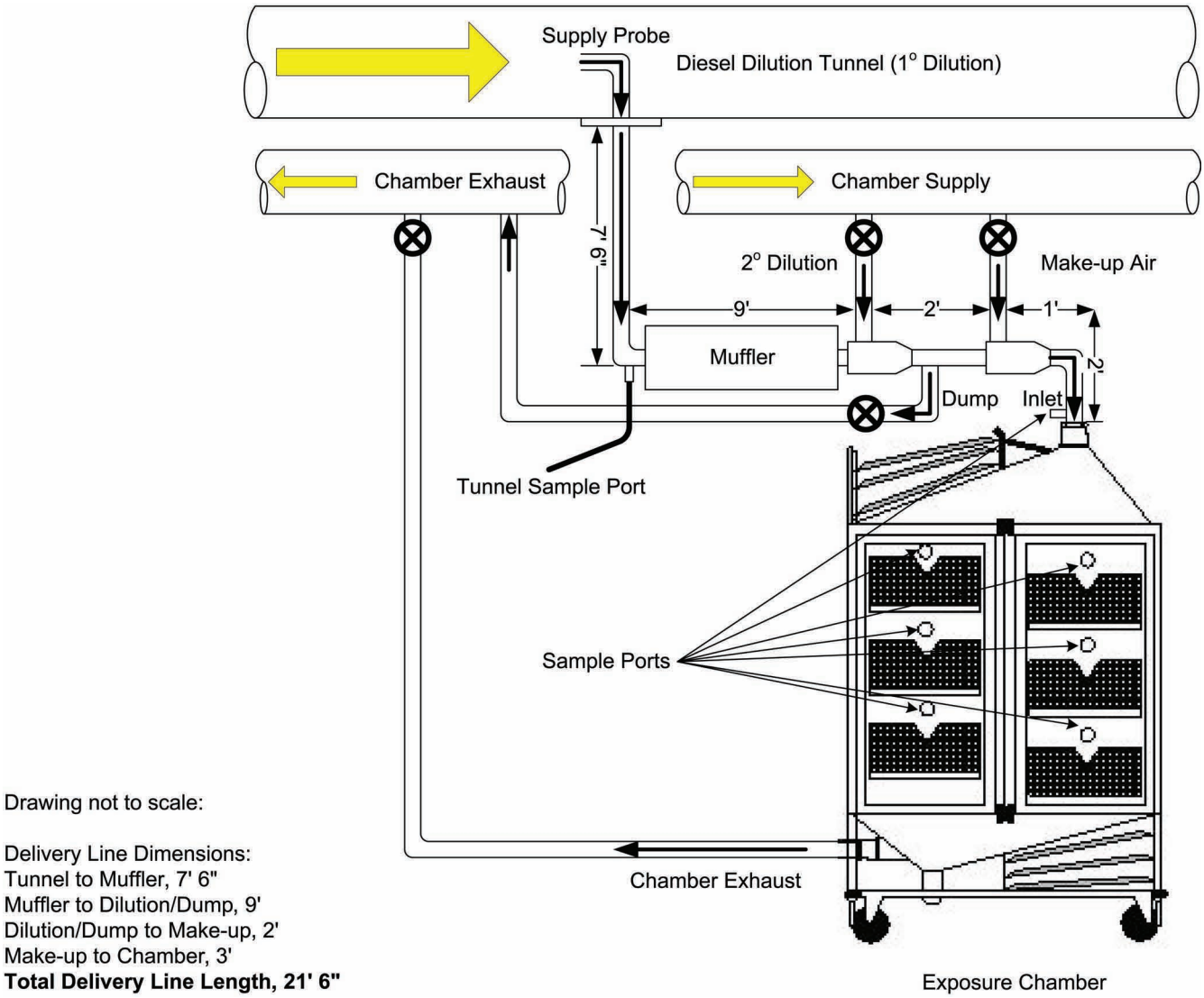

Figure B.4. Schematic (not to scale) of secondary and tertiary dilution system upstream of the inhalation exposure chamber. Diluted exhaust is extracted through the sampling probe shown on the left. The probe size ranges from 0.95 to 5 cm, with the smallest size extraction for the lowest exposure level and the largest for the highest level. Flows to the chambers are extracted by vacuum. Aerosol transits through a flow-through muffler before a series of two bypass and dilution legs.

allowed necropsies to be completed during the regular Monday–Friday work week. The daily schedule for operating the exposure system started with performance verifications and calibrations for monitoring and control equipment. The operator started the exposure system at approximately 1600 hours, after having conducted and documented the daily calibrations. Exposures were conducted for 16 hours plus the time to reach 90% of the target atmosphere (T_{90}). The system was operated without continuous operator presence between 1800 and 0800 hours and was programmed to automatically terminate the engine cycle and switch the exposure chambers to clean dilution air at 0800 hours.

Figure B.3 shows photographs of the aerosol extraction point, and Figure B.4 shows a schematic of the secondary and tertiary dilution system, along with the inhalation exposure chamber. Aerosol was extracted from the dilution tunnel through probes that ranged in diameter from 0.9 to 5 cm. Aerosol then transited through a flow-through muffler before a series of two bypass and dilution steps. The distance from the extraction point to the exposure chamber was approximately 6 m.

ATMOSPHERE CHARACTERIZATION

Methods

The analytical techniques and sampling equipment are summarized in Table B.1. The procedures for performing these measurements are described in this section. Note that all real-time measurements were recorded with a 1-second resolution.

Sample Collection

Sample tunnel emission measurements were obtained directly from the tunnel at the sample extraction point for the exposure chamber systems. Particle mass and size distribution measurements were obtained from the exposure chamber. Because of the contribution of animals to the PM measurement, samples were also obtained from the inlet of the chamber to define the contribution from DPM directly. Prestudy assessments showed that chamber inlet measurements adequately reflected the DPM measured in the exposure chamber. The exposure chambers contained multiple sample ports that allowed samples to be taken directly from the breathing zone of the animals (immediately above the wire cages at different levels in the chamber), with the exception of the Teflon-membrane filter for metals and the filter and XAD-4 cartridge for SVOCs. Samples from the Teflon-membrane filter and the SVOC filter and XAD-4 cartridge were taken out of an auxiliary sampling plenum, which was directly coupled to the chamber exhaust. Samples were pulled through stainless steel probes that were approximately 12 inches long and ¼ inch in diameter. Chamber uniformity assessments confirmed that samples could be collected in parallel ports and be representative of the same atmosphere.

Real-Time Mass and Size Distribution Real-time particle mass was measured using a Dekati mass monitor (DMM-230; Particle Instruments, Vadnais Heights, MN). Real-time measurements were begun a minimum of 10 minutes before the initiation of a test to collect data on background PM in the exposure chambers. Real-time soot (black carbon) was measured with the Photoacoustic Soot Spectrometer (PASS, Droplet Measurement Technologies, Boulder, CO).

Particle size was measured by a combination of a fast-response differential mobility analyzer (approximately 5–500 nm) and an aerodynamic particle sizer (0.5–20 μm). All analyzers were operated under default settings, assuming unit-density spherical particles. The fast-response differential mobility analyzer (DMA, Model 3091 Fast

Table B.1. Analytic Techniques and Sampling Equipment Used to Characterize Exposure Chamber Atmospheres[a]

Analysis	Collection Device	Collection Media	Analytical Instrument
Continuous PM mass	Dekati Mass Monitor	NA	NA
Nitric oxides (NO_x, NO_2)	Chemiluminescence analyzer	NA	NA
Black carbon/soot	Photo Acoustic Soot Spectrometer	NA	NA
Carbon monoxide/carbon dioxide	Infrared analyzer	NA	NA
Non-methane hydrocarbons	Heated flame ionization detector	NA	NA
Organic/elemental carbon	Aluminum in-line filter holder	Heat-treated quartz filter	TOR
Speciated metals	Teflon filter holder	Teflon-membrane filter	XRF/ICPMS
Speciated organic compounds			
Volatile hydrocarbons (C_1–C_{12})	Volatile organic sampler	Electropolished canister	GC/MS
Volatile carbonyls	Volatile organic sampler	DNPH cartridge	LC/UV
Semivolatile/fine particle organics	Tisch Environmental PUF sampler	TIGF filter/XAD-4	GC/MS
Size distribution			
0.5–20 μm aerodynamic distribution	Aerodynamic particle sizer	NA	NA
~5–500 nm particle number distribution/mass	Fast-mobility particle sizer	NA	NA

[a] DNPH indicates dinitrophyenylhydrazine; GC/MS, gas chromatography/mass spectrometry; ICPMS, inductively coupled plasma mass spectrometry; LC/UV, liquid chromatography/ultraviolet detection; PUF, polyurethane foam; NA, not applicable; TIGF, Teflon-impregnated glass fiber; TOR, thermal/optical reflectance; XAD-4, sorbent resin; XRF, X-ray fluorescence.

Response Particle Sizer Spectrometer, TSI, St. Paul, MN) also was used to measure particle number-based size distribution (in real time) with enhanced resolution for particle size (resolution, 1 second).

Chemical Characteristics of PM

Elemental and Organic Carbon Mass Elemental and organic carbon masses were determined at the Desert Research Institute (DRI) with prebaked quartz-fiber filters using the modified Interagency Monitoring of Protected Visual Environments thermal/optical reflectance method.

Organic Carbon Class and Species Particulate compounds and SVOCs were collected using Zefluor filters followed by XAD-4 resin cartridges. The target analytes included compounds that were statistically above detection limits during the Phase 1 component of the ACES program. Organic analyses for SVOCs were conducted at DRI by GC/MS.

Total Metals and Associated Elements Samples for metal analysis were collected on clean Teflon-membrane filters and analyzed at DRI by energy-dispersive X-ray fluorescence (EDXRF). After EDXRF analysis, the Teflon-membrane filters were returned to their petri dishes and stored under refrigeration until the XRF data validation was completed and had indicated that the runs were acceptable.

Inorganic Ions: Ammonium (and Ammonia), Sulfate, Nitrate One-half of the quartz filters (and blanks) collected for the carbon analysis were extracted and analyzed at DRI for water-soluble chloride, nitrite, nitrate, sulfate, and formic and acetic acid by ion chromatography. This extract was also analyzed for ammonium by the indolphenol colorimetric method.

Gases and Vapors

Nitrogen Oxides, Nonmethane Hydrocarbons, Sulfur Dioxide, and Carbon Monoxide Chemiluminescence analysis was used to measure NO_x (Teledyne Model 200 series, San Diego, CA; and Eco Physics 700 series, Ann Arbor, MI) and fluorescence analysis to measure SO_2 (Thermo Electron Corp.; Pulsed Fluorescence SO_2 Analyzer, Model 43i). CO and CO_2 concentrations were determined using a nondispersive infrared gas analyzer (California Analytical Instruments Model 600 series, California Analytical, Orange, CA). Nonmethane hydrocarbons (NMHC) were measured using a real-time flame ionization detector (Model 300H, California Analytical Instruments) calibrated against a certified propane standard. Analyzers were zeroed daily using ultra-zero air and calibrated with traceable span gases, as defined by the National Institute of Standards and Technology.

Gas Phase Hydrocarbon Speciation VOCs (except acids and carbonyls, which are too polar for collection in and analysis from a canister) were collected using a custom-designed canister sampler (L. Sheetz Enterprises, Reno, Nev.). Samples were collected downstream of an NO_x denuder in a precleaned Summa canister and analyzed within 30 days of collection to ensure accurate characterization of polar compounds that may "stick" to the walls of the canister. The NO_x denuder reduced NO_x and ameliorated NO_x–VOC reactions that can lead to false low readings of concentrations of reactive compounds such as 1,3-butadiene and styrene. Analysis was conducted at DRI by GC/MS.

Analysis of Carbonyl Compounds Carbonyl compounds were collected on dinitrophenylhydrazine (DNPH)-impregnated silica gel cartridges preceded by a commercially available oxidant scrubber and a Teflon-membrane prefilter to remove PM. In order to assess the trapping efficiency of the DNPH cartridge, two cartridges were used in series, and the backup cartridge was analyzed to ensure that all carbonyls were trapped on the first cartridge. Analysis was conducted at DRI by liquid chromatography/ultraviolet testing (photo-diode-array).

RESULTS: SUMMARY OF INTENSIVE CHARACTERIZATION

Exposure atmospheres were analyzed in detail as defined above. An overall description of the exposure atmosphere composition for the intensive characterization conducted during the first 13 weeks of exposure is provided in the main body of this report. Individual measurements from the characterization are summarized in Table B.2. Figures B.5 through B.8 provide representative particle number-based size distribution plots obtained at control, low, mid, and high exposure levels, respectively.

Table B.2. ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Dekati particle mass, chamber inlet	µg/m³	NA	NA	NA	NA
Dekati particle mass, inside chamber	µg/m³	0.12 ± 0.07	NA	NA	10.3 ± 5.2
Filter sample, chamber inlet	µg/m³	NA	1.0	3.0	8.3 ± 2.1
Filter sample, inside chamber	µg/m³	22.0	34.0	23.0	28.3 ± 23.0
Particle mass, FMPS	µg/m³	0.25 ± 0.03	0.42	0.81	2.93 ± 0.84
Particle count	Particle/cm³	370 ± 83.4	2,470	42,600	96,800 ± 16,600
NMAD (GSD)	nm	26.6 (2.0) ± 0.71 (0.08)	15.4 (1.59)	14.3 (1.50)	20.17 (1.55) ± 6.40 (0.05)
MMAD (GSD)	nm	419.6 (1.64) ± 6.22 (0.06)	351.8 (2.89)	45.4 (3.31)	44.17 (2.53) ± 1.72 (0.71)
Nitrogen oxide (NO)	µg/m³ (ppm)	0.5 (0.0005)	77.5 ± 35.3 (0.08 ± 0.03)	883 ± 226 (0.86 ± 0.22)	4875 ± 728 (4.75 ± 0.71)
Nitrogen dioxide (NO_2)	µg/m³ (ppm)	0.0	143 ± 59 (0.091 ± 0.038)	1306 ± 378 (0.83 ± 0.24)	6389 ± 1448 (4.06 ± 0.92)
Carbon monoxide (CO)	µg/m³ (ppm)	383 ± 287 (0.4 ± 0.3)	699 (0.73)	1839 (1.92)	5977 ± 785 (6.24 ± 0.82)
Carbon dioxide (CO_2)	mg/m³ (ppm)	1817 ± 54 (1207 ± 36)	1969 (1308)	3051 (2027)	5388 ± 343 (3580 ± 228)
Sulfur dioxide (SO_2)	mg/m³ (ppb)	6.1 ± 1.8 (2.7 ± 0.8)	6.8 (3)	19.4 (8.59)	53.2 ± 9 (23.6 ± 3.88)
Elemental carbon	µg/m³	1.0 ± 0.05	1.6 ± 0.08	1.0 ± 0.04	2.7 ± 0.15
Organic carbon	µg/m³	6.4 ± 0.22	6.6 ± 0.23	4.3 ± 0.13	6.1 ± 0.20
Ammonium	µg/m³	0.0	0.0	0.1	1.7
Sulfate	µg/m³	0.1	0.1	0.3	1.7
Nitrate	µg/m³	0.0	0.1	0.1	1.2
Elements (metals)	µg/m³	0.8 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	1.6 ± 0.2
Alkanes	µg/m³	3.4 ± 0.2	3.6 ± 0.2	12.7 ± 0.8	26.7 ± 1.5
Carbonyl	µg/m³	39.6 ± 2.6	129.0 ± 7.0	27.8 ± 2.0	0.3 ± 1.0
Polyaromatic hydrocarbon (PAH)	µg/m³	1.5 ± 0.1	1.4 ± 0.1	2.7 ± 0.1	6.2 ± 0.3
Nitro-PAH	ng/m³	0.7 ± 0.05	0.8 ± 0.05	2.4 ± 0.1	6.5 ± 0.4
Polars (acids)	µg/m³	4.2 ± 0.1	5.6 ± 0.1	6.4 ± 0.6	24.8 ± 2.2
Hopane and steranes	ng/m³	2.6 ± 0.2	2.4 ± 0.2	NA	0.9 ± 0.1
Volatile organic carbon (VOC)	µg/m³	19.1 ± 4.3	92.5 ± 7.8	33.1 ± 6.6	74.9 ± 12.8
Carbon					
Organic carbon fraction 1	µg/m³	0.5 ± 0.13	0.4 ± 0.11	0.3 ± 0.07	0.5 ± 0.13
Organic carbon fraction 2	µg/m³	1.6 ± 0.02	1.9 ± 0.02	1.7 ± 0.02	2.1 ± 0.02
Organic carbon fraction 3	µg/m³	3.1 ± 0.33	2.9 ± 0.29	1.8 ± 0.16	2.6 ± 0.26
Organic carbon fraction 4	µg/m³	0.8 ± 0.16	1.1 ± 0.23	0.6 ± 0.11	0.9 ± 0.18
Pyrolyzed organic carbon reflectance	µg/m³	0.4	0.4	0.0	0.0
Pyrolyzed organic carbon transmittance	µg/m³	0.6 ± 0.24	0.0	0.4 ± 0.13	1.1 ± 0.41
Organic carbon	µg/m³	6.4 ± 0.22	6.6 ± 0.23	4.3 ± 0.13	6.1 ± 0.2
Elemental carbon fraction 1	µg/m³	0.8	0.9	0.4	1.0
Elemental carbon fraction 2	µg/m³	0.5 ± 0.02	0.8 ± 0.04	0.6 ± 0.03	1.5 ± 0.11
Elemental carbon fraction 3	µg/m³	0.0	0.2 ± 0.07	0.0	0.1 ± 0.03
Elemental carbon	µg/m³	1 ± 0.04	1.6 ± 0.08	1 ± 0.04	2.7 ± 0.15
Total carbon	µg/m³	7.4 ± 0.29	8.3 ± 0.33	5.3 ± 0.18	8.7 ± 0.36
Elements (Metals)					
Sodium	ng/m³	0 ± 59.7	83.7 ± 61.1	185.7 ± 62.5	228.5 ± 63.4
Magnesium	ng/m³	7.3 ± 20.3	5.6 ± 19.7	18.5 ± 19.3	9.6 ± 19.2
Aluminum	ng/m³	14.3 ± 4	3.6 ± 3.8	34.7 ± 3.9	19.2 ± 3.8
Silicon	ng/m³	49.4 ± 2.6	8.9 ± 2.4	117.3 ± 2.7	14.2 ± 2.4
Phosphorous	ng/m³	125.4 ± 1.1	175.3 ± 1.3	107.4 ± 1	106.2 ± 1
Sulfur	ng/m³	70.7 ± 0.8	89.2 ± 0.9	158.4 ± 1.1	806.9 ± 3.3
Chlorine	ng/m³	103.9 ± 0.9	118.2 ± 0.9	109.3 ± 0.9	93.1 ± 0.9
Potassium	ng/m³	229.7 ± 1.2	267 ± 1.2	235.6 ± 1.2	186.9 ± 1.1
Calcium	ng/m³	142.5 ± 3	138.6 ± 2.9	177.1 ± 3	122.2 ± 2.8
Scandium	ng/m³	0 ± 12.3	0 ± 11.9	0 ± 11.6	0 ± 11.5
Titanium	ng/m³	3.2 ± 1	0 ± 1	9.4 ± 1	1.8 ± 0.9
Vanadium	ng/m³	0 ± 0.7	0 ± 0.6	0 ± 0.6	0 ± 0.6
Chromium	ng/m³	1 ± 0.7	0.4 ± 0.6	2.5 ± 0.6	1.1 ± 0.6
Manganese	ng/m³	1.4 ± 2.1	1.4 ± 2	3.8 ± 1.9	2.4 ± 1.9
Iron	ng/m³	29.5 ± 0.7	11.3 ± 0.7	46.5 ± 0.7	15 ± 0.7

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Elements (Metals) (*Continued*)					
Cobalt	ng/m³	0.1 ± 0.7	0 ± 0.6	0.6 ± 0.6	0.1 ± 0.6
Nickel	ng/m³	0.4 ± 1.9	0 ± 1.9	1.1 ± 1.8	0.6 ± 1.8
Copper	ng/m³	7.8 ± 2.4	3.4 ± 2.3	15.3 ± 2.3	5.4 ± 2.3
Zinc	ng/m³	7.5 ± 0.7	3.5 ± 0.6	13.9 ± 0.7	7.1 ± 0.6
Gallium	ng/m³	0 ± 3.3	0.2 ± 3.1	0 ± 3.1	0 ± 3.1
Arsenic	ng/m³	0 ± 0.7	0 ± 0.6	0 ± 0.6	0 ± 0.6
Selenium	ng/m³	0 ± 0.7	0 ± 0.6	0 ± 0.6	0 ± 0.6
Bromine	ng/m³	0.6 ± 0.7	0 ± 0.6	0.2 ± 0.6	0.2 ± 0.6
Rubidium	ng/m³	0 ± 0.7	0 ± 0.6	0 ± 0.6	0 ± 0.6
Strontium	ng/m³	0.6 ± 0.7	0 ± 0.6	2 ± 0.6	0.4 ± 0.6
Yttrium	ng/m³	0 ± 0.7	0.4 ± 0.6	0 ± 0.6	0.4 ± 0.6
Zirconium	ng/m³	0 ± 1.1	0 ± 1	0 ± 1	0 ± 1
Niobium	ng/m³	0.2 ± 0.9	0 ± 0.9	0 ± 0.9	0.2 ± 0.9
Molybdenum	ng/m³	0 ± 1.4	0 ± 1.3	0.7 ± 1.3	0.5 ± 1.3
Palladium	ng/m³	0 ± 1.6	0 ± 1.5	0 ± 1.5	0 ± 1.5
Silver	ng/m³	0.4 ± 1.5	0 ± 1.5	0 ± 1.4	0.1 ± 1.4
Cadmium	ng/m³	0.2 ± 1.9	0 ± 1.9	0 ± 1.8	0 ± 1.8
Indium	ng/m³	0.5 ± 2.1	0.4 ± 2	0 ± 1.9	0.9 ± 1.9
Tin	ng/m³	0.8 ± 2	0 ± 2	0 ± 1.9	0.1 ± 1.9
Antimony	ng/m³	0 ± 3.3	0 ± 3.1	0 ± 3.1	0 ± 3.1
Cesium	ng/m³	0 ± 6.6	0 ± 6.4	0 ± 6.3	0 ± 6.2
Barium	ng/m³	0 ± 7.6	2.2 ± 7.4	5.8 ± 7.2	1.8 ± 7.2
Lanthanum	ng/m³	1.1 ± 9.5	0 ± 9.2	1.7 ± 9	2.8 ± 9
Cerium	ng/m³	0 ± 9.1	0 ± 8.7	0 ± 8.6	0 ± 8.5
Samarium	ng/m³	0 ± 16.1	0 ± 15.5	0 ± 15.2	0 ± 15
Europium	ng/m³	0 ± 22.9	0 ± 22	0 ± 21.6	2 ± 21.5
Terbium	ng/m³	0 ± 17.4	0 ± 16.7	10.6 ± 16.6	0 ± 16.2
Hafnium	ng/m³	0 ± 4.8	0 ± 4.6	1 ± 4.5	0.3 ± 4.5
Tantalum	ng/m³	0.7 ± 2.7	0 ± 2.6	0 ± 2.5	0 ± 2.5
Wolfram	ng/m³	0 ± 7.7	0 ± 7.4	0 ± 7.2	0 ± 7.2
Iridium	ng/m³	0.6 ± 1.2	0.5 ± 1.2	0 ± 1.1	0.3 ± 1.1
Gold	ng/m³	0 ± 1.2	0 ± 1.2	0 ± 1.1	0 ± 1.1
Mercury	ng/m³	0 ± 0.7	0 ± 0.6	0 ± 0.6	0.1 ± 0.6
Thallium	ng/m³	0.7 ± 0.8	0 ± 0.8	0.4 ± 0.8	0.4 ± 0.8
Lead	ng/m³	0.4 ± 0.7	0 ± 0.7	3.7 ± 0.7	0.1 ± 0.7
Uranium	ng/m³	0.2 ± 1.1	0 ± 1	0 ± 1	0 ± 1
Alkanes					
Dodecane	ng/m³	324.2 ± 26.9	496.7 ± 41.1	1798.2 ± 148.4	2660.8 ± 219.6
Norfarnesane	ng/m³	208.9 ± 10.6	256 ± 12.9	826.5 ± 41.4	2172.1 ± 108.7
Tridecane	ng/m³	498.5 ± 25	463.4 ± 23.3	2071.7 ± 103.7	5360.8 ± 268.1
Heptylcyclohexane	ng/m³	55.2 ± 3.9	59.3 ± 4.1	231 ± 15.7	527.3 ± 35.8
Farnesane	ng/m³	127 ± 7.8	140.5 ± 8.6	668.4 ± 40.5	1294.6 ± 78.3
Tetradecane	ng/m³	515.4 ± 25.9	472.8 ± 23.7	1770.9 ± 88.6	3828.6 ± 191.5
Octylcyclohexane	ng/m³	51.5 ± 2.7	54.6 ± 2.8	157.1 ± 8	361 ± 18.1
Pentadecane	ng/m³	318.6 ± 16.1	391.7 ± 19.7	1449.3 ± 72.6	3456 ± 172.9
Nonylcyclohexane	ng/m³	42.3 ± 3.8	50.5 ± 4.5	135 ± 11.9	275.5 ± 24.1
Hexadecane	ng/m³	258.7 ± 13.1	274.7 ± 13.8	1035.7 ± 51.9	2247.3 ± 112.4
Norpristane	ng/m³	154.3 ± 7.9	132.2 ± 6.7	511.1 ± 25.7	972.5 ± 48.7
Decylcyclohexane	ng/m³	15.4 ± 1.8	30.4 ± 3.5	74.8 ± 8.4	106.3 ± 11.9
Heptadecane	ng/m³	235.4 ± 13.3	169.8 ± 9.6	666.8 ± 37.6	1131.4 ± 63.7
Heptadecane	ng/m³	152.9 ± 8.2	118.2 ± 6.4	354.4 ± 18.9	663 ± 35.2
Undecylcyclohexane	ng/m³	26.5 ± 2.2	31.2 ± 2.6	46.5 ± 3.8	96.9 ± 7.8
Octadecane	ng/m³	176.5 ± 8.9	127.1 ± 6.5	354.7 ± 17.8	640.3 ± 32.1
Phytane	ng/m³	122.1 ± 8.6	112.2 ± 7.9	213.6 ± 15	435.9 ± 30.5
Dodecylcyclohexane	ng/m³	9.6 ± 0.7	12.9 ± 1	28.9 ± 2	39 ± 2.7
Nonadecane	ng/m³	62.4 ± 6.56	63.9 ± 6.7	142.5 ± 14.9	184.9 ± 19.2
Tridecylcyclohexane	ng/m³	8.0 ± 0.6	10.1 ± 0.6	5.5 ± 0.4	18.7 ± 1.1

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a]		
			Low	Mid	High
Alkanes (*Continued*)					
Eicosane	ng/m³	21.4 ± 4.8	23.7 ± 5.2	68.8 ± 15.1	85.7 ± 18.7
Tetradecylcyclohexane	ng/m³	2.8 ± 0.3	1.8 ± 0.2	4.3 ± 0.3	7.1 ± 0.4
Heneicosane	ng/m³	5.5 ± 0.6	2.3 ± 0.3	23.8 ± 1.9	34.1 ± 2.6
Pentadecylcyclohexane	ng/m³	1.0 ± 0.3	1 ± 0.2	6 ± 0.7	4.6 ± 0.6
Docosane	ng/m³	1.7 ± 0.3	3.5 ± 0.6	6.6 ± 0.9	16.5 ± 2.1
Hexadecylcyclohexane	ng/m³	0.3 ± 0.2	0.1 ± 0.2	0.6 ± 0.2	1.3 ± 0.3
Tricosane	ng/m³	2.4 ± 0.4	2.4 ± 0.5	2.3 ± 0.6	7.8 ± 1.5
Heptadecylcyclohexane	ng/m³	0.1 ± 0.2	32.3 ± 1.7	0.3 ± 0.2	1 ± 0.2
Tetracosane	ng/m³	6.6 ± 0.5	7.5 ± 0.5	5.4 ± 0.3	4.4 ± 0.3
Octadecylcyclohexane	ng/m³	2.1 ± 0.4	0 ± 0.2	1.6 ± 0.3	1.4 ± 0.4
Pentacosane	ng/m³	11.8 ± 0.7	11.1 ± 0.6	9.7 ± 0.7	6.5 ± 0.6
Hexacosane	ng/m³	5.1 ± 0.4	5.2 ± 0.4	5 ± 0.3	3.7 ± 0.3
Nonadecylcyclohexane	ng/m³	1.1 ± 0.3	0 ± 0.2	0.6 ± 0.2	0.8 ± 0.2
Heptacosane	ng/m³	2.75 ± 0.6	2.5 ± 0.5	2.2 ± 0.5	1.9 ± 0.4
Eicosylcyclohexane	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0.2 ± 0.2
Octacosane	ng/m³	0.8 ± 0.3	0.3 ± 0.2	0.5 ± 0.2	1.5 ± 0.3
Nonacosane	ng/m³	0.2 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.9 ± 0.2
Heneicosylcyclohexane	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0.1 ± 0.2
Triacontane	ng/m³	0.1 ± 0.2	0 ± 0.2	0.4 ± 0.2	0.8 ± 0.2
Hentriacontane	ng/m³	0.1 ± 0.2	0 ± 0.2	0 ± 0.2	0.7 ± 0.2
Dotriacontane	ng/m³	0.2 ± 0.2	0 ± 0.2	0 ± 0.2	0.4 ± 0.2
Tritriacontane	ng/m³	0.2 ± 0.2	0 ± 0.2	0 ± 0.2	0.3 ± 0.2
Tetratriacontane	ng/m³	0.5 ± 0.2	0 ± 0.2	0 ± 0.2	0.5 ± 0.2
Pentatriacontane	ng/m³	0.5 ± 0.3	0 ± 0.2	0 ± 0.2	0.3 ± 0.2
Hexatriacontane	ng/m³	0.1 ± 0.2	0 ± 0.2	0 ± 0.2	0.3 ± 0.2
Heptatriacontane	ng/m³	0.3 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
Octatriacontane	ng/m³	0.1 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
Nonatriacontane	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
Tetracontane	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
Carbonyl					
Formaldehyde	µg/m³	2.7 ± 0.11	1.6 ± 0.11	0.9 ± 0.08	0 ± 0.07
Acetaldehyde	µg/m³	8.3 ± 0.3	17.3 ± 0.87	10.5 ± 0.54	0 ± 0.07
Acrolein	µg/m³	0.2 ± 0.06	0.1 ± 0.07	0.1 ± 0.07	0 ± 0.07
Glyoxal	µg/m³	0.1 ± 0.05	0 ± 0.07	0 ± 0.07	0.1 ± 0.07
Acetone	µg/m³	13.5 ± 0.45	10.1 ± 0.5	4.6 ± 0.27	0 ± 0.07
Propionaldehyde	µg/m³	1.5 ± 0.07	1.4 ± 0.1	1.6 ± 0.12	0 ± 0.07
Crotonaldehyde	µg/m³	0 ± 0.05	0 ± 0.07	0 ± 0.07	0 ± 0.07
Methacrolein	µg/m³	0 ± 0.05	0 ± 0.07	0 ± 0.07	0 ± 0.07
n-Butyraldehyde	µg/m³	1 ± 0.06	0.8 ± 0.08	0.6 ± 0.07	0 ± 0.07
2-Butanone (MEK)	µg/m³	1.9 ± 0.09	92 ± 4.6	3.7 ± 0.22	0 ± 0.07
Valeraldehyde	µg/m³	1.3 ± 0.07	1 ± 0.08	1 ± 0.08	0 ± 0.07
Hexaldehyde	µg/m³	8.5 ± 0.31	5.3 ± 0.3	4.7 ± 0.24	0.2 ± 0.07
Benzaldehyde	µg/m³	0.5 ± 0.05	0.1 ± 0.07	0 ± 0.07	0 ± 0.07
m-Tolualdehyde	µg/m³	0 ± 0.05	0 ± 0.07	0 ± 0.07	0 ± 0.07
Polyaromatic Hydrocarbon (PAH)					
1+2-Ethylnaphthalene	ng/m³	7.6 ± 0.5	6.3 ± 0.41	43.1 ± 2.84	81.6 ± 5.38
Naphthalene	ng/m³	37 ± 1.85	46 ± 2.3	231 ± 11.55	682.7 ± 34.14
Quinoline	ng/m³	0.5 ± 0.21	0 ± 0.12	0 ± 0.12	2.4 ± 0.76
2-Methylnaphthalene	ng/m³	29.6 ± 1.48	24 ± 1.2	109.7 ± 5.49	335 ± 16.75
1-Methylnaphthalene	ng/m³	20.4 ± 1.02	15 ± 0.75	82.9 ± 4.14	246.4 ± 12.32
Biphenyl	ng/m³	15 ± 0.75	14.8 ± 0.74	56.9 ± 2.84	166 ± 8.3
2-Methylbiphenyl	ng/m³	66.7 ± 3.68	86.9 ± 4.8	179.4 ± 9.9	468 ± 25.83
2,6+2,7-Dimethylnaphthalene	ng/m³	16.5 ± 0.82	14 ± 0.7	77.1 ± 3.86	246.2 ± 12.31
1,3+1,6+1,7-Dimethylnaphth	ng/m³	29.9 ± 1.49	24.5 ± 1.23	129.1 ± 6.46	465.8 ± 23.29
1,4+1,5+2,3-Dimethylnaphth	ng/m³	8.4 ± 0.42	5.8 ± 0.29	37.6 ± 1.88	147.8 ± 7.39

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Polyaromatic Hydrocarbon (PAH) (*Continued*)					
Acenaphthylene	ng/m³	0.4 ± 0.12	2.6 ± 0.34	15.5 ± 2.06	18 ± 2.4
1,2-Dimethylnaphthalene	ng/m³	2.4 ± 0.2	2.8 ± 0.21	9.6 ± 0.74	33.7 ± 2.58
1,8-Dimethylnaphthalene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Acenaphthene	ng/m³	0.5 ± 0.16	0.3 ± 0.12	0.7 ± 0.2	19.7 ± 5.21
4-Methylbiphenyl	ng/m³	300.5 ± 15.03	258.8 ± 12.94	382.6 ± 19.13	719.2 ± 35.96
3-Methylbiphenyl	ng/m³	827.8 ± 41.39	740.2 ± 37.01	1028.7 ± 51.43	1874.8 ± 93.74
Dibenzofuran	ng/m³	2.1 ± 0.21	2.2 ± 0.22	2.9 ± 0.29	13 ± 1.3
1-Ethyl-2-methylnaphthalene	ng/m³	5 ± 0.79	1.7 ± 0.26	11.5 ± 1.81	27.4 ± 4.31
2,3,5+I-Trimethylnaphthalene	ng/m³	9.8 ± 0.61	10.2 ± 0.63	20 ± 1.24	60.7 ± 3.76
B-Trimethylnaphthalene	ng/m³	11.6 ± 0.58	13.4 ± 0.67	45.5 ± 2.27	92.7 ± 4.63
A-Trimethylnaphthalene	ng/m³	14.3 ± 0.71	16.5 ± 0.82	46.9 ± 2.34	112 ± 5.6
C-Trimethylnaphthalene	ng/m³	11.6 ± 0.58	11.1 ± 0.56	38.9 ± 1.94	83.8 ± 4.19
2-Ethyl-1-methylnaphthalene	ng/m³	0.7 ± 0.12	0.1 ± 0.12	0.7 ± 0.12	3.8 ± 0.23
E-Trimethylnaphthalene	ng/m³	7.8 ± 0.41	7.7 ± 0.41	27.7 ± 1.45	56.8 ± 2.97
2,4,5-Trimethylnaphthalene	ng/m³	1.2 ± 0.22	0.4 ± 0.12	1.1 ± 0.16	7.6 ± 1.09
F-Trimethylnaphthalene	ng/m³	6 ± 0.63	6.9 ± 0.72	19.4 ± 2.02	45.5 ± 4.74
Fluorene	ng/m³	7.2 ± 0.38	3.1 ± 0.16	3.1 ± 0.16	19.4 ± 1
1,4,5-Trimethylnaphthalene	ng/m³	0.9 ± 0.12	0.7 ± 0.12	0.5 ± 0.12	4.1 ± 0.23
J-Trimethylnaphthalene	ng/m³	4.1 ± 0.24	4.6 ± 0.27	13.3 ± 0.79	29 ± 1.72
A-Methylfluorene	ng/m³	2.9 ± 0.2	3.4 ± 0.22	5.9 ± 0.39	11 ± 0.73
B-Methylfluorene	ng/m³	0.5 ± 0.12	0.3 ± 0.12	0.6 ± 0.12	1.7 ± 0.16
1-Methylfluorene	ng/m³	1 ± 0.21	2.1 ± 0.35	1.5 ± 0.25	6.6 ± 1.08
9-Fluorenone	ng/m³	7.6 ± 0.79	6.2 ± 0.65	9.9 ± 1.02	10.3 ± 1.06
Dibenzothiophene	ng/m³	0.3 ± 0.12	0.2 ± 0.12	0.1 ± 0.12	0.3 ± 0.12
Phenanthrene	ng/m³	8.6 ± 0.87	6.6 ± 0.67	16.6 ± 1.68	42.2 ± 4.26
Anthracene	ng/m³	1 ± 0.18	0.8 ± 0.13	0.8 ± 0.14	3.4 ± 0.57
Xanthone	ng/m³	0.3 ± 0.12	0.1 ± 0.12	0.1 ± 0.12	1.3 ± 0.22
Acenaphthenequinone	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
3-Methylphenanthrene	ng/m³	2.7 ± 0.15	1.8 ± 0.12	1.8 ± 0.12	4.1 ± 0.23
2-Methylphenanthrene	ng/m³	2 ± 0.16	1.6 ± 0.13	1 ± 0.12	4 ± 0.31
Perinaphthenone	ng/m³	0.6 ± 0.25	0.1 ± 0.12	0.9 ± 0.27	1.3 ± 0.41
2-Methylanthracene	ng/m³	0.1 ± 0.12	0.3 ± 0.12	0.9 ± 0.12	2.1 ± 0.27
4,5-Methylenephenanthrene	ng/m³	0.6 ± 0.12	1.1 ± 0.12	0.4 ± 0.12	1.2 ± 0.13
9-Methylphenanthrene	ng/m³	2.2 ± 0.33	0.6 ± 0.12	1 ± 0.15	3.9 ± 0.56
1-Methylphenanthrene	ng/m³	1.8 ± 0.27	0.3 ± 0.12	0.6 ± 0.12	2.3 ± 0.36
Anthrone	ng/m³	2.2 ± 0.17	0.9 ± 0.12	5.6 ± 0.28	4.7 ± 0.24
9-Methylanthracene	ng/m³	0.1 ± 0.12	0.2 ± 0.12	0.1 ± 0.12	0.6 ± 0.12
2-Phenylnaphthalene	ng/m³	0.2 ± 0.12	0.3 ± 0.12	0.3 ± 0.12	1.3 ± 0.14
Anthraquinone	ng/m³	0.4 ± 0.12	0.4 ± 0.12	0 ± 0.12	0.9 ± 0.13
A-Dimethylphenanthrene	ng/m³	0.3 ± 0.12	0.1 ± 0.12	0.1 ± 0.12	0.9 ± 0.15
B-Dimethylphenanthrene	ng/m³	0.2 ± 0.12	0 ± 0.12	0.1 ± 0.12	0.4 ± 0.12
1,7-Dimethylphenanthrene	ng/m³	0.2 ± 0.12	0.1 ± 0.12	0.2 ± 0.12	0.6 ± 0.12
3,6-Dimethylphenanthrene	ng/m³	0.2 ± 0.12	0 ± 0.12	0.1 ± 0.12	0.5 ± 0.12
D-Dimethylphenanthrene	ng/m³	0.2 ± 0.12	0 ± 0.12	0.1 ± 0.12	0.4 ± 0.12
E-Dimethylphenanthrene	ng/m³	0.1 ± 0.12	0.2 ± 0.12	0.1 ± 0.12	0.3 ± 0.12
C-Dimethylphenanthrene	ng/m³	0.7 ± 0.12	0.2 ± 0.12	0.1 ± 0.12	1.2 ± 0.18
Fluoranthene	ng/m³	0.4 ± 0.12	0.4 ± 0.12	0.4 ± 0.12	2.6 ± 0.32
Pyrene	ng/m³	0.6 ± 0.12	0.4 ± 0.12	1.2 ± 0.13	2.6 ± 0.25
9-Anthraaldehyde	ng/m³	0.2 ± 0.12	0 ± 0.12	0 ± 0.12	0.3 ± 0.12
Retene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Benzo[*a*]fluorene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.2 ± 0.12
Benzo[*b*]fluorene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.1 ± 0.12
B-MePy/MeFl	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.2 ± 0.12
1-MeFl+C-MeFl/Py	ng/m³	0 ± 0.12	0 ± 0.12	0.2 ± 0.12	0.2 ± 0.12
1,3-Methylfluoranthene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.1 ± 0.12

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Polyaromatic Hydrocarbon (PAH) (*Continued*)					
4-Methylpyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.2 ± 0.12
C-MePy/MeFl	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.1 ± 0.12
D-MePy/MeFl	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.2 ± 0.12
1-Methylpyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.1 ± 0.12
Benzonaphthothiophene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Benzo[*c*]phenanthrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Benzo[*g,h,i*]fluoranthene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.4 ± 0.13
9-Phenylanthracene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Cyclopenta[*c,d*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0.1 ± 0.12	0.1 ± 0.12
Benz[*a*]anthracene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Chrysene-triphenylene	ng/m³	0 ± 0.24	0 ± 0.24	0 ± 0.24	0 ± 0.24
Benzanthrone	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Benz[*a*]anthracene-7,12-dione	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
3-Methylchrysene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Chry56m	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
7-Methylbenz[*a*]anthracene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
7,12-Dimethylbenz[*a*]anthracene	ng/m³	0.1 ± 0.13	0.1 ± 0.13	0.1 ± 0.12	0.2 ± 0.13
Benzo[*b+j+k*]fluoranthene	ng/m³	0 ± 0.12	0.1 ± 0.12	0 ± 0.12	0 ± 0.12
Benzo[*a*]fluoranthene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
BeP	ng/m³	0 ± 0.12	0.1 ± 0.12	0 ± 0.12	0.1 ± 0.12
BaP	ng/m³	0 ± 0.12	0.2 ± 0.12	0.1 ± 0.12	0 ± 0.12
Perylene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.2 ± 0.12
Dibenz[*a,j*]acridine	ng/m³	0 ± 0.12	0.1 ± 0.12	0 ± 0.12	0.3 ± 0.12
7-Methylbenzo[*a*]pyrene	ng/m³	0 ± 0.12	0.2 ± 0.12	0.2 ± 0.12	0 ± 0.12
Bpy910dih	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Indeno[1,2,3-*cd*]fluoranthene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenz[*a,h*]acridine	ng/m³	0 ± 0.12	0.1 ± 0.12	0 ± 0.12	0 ± 0.12
Dbajacr	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Indeno[1,2,3-*cd*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*ah+ac*]anthracene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*a,j*]anthracene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Benzo[*b*]chrysene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Picene	ng/m³	0 ± 0.12	0 ± 0.12	0.1 ± 0.12	0.1 ± 0.12
Benzo[*g,h,i*]perylene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0.1 ± 0.12
Anthanthrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*a,l*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Coronene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*a,e*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*a,i*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*a,h*]pyrene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Dibenzo[*b,k*]fluoranthene	ng/m³	0 ± 0.12	0 ± 0.12	0 ± 0.12	0 ± 0.12
Nitro-PAH					
3-Nitrobenz[*e*]pryene	ng/m³	0.0	0.0	0.0	0.0
1-Nitronaphthalene	ng/m³	0.1	0.2 ± 0.01	0.6 ± 0.03	1.7 ± 0.08
1-Methyl-5-nitronaphthalene	ng/m³	0.0	0.0	0.1 ± 0.01	0.5 ± 0.03
2-Nitronaphthalene	ng/m³	0.1	0.0	0.5 ± 0.03	2.8 ± 0.14
2-Nitrobiphenyl	ng/m³	0.0	0.0	0.0	0.1 ± 0.01
2-Methyl-4-nitronaphthalene	ng/m³	0.0	0.0	0.2 ± 0.01	0.6 ± 0.03
1-Methyl-4-nitronaphthalene	ng/m³	0.0	0.0	0.0	0.4 ± 0.1
1-Methyl-6-nitronaphthalene	ng/m³	0.1	0.0	0.0	0.1 ± 0.01
3-Nitrobiphenyl	ng/m³	0.0	0.0	0.0	0.1
4-Nitrobiphenyl	ng/m³	0.0	0.0	0.0	0.0
1,3-Dinitronaphthalene	ng/m³	0.0	0.0	0.0	0.0
1,5-Dinitronaphthalene	ng/m³	0.0	0.0	0.0	0.0
5-Nitroacenaphthene	ng/m³	0.0	0.0	0.0	0.0
2-Nitrofluorene	ng/m³	0.0	0.0	0.0	0.0
4-Nitrophenanthrene	ng/m³	0.0	0.0	0.0	0.0

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Nitro-PAH (*Continued*)					
9-Nitroanthracene	ng/m³	0.0	0.0	0.0	0.1 ± 0.01
9-Nitrophenanthrene	ng/m³	0.0	0.0	0.0	0.0
1,8-Dinitronaphthalene	ng/m³	0.0	0.0	0.0	0.0
3-Nitrophenanthrene	ng/m³	0.4 ± 0.01	0.5 ± 0.03	0.9 ± 0.04	0.0
2-Nitrophenanthrene	ng/m³	0.0	0.0	0.0	0.0
2-Nitroanthracene	ng/m³	0.0	0.0	0.0	0.0
2-Nitrofluoranthene	ng/m³	0.0	0.0	0.0	0.0
3-Nitrofluoranthene	ng/m³	0.0	0.0	0.0	0.0
4-Nitropyrene	ng/m³	0.0	0.0	0.0	0.0
1-Nitropyrene	ng/m³	0.0	0.0	0.0	0.0
2-Nitropyrene	ng/m³	0.0	0.0	0.0	0.0
2,7-Dinitrofluorene	ng/m³	0.0	0.0	0.0	0.0
2,7-Dinitrofluoren-9-one	ng/m³	0.0	0.0	0.0	0.0
7-Nitrobenz[*a*]anthracene	ng/m³	0.0	0.0	0.0	0.0
6-Nitrochrysene	ng/m³	0.0	0.0	0.0	0.0
3-Nitrobenzanthrone	ng/m³	0.0	0.0	0.0	0.0
1,3-Dinitropyrene	ng/m³	0.0	0.0	0.0	0.0
1,6-Dinitropyrene	ng/m³	0.0	0.0	0.0	0.0
1,8-Dinitropyrene	ng/m³	0.0	0.0	0.0	0.0
6a+1e-Nitrobenzpyrene	ng/m³	0.0	0.0	0.0	0.0
Polars					
8,15-Pimaradien-18-oic acid	ng/m³	0.1 ± 0.2	0 ± 0.2	1.3 ± 0.2	2.3 ± 0.3
Maleic acid	ng/m³	130.8 ± 0.8	385.4 ± 0.8	330.6 ± 32.1	775.4 ± 85.1
Guaiacol	ng/m³	6.9 ± 0.2	1.2 ± 0.2	2.2 ± 0.3	11.6 ± 3.6
Salicylic acid	ng/m³	11.8 ± 0.4	58.3 ± 0.2	40.8 ± 3	133.3 ± 7.9
4-Me-guaiacol	ng/m³	1 ± 0.2	0.4 ± 0.2	1.4 ± 0.2	5.6 ± 0.6
2,3- and 3,5-Dimethylbenzoic acid	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
2,4-Dimethylbenzoic acid	ng/m³	0.4 ± 0.3	168.1 ± 0.2	413.2 ± 8.5	1697.9 ± 91.9
2,5-Dimethylbenzoic acid	ng/m³	0 ± 0.2	5.4 ± 0.2	53.6 ± 0.4	120.2 ± 7
2,6-Dimethylbenzoic acid	ng/m³	0.2 ± 0.2	6.8 ± 0.2	10.6 ± 0.5	59.5 ± 3.2
3,4-Dimethylbenzoic acid	ng/m³	4.6 ± 0.2	13.8 ± 0.3	57.1 ± 0.8	166.9 ± 9.5
4-Formyl-guaiacol (vanillin)	ng/m³	12.7 ± 0.2	16.3 ± 0.2	17.7 ± 1.5	92 ± 9.5
4-Ethyl-guaiacol	ng/m³	0.2 ± 0.2	0 ± 0.2	10 ± 0.2	8 ± 0.7
Syringol	ng/m³	1.5 ± 0.2	21.2 ± 0.2	55.9 ± 1.2	283.6 ± 15.2
Levoglucosan	ng/m³	2 ± 0.3	1.7 ± 0.2	2.1 ± 0.7	7.8 ± 1
4-Allyl-guaiacol (eugenol)	ng/m³	0 ± 0.2	0.1 ± 0.2	0.7 ± 0.2	3.3 ± 0.6
Isoeugenol	ng/m³	0.2 ± 0.2	0.7 ± 0.2	7.1 ± 0.2	7.3 ± 1.3
Isophthalic acid	ng/m³	16.7 ± 0.4	1.5 ± 0.3	1 ± 4.5	2011.2 ± 136.2
Phthalic acid	ng/m³	4.7 ± 0.2	0.3 ± 0.2	2.4 ± 0.2	38.8 ± 2.2
Acetovanillone	ng/m³	11.5 ± 0.2	11 ± 0.2	0.3 ± 1.1	198.2 ± 18.8
Vanillic acid	ng/m³	0.5 ± 0.2	4.6 ± 0.2	7.9 ± 0.4	47.7 ± 2.6
4-Methyl-syringol	ng/m³	0.3 ± 0.2	0.4 ± 0.2	0.9 ± 0.2	23.7 ± 7.3
2,3-Dimethoxybenzoic acid	ng/m³	123.5 ± 0.2	381 ± 0.2	631.1 ± 19.2	440.3 ± 38.7
2,4-Dimethoxybenzoic acid	ng/m³	0.8 ± 0.2	0.4 ± 0.2	12 ± 0.2	14.1 ± 1
2,5-Dimethoxybenzoic acid	ng/m³	0.6 ± 0.2	0.4 ± 0.2	1.2 ± 0.2	64.9 ± 10.1
2,6-Dimethoxybenzoic acid	ng/m³	1.3 ± 0.2	2.5 ± 0.2	0.4 ± 0.6	26 ± 5.7
3,4-Dimethoxybenzoic acid	ng/m³	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	2.5 ± 0.4
3,5-Dimethoxybenzoic acid	ng/m³	7.8 ± 0.2	30.2 ± 0.2	19.7 ± 3.1	23 ± 3.6
Docosanoic acid (c22)	ng/m³	1.5 ± 1.1	5.9 ± 0.4	1.5 ± 0.2	0.6 ± 0.2
Homovanillic acid	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	5.4 ± 0.4
Syringaldehyde	ng/m³	0.6 ± 0.2	7.2 ± 0.2	0.9 ± 0.5	31 ± 1.8
cis-Pinonic acid	ng/m³	43.8 ± 0.4	100.6 ± 0.2	37.2 ± 5.4	62.9 ± 6.2
Syringic acid	ng/m³	0 ± 0.2	0.3 ± 0.2	6.3 ± 0.2	2.9 ± 0.4
Myristoleic acid	ng/m³	1 ± 0.4	4.3 ± 0.2	24 ± 1.2	38.6 ± 11.1
Traumatic acid	ng/m³	2.8 ± 0.6	1.8 ± 0.3	2.5 ± 12.8	44.3 ± 0.9
1,11-Undecanedicarboxylic acid	ng/m³	0.4 ± 0.2	1 ± 0.2	1.4 ± 0.4	6.2 ± 1.9

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Polars (*Continued*)					
Palmitoleic acid	ng/m³	0 ± 0.2	0 ± 0.2	3.9 ± 0.4	39.1 ± 2
1,12-Dodecanedicarboxylic acid	ng/m³	0.9 ± 0.2	0 ± 0.2	0 ± 0.2	0.6 ± 0.2
Elaidic acid	ng/m³	0.5 ± 0.2	2.2 ± 0.4	2.4 ± 1	7.7 ± 0.2
Isostearic acid	ng/m³	0.2 ± 0.2	1.9 ± 0.2	2.9 ± 0.2	14.4 ± 0.8
Dehydroabietic acid	ng/m³	34.3 ± 14.5	0 ± 0.2	84.5 ± 19.2	42.6 ± 0.2
Pimaric acid	ng/m³	1.9 ± 0.2	3.9 ± 0.2	7.6 ± 0.3	26.2 ± 1.5
Sandaracopimaric acid	ng/m³	0.7 ± 0.2	1.5 ± 0.2	1.8 ± 0.2	7.4 ± 0.5
Abietic acid	ng/m³	0.2 ± 0.2	0.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
Isopimaric acid	ng/m³	1.4 ± 0.2	1.4 ± 0.2	4.9 ± 0.5	5.5 ± 0.5
7-Oxodehydroabietic acid	ng/m³	0 ± 0.2	0 ± 0.2	2 ± 0.2	1.9 ± 0.2
Heneicosanoic acid (c21)	ng/m³	1.7 ± 0.2	0 ± 0.2	0 ± 0.2	9.6 ± 0.6
Tricosanoic acid	ng/m³	0 ± 0.2	0.5 ± 0.2	0.8 ± 0.2	0.2 ± 0.2
Tetracosanoic acid (c24)	ng/m³	0 ± 0.2	0.6 ± 0.2	4.9 ± 0.2	2.4 ± 0.5
Cholesterol	ng/m³	11.8 ± 4.1	21.6 ± 2.6	18.2 ± 1.2	11.7 ± 0.2
β-Sitosterol	ng/m³	1.4 ± 0.3	1.4 ± 0.3	0.8 ± 0.3	2 ± 0.2
Hexanoic acid (c6)	ng/m³	730.6 ± 0.2	1357.9 ± 0.2	983.3 ± 147.9	6155.9 ± 784.9
Heptanoic acid (c7)	ng/m³	398.8 ± 0.2	981.3 ± 0.2	956.4 ± 52.5	6465.2 ± 383.6
Benzoic acid	ng/m³	254.6 ± 0.4	368.7 ± 0.2	747.3 ± 20.6	2416.6 ± 164.3
Octanoic acid (c8)	ng/m³	203.6 ± 0.2	484.9 ± 0.2	551.9 ± 37	976.4 ± 103.7
o-Toluic	ng/m³	4.6 ± 0.3	15.8 ± 0.2	71 ± 1.2	211.4 ± 16.1
m-Toluic	ng/m³	5.8 ± 0.2	42.9 ± 0.5	123.1 ± 2.4	317.5 ± 18.2
Nonanoic acid (c9)	ng/m³	374 ± 1.2	331.7 ± 0.2	195.5 ± 35.1	238.2 ± 70.9
p-Toluic	ng/m³	2.5 ± 0.2	28 ± 0.2	46 ± 2.3	154.6 ± 13.5
Decanoic acid (c10)	ng/m³	172.3 ± 5.8	366.5 ± 0.7	214.5 ± 57.9	199.5 ± 72.1
Undecanoic acid (c11)	ng/m³	32 ± 0.2	65.5 ± 0.2	56.3 ± 5.2	36 ± 6.7
Dodecanoic (lauric) acid (c12)	ng/m³	8.9 ± 1.9	26.6 ± 0.2	11.6 ± 4.8	8.1 ± 3.1
Tridecanoic acid (c13)	ng/m³	33.3 ± 0.2	113.4 ± 0.2	103.3 ± 5.8	90.1 ± 7.9
Myristic acid (c14)	ng/m³	3.7 ± 2.4	18.4 ± 0.2	45 ± 23.7	100.2 ± 8.6
Pentadecanoic acid (c15)	ng/m³	7.7 ± 0.9	6.8 ± 0.5	59.2 ± 19.3	184.9 ± 19.5
Palmitic acid (c16)	ng/m³	107.4 ± 16.5	95.9 ± 15.8	240.1 ± 31.5	346.9 ± 7
Heptadecanoic acid (c17)	ng/m³	3.8 ± 1.2	3.2 ± 0.4	4.1 ± 21.9	58.2 ± 0.6
Oleic acid	ng/m³	0 ± 5.1	0.7 ± 11.9	57.5 ± 25.9	73.4 ± 0.2
Stearic acid (c18)	ng/m³	28.1 ± 4.7	43.1 ± 3.6	60.4 ± 8.6	80.8 ± 0.2
Nonadecanoic acid (c19)	ng/m³	0 ± 0.2	0 ± 0.2	0 ± 0.2	0 ± 0.2
Eicosanoic acid (c20)	ng/m³	14.7 ± 0.4	5.9 ± 0.3	2.5 ± 0.9	13.3 ± 4.1
Hopane and Steranes					
17α(H),21β(H)-22,29,30-Trisnorhopane	ng/m³	0.1 ± 0.01	0.1 ± 0.01	NA	0.1 ± 0.01
17α(H),21β(H)-30-Norhopane	ng/m³	0.2 ± 0.01	0.3 ± 0.02	NA	0.2 ± 0.02
17α(H),21β(H)-Hopane	ng/m³	0.3 ± 0.02	0.3 ± 0.02	NA	0.2 ± 0.01
17β(H),21α(H)-Hopane	ng/m³	0 ± 0.01	0 ± 0.01	NA	0.0
22(*S*)-17α(H),21β(H)-30-Homohopane	ng/m³	0.1	0.1	NA	0.0
22(*R*)-17α(H),21β(H)-30-Homohopane	ng/m³	0.1 ± 0.01	0.1 ± 0.01	NA	0.0
17β(H),21β(H)-Hopane	ng/m³	0.0	0.0	NA	0.0
22(*S*)-17α(H),21β(H)-30,31-Bishomohopane	ng/m³	0.0	0.0	NA	0.0
22(*R*)-17α(H),21β(H)-30,31-Bishomohopane	ng/m³	0.0	0.0	NA	0.0
22(*S*)-17α(H),21β(H)-30,31,32-Trisomohopane	ng/m³	0.0	0.0	NA	0.0

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Hopane and Steranes (*Continued*)					
22(*R*)-17α(H),21β(H)-30,31,32-Trishomohopane	ng/m³	0.0	0.0	NA	0.0
C27-20(*S*)5α(H),14α(H)-Cholestane	ng/m³	0.2 ± 0.01	0.2 ± 0.02	NA	0.0
C27-20(*R*)5α(H),14β(H)-Cholestane	ng/m³	0.1 ± 0.01	0.1 ± 0.01	NA	0.0
C27-20(*S*)5α(H),14β(H),17β(H)-Cholestane	ng/m³	0.5 ± 0.02	0.3 ± 0.02	NA	0.2 ± 0.01
C27-20(*R*)5α(H),14α(H),17α(H)-Cholestane & C29-20S13β(H),17α(H)-dia	ng/m³	0.2 ± 0.01	0.1 ± 0.01	NA	0.1
C28-20(*S*)5α(H),14α(H),17α(H)-Ergostane	ng/m³	0.1	0.1 ± 0.01	NA	0.0
C28-20(*R*)5α(H),14β(H),17β(H)-Ergostane	ng/m³	0.1 ± 0.01	0.1 ± 0.01	NA	0.0
C28-20(*S*)5α(H),14β(H),17β(H)-Ergostane	ng/m³	0.1	0.1	NA	0.1 ± 0.01
C28-20(*R*)5α(H),14α(H),17α(H)-Ergostane	ng/m³	0.1 ± 0.01	0.1	NA	0.1
C29-20(*S*)5α(H),14α(H),17α(H)-Stigmastane	ng/m³	0.1	0.1	NA	0.0
C29-20(*R*)5α(H),14β(H),17β(H)-Stigmastane	ng/m³	0.3 ± 0.02	0.2 ± 0.01	NA	0.0
C29-20(*S*)5α(H),14β(H),17β(H)-Stigmastane	ng/m³	0.0	0.0	NA	0.0
C29-20(*R*)5α(H),14α(H),17α(H)-Stigmastane	ng/m³	0.1 ± 0.01	0.1 ± 0.01	NA	0.1 ± 0.01
Volatile Organic Compounds (VOCs)					
Acetylene	µg/m³	0.3 ± 0.05	0.3 ± 0.05	1.2 ± 0.21	2.9 ± 0.49
Ethene	µg/m³	0.3 ± 0.22	0.2 ± 0.12	0.8 ± 0.67	0.2 ± 0.17
Ethane	µg/m³	7.6 ± 0.38	5.9 ± 0.29	4.3 ± 0.22	6.4 ± 0.32
Propene	µg/m³	0.3 ± 0.22	0.2 ± 0.12	1 ± 0.69	3.5 ± 2.51
Propane	µg/m³	2.1 ± 0.14	2 ± 0.13	0.8 ± 0.05	1.1 ± 0.07
1,3-Butadiene	µg/m³	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02
1-Butene	µg/m³	0.1 ± 0.03	0.1 ± 0.03	0.2 ± 0.03	0.9 ± 0.05
Isobutylene	µg/m³	1.2 ± 0.06	3.2 ± 0.16	5.6 ± 0.28	6.1 ± 0.31
c-2-Butene	µg/m³	0.1 ± 0.03	0 ± 0.03	0 ± 0.03	0.3 ± 0.03
t-2-Butene	µg/m³	0.5 ± 0.3	0.2 ± 0.14	0.4 ± 0.29	0.5 ± 0.33
n-Butane	µg/m³	0.9 ± 0.05	0.8 ± 0.04	0.7 ± 0.04	1.3 ± 0.07
Isobutane	µg/m³	0.6 ± 0.09	0.5 ± 0.07	0.3 ± 0.05	0.5 ± 0.08
Isopentane	µg/m³	0.4 ± 0.07	0.4 ± 0.07	0.5 ± 0.09	0.9 ± 0.18
n-Pentane	µg/m³	0.2 ± 0.04	0.3 ± 0.03	0.3 ± 0.03	0.8 ± 0.09
1-Pentene	µg/m³	0 ± 0.03	0 ± 0.03	0.1 ± 0.03	0.2 ± 0.08
2-Methyl-1-butene	µg/m³	0 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03
Isoprene	µg/m³	0 ± 0.03	0 ± 0.03	0 ± 0.03	0 ± 0.03
t-2-Pentene	µg/m³	0 ± 0.03	0 ± 0.03	0 ± 0.03	0.1 ± 0.03
c-2-Pentene	µg/m³	0 ± 0.03	0 ± 0.03	0 ± 0.03	0.1 ± 0.03
2-Methyl-2-butene	µg/m³	0 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03
2,2-Dimethylbutane	µg/m³	0 ± 0.04	0.1 ± 0.04	0 ± 0.04	0 ± 0.04
Cyclopentene	µg/m³	0 ± 0.04	0 ± 0.04	0 ± 0.04	0.1 ± 0.04
Cyclopentane	µg/m³	0 ± 0.03	0.1 ± 0.03	0 ± 0.03	0.1 ± 0.04
2,3-Dimethylbutane	µg/m³	0 ± 0.04	1 ± 0.06	0 ± 0.04	0.1 ± 0.04
2-Methylpentane	µg/m³	0.1 ± 0.04	6.8 ± 0.59	0.1 ± 0.04	0.4 ± 0.04
3-Methylpentane	µg/m³	0.1 ± 0.04	7.5 ± 0.39	0.2 ± 0.04	0.3 ± 0.04
2-Methyl-1-pentene	µg/m³	0 ± 0.04	0 ± 0.04	0.1 ± 0.08	0.2 ± 0.22
n-Hexane	µg/m³	0.1 ± 0.04	15.3 ± 0.96	0.2 ± 0.04	0.4 ± 0.04
t-2-Hexene	µg/m³	0 ± 0.04	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.04
c-2-Hexene	µg/m³	0 ± 0.04	0 ± 0.04	0 ± 0.04	0 ± 0.04

(*Table continues on next page*)

[a] NA indicates sample not collected as defined by protocol.

Table B.2 (*Continued*). ACES Characterization of Exposure Atmosphere Composition in Mouse Chamber, April 2010

Exposure Atmosphere Composition	Units (± SD)	Control	Exposure Level[a] Low	Mid	High
Volatile Organic Compounds (VOCs) (*Continued*)					
1,3-Hexadiene (trans)	µg/m³	0 ± 0.04	0 ± 0.04	0 ± 0.04	0 ± 0.04
Methylcyclopentane	µg/m³	0.1 ± 0.04	7.8 ± 0.44	0.2 ± 0.04	0.5 ± 0.04
2,4-Dimethylpentane	µg/m³	0 ± 0.04	2.3 ± 0.17	0.1 ± 0.04	0.2 ± 0.04
Benzene	µg/m³	0.3 ± 0.03	0.5 ± 0.03	1.3 ± 0.06	4.1 ± 0.21
Cyclohexane	µg/m³	0.1 ± 0.04	1.8 ± 0.09	0.6 ± 0.04	1.5 ± 0.08
2-Methylhexane	µg/m³	0 ± 0.04	4.4 ± 0.29	0.1 ± 0.04	0.2 ± 0.04
2,3-Dimethylpentane	µg/m³	0.2 ± 0.04	2.4 ± 0.18	0.5 ± 0.04	0.6 ± 0.05
Cyclohexene	µg/m³	0 ± 0.04	0.1 ± 0.04	0.1 ± 0.04	0.2 ± 0.04
3-Methylhexane	µg/m³	0 ± 0.04	3 ± 0.15	0.1 ± 0.04	0.3 ± 0.04
1,3-Dimethylcyclopentane (cis)	µg/m³	0 ± 0.04	0.2 ± 0.04	0 ± 0.04	0.1 ± 0.04
1-Heptene	µg/m³	0 ± 0.04	0.4 ± 0.04	0.2 ± 0.04	0.6 ± 0.04
2,2,4-Trimethylpentane	µg/m³	0.1 ± 0.05	0.2 ± 0.05	0.2 ± 0.05	0.7 ± 0.05
n-Heptane	µg/m³	0.1 ± 0.04	0.2 ± 0.04	0.2 ± 0.04	0.6 ± 0.06
2,3-Dimethyl-2-pentene	µg/m³	0 ± 0.04	0 ± 0.04	0 ± 0.04	0 ± 0.04
Methylcyclohexane	µg/m³	0.1 ± 0.04	0.1 ± 0.04	1.1 ± 0.26	3.5 ± 0.81
2,3,4-Trimethylpentane	µg/m³	0 ± 0.05	0 ± 0.05	0 ± 0.05	0.1 ± 0.05
Toluene	µg/m³	0.5 ± 0.04	21 ± 1.05	0.9 ± 0.04	2.1 ± 0.11
2-Methylheptane	µg/m³	0 ± 0.05	0.1 ± 0.05	0.2 ± 0.05	0.5 ± 0.05
4-Methylheptane	µg/m³	0 ± 0.05	0 ± 0.05	0.1 ± 0.05	0.2 ± 0.05
3-Methylheptane	µg/m³	0 ± 0.05	0 ± 0.05	0.1 ± 0.05	0.4 ± 0.14
n-Octane	µg/m³	0 ± 0.05	0.1 ± 0.05	0.5 ± 0.05	1.5 ± 0.07
Ethylbenzene	µg/m³	0.4 ± 0.05	0.3 ± 0.05	0.3 ± 0.05	1 ± 0.05
m&*p*-Xylene	µg/m³	1.1 ± 0.07	0.9 ± 0.05	0.6 ± 0.05	1.7 ± 0.08
Styrene	µg/m³	0 ± 0.05	0 ± 0.05	0 ± 0.05	0 ± 0.05
o-Xylene	µg/m³	0.3 ± 0.05	0.3 ± 0.05	0.2 ± 0.05	0.6 ± 0.05
n-Nonane	µg/m³	0.1 ± 0.06	0.2 ± 0.06	0.8 ± 0.06	2.4 ± 0.12
Isopropylbenzene	µg/m³	0 ± 0.05	0 ± 0.05	0.1 ± 0.05	0.3 ± 0.05
n-Propylbenzene	µg/m³	0 ± 0.05	0 ± 0.05	0.2 ± 0.05	0.6 ± 0.05
Alpha-pinene	µg/m³	0 ± 0.06	0.2 ± 0.06	0.1 ± 0.06	0 ± 0.06
3-Ethyltoluene	µg/m³	0.1 ± 0.05	0.1 ± 0.05	0.5 ± 0.05	1.3 ± 0.07
4-Ethyltoluene	µg/m³	0 ± 0.05	0 ± 0.05	0.2 ± 0.05	0.7 ± 0.05
1,3,5-Trimethylbenzene	µg/m³	0 ± 0.05	0 ± 0.05	0.2 ± 0.06	0.6 ± 0.18
o-Ethyltoluene	µg/m³	0 ± 0.05	0 ± 0.05	0.2 ± 0.05	0.5 ± 0.05
1,2,4-Trimethylbenzene+ t-butylbenzene	µg/m³	0 ± 0.05	0 ± 0.05	0 ± 0.05	0 ± 0.05
n-Decane	µg/m³	0.1 ± 0.07	0.3 ± 0.1	2.5 ± 0.84	8.1 ± 2.72
1,2,3-Trimethylbenzene	µg/m³	0 ± 0.05	0.1 ± 0.05	0.3 ± 0.11	1 ± 0.33
Indan	µg/m³	0 ± 0.05	0 ± 0.05	0.1 ± 0.05	0.2 ± 0.05
1,3-Diethylbenzene	µg/m³	0 ± 0.06	0 ± 0.06	0.2 ± 0.06	0.7 ± 0.06
1,4-Diethylbenzene	µg/m³	0 ± 0.06	0 ± 0.06	0.2 ± 0.09	0.7 ± 0.27
n-Butylbenzene	µg/m³	0 ± 0.06	0 ± 0.06	0 ± 0.06	0 ± 0.06
n-Undecane	µg/m³	0.2 ± 0.07	0.4 ± 0.07	2.6 ± 0.24	8.6 ± 0.81

[a] NA indicates sample not collected as defined by protocol.

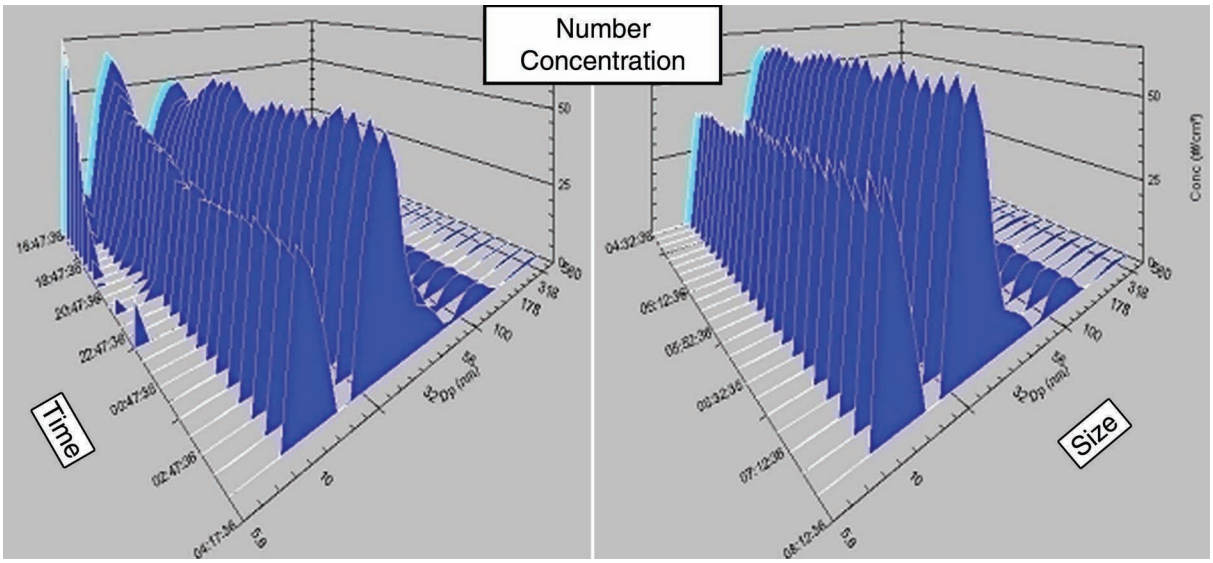

Figure B.5. Particle size distribution at the control exposure level chamber, April 7, 2010.

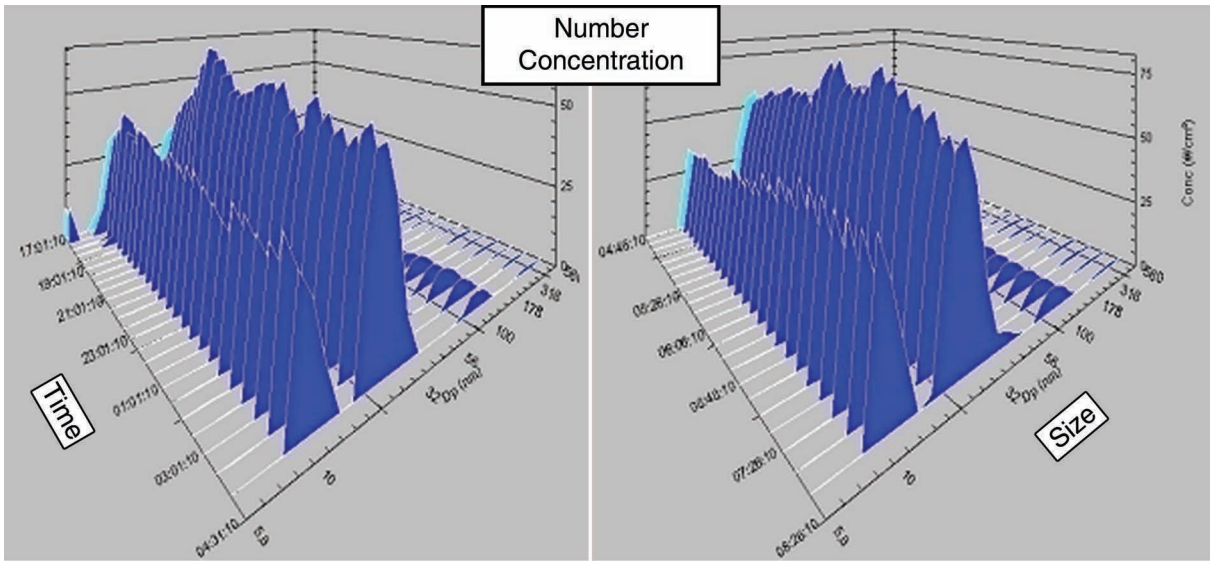

Figure B.6. Particle size distribution at the low-level exposure chamber, April 6, 2010.

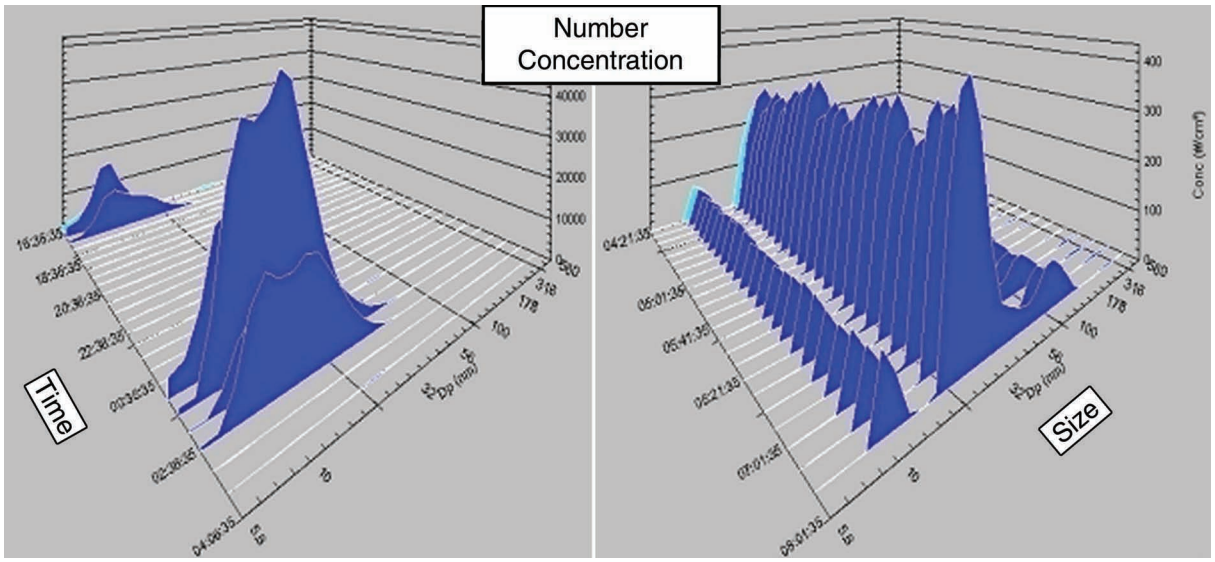

Figure B.7. Particle size distribution at the mid-level exposure chamber, April 11, 2010.

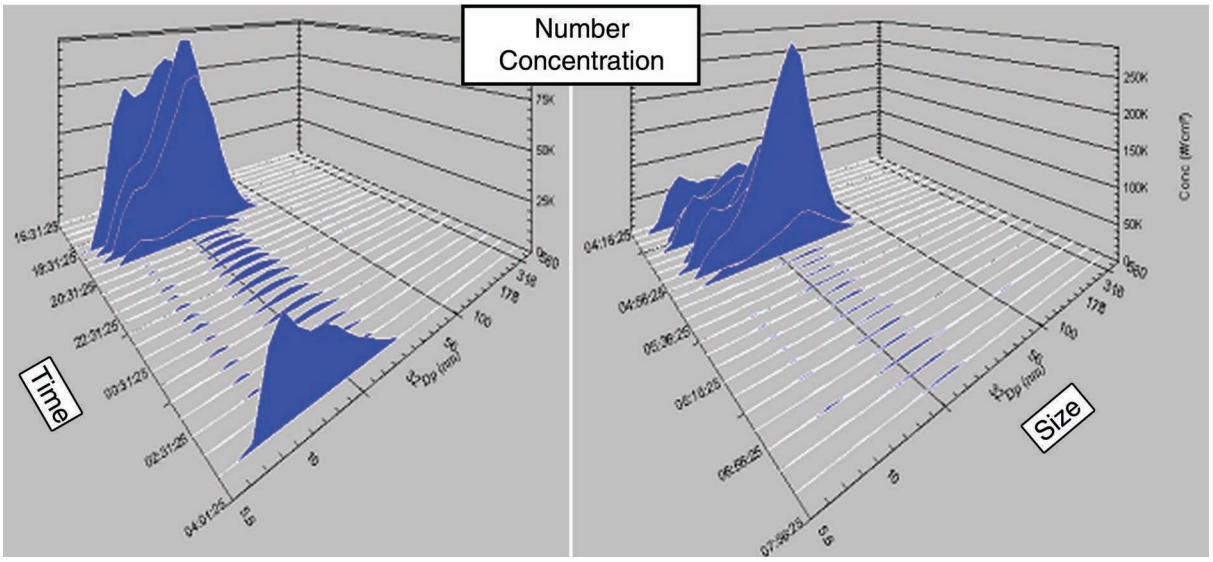

Figure B.8. Particle size distribution at the high-level exposure chamber, April 12, 2010.

APPENDIX C. Examples of Statistical Approach

EXAMPLE 1. ENDPOINT REQUIRING LOGARITHMIC TRANSFORMATION BEFORE ANOVA: TEAC IN RATS AT 13 WEEKS

Boxplots of the TEAC (μM) data for males and females are shown in Figure C.1. These plots indicate a higher degree of variation for control animals than for those with DE exposure. Standard deviations (SDs) relative to the exposure group means are shown in Figure C.2, which shows increasing variation with increasing mean for the raw data (left). The statistical evaluation of the equality of variances among exposure groups in each of the sexes indicated substantial evidence of heteroscedasticity (males $P = 0.010$, females $P = 0.007$; Levene's test). However, after logarithmic transformation of the data, the SD versus the mean values (shown on the right side of Figure C.2) exhibits a weak pattern of increasing variation with increasing mean, with the two largest exposure group means associated with the two largest SDs. However, there was no compelling statistical evidence of heterogeneity of variances (males, $P = 0.19$; females, $P = 0.34$). This result suggests that the normalization helped to satisfy the requirement for equal variance among groups.

The patterns of TEAC responses for males and females in the original and logarithmically transformed scales (shown in Figure C.3) are similar. When the logarithmically

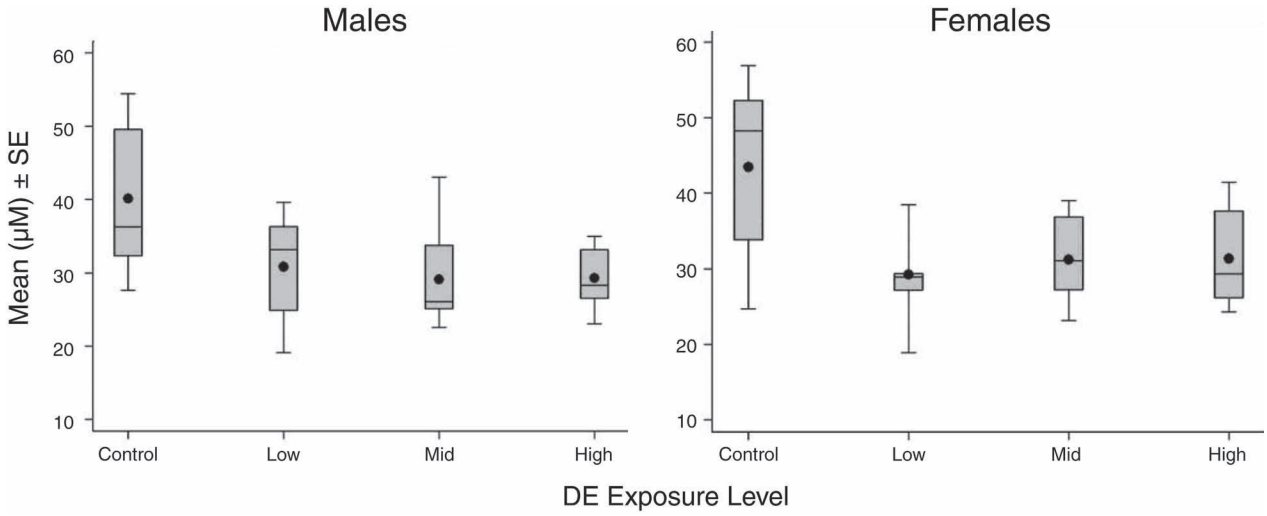

Figure C.1. Boxplots of TEAC data by sex.

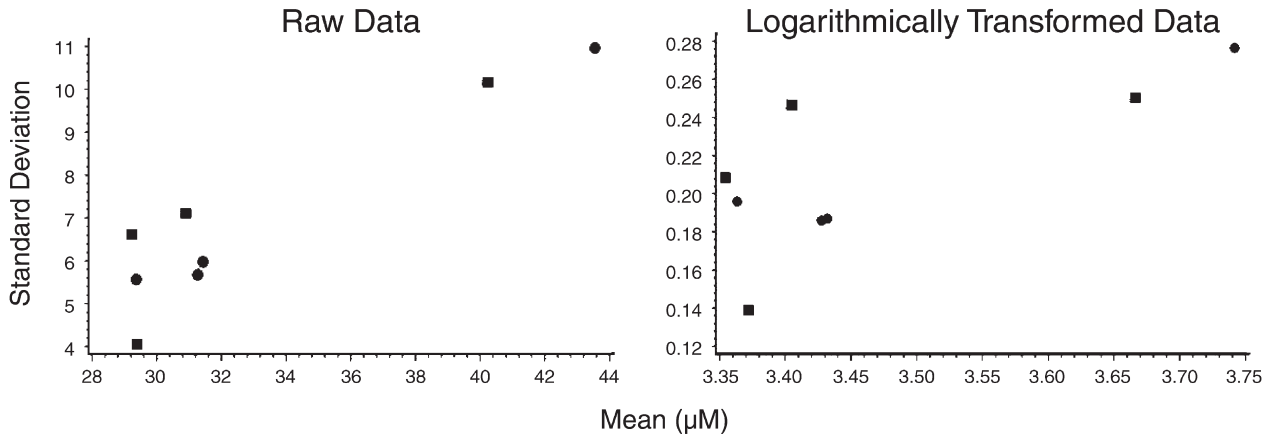

Figure C.2. Relation between exposure group standard deviations in TEAC and mean values for raw data (left) and logarithmically transformed data (right) in males (■) and females (●).

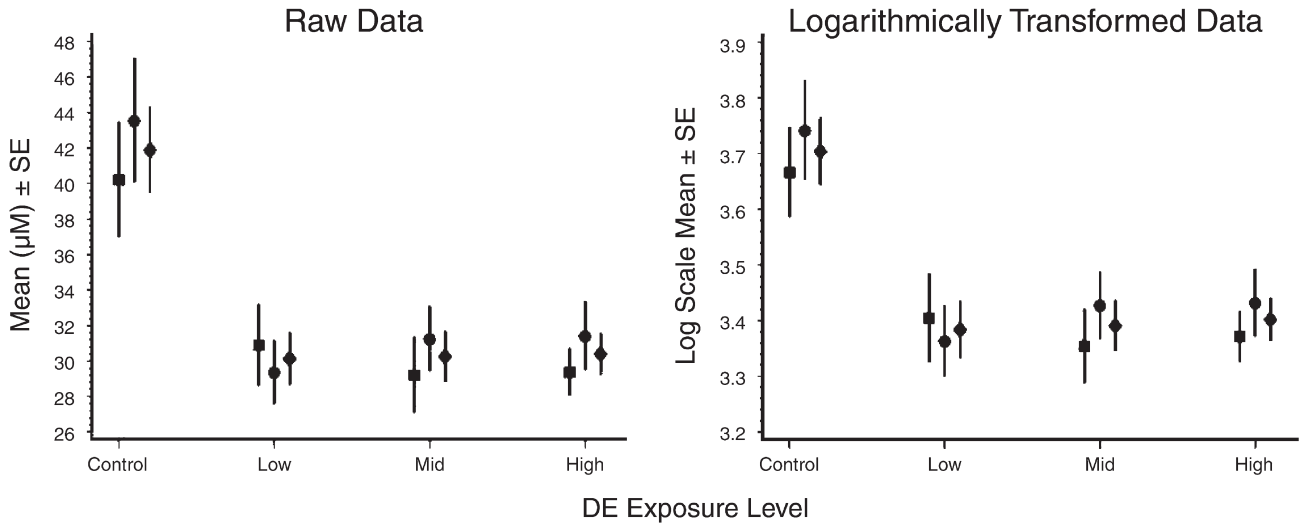

Figure C.3. Patterns of response in TEAC for males (■), females (●), and both sexes combined (◆) for raw data and logarithmically transformed data. Standard errors for combined sexes were calculated after adjustment for overall differences in response by sex.

transformed data were analyzed by sex, the ANOVA gave highly significant evidence of differences between exposure group responses ($P < 0.001$) for both males and females. Duncan's multiple comparison test (using the residual mean square from the sex-specific ANOVAs) indicated statistically significant ($P < 0.01$) differences between DE-exposed groups and controls for both sexes (with the exception of the males in the low- and high-level exposure groups, with $P < 0.05$).

A two-way ANOVA gave no substantive evidence to indicate differences in overall response between sexes (effect by sex, $P = 0.34$). The sex × exposure interaction term, which quantifies differential exposure-related response between sexes, also gave no indication that DE effects differed across sexes ($P = 0.81$). Consequently, a pooled analysis of the effects across sexes was considered to be appropriate. A two-way ANOVA (sex and exposure as factors with no interaction) of the logarithmically transformed data confirmed the strong evidence of exposure effects across sexes, with highly statistically significant ($P < 0.01$) differences between controls and all DE exposure group means.

EXAMPLE 2. ENDPOINT WITH EFFECTS POOLED ACROSS SEX: DL_{CO} IN RATS AT 13 WEEKS

Boxplots of DL_{CO} data from both sexes of rats at 13 weeks are shown in Figure C.4. These plots give no obvious indication of asymmetry or skewness in the data distributions around the exposure group means. For males, there appears to be little, if any, difference in variability relative to the observed mean values, but for females there is a suggestion of increasing variability with increasing magnitude of mean values. The SDs versus means plot for the exposure groups (Figure C.5) shows no consistent pattern across mean values (both sexes), although the two lowest mean values for females were associated with smaller SDs than observed for the rest of the exposure groups. Levene's homogeneity of variance test across all exposure groups provided no substantial evidence to suggest heterogeneity of variance ($P = 0.19$); consequently, the data were not transformed.

Further graphical representations of the summary DL_{CO} data (mean ± standard error [SE] for exposure groups) are provided in Figure C.6A and B. Figure C.6A (raw data) shows clear, but consistent (and approximately constant) differences between observed male and female mean values in all exposure groups. In this plot, mean values for males and females in each exposure group i($\bar{X}_{Mi}$, $\bar{X}_{Fi}$) were averaged to obtain an overall mean for exposure group i:

$$\bar{X}_{Bi} = \frac{\bar{X}_{Mi} + \bar{X}_{Fi}}{2}.$$

The exposure group means ($\bar{X}_{Bi}$) are shown with their corresponding SEs. These SEs do not encompass between-sex variation, but instead are limited to variation around sex-specific mean values. They are based on an estimate of a common variance for the two sexes in each exposure group (as is used in a t test), calculated as follows:

$$\hat{Var}(X_{Mi}) = \hat{Var}(X_{Fi}) = \hat{\sigma}^2 = \frac{(n_{Mi} - 1)s_{Mi}^2 + (n_{Fi} - 1)s_{Fi}^2}{n_{Mi} + n_{Fi} - 2}$$

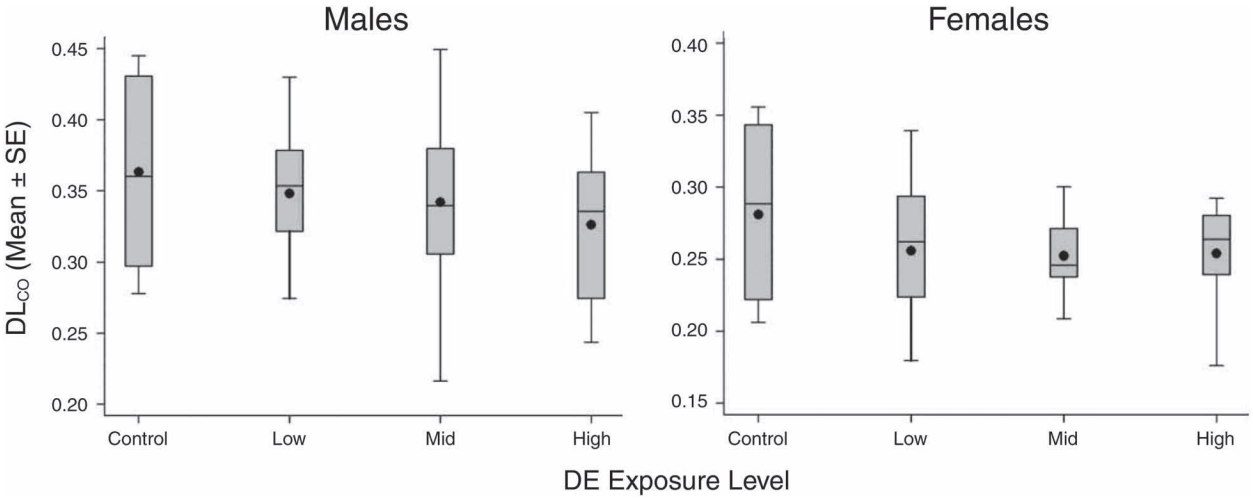

Figure C.4. Boxplots of DL_{CO} (mL/min/mm Hg) data by sex.

where n_{Mi} and n_{Fi} are the numbers of male and female animals in exposure group i, and s^2_{Mi} and s^2_{Fi} are the observed sample variances for the two sexes in exposure group i.

The SE of the mean $\bar{X}_{Bi}$ is calculated as follows:

$$\text{SE}\,\bar{X}_{Bi} = \tfrac{1}{2}\hat{\sigma}\sqrt{\frac{1}{n_{Mi}} + \frac{1}{n_{Fi}}}$$

Note that for $n_{Mi} = n_{Fi} = n_i$, SE $\bar{X}_{Bi} = \hat{\sigma}/\sqrt{2n_i}$. Thus, after adjustment for differences between sexes in mean values, the SE of the across-sex average will be approximately 30% smaller than the SEs of the sex-based means (under the assumption of equal variances across sexes), with the following calculation:

$$\text{SE}\,\bar{X}_{Mi} \approx \text{SE}\,\bar{X}_{Fi} \approx \frac{\hat{\sigma}}{\sqrt{n_i}}.$$

Figure C.6B shows the DL_{CO} data after normalizing for differences in mean values between males and females. This was accomplished by subtracting the overall response mean for each sex from each DL_{CO} value, which is essentially what is done in a two-way ANOVA with sex as one of the explanatory factors. The resulting gradients of exposure-related response (and magnitudes of the SE values) in Figure C.6B are identical (for each respective sex) to those in Figure C.6A. However, with the adjustment (or normalization) for overall differences in response between sexes, exposure-related effects for males and females can be more readily compared and assessed along with the combined evidence of effects across sexes shown in Figure C.6B.

Figure C.6B suggests virtually identical decreasing exposure-related trends in DL_{CO} in males and females. Individual ANOVAs for each of the sexes did not give statistically significant evidence of differences in mean values among exposure groups (males, $P = 0.55$; females, $P = 0.44$) or compelling evidence of exposure-related trends (males, $P = 0.16$; females, $P = 0.19$). A two-way ANOVA gave strong evidence of systematic differences in DL_{CO} values between sexes ($P < 0.001$), but no evidence of sex-based differences in exposure effects, as measured by the sex × exposure interaction term in the model ($P = 0.93$). However, based on a two-way analysis (sex and exposure with no interaction term), which adjusts for overall differences in response between sexes, there was statistically significant evidence of a decreasing trend in DL_{CO} across sexes ($P = 0.05$).

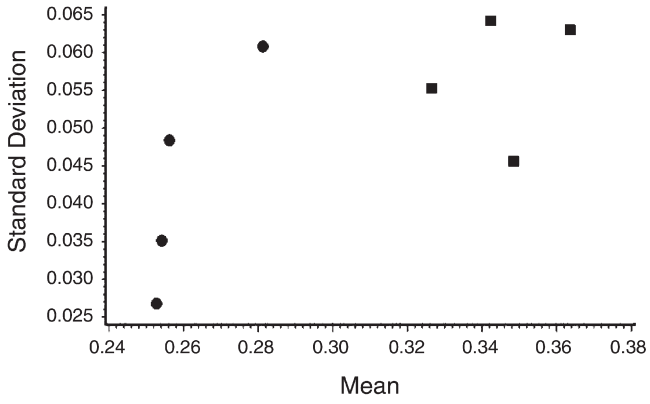

Figure C.5. Relation between exposure group DL_{CO} mean values and standard deviations in males (■) and females (●).

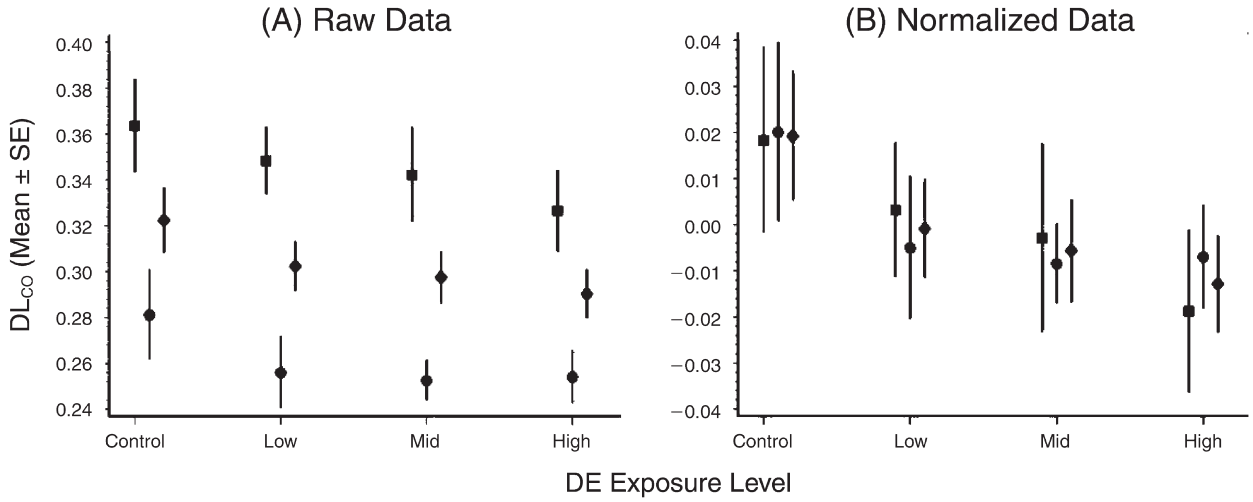

Figure C.6. Pattern of response in DL_{CO} (mL/min/mm Hg) for males (■), females (●), and both sexes combined (◆): (A) for raw data; and (B) for data normalized by the mean of overall response by sex. Standard errors for combined sexes were calculated after adjustment for overall differences in response by sex.

APPENDIX D. Biologic Response in Mice

Table D.1. Body Weights in Mice at End of 4 Weeks of Exposure*

Time Point / Group	Males		Females	
	n	Mean ± SEM	*n*	Mean ± SEM
Initial				
Control	30	19.2 ± 0.3	30	15.2 ± 0.2
Low	30	19.7 ± 0.3	30	15.2 ± 0.2
Mid	30	19.7 ± 0.3	30	15.4 ± 0.2
High	30	19.6 ± 0.3	30	15.4 ± 0.3
Trend[a]		0.456		0.541
4 Weeks				
Control	30	22.8 ± 0.4	30	19.7 ± 0.2
Low	30	23.8 ± 0.2	30	19.3 ± 0.2
Mid	30	23.5 ± 0.4	30	19.2 ± 0.3
High	30	23.2 ± 0.3	30	17.9 ± 0.5[b]
Trend[a]		0.536		<0.001 (−)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.01$.

* See Table 2 for units.

Table D.2. Body Weights in Mice at 1, 2, and 3 Months of Exposure*

Time Point / Group	Males		Females	
	n	Mean ± SEM	*n*	Mean ± SEM
Initial				
Control	30	19.5 ± 0.3	30	15.3 ± 0.2
Low	30	19.1 ± 0.3	30	15.2 ± 0.2
Mid	30	19.4 ± 0.2	30	15.1 ± 0.3
High	30	19.4 ± 0.3	30	15.1 ± 0.2
Trend[a]		0.931		0.430
1 Month				
Control	30	23.3 ± 0.3	30	19.8 ± 0.2
Low	30	23.1 ± 0.2	30	19.5 ± 0.2
Mid	30	23.0 ± 0.3	30	19.0 ± 0.2
High	30	23.1 ± 0.2	30	17.5 ± 0.5[b]
Trend[a]		0.593		0.000 (−)
2 Months				
Control	30	24.9 ± 0.3	30	21.3 ± 0.2
Low	30	24.8 ± 0.2	30	20.7 ± 0.2
Mid	30	25.2 ± 0.2	30	21.1 ± 0.2
High	30	25.1 ± 0.2	30	20.7 ± 0.2
Trend[a]		0.413		0.106
3 Months				
Control	30	25.9 ± 0.4	30	22.5 ± 0.2
Low	30	26.0 ± 0.2	30	22.3 ± 0.3
Mid	30	27.0 ± 0.2	30	22.8 ± 0.3
High	30	26.6 ± 0.2	30	22.1 ± 0.3
Trend[a]		0.020 (+)		0.470

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.01$.

* See Table 2 for units.

Table D.3. Organ Weights in Mice at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Brain						
Female						
Control	10	0.429 ± 0.004	10	0.0210 ± 0.0003	10	1.0000 ± 0.0000
Low	10	0.426 ± 0.007	10	0.0213 ± 0.0003	10	1.0000 ± 0.0000
Mid	10	0.428 ± 0.005	10	0.0218 ± 0.0005	10	1.0000 ± 0.0000
High	10	0.428 ± 0.004	10	0.0214 ± 0.0003	10	1.0000 ± 0.0000
Trend[a]		0.983		0.353		—
Male						
Control	10	0.428 ± 0.004	10	0.0186 ± 0.0002	10	1.0000 ± 0.0000
Low	10	0.441 ± 0.004	10	0.0184 ± 0.0002	10	1.0000 ± 0.0000
Mid	10	0.427 ± 0.008	10	0.0178 ± 0.0003	10	1.0000 ± 0.0000
High	10	0.426 ± 0.007	10	0.0181 ± 0.0003	10	1.0000 ± 0.0000
Trend[a]		0.442		0.100 (−)		—
Heart						
Female						
Control	10	0.124 ± 0.007	10	0.0060 ± 0.0003	10	0.288 ± 0.015
Low	10	0.117 ± 0.006	10	0.0058 ± 0.0002	10	0.273 ± 0.010
Mid	10	0.115 ± 0.003	10	0.0059 ± 0.0002	10	0.269 ± 0.006
High	10	0.118 ± 0.005	10	0.0058 ± 0.0002	10	0.275 ± 0.010
Trend[a]		0.409		0.595		0.371
Male						
Control	10	0.126 ± 0.004	10	0.0055 ± 0.0001	10	0.294 ± 0.009
Low	10	0.135 ± 0.006	10	0.0056 ± 0.0002	10	0.305 ± 0.012
Mid	10	0.137 ± 0.009	10	0.0057 ± 0.0003	10	0.320 ± 0.015
High	10	0.142 ± 0.006	10	0.0061 ± 0.0003	10	0.336 ± 0.018
Trend[a]		0.073 (+)		0.104		0.028 (+)
Kidneys						
Female						
Control	10	0.237 ± 0.005	10	0.0116 ± 0.0002	10	0.552 ± 0.008
Low	10	0.230 ± 0.004	10	0.0115 ± 0.0002	10	0.541 ± 0.010
Mid	10	0.233 ± 0.006	10	0.0119 ± 0.0003	10	0.545 ± 0.017
High	10	0.241 ± 0.005	10	0.0120 ± 0.0002	10	0.563 ± 0.009
Trend[a]		0.531		0.124		0.488
Male						
Control	10	0.302 ± 0.009	10	0.0131 ± 0.0003	10	0.707 ± 0.021
Low	10	0.334 ± 0.009	10	0.0139 ± 0.0003	10	0.757 ± 0.017
Mid	10	0.294 ± 0.014	10	0.0123 ± 0.0005	10	0.693 ± 0.036
High	10	0.318 ± 0.019	10	0.0134 ± 0.0005	10	0.745 ± 0.038
Trend[a]		0.895		0.657		0.701

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.3 (*Continued*). Organ Weights in Mice at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Liver						
Female						
Control	10	1.03 ± 0.03	10	0.051 ± 0.002	10	2.41 ± 0.09
Low	10	1.04 ± 0.03	10	0.052 ± 0.001	10	2.45 ± 0.07
Mid	10	0.94 ± 0.04	10	0.047 ± 0.002	10	2.19 ± 0.09
High	10	0.93 ± 0.04	10	0.046 ± 0.002	10	2.17 ± 0.10
Trend[a]		0.016 (−)		0.008 (−)		0.018 (−)
Male						
Control	9	1.15 ± 0.05	9	0.050 ± 0.002	9	2.68 ± 0.11
Low	10	1.18 ± 0.04	10	0.049 ± 0.001	10	2.68 ± 0.09
Mid	10	1.18 ± 0.04	10	0.049 ± 0.001	10	2.78 ± 0.09
High	10	1.14 ± 0.05	10	0.048 ± 0.002	10	2.67 ± 0.11
Trend[a]		0.911		0.423		0.863
Lung						
Female						
Control	9	0.189 ± 0.005	9	0.0092 ± 0.0002	9	0.44 ± 0.01
Low	10	0.189 ± 0.006	10	0.0095 ± 0.0003	10	0.45 ± 0.02
Mid	10	0.188 ± 0.007	10	0.0095 ± 0.0002	10	0.44 ± 0.02
High	10	0.204 ± 0.004	10	0.0102 ± 0.0003[b]	10	0.48 ± 0.01
Trend[a]		0.093 (+)		0.011 (+)		0.130
Male						
Control	9	0.195 ± 0.007	9	0.0085 ± 0.0002	9	0.46 ± 0.01
Low	10	0.204 ± 0.005	10	0.0085 ± 0.0002	10	0.46 ± 0.01
Mid	10	0.193 ± 0.005	10	0.0081 ± 0.0002	10	0.45 ± 0.01
High	10	0.207 ± 0.008	10	0.0088 ± 0.0003	10	0.49 ± 0.02
Trend[a]		0.378		0.652		0.193
Ovaries						
Female						
Control	10	0.014 ± 0.001	10	0.0007 ± 0.0000	10	0.033 ± 0.002
Low	10	0.011 ± 0.001	10	0.0006 ± 0.0001	10	0.026 ± 0.003
Mid	10	0.014 ± 0.001	10	0.0007 ± 0.0001	10	0.034 ± 0.004
High	10	0.014 ± 0.001	10	0.0007 ± 0.0001	10	0.032 ± 0.003
Trend[a]		0.694		0.540		0.686
Testes						
Male						
Control	10	0.170 ± 0.009	10	0.0074 ± 0.0004	10	0.40 ± 0.02
Low	10	0.154 ± 0.010	10	0.0064 ± 0.0004	10	0.35 ± 0.02
Mid	10	0.165 ± 0.006	10	0.0069 ± 0.0002	10	0.39 ± 0.01
High	10	0.152 ± 0.009	10	0.0064 ± 0.0003	10	0.35 ± 0.02
Trend[a]		0.244		0.087 (−)		0.269

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.3 (*Continued*). Organ Weights in Mice at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Thymus						
Female						
Control	10	0.076 ± 0.003	10	0.0037 ± 0.0001	10	0.177 ± 0.006
Low	10	0.075 ± 0.004	10	0.0038 ± 0.0002	10	0.177 ± 0.011
Mid	10	0.079 ± 0.003	10	0.0040 ± 0.0002	10	0.184 ± 0.007
High	10	0.079 ± 0.004	10	0.0039 ± 0.0002	10	0.185 ± 0.011
Trend[a]		0.429		0.255		0.443
Male						
Control	10	0.056 ± 0.002	10	0.0024 ± 0.0001	10	0.131 ± 0.005
Low	10	0.056 ± 0.003	10	0.0023 ± 0.0001	10	0.126 ± 0.006
Mid	10	0.060 ± 0.002	10	0.0025 ± 0.0001	10	0.140 ± 0.006
High	10	0.061 ± 0.003	10	0.0026 ± 0.0002	10	0.144 ± 0.010
Trend[a]		0.133		0.235		0.095 (+)
Urinary Bladder						
Female						
Control	10	0.021 ± 0.001	10	0.0010 ± 0.0001	10	0.048 ± 0.003
Low	10	0.019 ± 0.001	10	0.0010 ± 0.0001	10	0.045 ± 0.003
Mid	10	0.019 ± 0.002	10	0.0010 ± 0.0001	10	0.045 ± 0.005
High	10	0.020 ± 0.001	10	0.0010 ± 0.0001	10	0.047 ± 0.003
Trend[a]		0.824		0.999		0.849
Male						
Control	10	0.026 ± 0.001	10	0.0011 ± 0.0000	10	0.062 ± 0.002
Low	10	0.033 ± 0.001[b]	10	0.0014 ± 0.0001[b]	10	0.074 ± 0.003
Mid	10	0.026 ± 0.002	10	0.0011 ± 0.0001	10	0.062 ± 0.004
High	10	0.028 ± 0.002	10	0.0012 ± 0.0001	10	0.065 ± 0.005
Trend[a]		0.789		0.500		0.967

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.4. Organ Weights in Mice at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Brain						
Female						
Control	10	0.449 ± 0.011	10	0.0201 ± 0.0003	10	1.0000 ± 0.0000
Low	10	0.456 ± 0.006	10	0.0205 ± 0.0003	10	1.0000 ± 0.0000
Mid	10	0.450 ± 0.005	10	0.0196 ± 0.0004	10	1.0000 ± 0.0000
High	10	0.470 ± 0.009	10	0.0213 ± 0.0006	10	1.0000 ± 0.0000
Trend[a]		0.124		0.189		—
Male						
Control	10	0.453 ± 0.007	10	0.0174 ± 0.0002	10	1.0000 ± 0.0000
Low	10	0.454 ± 0.006	10	0.0172 ± 0.0004	10	1.0000 ± 0.0000
Mid	10	0.446 ± 0.004	10	0.0165 ± 0.0002	10	1.0000 ± 0.0000
High	10	0.446 ± 0.006	10	0.0170 ± 0.0003	10	1.0000 ± 0.0000
Trend[a]		0.229		0.146		—
Heart						
Female						
Control	10	0.134 ± 0.004	10	0.0060 ± 0.0002	10	0.300 ± 0.010
Low	10	0.130 ± 0.004	10	0.0058 ± 0.0002	10	0.285 ± 0.008
Mid	10	0.133 ± 0.005	10	0.0058 ± 0.0002	10	0.296 ± 0.009
High	10	0.132 ± 0.003	10	0.0060 ± 0.0001	10	0.282 ± 0.008
Trend[a]		0.890		0.783		0.289
Male						
Control	10	0.160 ± 0.007	10	0.0061 ± 0.0002	10	0.353 ± 0.013
Low	10	0.162 ± 0.007	10	0.0061 ± 0.0002	10	0.358 ± 0.015
Mid	10	0.155 ± 0.005	10	0.0057 ± 0.0001	10	0.347 ± 0.010
High	10	0.162 ± 0.006	10	0.0062 ± 0.0002	10	0.364 ± 0.016
Trend[a]		0.944		0.769		0.692
Kidneys						
Female						
Control	10	0.274 ± 0.004	10	0.0123 ± 0.0002	10	0.612 ± 0.015
Low	10	0.273 ± 0.008	10	0.0123 ± 0.0002	10	0.599 ± 0.014
Mid	10	0.265 ± 0.004	10	0.0115 ± 0.0002[b]	10	0.590 ± 0.009
High	10	0.269 ± 0.005	10	0.0121 ± 0.0001	10	0.574 ± 0.014
Trend[a]		0.362		0.137		0.048 (−)
Male						
Control	10	0.378 ± 0.013	10	0.0145 ± 0.0003	10	0.833 ± 0.020
Low	10	0.404 ± 0.014	10	0.0152 ± 0.0004	10	0.890 ± 0.034
Mid	10	0.397 ± 0.010	10	0.0147 ± 0.0002	10	0.889 ± 0.017
High	10	0.374 ± 0.005	10	0.0143 ± 0.0002	10	0.842 ± 0.021
Trend[a]		0.705		0.391		0.802

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.4 (*Continued*). Organ Weights in Mice at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Liver						
Female						
Control	10	1.08 ± 0.04	10	0.0484 ± 0.0011	10	2.41 ± 0.07
Low	10	1.08 ± 0.02	10	0.0487 ± 0.0005	10	2.38 ± 0.03
Mid	10	1.16 ± 0.05	10	0.0501 ± 0.0014	10	2.58 ± 0.11
High	10	1.08 ± 0.02	10	0.0489 ± 0.0008	10	2.31 ± 0.07
Trend[a]		0.590		0.495		0.815
Male						
Control	10	1.20 ± 0.05	10	0.0461 ± 0.0016	10	2.66 ± 0.13
Low	10	1.30 ± 0.06	10	0.0488 ± 0.0020	10	2.86 ± 0.15
Mid	10	1.35 ± 0.03	10	0.0501 ± 0.0011	10	3.03 ± 0.07[b]
High	10	1.18 ± 0.03	10	0.0451 ± 0.0012	10	2.65 ± 0.05
Trend[a]		0.998		0.838		0.753
Lung						
Female						
Control	9	0.204 ± 0.008	9	0.0091 ± 0.0003	9	0.456 ± 0.016
Low	10	0.216 ± 0.006	10	0.0097 ± 0.0002	10	0.473 ± 0.012
Mid	10	0.218 ± 0.011	10	0.0095 ± 0.0005	10	0.483 ± 0.021
High	10	0.211 ± 0.004	10	0.0096 ± 0.0002	10	0.450 ± 0.010
Trend[a]		0.504		0.473		0.906
Male						
Control	10	0.214 ± 0.008	10	0.0082 ± 0.0003	10	0.472 ± 0.016
Low	10	0.216 ± 0.008	10	0.0081 ± 0.0002	10	0.476 ± 0.020
Mid	10	0.214 ± 0.004	10	0.0079 ± 0.0002	10	0.480 ± 0.010
High	9	0.222 ± 0.005	9	0.0085 ± 0.0002	9	0.492 ± 0.009
Trend[a]		0.491		0.536		0.336
Ovaries						
Female						
Control	10	0.018 ± 0.002	10	0.0008 ± 0.0001	10	0.041 ± 0.006
Low	10	0.020 ± 0.003	10	0.0009 ± 0.0001	10	0.044 ± 0.006
Mid	10	0.017 ± 0.001	10	0.0007 ± 0.0000	10	0.037 ± 0.002
High	10	0.016 ± 0.001	10	0.0007 ± 0.0001	10	0.035 ± 0.003
Trend[a]		0.314		0.289		0.221
Testes						
Male						
Control	10	0.188 ± 0.006	10	0.0072 ± 0.0002	10	0.41 ± 0.01
Low	10	0.172 ± 0.009	10	0.0065 ± 0.0003	10	0.38 ± 0.02
Mid	10	0.177 ± 0.009	10	0.0066 ± 0.0003	10	0.40 ± 0.02
High	10	0.182 ± 0.007	10	0.0070 ± 0.0003	10	0.41 ± 0.02
Trend[a]		0.759		0.620		0.911

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.4 (*Continued*). Organ Weights in Mice at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Thymus						
Female						
Control	10	0.055 ± 0.002	10	0.0025 ± 0.0001	10	0.124 ± 0.005
Low	10	0.057 ± 0.003	10	0.0025 ± 0.0001	10	0.124 ± 0.006
Mid	10	0.065 ± 0.007	10	0.0028 ± 0.0003	10	0.145 ± 0.013
High	10	0.058 ± 0.002	10	0.0026 ± 0.0001	10	0.123 ± 0.006
Trend[a]		0.378		0.405		0.592
Male						
Control	10	0.048 ± 0.002	10	0.0019 ± 0.0001	10	0.106 ± 0.005
Low	10	0.045 ± 0.002	10	0.0017 ± 0.0001	10	0.100 ± 0.005
Mid	10	0.044 ± 0.003	10	0.0016 ± 0.0001	10	0.100 ± 0.008
High	10	0.044 ± 0.002	10	0.0017 ± 0.0001	10	0.100 ± 0.005
Trend[a]		0.252		0.224		0.441

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

* See Table 2 for units.

Table D.5. Bronchoalveolar Lavage Results in Mice at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Eosinophils Absolute Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.000 ± 0.000	18	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	9	0.000 ± 0.000	10	0.000 ± 0.000	19	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
Eosinophils Differential Cell Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.000 ± 0.000	18	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	9	0.000 ± 0.000	10	0.000 ± 0.000	19	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
Lymphocytes Absolute Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.003 ± 0.003	18	0.001 ± 0.001	0.205	Transformed
Low	10	0.003 ± 0.002	10	0.000 ± 0.000	20	0.002 ± 0.001		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.003 ± 0.003	20	0.001 ± 0.001		
Trend[c]		0.527		0.950		0.969		
13 Weeks								
Control	10	0.002 ± 0.002	10	0.006 ± 0.005	20	0.004 ± 0.003	0.535	Transformed
Low	9	0.000 ± 0.000	10	0.004 ± 0.002	19	0.002 ± 0.001		
Mid	10	0.008 ± 0.005	10	0.005 ± 0.004	20	0.007 ± 0.003		
High	10	0.004 ± 0.002	10	0.004 ± 0.003	20	0.004 ± 0.002		
Trend[c]		0.253		0.766		0.600		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.5 (*Continued*). Bronchoalveolar Lavage Results in Mice at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Lymphocytes Differential Cell Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.111 ± 0.111	18	0.056 ± 0.056	0.193	Categorical
Low	10	0.200 ± 0.133	10	0.000 ± 0.000	20	0.100 ± 0.067		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.100 ± 0.100	20	0.050 ± 0.050		
Trend[c]		0.116		0.937		0.994		
13 Weeks								
Control	10	0.100 ± 0.100	10	0.400 ± 0.221	20	0.250 ± 0.121	0.181	Categorical
Low	9	0.000 ± 0.000	10	0.500 ± 0.224	19	0.250 ± 0.118		
Mid	10	0.800 ± 0.512	10	0.300 ± 0.213	20	0.550 ± 0.277		
High	10	0.400 ± 0.221	10	0.300 ± 0.213	20	0.350 ± 0.154		
Trend[c]		0.045 (+)		0.588		0.402		
Macrophages Absolute Count								
4 Weeks								
Control	9	1.3 ± 0.2	9	1.4 ± 0.2	18	1.4 ± 0.1	0.057	ANOVA
Low	10	1.6 ± 0.2	10	1.2 ± 0.2	20	1.4 ± 0.1		
Mid	10	1.8 ± 0.2	10	1.2 ± 0.2	20	1.5 ± 0.2		
High	10	1.4 ± 0.2	10	1.8 ± 0.3	20	1.6 ± 0.2		
Trend[c]		0.721		0.288		0.316		
13 Weeks								
Control	10	1.0 ± 0.2	10	1.0 ± 0.2	20	1.0 ± 0.1	0.655	ANOVA
Low	9	0.7 ± 0.1	10	1.0 ± 0.2	19	0.9 ± 0.1		
Mid	10	0.8 ± 0.1	10	1.0 ± 0.2	20	0.9 ± 0.1		
High	10	1.0 ± 0.1	10	1.0 ± 0.1	20	1.0 ± 0.1		
Trend[c]		0.651		0.856		0.685		
Macrophages Differential Cell Count								
4 Weeks								
Control	9	99.67 ± 0.167	9	99.89 ± 0.111	18	99.78 ± 0.100	0.529	Categorical
Low	10	99.70 ± 0.153	10	99.90 ± 0.100	20	99.80 ± 0.091		
Mid	10	100.0 ± 0.000	10	90.00 ± 10.00	20	95.00 ± 5.000		
High	10	99.80 ± 0.200	10	99.20 ± 0.512	20	99.50 ± 0.275		
Trend[c]		0.353		0.218		0.854		
13 Weeks								
Control	10	99.70 ± 0.300	10	99.60 ± 0.221	20	99.65 ± 0.186	0.249	Categorical
Low	9	100.0 ± 0.000	10	99.40 ± 0.267	19	99.70 ± 0.141		
Mid	10	98.90 ± 0.547	10	99.50 ± 0.269	20	99.20 ± 0.305		
High	10	99.10 ± 0.526	10	98.80 ± 0.512	20	98.95 ± 0.367		
Trend[c]		0.107		0.158		0.031 (−)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.5 (*Continued*). Bronchoalveolar Lavage Results in Mice at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Number of Epithelials per 100 Cells Absolute Count								
4 Weeks								
Control	9	1.7 ± 0.4	9	3.2 ± 1.6	18	2.4 ± 0.8	0.533	Transformed
Low	10	5.0 ± 1.5	10	2.7 ± 1.4	20	3.9 ± 1.0		
Mid	10	2.9 ± 1.4	10	5.9 ± 2.2	20	4.4 ± 1.3		
High	10	3.6 ± 1.0	10	5.8 ± 3.1	20	4.7 ± 1.6		
Trend[c]		0.495		0.717		0.471		
13 Weeks								
Control	10	2.5 ± 2.1	10	7.0 ± 2.5	20	4.8 ± 1.6	0.564	ANOVA
Low	9	5.7 ± 2.6	10	5.1 ± 2.3	19	5.4 ± 1.7		
Mid	10	6.9 ± 3.4	10	4.0 ± 2.4	20	5.5 ± 2.1		
High	10	5.6 ± 3.8	10	4.8 ± 1.9	20	5.2 ± 2.1		
Trend[c]		0.444		0.456		0.866		
Other Absolute Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.000 ± 0.000	18	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	9	0.000 ± 0.000	10	0.000 ± 0.000	19	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
Other Differential Cell Count								
4 Weeks								
Control	9	0.000 ± 0.000	9	0.000 ± 0.000	18	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	9	0.000 ± 0.000	10	0.000 ± 0.000	19	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.5 (*Continued*). Bronchoalveolar Lavage Results in Mice at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
PMN Absolute Count								
4 Weeks								
Control	9	0.003 ± 0.002	9	0.000 ± 0.000	18	0.002 ± 0.001	0.209	Categorical
Low	10	0.003 ± 0.003	10	0.001 ± 0.001	20	0.002 ± 0.001		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.005 ± 0.005	10	0.018 ± 0.012	20	0.012 ± 0.006		
Trend[c]		0.858		0.110		0.166		
13 Weeks								
Control	10	0.004 ± 0.004	10	0.000 ± 0.000	20	0.002 ± 0.002	0.446	Categorical
Low	9	0.000 ± 0.000	10	0.001 ± 0.001	19	0.000 ± 0.000		
Mid	10	0.002 ± 0.002	10	0.003 ± 0.003	20	0.002 ± 0.002		
High	10	0.005 ± 0.003	10	0.014 ± 0.008	20	0.009 ± 0.004		
Trend[c]		0.844		0.051 (+)		0.015 (+)		
PMN Differential Cell Count								
4 Weeks								
Control	9	0.333 ± 0.167	9	0.000 ± 0.000	18	0.167 ± 0.083	0.131	Categorical
Low	10	0.100 ± 0.100	10	0.100 ± 0.100	20	0.100 ± 0.071		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.200 ± 0.200	10	0.700 ± 0.423	20	0.450 ± 0.234		
Trend[c]		0.502		0.098 (+)		0.151		
13 Weeks								
Control	10	0.200 ± 0.200	10	0.000 ± 0.000	20	0.100 ± 0.100	0.460	Categorical
Low	9	0.000 ± 0.000	10	0.100 ± 0.100	19	0.050 ± 0.053		
Mid	10	0.300 ± 0.300	10	0.200 ± 0.200	20	0.250 ± 0.180		
High	10	0.500 ± 0.307	10	0.900 ± 0.458	20	0.700 ± 0.276[d]		
Trend[c]		0.268		0.034 (+)		0.004 (+)		
Total Cells								
4 Weeks								
Control	9	1.3 ± 0.2	9	1.4 ± 0.2	18	1.4 ± 0.1	0.055	ANOVA
Low	10	1.6 ± 0.2	10	1.2 ± 0.2	20	1.4 ± 0.1		
Mid	10	1.8 ± 0.2	10	1.2 ± 0.2	20	1.5 ± 0.2		
High	10	1.4 ± 0.2	10	1.8 ± 0.3	20	1.6 ± 0.2		
Trend[c]		0.722		0.270		0.302		
13 Weeks								
Control	10	1.0 ± 0.2	10	1.0 ± 0.2	20	1.0 ± 0.1	0.667	ANOVA
Low	9	0.7 ± 0.1	10	1.0 ± 0.2	19	0.9 ± 0.1		
Mid	10	0.8 ± 0.1	10	1.0 ± 0.2	20	0.9 ± 0.1		
High	10	1.0 ± 0.1	10	1.1 ± 0.1	20	1.1 ± 0.1		
Trend[c]		0.636		0.818		0.648		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.6. Bronchoalveolar Biochemical Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Albumin								
4 Weeks								
Control	9	122 ± 9	9	161 ± 17	18	141 ± 10	0.243	ANOVA
Low	10	147 ± 9	10	158 ± 13	20	153 ± 8		
Mid	10	136 ± 8	10	133 ± 18	20	135 ± 10		
High	9	128 ± 16	10	178 ± 22	19	153 ± 14		
Trend[c]		0.847		0.742		0.674		
13 Weeks								
Control	10	177 ± 14	8	134 ± 16	18	156 ± 11	0.038	Transformed
Low	7	175 ± 12	10	154 ± 10	17	164 ± 8		
Mid	10	168 ± 12	9	161 ± 9	19	165 ± 7		
High	9	295 ± 50[d]	9	142 ± 10	18	218 ± 26		
Trend[c]		0.008 (+)		0.317		0.022 (+)		
Alkaline Phosphatase								
4 Weeks								
Control	9	0.019 ± 0.003	8	0.068 ± 0.051	17	0.043 ± 0.024	0.715	Transformed
Low	10	0.017 ± 0.002	10	0.023 ± 0.002	20	0.020 ± 0.001		
Mid	10	0.025 ± 0.008	8	0.025 ± 0.003	18	0.025 ± 0.005		
High	9	0.030 ± 0.009	9	0.026 ± 0.005	18	0.028 ± 0.005		
Trend[c]		0.143		0.702		0.622		
13 Weeks								
Control	9	0.017 ± 0.002	9	0.024 ± 0.003	18	0.020 ± 0.002	0.952	ANOVA
Low	7	0.013 ± 0.003	8	0.017 ± 0.003	15	0.015 ± 0.002		
Mid	10	0.017 ± 0.003	9	0.023 ± 0.002	19	0.020 ± 0.002		
High	10	0.019 ± 0.003	10	0.025 ± 0.003	20	0.022 ± 0.002		
Trend[c]		0.457		0.433		0.265		
GSH BALF								
4 Weeks								
Control	9	2.5 ± 0.8	8	3.2 ± 1.2	17	2.8 ± 0.7	0.610	Transformed
Low	9	2.8 ± 1.0	9	2.7 ± 0.5	18	2.8 ± 0.5		
Mid	10	3.6 ± 1.5	8	2.8 ± 0.6	18	3.2 ± 0.9		
High	9	3.4 ± 0.7	8	1.7 ± 0.3	17	2.6 ± 0.4		
Trend[c]		0.469		0.276		0.978		
13 Weeks								
Control	10	1.7 ± 0.4	10	1.6 ± 0.4	20	1.6 ± 0.3	0.453	Transformed
Low	8	1.1 ± 0.2	10	2.5 ± 0.4	18	1.8 ± 0.3		
Mid	10	2.5 ± 0.6	10	1.5 ± 0.3	20	2.0 ± 0.4		
High	10	2.8 ± 1.6	10	2.5 ± 0.6	20	2.6 ± 0.9		
Trend[c]		0.287		0.788		0.386		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.6 (*Continued*). Bronchoalveolar Biochemical Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
GSSG BALF								
4 Weeks								
Control	9	0.59 ± 0.08	8	0.55 ± 0.05	17	0.57 ± 0.05	0.361	Transformed
Low	9	1.02 ± 0.26	9	0.47 ± 0.06	18	0.74 ± 0.13		
Mid	10	0.56 ± 0.04	8	0.45 ± 0.05	18	0.51 ± 0.03		
High	9	0.64 ± 0.10	8	0.41 ± 0.03	17	0.52 ± 0.05		
Trend[c]		0.305		0.143		0.127		
13 Weeks								
Control	10	0.69 ± 0.27	10	0.63 ± 0.05	20	0.66 ± 0.14	0.089	Transformed
Low	8	0.35 ± 0.03	10	1.91 ± 1.22	18	1.13 ± 0.69		
Mid	10	0.56 ± 0.16	10	0.72 ± 0.09	20	0.64 ± 0.09		
High	10	0.32 ± 0.05	10	0.84 ± 0.12	20	0.58 ± 0.06		
Trend[c]		0.087 (−)		0.562		0.279		
Hemoglobin								
4 Weeks								
Control	9	0.0031 ± 0.0008	8	0.0029 ± 0.0008	17	0.0030 ± 0.0006	0.721	ANOVA
Low	10	0.0042 ± 0.0008	10	0.0041 ± 0.0014	20	0.0042 ± 0.0008		
Mid	10	0.0027 ± 0.0007	9	0.0036 ± 0.0007	19	0.0031 ± 0.0005		
High	8	0.0036 ± 0.0010	8	0.0024 ± 0.0006	16	0.0030 ± 0.0006		
Trend[c]		0.991		0.658		0.722		
13 Weeks								
Control	9	0.0061 ± 0.0007	8	0.0076 ± 0.0003	17	0.0069 ± 0.0004	0.503	ANOVA
Low	7	0.0050 ± 0.0012	10	0.0080 ± 0.0004	17	0.0065 ± 0.0005		
Mid	10	0.0067 ± 0.0008	9	0.0078 ± 0.0005	19	0.0072 ± 0.0005		
High	9	0.0058 ± 0.0004	9	0.0079 ± 0.0008	18	0.0068 ± 0.0004		
Trend[c]		0.837		0.818		0.801		
LDH								
4 Weeks								
Control	9	258 ± 39	9	257 ± 32	18	258 ± 25	0.787	ANOVA
Low	10	300 ± 47	10	301 ± 56	20	301 ± 37		
Mid	10	286 ± 45	10	357 ± 54	20	321 ± 35		
High	10	285 ± 28	10	284 ± 25	20	285 ± 19		
Trend[c]		0.723		0.498		0.452		
13 Weeks								
Control	10	375 ± 48	10	346 ± 43	20	361 ± 32	0.762	ANOVA
Low	10	177 ± 50	10	275 ± 69	20	226 ± 43		
Mid	10	505 ± 116	10	494 ± 65	20	499 ± 67		
High	10	440 ± 51	10	432 ± 51	20	436 ± 36		
Trend[c]		0.114		0.074 (+)		0.017 (+)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.6 (*Continued*). Bronchoalveolar Biochemical Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Protein								
4 Weeks								
Control	9	43 ± 6	9	39 ± 2	18	41 ± 3	0.539	ANOVA
Low	10	45 ± 4	10	43 ± 3	20	44 ± 2		
Mid	10	41 ± 6	10	37 ± 7	20	39 ± 5		
High	10	38 ± 5	10	46 ± 6	20	42 ± 4		
Trend[c]		0.373		0.478		0.898		
13 Weeks								
Control	10	72 ± 7	10	77 ± 4	20	74 ± 4	0.307	Transformed
Low	9	53 ± 6	10	92 ± 18	19	72 ± 10		
Mid	10	89 ± 13	10	80 ± 10	20	84 ± 8		
High	10	86 ± 13	10	83 ± 7	20	85 ± 7		
Trend[c]		0.131		0.721		0.187		
TEAC								
4 Weeks								
Control	9	36 ± 5	9	11 ± 1	18	23 ± 2	0.369	Transformed
Low	9	28 ± 3	10	13 ± 2	19	20 ± 2		
Mid	10	33 ± 3	8	15 ± 2	18	24 ± 2		
High	10	31 ± 3	9	10 ± 1	19	21 ± 2		
Trend[c]		0.764		0.904		0.782		
13 Weeks								
Control	10	31 ± 3	10	29 ± 4	20	30 ± 2	0.015	ANOVA
Low	8	22 ± 1	10	34 ± 4	18	28 ± 2		
Mid	10	37 ± 3	9	29 ± 3	19	33 ± 2		
High	10	27 ± 3	10	31 ± 2	20	29 ± 2		
Trend[c]		0.804		0.951		0.874		
Total Glutathione BALF								
4 Weeks								
Control	8	3.3 ± 0.9	8	3.7 ± 1.2	16	3.5 ± 0.8	0.632	ANOVA
Low	9	3.7 ± 0.9	9	3.2 ± 0.5	18	3.5 ± 0.5		
Mid	10	4.2 ± 1.5	8	3.3 ± 0.6	18	3.7 ± 0.9		
High	8	4.5 ± 0.7	7	2.4 ± 0.2	15	3.4 ± 0.4		
Trend[c]		0.426		0.252		0.951		
13 Weeks								
Control	10	2.3 ± 0.4	10	2.2 ± 0.4	20	2.2 ± 0.3	0.400	ANOVA
Low	8	1.5 ± 0.2	10	3.2 ± 0.5	18	2.3 ± 0.3		
Mid	10	3.1 ± 0.8	10	2.2 ± 0.4	20	2.7 ± 0.4		
High	10	3.1 ± 1.6	10	3.3 ± 0.6	20	3.2 ± 0.8		
Trend[c]		0.329		0.284		0.178		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.7. Lung Tissue Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint / TIme Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Cytokine IL-1β Lung								
4 Weeks								
Control	10	21 ± 2	10	30 ± 3	20	25 ± 2	0.636	ANOVA
Low	10	20 ± 2	9	32 ± 3	19	26 ± 2		
Mid	10	22 ± 3	10	33 ± 2	20	28 ± 2		
High	10	18 ± 3	10	34 ± 3	20	26 ± 2		
Trend[c]		0.632		0.271		0.607		
13 Weeks								
Control	10	223 ± 8	10	75 ± 5	20	149 ± 5	0.748	Transformed
Low	10	255 ± 12	10	77 ± 3	20	166 ± 6		
Mid	10	241 ± 8	10	79 ± 4	20	160 ± 4		
High	10	247 ± 9	10	79 ± 4	20	163 ± 5		
Trend[c]		0.141		0.404		0.111		
Cytokine IL-6 Lung								
4 Weeks								
Control	10	3.4 ± 0.6	10	3.3 ± 0.6	20	3.3 ± 0.4	0.254	Transformed
Low	10	3.8 ± 1.1	9	4.8 ± 1.1	19	4.3 ± 0.8		
Mid	10	2.6 ± 0.5	10	3.1 ± 0.4	20	2.9 ± 0.3		
High	10	11.5 ± 3.8[d]	10	5.5 ± 1.3	20	8.5 ± 2.0[e]		
Trend[c]		0.035 (+)		0.093 (+)		0.007 (+)		
13 Weeks								
Control	10	43.0 ± 13.1	10	9.8 ± 0.7	20	26.4 ± 6.6	0.413	Transformed
Low	10	36.7 ± 3.2	10	11.5 ± 1.6	20	24.1 ± 1.8		
Mid	10	36.1 ± 6.3	10	8.6 ± 0.8	20	22.4 ± 3.2		
High	10	45.4 ± 15.9	10	18.6 ± 6.2	20	32.0 ± 8.5		
Trend[c]		0.913		0.090 (+)		0.261		
Cytokine KC Lung								
4 Weeks								
Control	10	82 ± 56	10	14 ± 1	20	48 ± 28	0.368	Transformed
Low	10	61 ± 33	9	17 ± 2	19	39 ± 17		
Mid	10	13 ± 2	10	16 ± 2	20	14 ± 2		
High	10	50 ± 26	10	15 ± 1	20	33 ± 13		
Trend[c]		0.708		0.705		0.813		
13 Weeks								
Control	10	88 ± 40	10	95 ± 38	20	92 ± 28	0.551	Transformed
Low	10	38 ± 4	10	35 ± 4	20	36 ± 3		
Mid	10	49 ± 9	10	46 ± 9	20	48 ± 6		
High	10	40 ± 6	10	112 ± 62	20	76 ± 31		
Trend[c]		0.371		0.785		0.675		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table D.7 (*Continued*). Lung Tissue Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint / TIme Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Cytokine MIP-2 Lung								
4 Weeks								
Control	8	31 ± 4	8	34 ± 7	16	33 ± 4	0.364	Transformed
Low	7	29 ± 6	8	52 ± 10	15	41 ± 6		
Mid	8	39 ± 6	6	44 ± 12	14	42 ± 6		
High	9	32 ± 4	7	34 ± 7	16	33 ± 4		
Trend[c]		0.620		0.947		0.804		
13 Weeks								
Control	3	22 ± 5	4	0 ± 0		—	—	No analysis
Low	2	22 ± 8	4	0 ± 0		—		
Mid	3	16 ± 1	4	0 ± 0		—		
High	1	15	4	0 ± 0		—		
Trend[c]		0.349		—		—		
Cytokine TNF-α Lung								
4 Weeks								
Control	2	0.55 ± 0.06	5	0.67 ± 0.19	7	0.61 ± 0.16	0.884	Transformed
Low	0	0.00 ± 0.00	3	0.88 ± 0.32	3	0.88 ± 0.23		
Mid	1	0.22	2	0.31 ± 0.04	3	0.26 ± 0.03		
High	1	0.39	5	0.69 ± 0.13	6	0.54 ± 0.16		
Trend[c]		—		0.721		0.471		
13 Weeks								
Control	3	0.58 ± 0.29	10	1.36 ± 0.16	13	0.97 ± 0.17	0.751	ANOVA
Low	3	0.79 ± 0.34	10	1.46 ± 0.12	13	1.13 ± 0.14		
Mid	0	0.00 ± 0.00	10	1.49 ± 0.06	10	1.49 ± 0.03		
High	0	0.00 ± 0.00	10	1.59 ± 0.13	10	1.59 ± 0.07		
Trend[c]		—		0.214		0.205		
GSH Lung								
4 Weeks								
Control	10	0.072 ± 0.013	10	0.166 ± 0.017	20	0.119 ± 0.011	0.065	Transformed
Low	10	0.118 ± 0.018	10	0.161 ± 0.026	20	0.139 ± 0.016		
Mid	10	0.084 ± 0.010	10	0.148 ± 0.028	20	0.116 ± 0.015		
High	10	0.143 ± 0.024[d]	10	0.161 ± 0.024	20	0.152 ± 0.017		
Trend[c]		0.017 (+)		0.603		0.130		
13 Weeks								
Control	10	0.063 ± 0.010	10	0.062 ± 0.013	20	0.062 ± 0.008	0.185	ANOVA
Low	10	0.081 ± 0.011	10	0.064 ± 0.009	20	0.072 ± 0.007		
Mid	10	0.088 ± 0.012	10	0.046 ± 0.007	20	0.067 ± 0.007		
High	10	0.082 ± 0.004	10	0.056 ± 0.008	20	0.069 ± 0.004		
Trend[c]		0.137		0.400		0.611		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table D.7 (*Continued*). Lung Tissue Results in Mice at End of 4 and 13 Weeks of Exposure*

Endpoint/ TIme Point/Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
GSSG Lung								
4 Weeks								
Control	10	0.010 ± 0.002	10	0.014 ± 0.002	20	0.012 ± 0.002	0.445	ANOVA
Low	10	0.016 ± 0.004	10	0.016 ± 0.004	20	0.016 ± 0.003		
Mid	10	0.010 ± 0.002	10	0.018 ± 0.003	20	0.014 ± 0.002		
High	10	0.017 ± 0.003	10	0.016 ± 0.002	20	0.017 ± 0.002		
Trend[c]		0.181		0.469		0.148		
13 Weeks								
Control	10	0.027 ± 0.003	10	0.041 ± 0.005	20	0.034 ± 0.003	0.428	Transformed
Low	10	0.034 ± 0.004	10	0.035 ± 0.003	20	0.035 ± 0.002		
Mid	10	0.037 ± 0.006	10	0.029 ± 0.004	20	0.033 ± 0.004		
High	10	0.029 ± 0.003	10	0.037 ± 0.005	20	0.033 ± 0.003		
Trend[c]		0.893		0.340		0.660		
HO-1								
4 Weeks								
Control	10	6.3 ± 0.8	10	8.1 ± 1.3	20	7.2 ± 0.8	0.568	ANOVA
Low	10	8.4 ± 1.7	9	7.7 ± 1.0	19	8.0 ± 1.0		
Mid	10	6.7 ± 0.7	10	6.1 ± 0.7	20	6.4 ± 0.5		
High	10	8.1 ± 1.0	10	7.6 ± 0.5	20	7.8 ± 0.6		
Trend[c]		0.475		0.466		0.932		
13 Weeks								
Control	10	6.1 ± 0.4	10	5.0 ± 0.3	20	5.5 ± 0.3	0.384	ANOVA
Low	10	6.7 ± 0.7	10	5.3 ± 0.4	20	6.0 ± 0.4		
Mid	10	5.8 ± 0.3	10	5.9 ± 0.6	20	5.9 ± 0.3		
High	10	6.1 ± 0.3	10	5.2 ± 0.4	20	5.6 ± 0.2		
Trend[c]		0.685		0.576		0.940		
Total Glutathione Lung								
4 Weeks								
Control	9	0.090 ± 0.013	10	0.179 ± 0.017	19	0.135 ± 0.011	0.175	Transformed
Low	10	0.134 ± 0.021	10	0.177 ± 0.029	20	0.155 ± 0.018		
Mid	10	0.094 ± 0.011	10	0.166 ± 0.028	20	0.130 ± 0.015		
High	10	0.160 ± 0.026[d]	10	0.177 ± 0.025	20	0.168 ± 0.018		
Trend[c]		0.052 (+)		0.700		0.267		
13 Weeks								
Control	10	0.090 ± 0.011	10	0.102 ± 0.014	20	0.096 ± 0.009	0.049	ANOVA
Low	10	0.114 ± 0.012	10	0.099 ± 0.011	20	0.107 ± 0.008		
Mid	10	0.125 ± 0.017	10	0.075 ± 0.007	20	0.100 ± 0.009		
High	10	0.112 ± 0.005	10	0.093 ± 0.007	20	0.102 ± 0.004		
Trend[c]		0.164		0.249		0.740		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table D.8. Hematology Results in Mice at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Basophils								
Control	9	0.3 ± 0.1	10	0.4 ± 0.1	19	0.3 ± 0.1	0.555	ANOVA
Low	10	0.4 ± 0.1	10	0.7 ± 0.1	20	0.6 ± 0.1		
Mid	10	0.2 ± 0.1	9	0.7 ± 0.2	19	0.4 ± 0.1		
High	10	0.3 ± 0.1	10	0.6 ± 0.2	20	0.5 ± 0.1		
Trend[c]		0.568		0.274		0.543		
Basophils Absolute								
Control	9	0.003 ± 0.003	10	0.004 ± 0.002	19	0.004 ± 0.002	0.071	Categorical
Low	10	0.004 ± 0.002	10	0.012 ± 0.001[d]	20	0.008 ± 0.001[d]		
Mid	10	0.003 ± 0.002	9	0.004 ± 0.002	19	0.004 ± 0.001		
High	10	0.003 ± 0.002	10	0.005 ± 0.002	20	0.004 ± 0.001		
Trend[c]		0.850		0.513		0.918		
Eosinophils								
Control	9	1.5 ± 0.2	10	2.8 ± 0.9	19	2.2 ± 0.5	0.671	ANOVA
Low	10	2.5 ± 0.7	10	2.7 ± 0.4	20	2.6 ± 0.4		
Mid	10	2.0 ± 0.3	9	3.4 ± 0.5	19	2.7 ± 0.3		
High	10	1.6 ± 0.4	10	2.6 ± 0.4	20	2.1 ± 0.3		
Trend[c]		0.910		0.999		0.925		
Eosinophils Absolute								
Control	9	0.028 ± 0.004	10	0.032 ± 0.009	19	0.030 ± 0.005	0.676	ANOVA
Low	10	0.039 ± 0.015	10	0.035 ± 0.006	20	0.037 ± 0.008		
Mid	10	0.029 ± 0.007	9	0.040 ± 0.003	19	0.035 ± 0.004		
High	10	0.030 ± 0.008	10	0.023 ± 0.004	20	0.027 ± 0.005		
Trend[c]		0.940		0.416		0.602		
Hematocrit								
Control	9	53.3 ± 0.5	10	51.6 ± 0.9	19	52.4 ± 0.5	0.023	ANOVA
Low	10	51.8 ± 0.3	10	53.3 ± 0.6	20	52.5 ± 0.3		
Mid	10	51.0 ± 0.7[e]	9	52.5 ± 0.5	19	51.8 ± 0.4		
High	10	52.7 ± 0.5	10	52.0 ± 0.7	20	52.4 ± 0.4		
Trend[c]		0.304		0.846		0.686		
Hemoglobin Measured								
Control	9	15.9 ± 0.1	10	15.4 ± 0.3	19	15.7 ± 0.2	0.002	ANOVA
Low	10	15.5 ± 0.1	10	16.1 ± 0.2	20	15.8 ± 0.1		
Mid	10	15.3 ± 0.2[e]	9	15.9 ± 0.1	19	15.6 ± 0.1		
High	10	15.8 ± 0.2	10	15.5 ± 0.2	20	15.7 ± 0.1		
Trend[c]		0.379		0.797		0.766		
Large Unstained Cells								
Control	9	1.2 ± 0.2	10	1.4 ± 0.2	19	1.3 ± 0.2	0.757	ANOVA
Low	10	1.6 ± 0.3	10	1.7 ± 0.2	20	1.6 ± 0.2		
Mid	10	1.0 ± 0.3	9	1.3 ± 0.2	19	1.1 ± 0.2		
High	10	0.8 ± 0.2	10	1.4 ± 0.3	20	1.1 ± 0.2		
Trend[c]		0.128		0.701		0.153		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.8 (*Continued*). Hematology Results in Mice at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Large Unstained Cells Absolute								
Control	9	0.023 ± 0.008	10	0.018 ± 0.003	19	0.021 ± 0.004	0.839	ANOVA
Low	10	0.023 ± 0.005	10	0.025 ± 0.004	20	0.024 ± 0.003		
Mid	10	0.015 ± 0.005	9	0.018 ± 0.003	19	0.016 ± 0.003		
High	10	0.016 ± 0.005	10	0.014 ± 0.005	20	0.015 ± 0.004		
Trend[c]		0.269		0.265		0.121		
Lymphocytes								
Control	9	56 ± 8	10	78 ± 1	19	67 ± 4	0.062	Transformed
Low	10	68 ± 5	10	71 ± 3	20	70 ± 3		
Mid	10	65 ± 4	9	77 ± 2	19	71 ± 2		
High	10	69 ± 5	10	75 ± 2	20	72 ± 3		
Trend[c]		0.056 (−)		0.652		0.084 (−)		
Lymphocytes Absolute								
Control	9	1.1 ± 0.3	10	1.0 ± 0.1	19	1.1 ± 0.1	0.498	Transformed
Low	10	0.9 ± 0.1	10	1.0 ± 0.1	20	1.0 ± 0.1		
Mid	10	0.9 ± 0.1	9	1.0 ± 0.1	19	0.9 ± 0.1		
High	10	1.2 ± 0.3	10	0.7 ± 0.1	20	0.9 ± 0.1		
Trend[c]		0.925		0.048 (−)		0.362		
Mean Corpuscular Hemoglobin								
Control	9	14.6 ± 0.12	10	15.0 ± 0.09	19	14.8 ± 0.08	0.590	ANOVA
Low	10	14.6 ± 0.06	10	15.0 ± 0.08	20	14.8 ± 0.05		
Mid	10	14.5 ± 0.06	9	15.1 ± 0.08	19	14.8 ± 0.05		
High	10	14.5 ± 0.08	10	15.0 ± 0.08	20	14.7 ± 0.06		
Trend[c]		0.423		0.441		0.982		
Mean Corpuscular Hemoglobin Concentration								
Control	9	30.0 ± 0.2	10	29.9 ± 0.2	19	29.9 ± 0.1	0.492	ANOVA
Low	10	30.0 ± 0.2	10	30.1 ± 0.1	20	30.1 ± 0.1		
Mid	10	30.0 ± 0.2	9	30.3 ± 0.2	19	30.1 ± 0.1		
High	10	30.0 ± 0.2	10	29.8 ± 0.2	20	29.9 ± 0.1		
Trend[c]		0.942		0.819		0.921		
Mean Corpuscular Volume								
Control	9	48.6 ± 0.5	10	50.1 ± 0.3	19	49.4 ± 0.3	0.387	ANOVA
Low	10	48.6 ± 0.2	10	49.6 ± 0.3	20	49.1 ± 0.2		
Mid	10	48.4 ± 0.2	9	49.8 ± 0.2	19	49.1 ± 0.1		
High	10	48.2 ± 0.3	10	50.3 ± 0.3	20	49.2 ± 0.2		
Trend[c]		0.333		0.555		0.699		
Monocytes								
Control	9	0.6 ± 0.1	10	1.0 ± 0.3	19	0.8 ± 0.2	0.226	Transformed
Low	10	0.7 ± 0.1	10	0.6 ± 0.1	20	0.7 ± 0.1		
Mid	10	1.1 ± 0.2	9	0.9 ± 0.2	19	1.0 ± 0.2		
High	10	0.4 ± 0.1	10	0.7 ± 0.1	20	0.6 ± 0.1		
Trend[c]		0.500		0.740		0.448		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.8 (*Continued*). Hematology Results in Mice at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Monocytes Absolute								
Control	9	0.014 ± 0.003	10	0.012 ± 0.002	19	0.013 ± 0.002	0.962	Categorical
Low	10	0.011 ± 0.002	10	0.007 ± 0.002	20	0.009 ± 0.002		
Mid	10	0.014 ± 0.004	9	0.012 ± 0.002	19	0.013 ± 0.002		
High	10	0.008 ± 0.002	10	0.006 ± 0.002	20	0.007 ± 0.002[e]		
Trend[c]		0.196		0.198		0.066 (−)		
Neutrophils								
Control	9	41 ± 8	10	17 ± 1	19	29 ± 4	0.084	Transformed
Low	10	27 ± 5	10	24 ± 2[e]	20	25 ± 3		
Mid	10	31 ± 5	9	18 ± 1	19	24 ± 3		
High	10	28 ± 6	10	21 ± 2	20	24 ± 3		
Trend[c]		0.258		0.381		0.550		
Neutrophils Absolute								
Control	9	0.71 ± 0.16	10	0.22 ± 0.03	19	0.46 ± 0.08	0.107	Transformed
Low	10	0.40 ± 0.10	10	0.34 ± 0.06	20	0.37 ± 0.06		
Mid	10	0.42 ± 0.09	9	0.24 ± 0.05	19	0.33 ± 0.05		
High	10	0.32 ± 0.02	10	0.18 ± 0.02	20	0.25 ± 0.02		
Trend[c]		0.122		0.248		0.064 (−)		
Platelet								
Control	9	1355 ± 80	10	999 ± 47	19	1177 ± 45	0.797	Transformed
Low	10	1345 ± 166	10	1067 ± 61	20	1206 ± 88		
Mid	10	1299 ± 45	9	1044 ± 23	19	1171 ± 26		
High	10	1476 ± 138	10	1109 ± 57	20	1293 ± 75		
Trend[c]		0.515		0.130		0.171		
Red Blood Cell Count								
Control	9	11.0 ± 0.1	10	10.3 ± 0.2	19	10.6 ± 0.1	0.002	ANOVA
Low	10	10.7 ± 0.1	10	10.7 ± 0.1[e]	20	10.7 ± 0.1		
Mid	10	10.6 ± 0.2[e]	9	10.5 ± 0.1	19	10.5 ± 0.1		
High	10	10.9 ± 0.1	10	10.3 ± 0.1	20	10.6 ± 0.1		
Trend[c]		0.738		0.961		0.846		
Reticulocytes								
Control	9	2.5 ± 0.2	10	2.8 ± 0.3	19	2.7 ± 0.2	0.722	ANOVA
Low	10	2.8 ± 0.2	10	2.7 ± 0.1	20	2.8 ± 0.1		
Mid	10	2.6 ± 0.1	9	2.7 ± 0.1	19	2.6 ± 0.1		
High	10	2.8 ± 0.1	10	2.8 ± 0.3	20	2.8 ± 0.1		
Trend[c]		0.464		0.924		0.775		
White Blood Cell Count								
Control	9	1.9 ± 0.3	10	1.3 ± 0.1	19	1.6 ± 0.2	0.419	Transformed
Low	10	1.4 ± 0.2	10	1.4 ± 0.2	20	1.4 ± 0.1		
Mid	10	1.4 ± 0.2	9	1.3 ± 0.2	19	1.3 ± 0.1		
High	10	1.6 ± 0.3	10	0.9 ± 0.1	20	1.2 ± 0.1		
Trend[c]		0.415		0.050 (−)		0.077 (−)		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized weighted least squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table D.9. Serum Chemistry Results in Mice at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both		Interaction[a]	Method[b]
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM		
A/G Ratio								
Control	10	1.95 ± 0.06	7	2.60 ± 0.07	17	2.28 ± 0.05	0.080	ANOVA
Low	9	2.02 ± 0.08	7	2.37 ± 0.10	16	2.20 ± 0.06		
Mid	10	1.99 ± 0.08	8	2.68 ± 0.10	18	2.33 ± 0.06		
High	10	2.10 ± 0.06	9	2.46 ± 0.11	19	2.28 ± 0.06		
Trend[c]		0.189		0.773		0.522		
ALB								
Control	10	3.00 ± 0.03	7	3.33 ± 0.03	17	3.16 ± 0.02	0.236	ANOVA
Low	9	2.94 ± 0.04	7	3.37 ± 0.04	16	3.16 ± 0.03		
Mid	10	2.96 ± 0.05	8	3.33 ± 0.03	18	3.14 ± 0.03		
High	10	3.10 ± 0.04	9	3.37 ± 0.04	19	3.23 ± 0.03		
Trend[c]		0.084 (−)		0.682		0.115		
ALT								
Control	10	49.1 ± 7.1	8	29.4 ± 1.4	18	39.2 ± 4.0	0.633	Transformed
Low	10	39.4 ± 2.6	10	29.5 ± 0.8	20	34.5 ± 1.4		
Mid	10	45.0 ± 7.8	9	30.8 ± 2.1	19	37.9 ± 4.2		
High	10	40.2 ± 1.9	10	33.0 ± 3.4	20	36.6 ± 1.9		
Trend[c]		0.463		0.346		0.869		
AST								
Control	10	133 ± 21	8	101 ± 11	18	117 ± 13	0.822	Transformed
Low	10	97 ± 12	9	88 ± 10	19	92 ± 8		
Mid	10	106 ± 14	9	98 ± 11	19	102 ± 9		
High	10	90 ± 7	10	96 ± 14	20	93 ± 8		
Trend[c]		0.138		0.790		0.177		
BUN								
Control	10	20.5 ± 1.0	7	20.3 ± 2.1	17	20.4 ± 1.1	0.344	ANOVA
Low	10	24.0 ± 2.1	7	19.1 ± 1.4	17	21.6 ± 1.4		
Mid	10	22.9 ± 2.1	8	22.9 ± 2.3	18	22.9 ± 1.6		
High	10	21.0 ± 0.6	9	21.9 ± 1.3	19	21.4 ± 0.7		
Trend[c]		0.955		0.300		0.440		
BUN/CR								
Control	10	117 ± 18	7	86 ± 9	17	101 ± 11	0.958	Transformed
Low	10	150 ± 31	7	105 ± 27	17	127 ± 22		
Mid	10	151 ± 23	8	117 ± 27	18	134 ± 18		
High	9	142 ± 20	9	133 ± 29	18	137 ± 18		
Trend[c]		0.346		0.305		0.144		
CREA								
Control	10	0.20 ± 0.02	8	0.29 ± 0.05	18	0.24 ± 0.02	0.674	Transformed
Low	10	0.20 ± 0.03	9	0.24 ± 0.03	19	0.22 ± 0.02		
Mid	10	0.18 ± 0.02	10	0.31 ± 0.06	20	0.25 ± 0.03		
High	10	0.15 ± 0.02	10	0.26 ± 0.05	20	0.21 ± 0.03		
Trend[c]		0.091 (−)		0.637		0.104		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

Table D.9 (*Continued*). Serum Chemistry Results in Mice at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both		Interaction[a]	Method[b]
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM		
GLOB								
Control	10	1.55 ± 0.05	7	1.29 ± 0.03	17	1.42 ± 0.03	0.048	ANOVA
Low	9	1.47 ± 0.05	7	1.43 ± 0.06	16	1.45 ± 0.04		
Mid	10	1.50 ± 0.04	8	1.26 ± 0.05	18	1.38 ± 0.03		
High	10	1.48 ± 0.02	9	1.40 ± 0.06	19	1.44 ± 0.03		
Trend[c]		0.348		0.458		0.881		
TP								
Control	10	4.55 ± 0.05	7	4.61 ± 0.04	17	4.58 ± 0.04	0.025	ANOVA
Low	9	4.41 ± 0.05	7	4.80 ± 0.06	16	4.61 ± 0.04		
Mid	10	4.46 ± 0.03	8	4.59 ± 0.08	18	4.52 ± 0.04		
High	10	4.58 ± 0.04	9	4.77 ± 0.06	19	4.67 ± 0.04		
Trend[c]		0.472		0.392		0.312		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

APPENDIX E. Biologic Response in Rats

Table E.1. Body Weights in Rats at End of 4 Weeks of Exposure*

Time Point / Group	Males		Females	
	n	Mean ± SEM	*n*	Mean ± SEM
Initial				
Control	10	147 ± 5	10	123 ± 2
Low	10	160 ± 7	10	119 ± 2
Mid	10	159 ± 6	10	124 ± 2
High	10	149 ± 3	10	129 ± 3
Trend[a]		0.898		0.054 (+)
4 Weeks				
Control	10	297 ± 8	10	195 ± 3
Low	10	312 ± 9	10	191 ± 4
Mid	10	315 ± 7	10	198 ± 5
High	10	303 ± 6	10	201 ± 4
Trend[a]		0.534		0.164

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

Table E.2. Body Weights in Rats at End of 1, 2, and 3 Months of Exposure*

Time Point / Group	Males		Females	
	n	Mean ± SEM	*n*	Mean ± SEM
Initial				
Control	10	158 ± 6	10	126 ± 3
Low	10	153 ± 5	10	125 ± 3
Mid	10	156 ± 5	10	126 ± 2
High	10	150 ± 4	10	122 ± 3
Trend[a]		0.338		0.231
1 Month				
Control	10	314 ± 6	10	206 ± 5
Low	10	304 ± 3	10	202 ± 3
Mid	10	307 ± 7	10	203 ± 5
High	10	300 ± 5	10	197 ± 4
Trend[a]		0.105		0.129
2 Months				
Control	10	403 ± 8	10	239 ± 6
Low	10	393 ± 8	10	232 ± 3
Mid	10	394 ± 9	10	233 ± 6
High	10	389 ± 7	10	236 ± 6
Trend[a]		0.280		0.707
3 Months				
Control	10	444 ± 9	10	251 ± 7
Low	10	435 ± 8	10	246 ± 4
Mid	10	434 ± 11	10	246 ± 6
High	10	419 ± 11	10	244 ± 8
Trend[a]		0.075 (−)		0.501

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

Table E.3. Organ Weights in Rats at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Brain						
Female						
Control	10	1.72 ± 0.02	10	0.0090 ± 0.0002	10	1.0000 ± 0.0000
Low	10	1.73 ± 0.03	10	0.0090 ± 0.0002	10	1.0000 ± 0.0000
Mid	10	1.77 ± 0.03	10	0.0089 ± 0.0002	10	1.0000 ± 0.0000
High	10	1.77 ± 0.03	10	0.0087 ± 0.0002	10	1.0000 ± 0.0000
Trend[a]		0.110		0.310		—
Male						
Control	10	1.89 ± 0.03	10	0.0063 ± 0.0002	10	1.0000 ± 0.0000
Low	10	1.91 ± 0.03	10	0.0060 ± 0.0001	10	1.0000 ± 0.0000
Mid	10	1.86 ± 0.04	10	0.0059 ± 0.0002	10	1.0000 ± 0.0000
High	10	1.89 ± 0.02	10	0.0061 ± 0.0001	10	1.0000 ± 0.0000
Trend[a]		0.636		0.399		—
Heart						
Female						
Control	10	0.62 ± 0.03	10	0.0032 ± 0.0001	10	0.361 ± 0.017
Low	10	0.66 ± 0.03	10	0.0034 ± 0.0001	10	0.378 ± 0.010
Mid	10	0.68 ± 0.02	10	0.0034 ± 0.0001	10	0.385 ± 0.011
High	10	0.63 ± 0.02	10	0.0031 ± 0.0001	10	0.356 ± 0.014
Trend[a]		0.682		0.412		0.873
Male						
Control	10	0.89 ± 0.09	10	0.0029 ± 0.0002	10	0.468 ± 0.045
Low	10	0.87 ± 0.03	10	0.0028 ± 0.0001	10	0.458 ± 0.013
Mid	10	0.93 ± 0.04	10	0.0029 ± 0.0001	10	0.501 ± 0.028
High	10	0.91 ± 0.04	10	0.0029 ± 0.0001	10	0.484 ± 0.019
Trend[a]		0.638		0.590		0.499
Kidneys						
Female						
Control	10	1.16 ± 0.02	10	0.0061 ± 0.0001	10	0.68 ± 0.01
Low	10	1.19 ± 0.04	10	0.0062 ± 0.0001	10	0.69 ± 0.02
Mid	10	1.23 ± 0.04	10	0.0062 ± 0.0001	10	0.69 ± 0.01
High	10	1.26 ± 0.04	10	0.0062 ± 0.0002	10	0.71 ± 0.02
Trend[a]		0.040 (+)		0.562		0.154
Male						
Control	10	1.74 ± 0.10	10	0.0057 ± 0.0002	10	0.92 ± 0.04
Low	10	1.82 ± 0.07	10	0.0057 ± 0.0001	10	0.95 ± 0.03
Mid	10	1.84 ± 0.04	10	0.0058 ± 0.0001	10	0.99 ± 0.04
High	10	1.88 ± 0.19	10	0.0061 ± 0.0006	10	1.00 ± 0.10
Trend[a]		0.406		0.436		0.314

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

Table E.3 (*Continued*). Organ Weights in Rats at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Liver						
Female						
Control	10	6.60 ± 0.25	10	0.035 ± 0.001	10	3.85 ± 0.15
Low	10	6.97 ± 0.26	10	0.036 ± 0.001	10	4.03 ± 0.12
Mid	10	7.13 ± 0.26	10	0.036 ± 0.001	10	4.02 ± 0.12
High	10	6.79 ± 0.25	10	0.033 ± 0.001	10	3.85 ± 0.15
Trend[a]		0.520		0.425		0.996
Male						
Control	10	11.4 ± 0.59	10	0.037 ± 0.001	10	6.00 ± 0.27
Low	10	11.8 ± 0.30	10	0.037 ± 0.001	10	6.18 ± 0.15
Mid	10	11.7 ± 0.28	10	0.037 ± 0.001	10	6.29 ± 0.25
High	10	10.8 ± 0.44	10	0.035 ± 0.001	10	5.75 ± 0.25
Trend[a]		0.343		0.088 (−)		0.539
Lungs						
Female						
Control	10	1.20 ± 0.03	10	0.0063 ± 0.0002	10	0.70 ± 0.02
Low	10	1.19 ± 0.05	10	0.0062 ± 0.0002	10	0.69 ± 0.03
Mid	10	1.24 ± 0.04	10	0.0062 ± 0.0002	10	0.70 ± 0.02
High	10	1.26 ± 0.03	10	0.0062 ± 0.0001	10	0.71 ± 0.02
Trend[a]		0.221		0.754		0.624
Male						
Control	10	1.59 ± 0.03	10	0.0053 ± 0.0002	10	0.84 ± 0.02
Low	10	1.64 ± 0.03	10	0.0052 ± 0.0001	10	0.86 ± 0.02
Mid	10	1.67 ± 0.04	10	0.0053 ± 0.0002	10	0.90 ± 0.03
High	10	1.64 ± 0.04	10	0.0053 ± 0.0001	10	0.87 ± 0.02
Trend[a]		0.231		0.709		0.138
Ovaries						
Female						
Control	10	0.123 ± 0.006	10	0.0006 ± 0.0000	10	0.072 ± 0.004
Low	10	0.119 ± 0.010	10	0.0006 ± 0.0000	10	0.068 ± 0.005
Mid	10	0.137 ± 0.008	10	0.0007 ± 0.0000	10	0.077 ± 0.004
High	10	0.137 ± 0.009	10	0.0007 ± 0.0000	10	0.077 ± 0.005
Trend[a]		0.108		0.349		0.206
Testes						
Male						
Control	10	3.44 ± 0.07	10	0.0114 ± 0.0004	10	1.82 ± 0.04
Low	10	3.44 ± 0.07	10	0.0109 ± 0.0003	10	1.81 ± 0.04
Mid	10	3.47 ± 0.08	10	0.0109 ± 0.0003	10	1.87 ± 0.07
High	10	3.21 ± 0.18	10	0.0104 ± 0.0007	10	1.70 ± 0.10
Trend[a]		0.173		0.150		0.319

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

* See Table 2 for units.

Table E.3 (*Continued*). Organ Weights in Rats at End of 4 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Thymus						
Female						
Control	10	0.42 ± 0.02	10	0.0022 ± 0.0001	10	0.24 ± 0.01
Low	10	0.44 ± 0.02	10	0.0023 ± 0.0001	10	0.26 ± 0.01
Mid	10	0.46 ± 0.02	10	0.0023 ± 0.0001	10	0.26 ± 0.01
High	10	0.47 ± 0.02	10	0.0023 ± 0.0001	10	0.27 ± 0.01
Trend[a]		0.073 (+)		0.354		0.199
Male						
Control	10	0.58 ± 0.04	10	0.0019 ± 0.0001	10	0.30 ± 0.02
Low	10	0.57 ± 0.03	10	0.0018 ± 0.0001	10	0.30 ± 0.01
Mid	10	0.63 ± 0.05	10	0.0020 ± 0.0001	10	0.34 ± 0.04
High	10	0.58 ± 0.03	10	0.0019 ± 0.0001	10	0.31 ± 0.02
Trend[a]		0.636		0.667		0.564

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.
* See Table 2 for units.

Table E.4. Organ Weights in Rats at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Brain						
Female						
Control	10	1.85 ± 0.04	10	0.0074 ± 0.0002	10	1.0000 ± 0.0000
Low	10	1.85 ± 0.02	10	0.0076 ± 0.0001	10	1.0000 ± 0.0000
Mid	10	1.82 ± 0.02	10	0.0074 ± 0.0002	10	1.0000 ± 0.0000
High	10	1.84 ± 0.02	10	0.0076 ± 0.0002	10	1.0000 ± 0.0000
Trend[a]		0.531		0.660		—
Male						
Control	10	2.04 ± 0.05	10	0.0047 ± 0.0002	10	1.0000 ± 0.0000
Low	10	2.06 ± 0.03	10	0.0048 ± 0.0001	10	1.0000 ± 0.0000
Mid	10	1.99 ± 0.04	10	0.0047 ± 0.0001	10	1.0000 ± 0.0000
High	10	2.04 ± 0.02	10	0.0049 ± 0.0001	10	1.0000 ± 0.0000
Trend[a]		0.795		0.374		—

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.
[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.
[c] Significantly different from control group; $P < 0.01$.
* See Table 2 for units.

Table E.4 (*Continued*). Organ Weights in Rats at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Heart						
Female						
Control	10	0.77 ± 0.03	10	0.0030 ± 0.0001	10	0.414 ± 0.015
Low	10	0.70 ± 0.02	10	0.0029 ± 0.0001	10	0.377 ± 0.010
Mid	10	0.75 ± 0.02	10	0.0030 ± 0.0001	10	0.410 ± 0.012
High	10	0.69 ± 0.01[b]	10	0.0029 ± 0.0001	10	0.377 ± 0.006
Trend[a]		0.066 (−)		0.271		0.126
Male						
Control	10	1.11 ± 0.03	10	0.0025 ± 0.0001	10	0.548 ± 0.022
Low	10	1.02 ± 0.01[b]	10	0.0024 ± 0.0000	10	0.495 ± 0.007[b]
Mid	10	1.01 ± 0.02[c]	10	0.0024 ± 0.0001	10	0.509 ± 0.012
High	10	1.01 ± 0.02[b]	10	0.0024 ± 0.0000	10	0.495 ± 0.011[b]
Trend[a]		0.005 (−)		0.143		0.026 (−)
Kidneys						
Female						
Control	10	1.38 ± 0.04	10	0.0055 ± 0.0001	10	0.75 ± 0.02
Low	10	1.36 ± 0.06	10	0.0056 ± 0.0003	10	0.73 ± 0.03
Mid	10	1.35 ± 0.05	10	0.0055 ± 0.0002	10	0.74 ± 0.02
High	10	1.33 ± 0.03	10	0.0055 ± 0.0001	10	0.72 ± 0.02
Trend[a]		0.411		0.856		0.528
Male						
Control	10	2.16 ± 0.05	10	0.0049 ± 0.0001	10	1.06 ± 0.03
Low	10	2.13 ± 0.08	10	0.0050 ± 0.0001	10	1.04 ± 0.05
Mid	10	2.16 ± 0.07	10	0.0051 ± 0.0002	10	1.08 ± 0.04
High	10	2.03 ± 0.06	10	0.0048 ± 0.0001	10	0.99 ± 0.02
Trend[a]		0.231		0.785		0.309
Liver						
Female						
Control	10	7.52 ± 0.24	10	0.030 ± 0.001	10	4.07 ± 0.15
Low	10	7.56 ± 0.32	10	0.031 ± 0.001	10	4.07 ± 0.15
Mid	10	7.40 ± 0.28	10	0.030 ± 0.001	10	4.06 ± 0.15
High	10	7.28 ± 0.24	10	0.030 ± 0.001	10	3.97 ± 0.14
Trend[a]		0.480		0.784		0.616
Male						
Control	10	12.2 ± 0.35	10	0.028 ± 0.001	10	6.01 ± 0.24
Low	10	12.2 ± 0.43	10	0.029 ± 0.001	10	5.97 ± 0.23
Mid	10	12.2 ± 0.45	10	0.029 ± 0.001	10	6.14 ± 0.17
High	10	11.7 ± 0.30	10	0.028 ± 0.001	10	5.75 ± 0.15
Trend[a]		0.466		0.730		0.503

(*Table continues on next page*)

[a] *P* value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

[c] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.4 (*Continued*). Organ Weights in Rats at End of 13 Weeks of Exposure*

Organ / Sex / Exposure Group	Organ Weight		Organ-to-Body Weight Ratio		Organ-to-Brain Weight Ratio	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
Lungs						
Female						
Control	10	1.34 ± 0.03	10	0.0054 ± 0.0001	10	0.73 ± 0.01
Low	10	1.34 ± 0.03	10	0.0055 ± 0.0001	10	0.72 ± 0.02
Mid	10	1.35 ± 0.05	10	0.0055 ± 0.0001	10	0.74 ± 0.02
High	10	1.33 ± 0.03	10	0.0055 ± 0.0002	10	0.73 ± 0.02
Trend[a]		0.893		0.482		0.843
Male						
Control	10	1.83 ± 0.05	10	0.0042 ± 0.0001	10	0.90 ± 0.03
Low	10	1.85 ± 0.04	10	0.0043 ± 0.0001	10	0.90 ± 0.03
Mid	10	1.82 ± 0.06	10	0.0043 ± 0.0001	10	0.91 ± 0.02
High	10	1.91 ± 0.04	10	0.0045 ± 0.0001[b]	10	0.94 ± 0.02
Trend[a]		0.294		0.026 (+)		0.256
Ovaries						
Female						
Control	10	0.133 ± 0.009	10	0.0005 ± 0.0000	10	0.072 ± 0.005
Low	10	0.117 ± 0.009	10	0.0005 ± 0.0000	10	0.064 ± 0.005
Mid	10	0.134 ± 0.005	10	0.0005 ± 0.0000	10	0.073 ± 0.003
High	10	0.143 ± 0.007	10	0.0006 ± 0.0000	10	0.078 ± 0.004
Trend[a]		0.202		0.083 (+)		0.181
Testes						
Male						
Control	10	3.52 ± 0.08	10	0.0080 ± 0.0002	10	1.73 ± 0.05
Low	10	3.82 ± 0.10	10	0.0089 ± 0.0002	10	1.86 ± 0.06
Mid	10	3.40 ± 0.20	10	0.0081 ± 0.0005	10	1.71 ± 0.10
High	10	3.71 ± 0.08	10	0.0088 ± 0.0002	10	1.82 ± 0.04
Trend[a]		0.764		0.288		0.731
Thymus						
Female						
Control	10	0.339 ± 0.017	10	0.0014 ± 0.0001	10	0.185 ± 0.012
Low	10	0.310 ± 0.010	10	0.0013 ± 0.0000	10	0.168 ± 0.007
Mid	10	0.321 ± 0.020	10	0.0013 ± 0.0001	10	0.176 ± 0.010
High	10	0.315 ± 0.022	10	0.0013 ± 0.0001	10	0.172 ± 0.013
Trend[a]		0.437		0.604		0.531
Male						
Control	10	0.422 ± 0.016	10	0.0010 ± 0.0000	10	0.209 ± 0.010
Low	10	0.408 ± 0.053	10	0.0009 ± 0.0001	10	0.200 ± 0.026
Mid	10	0.421 ± 0.032	10	0.0010 ± 0.0001	10	0.211 ± 0.015
High	10	0.450 ± 0.030	10	0.0011 ± 0.0001	10	0.220 ± 0.015
Trend[a]		0.539		0.271		0.562

[a] P value and direction of exposure–response trend. Trend directions (+ or −) reported for $P < 0.1$.

[b] Significantly different from control group by Dunnett's multiple comparison procedure; $P < 0.05$.

[c] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5. Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
ALP								
4 Weeks								
Control	10	36 ± 4	10	34 ± 2	20	35 ± 2	0.919	ANOVA
Low	10	34 ± 3	10	30 ± 2	20	32 ± 2		
Mid	10	35 ± 3	10	33 ± 2	20	34 ± 2		
High	10	41 ± 2	10	36 ± 2	20	39 ± 2		
Trend[c]		0.221		0.309		0.107		
13 Weeks								
Control	10	38 ± 2	10	35 ± 2	20	36 ± 2	0.691	ANOVA
Low	10	37 ± 4	10	40 ± 2	20	39 ± 2		
Mid	10	35 ± 1	10	34 ± 5	20	34 ± 3		
High	10	40 ± 4	10	45 ± 4	20	43 ± 3		
Trend[c]		0.718		0.126		0.162		
Eosinophils Absolute Count								
4 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	0.775	Categorical
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.007 ± 0.007	10	0.000 ± 0.000	20	0.003 ± 0.003		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		0.297		—		0.989		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.012 ± 0.012	20	0.006 ± 0.006	0.532	Transformed
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.016 ± 0.016	20	0.008 ± 0.008		
Trend[c]		—		0.817		0.991		
Eosinophils Differential Cell Count								
4 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	0.775	Categorical
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.100 ± 0.100	10	0.000 ± 0.000	20	0.050 ± 0.050		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		0.297		—		0.989		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.100 ± 0.100	20	0.050 ± 0.050	0.529	Transformed
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.100 ± 0.100	20	0.050 ± 0.050		
Trend[c]		—		1.000		1.000		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5 (*Continued*). Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
LDH								
4 Weeks								
Control	10	41 ± 7	10	46 ± 4	20	44 ± 4	0.586	ANOVA
Low	10	30 ± 3	10	52 ± 6	20	41 ± 3		
Mid	10	34 ± 8	10	51 ± 8	20	43 ± 6		
High	10	29 ± 5	10	45 ± 6	20	37 ± 4		
Trend[c]		0.241		0.880		0.348		
13 Weeks								
Control	10	45 ± 9	10	44 ± 4	20	45 ± 5	0.384	ANOVA
Low	10	48 ± 11	10	42 ± 5	20	45 ± 6		
Mid	10	41 ± 8	10	45 ± 8	20	43 ± 6		
High	10	42 ± 6	10	62 ± 10	20	52 ± 6		
Trend[c]		0.651		0.085 (+)		0.440		
Lymphocytes Absolute Count								
4 Weeks								
Control	10	0.040 ± 0.015	10	0.006 ± 0.006	20	0.023 ± 0.008	0.071	Categorical
Low	10	0.000 ± 0.000[d]	10	0.016 ± 0.016	20	0.008 ± 0.008		
Mid	10	0.095 ± 0.077	10	0.000 ± 0.000	20	0.048 ± 0.038		
High	10	0.009 ± 0.009	10	0.010 ± 0.010	20	0.009 ± 0.007		
Trend[c]		0.994		0.940		0.821		
13 Weeks								
Control	10	0.019 ± 0.019	10	0.007 ± 0.007	20	0.013 ± 0.010	0.089	Categorical
Low	10	0.077 ± 0.040	10	0.000 ± 0.000	20	0.038 ± 0.020		
Mid	10	0.024 ± 0.016	10	0.000 ± 0.000	20	0.012 ± 0.008		
High	10	0.007 ± 0.007	10	0.031 ± 0.021	20	0.019 ± 0.011		
Trend[c]		0.212		0.234		0.459		
Lymphocytes Differential Cell Count								
4 Weeks								
Control	10	0.600 ± 0.221	10	0.100 ± 0.100	20	0.350 ± 0.121	0.055	Categorical
Low	10	0.000 ± 0.000[d]	10	0.200 ± 0.200	20	0.100 ± 0.100		
Mid	10	1.000 ± 0.699	10	0.000 ± 0.000	20	0.500 ± 0.350		
High	10	0.100 ± 0.100	10	0.100 ± 0.100	20	0.100 ± 0.071		
Trend[c]		0.603		0.654		0.433		
13 Weeks								
Control	10	0.200 ± 0.200	10	0.100 ± 0.100	20	0.150 ± 0.112	0.150	Categorical
Low	10	0.500 ± 0.224	10	0.000 ± 0.000	20	0.250 ± 0.112		
Mid	10	0.200 ± 0.133	10	0.000 ± 0.000	20	0.100 ± 0.067		
High	10	0.100 ± 0.100	10	0.300 ± 0.213	20	0.200 ± 0.118		
Trend[c]		0.380		0.372		0.497		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5 (*Continued*). Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Macrophages Absolute Count								
4 Weeks								
Control	10	7.2 ± 0.9	10	6.3 ± 0.9	20	6.8 ± 0.6	0.202	Transformed
Low	10	7.6 ± 0.8	10	4.8 ± 0.4	20	6.2 ± 0.4		
Mid	10	9.3 ± 1.3	10	6.6 ± 0.9	20	8.0 ± 0.8		
High	10	11.3 ± 2.1	10	5.4 ± 0.8	20	8.3 ± 1.1		
Trend[c]		0.033 (+)		0.692		0.196		
13 Weeks								
Control	10	11.2 ± 1.5	10	9.2 ± 1.2	20	10.2 ± 1.0	0.441	Transformed
Low	10	12.1 ± 1.6	10	6.5 ± 0.6	20	9.3 ± 0.9		
Mid	10	15.5 ± 2.2	10	8.2 ± 0.9	20	11.9 ± 1.2		
High	10	11.3 ± 1.9	10	7.7 ± 1.3	20	9.5 ± 1.1		
Trend[c]		0.812		0.474		0.746		
Macrophages Differential Cell Count								
4 Weeks								
Control	10	97.5 ± 1.5	10	99.1 ± 0.5	20	98.3 ± 0.8	0.356	Categorical
Low	10	99.0 ± 0.4	10	98.9 ± 0.6	20	99.0 ± 0.4		
Mid	10	98.7 ± 0.7	10	98.7 ± 0.8	20	98.7 ± 0.5		
High	10	99.6 ± 0.3	10	95.3 ± 3.1	20	97.5 ± 1.5		
Trend[c]		0.183		0.195		0.485		
13 Weeks								
Control	10	99.2 ± 0.4	10	99.1 ± 0.4	20	99.2 ± 0.3	0.442	Categorical
Low	10	98.1 ± 0.6	10	94.4 ± 3.2	20	96.3 ± 1.7		
Mid	10	92.2 ± 5.6	10	98.5 ± 1.4	20	95.4 ± 2.9		
High	10	97.2 ± 2.6	10	97.6 ± 1.3	20	97.4 ± 1.5		
Trend[c]		0.196		0.939		0.253		
Number of Epithelials per 100 Cells Absolute Count								
4 Weeks								
Control	10	1.900 ± 1.286	10	1.100 ± 0.823	20	1.500 ± 0.763	0.510	Categorical
Low	10	0.000 ± 0.000	10	0.200 ± 0.200	20	0.100 ± 0.100		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.200 ± 0.200	10	0.000 ± 0.000	20	0.100 ± 0.100		
Trend[c]		0.169		0.137		0.045 (−)		
13 Weeks								
Control	10	1.600 ± 0.686	10	1.500 ± 0.703	20	1.550 ± 0.491	0.623	Categorical
Low	10	0.400 ± 0.163	10	1.400 ± 1.087	20	0.900 ± 0.550		
Mid	10	0.800 ± 0.359	10	0.700 ± 0.335	20	0.750 ± 0.246		
High	10	1.200 ± 0.490	10	2.200 ± 0.904	20	1.700 ± 0.514		
Trend[c]		0.742		0.684		0.999		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5 (*Continued*). Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Number of Epithelials per 100 Cells Differential Cell Count								
4 Weeks								
Control	10	1.900 ± 1.286	10	1.100 ± 0.823	20	1.500 ± 0.763	0.510	Categorical
Low	10	0.000 ± 0.000	10	0.200 ± 0.200	20	0.100 ± 0.100		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.200 ± 0.200	10	0.000 ± 0.000	20	0.100 ± 0.100		
Trend[c]		0.169		0.137		0.045 (−)		
13 Weeks								
Control	10	1.600 ± 0.686	10	1.500 ± 0.703	20	1.550 ± 0.491	0.623	Categorical
Low	10	0.400 ± 0.163	10	1.400 ± 1.087	20	0.900 ± 0.550		
Mid	10	0.800 ± 0.359	10	0.700 ± 0.335	20	0.750 ± 0.246		
High	10	1.200 ± 0.490	10	2.200 ± 0.904	20	1.700 ± 0.514		
Trend[c]		0.742		0.684		0.999		
Other Absolute Count								
4 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
Other Differential Cell Count								
4 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		
13 Weeks								
Control	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000	—	No Analysis
Low	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Mid	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
High	10	0.000 ± 0.000	10	0.000 ± 0.000	20	0.000 ± 0.000		
Trend[c]		—		—		—		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5 (*Continued*). Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
PMN Absolute Count								
4 Weeks								
Control	10	0.17 ± 0.15	10	0.05 ± 0.03	20	0.11 ± 0.08	0.388	Transformed
Low	10	0.08 ± 0.03	10	0.04 ± 0.02	20	0.06 ± 0.02		
Mid	10	0.02 ± 0.01	10	0.11 ± 0.07	20	0.06 ± 0.03		
High	10	0.03 ± 0.03	10	0.44 ± 0.35	20	0.23 ± 0.17		
Trend[c]		0.081 (−)		0.672		0.444		
13 Weeks								
Control	10	0.09 ± 0.05	10	0.08 ± 0.04	20	0.08 ± 0.03	0.678	Transformed
Low	10	0.18 ± 0.08	10	0.54 ± 0.33	20	0.36 ± 0.17		
Mid	10	2.48 ± 2.25	10	0.14 ± 0.13	20	1.31 ± 1.13		
High	10	0.55 ± 0.55	10	0.20 ± 0.15	20	0.38 ± 0.28		
Trend[c]		0.838		0.852		0.779		
PMN Differential Cell Count								
4 Weeks								
Control	10	1.9 ± 1.6	10	0.8 ± 0.5	20	1.4 ± 0.8	0.259	Categorical
Low	10	1.0 ± 0.4	10	0.9 ± 0.5	20	1.0 ± 0.3		
Mid	10	0.2 ± 0.1	10	1.3 ± 0.8	20	0.8 ± 0.4		
High	10	0.3 ± 0.3	10	4.6 ± 3.1	20	2.5 ± 1.6		
Trend[c]		0.222		0.190		0.540		
13 Weeks								
Control	10	0.6 ± 0.3	10	0.7 ± 0.3	20	0.7 ± 0.2	0.699	Transformed
Low	10	1.4 ± 0.6	10	5.6 ± 3.2	20	3.5 ± 1.6		
Mid	10	7.6 ± 5.6	10	1.5 ± 1.4	20	4.6 ± 2.9		
High	10	2.7 ± 2.6	10	2.0 ± 1.4	20	2.4 ± 1.5		
Trend[c]		0.649		0.877		0.663		
Total Cells								
4 Weeks								
Control	10	7.4 ± 0.9	10	6.3 ± 0.9	20	6.9 ± 0.6	0.329	Transformed
Low	10	7.6 ± 0.8	10	4.9 ± 0.5	20	6.3 ± 0.4		
Mid	10	9.4 ± 1.3	10	6.7 ± 0.9	20	8.1 ± 0.8		
High	10	11.3 ± 2.1	10	5.9 ± 1.1	20	8.6 ± 1.2		
Trend[c]		0.044 (+)		0.882		0.175		
13 Weeks								
Control	10	11.3 ± 1.5	10	9.3 ± 1.2	20	10.3 ± 1.0	0.393	Transformed
Low	10	12.3 ± 1.7	10	7.0 ± 0.8	20	9.7 ± 0.9		
Mid	10	18.1 ± 3.3	10	8.3 ± 0.9	20	13.2 ± 1.7		
High	10	11.9 ± 2.1	10	7.9 ± 1.3	20	9.9 ± 1.2		
Trend[c]		0.654		0.492		0.891		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.5 (*Continued*). Bronchoalveolar Lavage Results in Rats at End of 4 and 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Time Point / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
µTP								
4 Weeks								
Control	10	6.0 ± 0.9	10	7.3 ± 1.0	20	6.7 ± 0.7	0.470	Transformed
Low	10	5.4 ± 0.5	10	7.6 ± 1.2	20	6.5 ± 0.6		
Mid	10	7.7 ± 1.1	10	7.4 ± 0.8	20	7.6 ± 0.7		
High	10	7.0 ± 0.8	10	6.9 ± 1.0	20	7.0 ± 0.6		
Trend[c]		0.140		0.536		0.677		
13 Weeks								
Control	10	6.0 ± 1.0	10	7.8 ± 0.6	20	6.9 ± 0.6	0.562	Transformed
Low	10	5.8 ± 0.7	10	6.7 ± 0.5	20	6.3 ± 0.4		
Mid	10	5.0 ± 1.0	10	6.8 ± 0.8	20	5.9 ± 0.6		
High	10	10.8 ± 1.8[d]	10	11.2 ± 1.5	20	11.0 ± 1.2[e]		
Trend[c]		0.030 (+)		0.072 (+)		0.004 (+)		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 10 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

[e] Significantly different from control group; $P < 0.01$.

* See Table 2 for units.

Table E.6. Bronchoalveolar Biochemical Results in Rats at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both		Interaction[a]	Method[b]
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM		
Albumin								
4 Weeks								
Control	10	49 ± 8	10	74 ± 12	20	61 ± 7	0.657	ANOVA
Low	10	47 ± 3	10	69 ± 10	20	58 ± 5		
Mid	10	68 ± 14	10	73 ± 6	20	71 ± 8		
High	10	56 ± 6	10	66 ± 5	20	61 ± 4		
Trend[c]		0.307		0.667		0.663		
13 Weeks								
Control	9	43 ± 4	10	64 ± 5	19	54 ± 3	0.566	Transformed
Low	10	41 ± 3	10	53 ± 5	20	47 ± 3		
Mid	10	38 ± 4	10	50 ± 5	20	44 ± 3		
High	10	79 ± 15[d]	10	81 ± 10	20	80 ± 9[d]		
Trend[c]		0.007 (+)		0.319		0.007 (+)		
GSH BALF								
4 Weeks								
Control	10	1.6 ± 0.4	10	1.1 ± 0.1	20	1.3 ± 0.2	0.653	Transformed
Low	10	1.0 ± 0.2	10	1.2 ± 0.2	20	1.1 ± 0.2		
Mid	10	1.3 ± 0.3	10	1.2 ± 0.2	20	1.2 ± 0.2		
High	10	1.6 ± 0.4	10	1.2 ± 0.3	20	1.4 ± 0.3		
Trend[c]		0.894		0.378		0.518		
13 Weeks								
Control	10	0.9 ± 0.2	10	0.6 ± 0.2	20	0.7 ± 0.2	0.748	Transformed
Low	10	0.7 ± 0.3	10	0.4 ± 0.1	20	0.5 ± 0.2		
Mid	10	0.5 ± 0.2	10	1.9 ± 1.3	20	1.2 ± 0.6		
High	10	0.5 ± 0.1	10	0.7 ± 0.2	20	0.6 ± 0.1		
Trend[c]		0.495		0.506		0.984		
GSSG BALF								
4 Weeks								
Control	10	0.39 ± 0.03	10	0.29 ± 0.06	20	0.34 ± 0.03	0.920	ANOVA
Low	10	0.43 ± 0.10	10	0.26 ± 0.03	20	0.35 ± 0.05		
Mid	10	0.35 ± 0.04	10	0.20 ± 0.03	20	0.27 ± 0.02		
High	10	0.35 ± 0.04	9	0.22 ± 0.05	19	0.29 ± 0.03		
Trend[c]		0.490		0.188		0.173		
13 Weeks								
Control	10	0.13 ± 0.04	10	0.20 ± 0.06	20	0.16 ± 0.04	0.167	Transformed
Low	10	0.11 ± 0.03	10	0.11 ± 0.03	20	0.11 ± 0.02		
Mid	10	0.06 ± 0.02	10	0.17 ± 0.03	20	0.12 ± 0.02		
High	10	0.10 ± 0.03	10	0.28 ± 0.08	20	0.19 ± 0.04		
Trend[c]		0.537		0.258		0.752		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.6 (*Continued*). Bronchoalveolar Biochemical Results in Rats at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Hemoglobin								
4 Weeks								
Control	9	0.0022 ± 0.0005	10	0.0016 ± 0.0005	19	0.0019 ± 0.0003	0.284	ANOVA
Low	10	0.0023 ± 0.0004	10	0.0027 ± 0.0008	20	0.0025 ± 0.0004		
Mid	10	0.0038 ± 0.0008	10	0.0026 ± 0.0003	20	0.0032 ± 0.0004		
High	10	0.0025 ± 0.0007	10	0.0033 ± 0.0005	20	0.0029 ± 0.0004		
Trend[c]		0.401		0.046 (+)		0.047 (+)		
13 Weeks								
Control	9	0.0049 ± 0.0009	10	0.0056 ± 0.0007	19	0.0052 ± 0.0006	0.901	ANOVA
Low	10	0.0054 ± 0.0008	10	0.0049 ± 0.0006	20	0.0052 ± 0.0005		
Mid	10	0.0045 ± 0.0009	10	0.0045 ± 0.0007	20	0.0045 ± 0.0006		
High	10	0.0058 ± 0.0007	10	0.0057 ± 0.0011	20	0.0058 ± 0.0006		
Trend[c]		0.624		0.977		0.747		
TEAC								
4 Weeks								
Control	10	39.7 ± 4.1	10	33.8 ± 3.1	20	36.8 ± 2.5	0.585	Transformed
Low	10	31.9 ± 1.5	10	23.5 ± 1.0[d]	20	27.7 ± 0.9[d]		
Mid	10	34.7 ± 3.2	10	23.8 ± 1.1[d]	20	29.3 ± 1.7[e]		
High	10	36.0 ± 3.3	10	28.1 ± 1.4	20	32.0 ± 1.8		
Trend[c]		0.617		0.141		0.192		
13 Weeks								
Control	10	40.2 ± 3.2	10	43.6 ± 3.5	20	41.9 ± 2.4	0.799	Transformed
Low	10	30.9 ± 2.2[e]	10	29.4 ± 1.8[d]	20	30.1 ± 1.4[d]		
Mid	10	29.2 ± 2.1[d]	10	31.3 ± 1.8[d]	20	30.3 ± 1.4[d]		
High	10	29.4 ± 1.3[e]	10	31.4 ± 1.9[d]	20	30.4 ± 1.1[d]		
Trend[c]		0.004 (−)		0.007 (−)		<0.001 (−)		
Total Glutathione BALF								
4 Weeks								
Control	10	2.0 ± 0.4	10	1.4 ± 0.1	20	1.7 ± 0.2	0.623	Transformed
Low	10	1.3 ± 0.2	10	1.5 ± 0.2	20	1.4 ± 0.2		
Mid	10	1.7 ± 0.3	10	1.4 ± 0.3	20	1.5 ± 0.2		
High	10	1.9 ± 0.5	10	1.4 ± 0.3	20	1.7 ± 0.3		
Trend[c]		0.738		0.268		0.343		
13 Weeks								
Control	10	1.0 ± 0.3	10	0.8 ± 0.2	20	0.9 ± 0.2	0.585	Transformed
Low	10	0.7 ± 0.3	10	0.5 ± 0.2	20	0.6 ± 0.2		
Mid	10	0.6 ± 0.2	10	2.0 ± 1.3	20	1.3 ± 0.6		
High	10	0.6 ± 0.1	10	0.9 ± 0.2	20	0.8 ± 0.1		
Trend[c]		0.485		0.301		0.839		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.7. Lung Tissue Results in Rats at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Cytokine CINC-3 Lung								
4 Weeks								
Control	10	4.5 ± 0.7	10	5.9 ± 0.8	20	5.2 ± 0.6	0.461	Transformed
Low	10	4.3 ± 0.5	10	8.2 ± 2.1	20	6.2 ± 1.1		
Mid	10	4.0 ± 0.3	9	4.5 ± 0.5	19	4.3 ± 0.3		
High	10	3.9 ± 0.5	10	4.7 ± 0.7	20	4.3 ± 0.4		
Trend[c]		0.615		0.107		0.110		
13 Weeks								
Control	10	5.1 ± 0.6	10	7.0 ± 1.4	20	6.1 ± 0.8	0.890	Transformed
Low	10	5.2 ± 0.4	10	7.1 ± 1.5	20	6.1 ± 0.8		
Mid	10	5.6 ± 1.1	10	9.4 ± 5.1	20	7.5 ± 2.6		
High	10	4.3 ± 0.4	10	7.6 ± 2.3	20	5.9 ± 1.2		
Trend[c]		0.318		0.907		0.557		
Cytokine IL-1β Lung								
4 Weeks								
Control	10	90 ± 20	10	49 ± 6	20	69 ± 11	0.329	Transformed
Low	10	65 ± 7	10	47 ± 7	20	56 ± 5		
Mid	10	53 ± 5	9	55 ± 7	19	54 ± 4		
High	10	61 ± 4	10	58 ± 9	20	59 ± 5		
Trend[c]		0.209		0.337		0.904		
13 Weeks								
Control	10	56 ± 6	10	38 ± 2	20	47 ± 3	0.013	Transformed
Low	10	52 ± 5	10	48 ± 6	20	50 ± 4		
Mid	10	59 ± 6	10	41 ± 4	20	50 ± 4		
High	10	50 ± 3	10	65 ± 10[d]	20	57 ± 5		
Trend[c]		0.695		0.011 (+)		0.104		
Cytokine IL-6 Lung								
4 Weeks								
Control	10	20.8 ± 2.9	10	21.4 ± 0.7	20	21.1 ± 1.5	0.937	ANOVA
Low	10	22.6 ± 2.0	10	23.3 ± 2.0	20	23.0 ± 1.4		
Mid	10	20.9 ± 1.8	9	23.5 ± 1.5	19	22.2 ± 1.2		
High	10	24.3 ± 2.4	10	24.5 ± 1.4	20	24.4 ± 1.4		
Trend[c]		0.407		0.151		0.136		
13 Weeks								
Control	10	14.3 ± 2.6	10	11.5 ± 2.4	20	12.9 ± 1.7	0.506	Transformed
Low	10	15.4 ± 3.2	10	16.8 ± 3.6	20	16.1 ± 2.4		
Mid	10	17.5 ± 3.1	10	11.8 ± 1.7	20	14.7 ± 1.8		
High	10	15.7 ± 2.5	10	31.7 ± 6.7[e]	20	23.7 ± 3.6		
Trend[c]		0.288		0.036 (+)		0.038 (+)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.7 (*Continued*). Lung Tissue Results in Rats at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Cytokine KC Lung								
4 Weeks								
Control	10	403 ± 84	10	394 ± 58	20	398 ± 51	0.576	Transformed
Low	10	275 ± 31	10	526 ± 126	20	400 ± 65		
Mid	10	265 ± 19	9	357 ± 52	19	311 ± 27		
High	10	325 ± 37	10	416 ± 71	20	371 ± 40		
Trend[c]		0.525		0.868		0.606		
13 Weeks								
Control	10	262 ± 29	10	245 ± 23	20	254 ± 19	0.414	Transformed
Low	10	261 ± 33	10	355 ± 81	20	308 ± 44		
Mid	10	339 ± 76	10	271 ± 48	20	305 ± 45		
High	10	271 ± 37	10	383 ± 57	20	327 ± 34		
Trend[c]		0.738		0.289		0.302		
Cytokine TNF-α Lung								
4 Weeks								
Control	10	2.9 ± 0.2	10	3.3 ± 0.2	20	3.1 ± 0.1	0.053	ANOVA
Low	10	3.9 ± 0.2	10	3.3 ± 0.2	20	3.6 ± 0.1		
Mid	10	3.2 ± 0.2	9	3.6 ± 0.3	19	3.4 ± 0.2		
High	10	3.9 ± 0.5	10	3.2 ± 0.2	20	3.5 ± 0.3		
Trend[c]		0.087 (+)		0.773		0.258		
13 Weeks								
Control	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	—	No Analysis
Low	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0		
Mid	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0		
High	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0		
Trend[c]		—		—		—		
GSH Lung								
4 Weeks								
Control	10	0.031 ± 0.004	10	0.024 ± 0.002	20	0.027 ± 0.002	0.506	Transformed
Low	10	0.033 ± 0.004	10	0.028 ± 0.004	20	0.031 ± 0.003		
Mid	10	0.029 ± 0.004	9	0.030 ± 0.004	19	0.029 ± 0.003		
High	10	0.036 ± 0.005	10	0.045 ± 0.006[e]	20	0.040 ± 0.004[e]		
Trend[c]		0.560		0.009 (+)		0.023 (+)		
13 Weeks								
Control	10	0.021 ± 0.004	10	0.016 ± 0.005	20	0.019 ± 0.003	0.977	ANOVA
Low	10	0.023 ± 0.003	10	0.020 ± 0.005	20	0.021 ± 0.003		
Mid	10	0.024 ± 0.004	10	0.022 ± 0.004	20	0.023 ± 0.003		
High	10	0.031 ± 0.007	10	0.026 ± 0.003	20	0.028 ± 0.004		
Trend[c]		0.188		0.109		0.036 (+)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.7 (*Continued*). Lung Tissue Results in Rats at End of 4 and 13 Weeks of Exposure*

Endpoint / Time Point / Group	Males		Females		Both		Interaction[a]	Method[b]
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM		
GSSG Lung								
4 Weeks								
Control	10	0.0049 ± 0.0014	10	0.0041 ± 0.0006	20	0.0045 ± 0.0008	0.592	Transformed
Low	10	0.0039 ± 0.0008	10	0.0067 ± 0.0020	20	0.0053 ± 0.0011		
Mid	10	0.0033 ± 0.0008	9	0.0040 ± 0.0009	19	0.0037 ± 0.0006		
High	10	0.0026 ± 0.0003	10	0.0042 ± 0.0009	20	0.0034 ± 0.0005		
Trend[c]		0.128		0.644		0.166		
13 Weeks								
Control	10	0.0164 ± 0.0011	10	0.0168 ± 0.0041	20	0.0166 ± 0.0021	0.783	ANOVA
Low	10	0.0188 ± 0.0014	10	0.0246 ± 0.0088	20	0.0217 ± 0.0044		
Mid	10	0.0204 ± 0.0033	10	0.0227 ± 0.0024	20	0.0216 ± 0.0020		
High	10	0.0206 ± 0.0036	10	0.0183 ± 0.0017	20	0.0195 ± 0.0020		
Trend[c]		0.228		0.909		0.505		
HO-1								
4 Weeks								
Control	10	1.4 ± 0.1	10	1.4 ± 0.1	20	1.4 ± 0.1	0.718	Transformed
Low	10	1.8 ± 0.2	10	1.5 ± 0.1	20	1.6 ± 0.1		
Mid	10	1.5 ± 0.1	9	1.6 ± 0.1	19	1.6 ± 0.1		
High	10	1.7 ± 0.1	10	1.7 ± 0.2	20	1.7 ± 0.1		
Trend[c]		0.139		0.131		0.034 (+)		
13 Weeks								
Control	10	1.2 ± 0.1	10	1.1 ± 0.1	20	1.1 ± 0.1	0.869	Transformed
Low	10	1.3 ± 0.1	10	1.2 ± 0.1	20	1.3 ± 0.1		
Mid	10	1.4 ± 0.1	10	1.4 ± 0.1	20	1.4 ± 0.1[e]		
High	10	1.7 ± 0.2[d]	10	1.7 ± 0.1[d]	20	1.7 ± 0.1[d]		
Trend[c]		0.002 (+)		<0.001 (+)		<0.001 (+)		
Total Glutathione Lung								
4 Weeks								
Control	10	0.036 ± 0.004	10	0.028 ± 0.002	20	0.032 ± 0.002	0.468	Transformed
Low	10	0.037 ± 0.005	10	0.035 ± 0.004	20	0.036 ± 0.003		
Mid	10	0.032 ± 0.005	9	0.034 ± 0.004	19	0.033 ± 0.003		
High	10	0.039 ± 0.005	10	0.049 ± 0.007[e]	20	0.044 ± 0.004		
Trend[c]		0.945		0.025 (+)		0.098 (+)		
13 Weeks								
Control	10	0.038 ± 0.004	10	0.033 ± 0.004	20	0.035 ± 0.003	0.888	ANOVA
Low	10	0.042 ± 0.004	10	0.036 ± 0.005	20	0.039 ± 0.003		
Mid	10	0.044 ± 0.007	10	0.045 ± 0.004	20	0.045 ± 0.004		
High	10	0.051 ± 0.010	10	0.044 ± 0.004	20	0.048 ± 0.005		
Trend[c]		0.165		0.033 (+)		0.017 (+)		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.01$.

[e] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.8. Hematology Results in Rats at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Basophils								
Control	10	0.25 ± 0.05	10	0.39 ± 0.08	20	0.32 ± 0.05	0.373	Categorical
Low	10	0.28 ± 0.04	10	0.35 ± 0.08	20	0.32 ± 0.05		
Mid	10	0.15 ± 0.04	10	0.40 ± 0.06	20	0.28 ± 0.04		
High	10	0.23 ± 0.03	10	0.36 ± 0.05	20	0.30 ± 0.03		
Trend[c]		0.229		0.887		0.263		
Basophils Absolute								
Control	10	0.007 ± 0.002	10	0.006 ± 0.002	20	0.007 ± 0.001	0.200	Categorical
Low	10	0.008 ± 0.001	10	0.006 ± 0.002	20	0.007 ± 0.001		
Mid	10	0.004 ± 0.002	10	0.007 ± 0.002	20	0.006 ± 0.001		
High	10	0.007 ± 0.002	10	0.004 ± 0.002	20	0.006 ± 0.001		
Trend[c]		0.536		0.537		0.383		
Eosinophils								
Control	10	2.3 ± 0.3	10	2.6 ± 0.3	20	2.4 ± 0.2	0.889	Transformed
Low	10	1.9 ± 0.2	10	2.5 ± 0.3	20	2.2 ± 0.2		
Mid	10	1.9 ± 0.2	10	2.7 ± 0.5	20	2.3 ± 0.3		
High	10	1.9 ± 0.2	10	2.7 ± 0.4	20	2.3 ± 0.2		
Trend[c]		0.343		0.935		0.596		
Eosinophils Absolute								
Control	10	0.065 ± 0.010	10	0.043 ± 0.007	20	0.054 ± 0.006	0.889	Categorical
Low	10	0.057 ± 0.006	10	0.046 ± 0.006	20	0.052 ± 0.004		
Mid	10	0.059 ± 0.004	10	0.045 ± 0.005	20	0.052 ± 0.003		
High	10	0.055 ± 0.007	10	0.041 ± 0.005	20	0.048 ± 0.004		
Trend[c]		0.428		0.768		0.512		
Hematocrit								
Control	10	45.9 ± 0.5	10	43.2 ± 0.7	20	44.5 ± 0.4	0.969	ANOVA
Low	10	46.3 ± 0.8	10	43.9 ± 0.9	20	45.1 ± 0.6		
Mid	10	45.5 ± 0.6	10	42.7 ± 0.6	20	44.1 ± 0.4		
High	10	44.8 ± 0.5	10	42.7 ± 1.1	20	43.8 ± 0.6		
Trend[c]		0.171		0.485		0.160		
Hemoglobin Measured								
Control	10	15.1 ± 0.2	10	14.3 ± 0.2	20	14.7 ± 0.1	0.955	ANOVA
Low	10	15.4 ± 0.3	10	14.7 ± 0.3	20	15.1 ± 0.2		
Mid	10	15.1 ± 0.2	10	14.3 ± 0.1	20	14.7 ± 0.1		
High	10	14.9 ± 0.1	10	14.4 ± 0.3	20	14.7 ± 0.2		
Trend[c]		0.460		0.885		0.548		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.8 (*Continued*). Hematology Results in Rats at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Large Unstained Cells								
Control	10	1.1 ± 0.1	10	1.3 ± 0.2	20	1.2 ± 0.1	0.402	Transformed
Low	10	1.1 ± 0.2	10	1.1 ± 0.1	20	1.1 ± 0.1		
Mid	10	0.8 ± 0.1	10	1.1 ± 0.1	20	0.9 ± 0.1		
High	10	0.7 ± 0.1	10	1.2 ± 0.2	20	1.0 ± 0.1		
Trend[c]		0.007 (−)		0.748		0.045 (−)		
Large Unstained Cells Absolute								
Control	10	0.031 ± 0.004	10	0.022 ± 0.004	20	0.027 ± 0.003	0.325	Categorical
Low	10	0.033 ± 0.004	10	0.021 ± 0.002	20	0.027 ± 0.002		
Mid	10	0.024 ± 0.003	10	0.021 ± 0.002	20	0.023 ± 0.002		
High	10	0.022 ± 0.005	10	0.022 ± 0.005	20	0.022 ± 0.004		
Trend[c]		0.062 (−)		1.000		0.242		
Lymphocytes								
Control	10	76 ± 2	10	74 ± 2	20	75 ± 1	0.261	ANOVA
Low	10	75 ± 2	10	70 ± 2	20	73 ± 2		
Mid	10	74 ± 2	10	75 ± 2	20	74 ± 1		
High	10	79 ± 2	10	72 ± 2	20	75 ± 1		
Trend[c]		0.424		0.763		0.740		
Lymphocytes Absolute								
Control	10	2.1 ± 0.2	10	1.3 ± 0.1	20	1.7 ± 0.1	0.950	ANOVA
Low	10	2.2 ± 0.2	10	1.4 ± 0.1	20	1.8 ± 0.1		
Mid	10	2.3 ± 0.2	10	1.3 ± 0.1	20	1.8 ± 0.1		
High	10	2.3 ± 0.2	10	1.3 ± 0.2	20	1.8 ± 0.1		
Trend[c]		0.581		0.970		0.678		
Mean Corpuscular Hemoglobin								
Control	10	17.9 ± 0.2	10	18.5 ± 0.2	20	18.2 ± 0.2	0.373	ANOVA
Low	10	18.7 ± 0.2	10	18.8 ± 0.2	20	18.8 ± 0.2		
Mid	10	18.4 ± 0.4	10	18.7 ± 0.2	20	18.6 ± 0.2		
High	10	18.3 ± 0.3	10	19.2 ± 0.2	20	18.8 ± 0.2		
Trend[c]		0.383		0.045 (+)		0.060 (+)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.8 (*Continued*). Hematology Results in Rats at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint/Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Mean Corpuscular Hemoglobin Concentration								
Control	10	32.9 ± 0.2	10	33.1 ± 0.3	20	33.0 ± 0.2	0.991	ANOVA
Low	10	33.3 ± 0.5	10	33.5 ± 0.4	20	33.4 ± 0.3		
Mid	10	33.2 ± 0.3	10	33.5 ± 0.4	20	33.4 ± 0.2		
High	10	33.3 ± 0.3	10	33.7 ± 0.3	20	33.5 ± 0.2		
Trend[c]		0.450		0.250		0.169		
Mean Corpuscular Volume								
Control	10	54.2 ± 0.6	10	55.9 ± 0.5	20	55.1 ± 0.4	0.203	ANOVA
Low	10	56.4 ± 0.7	10	56.1 ± 0.3	20	56.2 ± 0.4		
Mid	10	55.3 ± 0.7	10	55.9 ± 0.5	20	55.6 ± 0.4		
High	10	55.0 ± 0.4	10	56.8 ± 0.6	20	55.9 ± 0.4		
Trend[c]		0.647		0.247		0.284		
Monocytes								
Control	10	2.3 ± 0.2	10	3.1 ± 0.4	20	2.7 ± 0.2	0.947	Transformed
Low	10	2.7 ± 0.3	10	3.1 ± 0.4	20	2.9 ± 0.3		
Mid	10	2.3 ± 0.2	10	3.0 ± 0.4	20	2.6 ± 0.2		
High	10	2.5 ± 0.2	10	3.0 ± 0.4	20	2.7 ± 0.3		
Trend[c]		0.874		0.812		0.770		
Monocytes Absolute								
Control	10	0.07 ± 0.01	10	0.05 ± 0.01	20	0.06 ± 0.01	1.000	ANOVA
Low	10	0.08 ± 0.01	10	0.06 ± 0.01	20	0.07 ± 0.01		
Mid	10	0.07 ± 0.01	10	0.06 ± 0.01	20	0.06 ± 0.01		
High	10	0.07 ± 0.01	10	0.06 ± 0.01	20	0.06 ± 0.01		
Trend[c]		0.902		0.984		0.941		
Neutrophils								
Control	10	18 ± 1	10	18 ± 2	20	18 ± 1	0.141	Transformed
Low	10	19 ± 2	10	23 ± 3	20	21 ± 2		
Mid	10	21 ± 2	10	18 ± 1	20	19 ± 1		
High	10	16 ± 2	10	21 ± 2	20	19 ± 1		
Trend[c]		0.513		0.611		0.913		
Neutrophils Absolute								
Control	10	0.51 ± 0.06	10	0.31 ± 0.04	20	0.41 ± 0.04	0.148	Transformed
Low	10	0.55 ± 0.05	10	0.45 ± 0.08	20	0.50 ± 0.05		
Mid	10	0.71 ± 0.15	10	0.31 ± 0.05	20	0.51 ± 0.08		
High	10	0.47 ± 0.07	10	0.36 ± 0.05	20	0.42 ± 0.04		
Trend[c]		0.751		0.820		0.947		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.8 (*Continued*). Hematology Results in Rats at End of 13 Weeks of Exposure*

	Males		Females		Both			
Endpoint / Group	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
Platelet								
Control	10	784 ± 27	9	832 ± 46	19	808 ± 26	0.867	ANOVA
Low	10	835 ± 35	10	826 ± 36	20	831 ± 25		
Mid	10	814 ± 37	10	814 ± 31	20	814 ± 24		
High	10	841 ± 26	10	845 ± 48	20	843 ± 27		
Trend[c]		0.293		0.880		0.418		
Red Blood Cell Count								
Control	10	8.5 ± 0.1	10	7.7 ± 0.2	20	8.1 ± 0.1	0.741	ANOVA
Low	10	8.2 ± 0.2	10	7.8 ± 0.2	20	8.0 ± 0.1		
Mid	10	8.2 ± 0.2	10	7.6 ± 0.1	20	7.9 ± 0.1		
High	10	8.2 ± 0.1	10	7.5 ± 0.2	20	7.8 ± 0.1		
Trend[c]		0.186		0.264		0.081 (−)		
Reticulocytes								
Control	10	1.8 ± 0.1	10	2.4 ± 0.1	20	2.1 ± 0.1	0.068	Transformed
Low	10	2.8 ± 0.6[d]	10	2.3 ± 0.1	20	2.5 ± 0.3		
Mid	10	2.2 ± 0.1	10	2.3 ± 0.1	20	2.2 ± 0.1		
High	10	1.9 ± 0.1	10	2.5 ± 0.2	20	2.2 ± 0.1		
Trend[c]		0.910		0.778		0.802		
White Blood Cell Count								
Control	10	2.8 ± 0.2	10	1.7 ± 0.2	20	2.3 ± 0.1	0.751	ANOVA
Low	10	2.9 ± 0.2	10	2.0 ± 0.2	20	2.5 ± 0.1		
Mid	10	3.2 ± 0.3	10	1.8 ± 0.2	20	2.5 ± 0.2		
High	10	2.9 ± 0.3	10	1.8 ± 0.2	20	2.3 ± 0.2		
Trend[c]		0.692		0.974		0.765		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data; Categorical indicates categorized, weighted least-squares ANOVA of data with less than 6 different values.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.9. Serum Chemistry Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
A/G Ratio								
Control	10	1.86 ± 0.06	10	2.67 ± 0.06	20	2.27 ± 0.04	0.093	ANOVA
Low	10	1.88 ± 0.07	10	2.74 ± 0.08	20	2.31 ± 0.05		
Mid	10	1.92 ± 0.06	10	2.59 ± 0.09	20	2.26 ± 0.06		
High	10	1.96 ± 0.06	10	2.49 ± 0.07	20	2.22 ± 0.05		
Trend[c]		0.231		0.055 (−)		0.446		
ALB								
Control	10	3.96 ± 0.05	10	5.11 ± 0.14	20	4.54 ± 0.08	0.313	Transformed
Low	10	4.07 ± 0.06	10	5.00 ± 0.10	20	4.54 ± 0.06		
Mid	10	4.01 ± 0.03	10	4.92 ± 0.10	20	4.47 ± 0.05		
High	10	4.02 ± 0.04	10	4.83 ± 0.13	20	4.43 ± 0.07		
Trend[c]		0.546		0.104		0.219		
ALP								
Control	10	83 ± 4	10	40 ± 3	20	61 ± 3	0.708	ANOVA
Low	10	81 ± 4	10	41 ± 3	20	61 ± 2		
Mid	10	71 ± 6	10	37 ± 4	20	54 ± 4		
High	10	78 ± 7	10	34 ± 3	20	56 ± 4		
Trend[c]		0.333		0.153		0.117		
ALT								
Control	10	50 ± 1	10	84 ± 13	20	67 ± 7	0.804	Transformed
Low	10	64 ± 11	10	84 ± 11	20	74 ± 8		
Mid	10	50 ± 5	10	75 ± 15	20	63 ± 8		
High	10	50 ± 2	10	65 ± 9	20	57 ± 5		
Trend[c]		0.547		0.181		0.137		
AST								
Control	10	143 ± 14	10	217 ± 42	20	180 ± 22	0.719	Transformed
Low	10	135 ± 8	10	196 ± 25	20	166 ± 13		
Mid	10	149 ± 17	10	280 ± 59	20	215 ± 31		
High	10	115 ± 6	10	308 ± 121	20	211 ± 60		
Trend[c]		0.159		0.593		0.950		
BILI-T								
Control	10	0.13 ± 0.02	10	0.21 ± 0.01	20	0.17 ± 0.01	0.076	Categorical
Low	10	0.15 ± 0.02	10	0.19 ± 0.01	20	0.17 ± 0.01		
Mid	10	0.16 ± 0.02	10	0.20 ± 0.02	20	0.18 ± 0.01		
High	10	0.16 ± 0.02	10	0.17 ± 0.02	20	0.17 ± 0.01		
Trend[c]		0.138		0.052 (−)		0.543		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.9 (*Continued*). Serum Chemistry Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
BUN								
Control	10	20.4 ± 0.6	10	20.9 ± 0.8	20	20.7 ± 0.5	0.839	ANOVA
Low	10	19.0 ± 0.8	10	20.0 ± 0.6	20	19.5 ± 0.5		
Mid	10	19.8 ± 1.0	10	21.5 ± 1.1	20	20.7 ± 0.7		
High	10	18.5 ± 0.7	10	20.5 ± 1.4	20	19.5 ± 0.8		
Trend[c]		0.168		0.948		0.417		
BUN/CR								
Control	10	48 ± 2	10	58 ± 3	20	53 ± 2	0.785	ANOVA
Low	10	48 ± 2	10	52 ± 2	20	50 ± 1		
Mid	10	48 ± 3	10	57 ± 6	20	52 ± 3		
High	10	44 ± 4	10	54 ± 5	20	49 ± 3		
Trend[c]		0.279		0.741		0.374		
CA								
Control	10	10.7 ± 0.1	10	11.1 ± 0.2	20	10.9 ± 0.1	0.502	ANOVA
Low	10	11.0 ± 0.1	10	11.0 ± 0.1	20	11.0 ± 0.1		
Mid	10	10.7 ± 0.1	10	11.0 ± 0.1	20	10.8 ± 0.1		
High	10	10.7 ± 0.1	10	11.0 ± 0.2	20	10.8 ± 0.1		
Trend[c]		0.629		0.476		0.386		
CHOL								
Control	10	71 ± 2	10	81 ± 5	20	76 ± 3	0.018	ANOVA
Low	10	81 ± 3[d]	10	71 ± 3	20	76 ± 2		
Mid	10	71 ± 3	10	65 ± 3[d]	20	68 ± 2		
High	10	74 ± 3	10	78 ± 3	20	76 ± 2		
Trend[c]		0.966		0.323		0.473		
CK								
Control	2	1087 ± 394	1	2427	3	1757 ± 341	0.250	ANOVA
Low	2	843 ± 265	1	742	3	793 ± 229		
Mid	2	1160 ± 86	1	2101	3	1630 ± 74		
High	2	687 ± 292	1	724	3	705 ± 252		
Trend[c]		0.521		—		0.181		
CL-S								
Control	10	102 ± 0.4	10	103 ± 0.5	20	103 ± 0.3	0.683	Transformed
Low	10	102 ± 0.3	10	104 ± 0.8	20	103 ± 0.4		
Mid	10	103 ± 0.8	10	105 ± 1.1	20	104 ± 0.7[d]		
High	10	102 ± 0.5	10	104 ± 0.4	20	103 ± 0.3		
Trend[c]		0.775		0.247		0.259		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.9 (*Continued*). Serum Chemistry Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
CREA								
Control	10	0.43 ± 0.02	10	0.37 ± 0.02	20	0.40 ± 0.02	0.486	Categorical
Low	10	0.40 ± 0.01	10	0.39 ± 0.02	20	0.40 ± 0.01		
Mid	10	0.42 ± 0.01	10	0.40 ± 0.03	20	0.41 ± 0.01		
High	10	0.46 ± 0.06	10	0.39 ± 0.02	20	0.43 ± 0.03		
Trend[c]		0.557		0.409		0.594		
GGT								
Control	10	0.0000 ± 0.0000	10	0.0000 ± 0.0000	20	0.0000 ± 0.0000	0.476	Categorical
Low	10	0.0000 ± 0.0000	10	0.0000 ± 0.0000	20	0.0000 ± 0.0000		
Mid	10	0.0000 ± 0.0000	10	0.2000 ± 0.1333	20	0.1000 ± 0.0667		
High	10	0.0000 ± 0.0000	10	0.0000 ± 0.0000	20	0.0000 ± 0.0000		
Trend[c]		—		0.116		0.987		
GLOB								
Control	10	2.16 ± 0.06	10	1.92 ± 0.04	20	2.04 ± 0.04	0.282	ANOVA
Low	10	2.20 ± 0.06	10	1.84 ± 0.05	20	2.02 ± 0.04		
Mid	10	2.11 ± 0.06	10	1.92 ± 0.07	20	2.02 ± 0.05		
High	10	2.08 ± 0.05	10	1.93 ± 0.05	20	2.01 ± 0.03		
Trend[c]		0.211		0.659		0.543		
GLU								
Control	10	249 ± 7	10	228 ± 15	20	239 ± 8	0.388	ANOVA
Low	10	280 ± 17	10	226 ± 13	20	253 ± 11		
Mid	10	238 ± 10	10	217 ± 12	20	227 ± 8		
High	10	247 ± 16	10	194 ± 11	20	220 ± 10		
Trend[c]		0.402		0.061 (−)		0.055 (−)		
K-S								
Control	10	5.5 ± 0.2	10	4.8 ± 0.3	20	5.1 ± 0.2	0.756	ANOVA
Low	10	5.3 ± 0.2	10	4.9 ± 0.1	20	5.1 ± 0.1		
Mid	10	5.5 ± 0.2	10	5.3 ± 0.2	20	5.4 ± 0.2		
High	10	5.3 ± 0.2	10	5.0 ± 0.3	20	5.2 ± 0.2		
Trend[c]		0.774		0.391		0.659		
LDH								
Control	2	1483 ± 607	1	2131	3	1807 ± 526	0.719	ANOVA
Low	2	1081 ± 390	1	920	3	1001 ± 338		
Mid	2	1641 ± 287	1	1148	3	1395 ± 249		
High	2	659 ± 267	1	843	3	751 ± 231		
Trend[c]		0.356		—		0.101		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.9 (*Continued*). Serum Chemistry Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
NA-S								
Control	10	146 ± 0.7	10	146 ± 0.4	20	146 ± 0.4	0.981	ANOVA
Low	10	145 ± 0.6	10	145 ± 0.8	20	145 ± 0.5		
Mid	10	146 ± 0.7	10	146 ± 1.3	20	146 ± 0.7		
High	10	144 ± 0.6	10	145 ± 0.7	20	145 ± 0.5		
Trend[c]		0.314		0.360		0.173		
PHOS								
Control	10	6.6 ± 0.4	10	6.1 ± 0.5	20	6.4 ± 0.3	0.731	ANOVA
Low	10	6.6 ± 0.3	10	6.1 ± 0.2	20	6.3 ± 0.2		
Mid	10	6.8 ± 0.4	10	6.3 ± 0.4	20	6.6 ± 0.3		
High	10	6.8 ± 0.3	10	5.6 ± 0.5	20	6.2 ± 0.3		
Trend[c]		0.639		0.450		0.774		
TP								
Control	10	6.1 ± 0.1	10	7.0 ± 0.2	20	6.6 ± 0.1	0.511	Transformed
Low	10	6.3 ± 0.1	10	6.8 ± 0.1	20	6.6 ± 0.1		
Mid	10	6.1 ± 0.1	10	6.8 ± 0.1	20	6.5 ± 0.1		
High	10	6.1 ± 0.1	10	6.8 ± 0.2	20	6.4 ± 0.1		
Trend[c]		0.497		0.235		0.168		
TRIG								
Control	10	140 ± 19	10	79 ± 18	20	110 ± 13	0.071	ANOVA
Low	10	149 ± 15	10	75 ± 11	20	112 ± 9		
Mid	10	91 ± 13	10	107 ± 24	20	99 ± 14		
High	10	120 ± 18	10	91 ± 23	20	105 ± 14		
Trend[c]		0.112		0.459		0.649		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P < 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.10. Pulmonary Function Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both		Interaction[a]	Method[b]
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM		
SVC								
Control	10	18.0 ± 0.8	10	13.6 ± 0.5	20	15.8 ± 0.5	0.912	ANOVA
Low	10	17.1 ± 0.4	10	13.2 ± 0.4	20	15.1 ± 0.3		
Mid	10	17.3 ± 0.4	10	13.6 ± 0.3	20	15.5 ± 0.2		
High	10	16.8 ± 0.7	10	12.7 ± 0.4	20	14.8 ± 0.4		
Trend[c]		0.213		0.297		0.099 (−)		
Cqs								
Control	10	0.95 ± 0.04	10	0.74 ± 0.04	20	0.85 ± 0.03	0.794	ANOVA
Low	10	0.92 ± 0.02	10	0.74 ± 0.02	20	0.83 ± 0.01		
Mid	10	0.96 ± 0.04	10	0.81 ± 0.04	20	0.89 ± 0.03		
High	10	0.90 ± 0.04	10	0.70 ± 0.03	20	0.80 ± 0.02		
Trend[c]		0.518		0.713		0.461		
FVC								
Control	10	17.4 ± 0.8	10	12.8 ± 0.5	20	15.1 ± 0.4	0.743	ANOVA
Low	10	16.2 ± 0.4	10	12.5 ± 0.5	20	14.4 ± 0.3		
Mid	10	16.6 ± 0.3	10	12.8 ± 0.3	20	14.7 ± 0.2		
High	10	16.1 ± 0.6	10	11.8 ± 0.3	20	13.9 ± 0.3[d]		
Trend[c]		0.172		0.125		0.042 (−)		
FVC/kg								
Control	10	39.2 ± 1.5	10	51.6 ± 2.2	20	45.4 ± 1.3	0.461	ANOVA
Low	10	37.2 ± 1.6	10	51.4 ± 2.1	20	44.3 ± 1.3		
Mid	10	38.5 ± 0.7	10	52.5 ± 1.4	20	45.5 ± 0.8		
High	10	38.4 ± 0.7	10	48.3 ± 0.9	20	43.4 ± 0.6		
Trend[c]		0.813		0.273		0.295		
$FEV_{0.1}$								
Control	10	6.4 ± 0.2	10	5.8 ± 0.2	20	6.1 ± 0.2	0.379	ANOVA
Low	10	6.4 ± 0.2	10	5.5 ± 0.3	20	5.9 ± 0.2		
Mid	10	6.1 ± 0.3	10	5.9 ± 0.2	20	6.0 ± 0.2		
High	10	6.4 ± 0.2	10	5.6 ± 0.1	20	6.0 ± 0.1		
Trend[c]		0.861		0.798		0.762		
$FEV_{0.1}$/FVC (%)								
Control	10	37.2 ± 1.3	10	45.5 ± 2.3	20	41.3 ± 1.3	0.536	ANOVA
Low	10	39.8 ± 2.3	10	44.2 ± 2.3	20	42.0 ± 1.6		
Mid	10	36.8 ± 1.6	10	46.1 ± 1.4	20	41.4 ± 1.0		
High	10	40.3 ± 1.4	10	47.3 ± 0.9	20	43.8 ± 0.8		
Trend[c]		0.419		0.383		0.230		
PEFR								
Control	10	82 ± 2	10	75 ± 2	20	78 ± 1	0.262	ANOVA
Low	10	81 ± 2	10	67 ± 4	20	74 ± 2		
Mid	10	81 ± 2	10	71 ± 3	20	76 ± 2		
High	10	80 ± 2	10	65 ± 1[d]	20	73 ± 1		
Trend[c]		0.55		0.039 (−)		0.045 (−)		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.10 (*Continued*). Pulmonary Function Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
PF/FVC								
Control	10	4.7 ± 0.1	10	5.8 ± 0.1	20	5.3 ± 0.1	0.117	ANOVA
Low	10	5.1 ± 0.2	10	5.4 ± 0.3	20	5.2 ± 0.2		
Mid	10	4.9 ± 0.1	10	5.5 ± 0.2	20	5.2 ± 0.1		
High	10	5.0 ± 0.2	10	5.6 ± 0.1	20	5.3 ± 0.1		
Trend[c]		0.256		0.423		0.978		
FEF_{75}								
Control	10	76.4 ± 2.2	10	69.7 ± 1.6	20	73.0 ± 1.3	0.454	ANOVA
Low	10	75.8 ± 1.5	10	63.3 ± 4.4	20	69.5 ± 2.3		
Mid	10	74.8 ± 2.2	10	67.9 ± 2.2	20	71.4 ± 1.6		
High	10	74.7 ± 1.8	10	63.2 ± 1.0	20	68.9 ± 1.0		
Trend[c]		0.49		0.212		0.154		
FEF_{75}/FVC								
Control	10	4.4 ± 0.1	10	5.5 ± 0.1	20	4.9 ± 0.1	0.302	ANOVA
Low	10	4.7 ± 0.2	10	5.1 ± 0.4	20	4.9 ± 0.2		
Mid	10	4.5 ± 0.1	10	5.3 ± 0.2	20	4.9 ± 0.1		
High	10	4.7 ± 0.2	10	5.4 ± 0.1	20	5.0 ± 0.1		
Trend[c]		0.384		0.955		0.673		
FEF_{50}								
Control	10	76 ± 4	10	66 ± 2	20	71 ± 2	0.229	ANOVA
Low	10	77 ± 2	10	56 ± 5	20	67 ± 3		
Mid	10	73 ± 2	10	63 ± 3	20	68 ± 2		
High	10	72 ± 3	10	54 ± 3	20	63 ± 2[d]		
Trend[c]		0.18		0.071 (−)		0.025 (−)		
FEF_{50}/FVC								
Control	10	4.4 ± 0.1	10	5.2 ± 0.2	20	4.8 ± 0.1	0.074	ANOVA
Low	10	4.8 ± 0.1	10	4.5 ± 0.4	20	4.6 ± 0.2		
Mid	10	4.4 ± 0.2	10	4.9 ± 0.2	20	4.6 ± 0.1		
High	10	4.5 ± 0.1	10	4.5 ± 0.2	20	4.5 ± 0.1		
Trend[c]		0.841		0.223		0.245		
FEF_{25}								
Control	10	44 ± 3	10	37 ± 2	20	41 ± 2	0.209	ANOVA
Low	10	47 ± 3	10	33 ± 3	20	40 ± 2		
Mid	10	42 ± 3	10	36 ± 3	20	39 ± 2		
High	10	42 ± 3	10	28 ± 2[d]	20	35 ± 2		
Trend[c]		0.458		0.030 (−)		0.045 (−)		
FEF_{25}/FVC								
Control	10	2.5 ± 0.2	10	2.9 ± 0.2	20	2.7 ± 0.1	0.087	ANOVA
Low	10	2.9 ± 0.2	10	2.6 ± 0.2	20	2.8 ± 0.1		
Mid	10	2.5 ± 0.2	10	2.8 ± 0.2	20	2.7 ± 0.1		
High	10	2.6 ± 0.1	10	2.3 ± 0.2	20	2.5 ± 0.1		
Trend[c]		0.819		0.067 (−)		0.119		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

Table E.10 (*Continued*). Pulmonary Function Results in Rats at End of 13 Weeks of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
FEF_{10}								
Control	10	22.0 ± 4.4	10	14.9 ± 2.4	20	18.4 ± 2.5	0.842	Transformed
Low	10	17.1 ± 1.9	10	13.0 ± 1.4	20	15.0 ± 1.2		
Mid	10	15.4 ± 1.8	10	12.5 ± 1.5	20	13.9 ± 1.2		
High	10	17.0 ± 3.2	10	9.3 ± 0.7	20	13.1 ± 1.6		
Trend[c]		0.292		0.107		0.053 (−)		
FEF_{10}/FVC								
Control	10	1.3 ± 0.3	10	1.2 ± 0.2	20	1.2 ± 0.2	0.870	Transformed
Low	10	1.1 ± 0.1	10	1.0 ± 0.1	20	1.0 ± 0.1		
Mid	10	0.9 ± 0.1	10	1.0 ± 0.1	20	0.9 ± 0.1		
High	10	1.0 ± 0.1	10	0.8 ± 0.1	20	0.9 ± 0.1		
Trend[c]		0.402		0.176		0.110		
MMEF								
Control	10	60 ± 2	10	54 ± 1	20	57 ± 1	0.149	ANOVA
Low	10	61 ± 2	10	48 ± 4	20	55 ± 2		
Mid	10	58 ± 1	10	52 ± 2	20	55 ± 1		
High	10	58 ± 2	10	45 ± 1[d]	20	52 ± 1		
Trend[c]		0.329		0.054 (−)		0.035 (−)		
MF/FVC								
Control	10	3.47 ± 0.08	10	4.21 ± 0.12	20	3.84 ± 0.07	0.063	ANOVA
Low	10	3.81 ± 0.14[d]	10	3.84 ± 0.29	20	3.82 ± 0.16		
Mid	10	3.51 ± 0.08	10	4.06 ± 0.13	20	3.79 ± 0.08		
High	10	3.63 ± 0.04	10	3.85 ± 0.08	20	3.74 ± 0.05		
Trend[c]		0.648		0.292		0.482		
DL_{CO}								
Control	10	0.36 ± 0.02	10	0.28 ± 0.02	20	0.32 ± 0.01	0.929	ANOVA
Low	10	0.35 ± 0.01	10	0.26 ± 0.02	20	0.30 ± 0.01		
Mid	10	0.34 ± 0.02	10	0.25 ± 0.01	20	0.30 ± 0.01		
High	10	0.33 ± 0.02	10	0.25 ± 0.01	20	0.29 ± 0.01		
Trend[c]		0.158		0.189		0.050 (−)		
DL_{CO}/kg								
Control	10	0.82 ± 0.05	10	1.14 ± 0.08	20	0.98 ± 0.05	0.983	Transformed
Low	10	0.79 ± 0.03	10	1.05 ± 0.06	20	0.92 ± 0.03		
Mid	10	0.79 ± 0.05	10	1.04 ± 0.05	20	0.91 ± 0.03		
High	10	0.79 ± 0.05	10	1.04 ± 0.05	20	0.92 ± 0.04		
Trend[c]		0.595		0.440		0.343		
DL_{CO}/VA								
Control	10	0.017 ± 0.001	10	0.016 ± 0.001	20	0.017 ± 0.001	0.851	ANOVA
Low	10	0.017 ± 0.001	10	0.015 ± 0.001	20	0.016 ± 0.001		
Mid	10	0.016 ± 0.001	10	0.016 ± 0.001	20	0.016 ± 0.001		
High	10	0.016 ± 0.001	10	0.015 ± 0.001	20	0.016 ± 0.001		
Trend[c]		0.236		0.811		0.289		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

[d] Significantly different from control group; $P < 0.05$.

* See Table 2 for units.

APPENDIX F. Histopathology and Pulmonary Function Findings in Rats at 12 Months

Although not a focus of this report, the results of biologic responses for pulmonary function and histopathology in rats at 12 months are reported here. These endpoints are included because the evaluation of their potential progression may help to place changes at after 3 months of exposure in better perspective. The histologic findings at 12 months showed a small increase in the extent of the tissue changes in the respiratory tract, but the severity of the changes remained mostly minimal. Tables F.1 and F.2 provide a summary of the incidence, severity, and statistical significance of tissue changes in the lungs and nasal regions, respectively, and can be compared with Tables 7 and 8 (showing 3-month results) in the text of the main report. Figures F.1 through F.3 provide representative photomicrographs of findings in the lungs at the high exposure level. As with the 3-month tissue findings, these findings were limited to the respiratory tract and were characterized by an increased number and prominence of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli that were in close proximity to the terminal bronchial and alveolar ducts. The distribution was rather uniform and focused at the central acinus. The animals exposed for 12 months showed tissue changes remaining mostly minimal and restricted to the central acinar region (albeit a larger portion of that region compared with the animals exposed for 3 months). Furthermore, the incidence of fibrotic tissue at 12 months was found across all animals at the high-exposure level, compared with only scattered observations of this finding at 3 months. In addition to the epithelial proliferation, there was a subtle accumulation after both 3 months and 12 months of exposure of pulmonary alveolar macrophages and minimal bronchiolization in a few animals. The nasal findings showed slight degeneration of the olfactory epithelium. The finding was observed in some animals in each of the DE exposure levels. At 3 months these findings were observed only at the high-exposure level. The pattern of olfactory lesion distribution, while increased in exposed rats, was not completely consistent with what might be expected with an olfactory epithelial toxicant.

The pulmonary function changes (Table F.3) observed at 3 months in the rats persisted only for DL_{CO} at 12 months. The changes in DL_{CO} remained small and did not progress compared with the 3-month observations. Similar to the 3-month findings, the 12-month results were statistically significant only when sexes were combined and also normalized for body weight. Overall, the lack of observed findings or progression in the pulmonary function parameters suggests that the biologic significance of these observations is small.

Table F.1. Lung Tissue Findings in Wistar Han Rats After 12 Months of Exposure

	Incidence[a] of Lesions with Severity Score > 0[b] (*P* Value)[c]		
Finding / Group	Males	Females	Both
Accumulation; macrophage			
Control	0/10 (0.012)	0/10 (0.012)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	3/10 (0.105)	3/10 (0.105)	6/20 (0.010)
Bronchiolization			
Control	0/10 (0.250)	0/10 (0.058)	0/20 (0.014)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	1/10 (0.500)	2/10 (0.237)	3/20 (0.115)
Fibrosis; interstitial			
Control	0/10 (<0.001)	0/10 (<0.001)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	8/10 (<0.001)	10/10 (<0.001)	18/20 (<0.001)
Histiocytosis; alveolar; focal			
Control	6/10[d] (0.160)	9/10[d] (0.036N)	15/20[e] (0.290N)
Low	9/10[e] (0.152)	7/10[d] (0.291)	16/20[f] (0.500)
Mid	7/10[d] (0.500)	6/10[d] (0.152)	13/20[e] (0.366)
High	9/10 (0.152)	5/10 (0.070)	14/20 (0.500)
Hyperplasia; epithelium; periacinar			
Control	0/10 (<0.001)	0/10 (<0.001)	0/20 (<0.001)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	10/10[d] (<0.001)	10/10[d] (<0.001)	20/20[e] (<0.001)
Infiltrate; mixed cell			
Control	4/10 (0.125N)	4/10 (0.390N)	8/20 (0.133N)
Low	6/10[d] (0.328)	5/10 (0.500)	11/20[d] (0.264)
Mid	3/10 (0.500)	5/10 (0.500)	8/20 (0.626)
High	2/10 (0.314)	3/10 (0.500)	5/20 (0.250)

[a] Incidences reported where there was more than one lesion-bearing animal across sexes.

[b] Lesion-bearing animals/total number of animals necropsied. Unless otherwise indicated, reported incidence reflects number of animals with a severity score of 1.

[c] *P* values for treated groups reflect Fisher two-sided exact tests against control incidence. *P* value listed for control group is for Cochran-Armitage one-sided trend test; N denotes a decreasing trend across exposures.

[d] One animal had a severity score of 2.

[e] Two animals had a severity score of 2.

[f] Three animals had a severity score of 2.

Table F.2. Nasal Tissue Findings in Wistar Han Rats After 12 Months of Exposure

Finding/Group	Incidence[a] of Lesions with Severity Score > 0[b] (*P* Value)[c]		
	Males	Females	Both
Turbinate 1			
Degeneration; olfactory epithelium			
Control	0/10 (0.250)	0/10 (0.058)	0/20 (0.014)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	1/10 (0.500)	2/10 (0.237)	3/20 (0.115)
Metaplasia; squamous; lateral wall			
Control	0/10 (0.372)	0/10 (0.250)	0/20 (0.153)
Low	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	1/10 (0.500)	1/10 (0.500)	2/20 (0.244)
Turbinate 2			
Degeneration; olfactory epithelium			
Control	0/10 (0.003)	0/10 (0.071)	0/20 (0.001)
Low	1/10 (0.500)	1/10 (0.500)	2/20 (0.244)
Mid	2/10 (0.237)	4/10[d] (0.043)	6/20[d] (0.010)
High	5/10[d] (0.016)	2/10[e] (0.237)	7/20[f] (0.004)
Metaplasia; squamous; olfactory epithelium			
Control	0/10 (0.372)	0/10 (1.000)	0/20 (0.373)
Low	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
Mid	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
High	1/10[d] (0.500)	0/10 (1.000)	1/20[d] (0.500)
Respiratory metaplasia; olfactory epithelium			
Control	0/10 (0.015)	0/10 (0.250)	0/20 (0.005)
Low	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)
Mid	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
High	3/10 (0.105)	1/10 (0.500)	4/20 (0.053)
Turbinate 3			
Degeneration; olfactory epithelium			
Control	0/10 (0.308N)	0/10 (0.051)	0/20 (0.244)
Low	3/10[d] (0.105)	0/10 (1.000)	3/20[d] (0.115)
Mid	0/10 (1.000)	2/10[d] (0.237)	2/20[d] (0.244)
High	0/10 (1.000)	2/10 (0.237)	2/20 (0.244)
Metaplasia; squamous; olfactory epithelium			
Control	0/10 (0.500N)	0/10 (0.500)	0/20 (0.627)
Low	1/10 (0.500)	0/10 (1.000)	1/20 (0.500)
Mid	0/10 (1.000)	1/10 (0.500)	1/20 (0.500)
High	0/10 (1.000)	0/10 (1.000)	0/20 (1.000)

[a] Incidences reported where there was more than one lesion-bearing animal across sexes.

[b] Lesion-bearing animals/total number of animals necropsied. Unless otherwise indicated, reported incidence reflects number of animals with a severity score of 1.

[c] *P* values for treated groups reflect Fisher two-sided exact tests against control incidence. *P* value listed for control group is for Cochran-Armitage one-sided trend test; N denotes a decreasing trend across exposures.

[d] One animal had a severity score of 2.

[e] Two animals had a severity score of 2.

[f] Three animals had a severity score of 2.

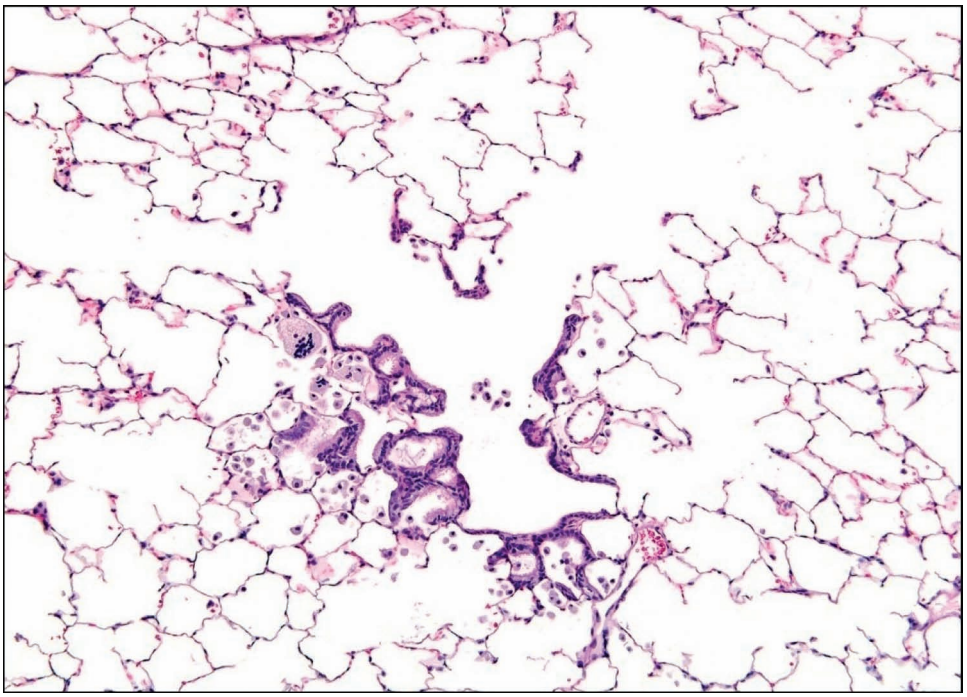

Figure F.1. Photomicrograph (original magnification ×100) of lung tissue from a representative Wistar Han rat (female) after 12 months of exposure to the highest level of DE from 2007-compliant engines. This photomicrograph illustrates typical findings of periacinar epithelial hyperplasia at the high exposure level. Compared with the 3-month findings, the hyperplasia extends further into the acinus, but the severity of the findings is scored the same at 12 months as at 3 months for most animals.

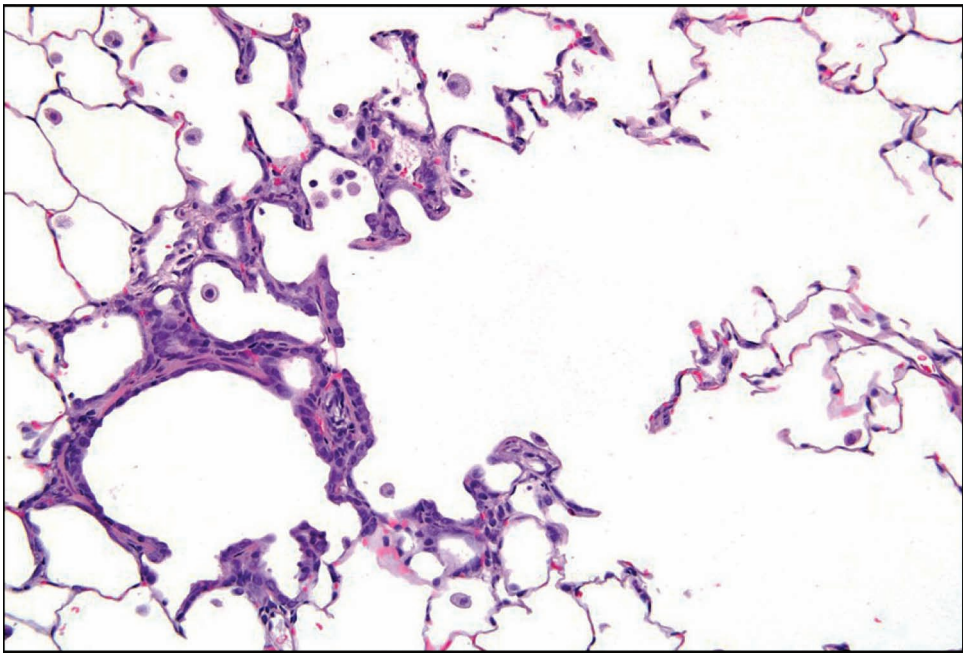

Figure F.2. Photomicrograph (original magnification ×200) of lung tissue from a representative Wistar Han rat (male) after 12 months of exposure to the highest level of DE from 2007-compliant engines. This photomicrograph illustrates typical findings of enhanced thickened alveolar septae with an increase in fibrous tissue. Compared with the 3-month findings, the septae show increased thickness. Fibrous tissue was found in all animals exposed to high levels of DE at 12 months versus a smaller number of animals at 3 months.

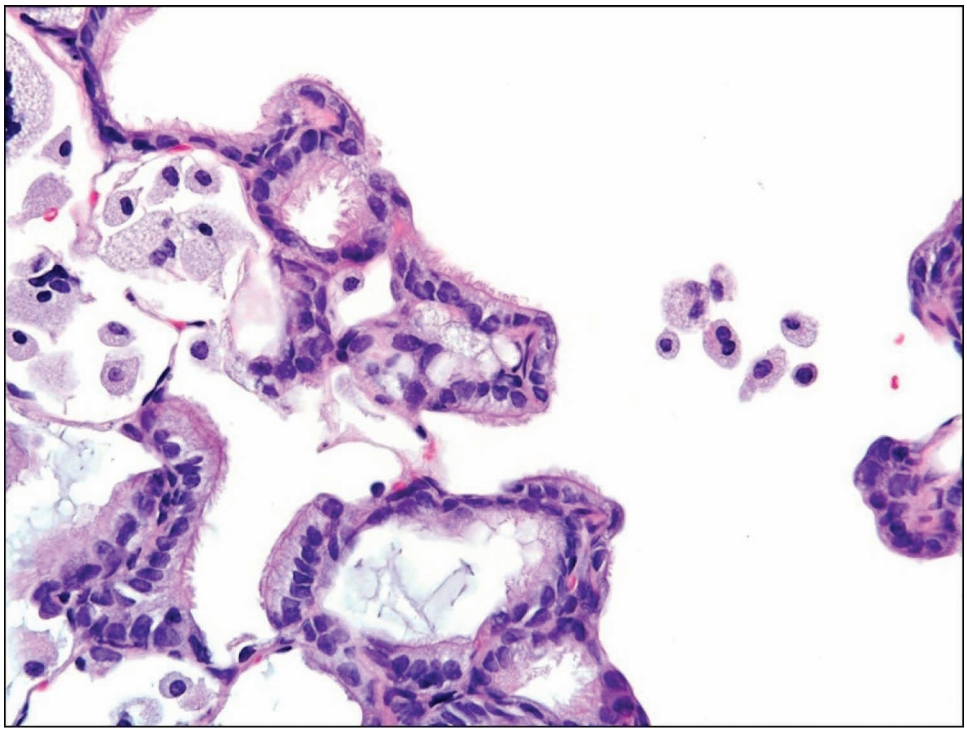

Figure F.3. Photomicrograph (original magnification ×400) of lung tissue from a representative Wistar Han rat (female) after 12 months of exposure to the highest level of DE from 2007-compliant engines. This photomicrograph illustrates bronchiolization, with the observation of cuboidal ciliated cells in the alveolar area. Bronchiolization was noted in 3 out of 20 animals at 12 months, but was not observed at 3 months. This photomicrograph is a close-up of Figure F.1.

Table F.3. Pulmonary Function Findings in Wistar Han Rats After 12 Months of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
SVC								
Control	10	22.3 ± 0.6	10	15.5 ± 0.8	20	18.9 ± 0.5	0.362	ANOVA
Low	10	21.9 ± 1.0	10	14.8 ± 0.7	20	18.3 ± 0.6		
Mid	10	21.3 ± 0.7	10	16.5 ± 0.8	20	18.9 ± 0.5		
High	10	21.9 ± 0.8	10	14.9 ± 0.5	20	18.4 ± 0.5		
Trend[c]		0.646		0.982		0.718		
Cqs								
Control	10	1.26 ± 0.08	10	0.92 ± 0.04	20	1.09 ± 0.05	0.792	Transformed
Low	10	1.22 ± 0.07	10	0.93 ± 0.05	20	1.07 ± 0.05		
Mid	10	1.20 ± 0.07	10	0.98 ± 0.07	20	1.09 ± 0.05		
High	10	1.23 ± 0.06	10	0.89 ± 0.03	20	1.06 ± 0.04		
Trend[c]		0.867		0.917		0.843		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

* See Table 2 for units.

Table F.3 (*Continued*). Pulmonary Function Findings in Wistar Han Rats After 12 Months of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
FVC								
Control	10	20.3 ± 0.5	10	14.6 ± 0.5	20	17.5 ± 0.3	0.149	ANOVA
Low	10	19.9 ± 0.8	10	14.1 ± 0.5	20	17.0 ± 0.5		
Mid	10	19.3 ± 0.6	10	15.3 ± 0.7	20	17.3 ± 0.5		
High	10	20.3 ± 0.7	10	13.5 ± 0.4	20	16.9 ± 0.4		
Trend[c]		0.789		0.415		0.475		
FVC/kg								
Control	10	35 ± 1	10	46 ± 3	20	40 ± 1	0.181	Transformed
Low	10	34 ± 2	10	44 ± 3	20	39 ± 2		
Mid	10	33 ± 2	10	45 ± 2	20	39 ± 1		
High	10	36 ± 1	10	40 ± 2	20	38 ± 1		
Trend[c]		0.861		0.130		0.309		
$FEV_{0.1}$								
Control	10	6.3 ± 0.4	10	4.3 ± 0.4	20	5.3 ± 0.3	0.235	ANOVA
Low	10	6.3 ± 0.5	10	4.5 ± 0.3	20	5.4 ± 0.3		
Mid	10	5.3 ± 0.4	10	4.8 ± 0.6	20	5.1 ± 0.3		
High	10	7.1 ± 0.8	10	4.7 ± 0.5	20	5.9 ± 0.5		
Trend[c]		0.554		0.484		0.369		
$FEV_{0.1\%}$								
Control	10	31 ± 2	10	30 ± 3	20	30 ± 2	0.746	ANOVA
Low	10	31 ± 2	10	32 ± 2	20	32 ± 1		
Mid	10	28 ± 2	10	31 ± 3	20	30 ± 2		
High	10	35 ± 3	10	34 ± 3	20	34 ± 2		
Trend[c]		0.503		0.329		0.229		
PEFR								
Control	10	125 ± 4	10	99 ± 3	20	112 ± 2	0.897	ANOVA
Low	10	127 ± 4	10	96 ± 3	20	112 ± 2		
Mid	10	127 ± 4	10	101 ± 6	20	114 ± 4		
High	10	120 ± 4	10	93 ± 3	20	106 ± 2		
Trend[c]		0.325		0.454		0.214		
PF/FVC								
Control	10	6.2 ± 0.2	10	6.8 ± 0.1	20	6.5 ± 0.1	0.210	ANOVA
Low	10	6.4 ± 0.1	10	6.9 ± 0.2	20	6.6 ± 0.1		
Mid	10	6.6 ± 0.2	10	6.6 ± 0.3	20	6.6 ± 0.2		
High	10	6.0 ± 0.3	10	6.9 ± 0.2	20	6.4 ± 0.2		
Trend[c]		0.741		0.987		0.804		
FEF_{75}								
Control	10	123 ± 3	10	94 ± 3	20	109 ± 2	0.983	ANOVA
Low	10	124 ± 3	10	94 ± 3	20	109 ± 2		
Mid	10	125 ± 4	10	97 ± 6	20	111 ± 3		
High	10	119 ± 4	10	88 ± 3	20	104 ± 2		
Trend[c]		0.442		0.418		0.253		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

* See Table 2 for units.

Table F.3 (*Continued*). Pulmonary Function Findings in Wistar Han Rats After 12 Months of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
F_{75}/FVC								
Control	10	6.1 ± 0.2	10	6.5 ± 0.2	20	6.3 ± 0.1	0.335	ANOVA
Low	10	6.3 ± 0.2	10	6.7 ± 0.2	20	6.5 ± 0.1		
Mid	10	6.5 ± 0.2	10	6.4 ± 0.3	20	6.5 ± 0.2		
High	10	5.9 ± 0.3	10	6.6 ± 0.2	20	6.3 ± 0.2		
Trend[c]		0.892		0.933		0.877		
FEF_{50}								
Control	10	105 ± 4	10	86 ± 3	20	95 ± 2	0.904	ANOVA
Low	10	103 ± 3	10	78 ± 3	20	90 ± 2		
Mid	10	107 ± 4	10	85 ± 6	20	96 ± 4		
High	10	98 ± 4	10	76 ± 4	20	87 ± 3		
Trend[c]		0.294		0.259		0.116		
F_{50}/FVC								
Control	10	4.8 ± 0.4	10	5.9 ± 0.1	20	5.3 ± 0.2	0.431	Transformed
Low	10	4.9 ± 0.4	10	5.6 ± 0.3	20	5.2 ± 0.2		
Mid	10	5.6 ± 0.1	10	5.5 ± 0.3	20	5.6 ± 0.2		
High	10	4.9 ± 0.3	10	5.6 ± 0.3	20	5.2 ± 0.2		
Trend[c]		0.318		0.407		0.459		
FEF_{25}								
Control	10	56 ± 3	10	45 ± 2	20	51 ± 2	0.875	ANOVA
Low	10	56 ± 3	10	43 ± 2	20	49 ± 2		
Mid	10	57 ± 2	10	48 ± 3	20	53 ± 2		
High	10	52 ± 2	10	39 ± 2	20	45 ± 2		
Trend[c]		0.284		0.204		0.092 (−)		
F_{25}/FVC								
Control	10	2.8 ± 0.2	10	3.1 ± 0.1	20	2.9 ± 0.1	0.935	ANOVA
Low	10	2.8 ± 0.1	10	3.1 ± 0.2	20	2.9 ± 0.1		
Mid	10	3.0 ± 0.1	10	3.1 ± 0.2	20	3.1 ± 0.1		
High	10	2.6 ± 0.1	10	2.9 ± 0.2	20	2.7 ± 0.1		
Trend[c]		0.360		0.381		0.202		
F_{10}								
Control	10	18.0 ± 1.4	10	15.1 ± 1.2	20	16.6 ± 0.9	0.885	ANOVA
Low	10	17.7 ± 1.0	10	15.7 ± 0.7	20	16.7 ± 0.6		
Mid	10	19.5 ± 1.5	10	17.4 ± 0.9	20	18.5 ± 0.8		
High	10	17.0 ± 1.0	10	13.5 ± 1.0	20	15.3 ± 0.7		
Trend[c]		0.839		0.471		0.539		
F_{10}/FVC								
Control	10	0.89 ± 0.08	10	1.03 ± 0.06	20	0.96 ± 0.05	0.841	ANOVA
Low	10	0.89 ± 0.05	10	1.13 ± 0.06	20	1.01 ± 0.04		
Mid	10	1.01 ± 0.07	10	1.15 ± 0.05	20	1.08 ± 0.04		
High	10	0.84 ± 0.04	10	1.00 ± 0.07	20	0.92 ± 0.04		
Trend[c]		0.899		0.817		0.795		

(*Table continues on next page*)

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

* See Table 2 for units.

Table F.3 (*Continued*). Pulmonary Function Findings in Wistar Han Rats After 12 Months of Exposure*

Endpoint / Group	Males		Females		Both			
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	Interaction[a]	Method[b]
MMEF								
Control	10	90 ± 3	10	70 ± 3	20	80 ± 2	0.937	ANOVA
Low	10	90 ± 3	10	68 ± 2	20	79 ± 2		
Mid	10	91 ± 3	10	73 ± 4	20	82 ± 2		
High	10	85 ± 2	10	64 ± 3	20	74 ± 2		
Trend[c]		0.305		0.277		0.127		
MF/FVC								
Control	10	4.4 ± 0.1	10	4.8 ± 0.1	20	4.6 ± 0.1	0.480	ANOVA
Low	10	4.5 ± 0.1	10	4.9 ± 0.2	20	4.7 ± 0.1		
Mid	10	4.8 ± 0.1	10	4.8 ± 0.2	20	4.8 ± 0.1		
High	10	4.3 ± 0.2	10	4.7 ± 0.2	20	4.5 ± 0.1		
Trend[c]		0.643		0.638		0.506		
DL_{CO}								
Control	10	0.35 ± 0.02	10	0.25 ± 0.02	20	0.30 ± 0.01	0.989	Transformed
Low	10	0.34 ± 0.02	10	0.23 ± 0.02	20	0.28 ± 0.02		
Mid	10	0.33 ± 0.02	10	0.23 ± 0.01	20	0.28 ± 0.01		
High	10	0.31 ± 0.02	10	0.22 ± 0.02	20	0.26 ± 0.01		
Trend[c]		0.135		0.138		0.031 (−)		
DL_{CO}/kg								
Control	10	0.61 ± 0.04	10	0.79 ± 0.06	20	0.70 ± 0.04	0.914	Transformed
Low	10	0.57 ± 0.04	10	0.72 ± 0.06	20	0.65 ± 0.04		
Mid	10	0.56 ± 0.04	10	0.68 ± 0.06	20	0.62 ± 0.04		
High	10	0.54 ± 0.03	10	0.64 ± 0.05	20	0.59 ± 0.03		
Trend[c]		0.277		0.056 (−)		0.028 (−)		
DL_{CO}/VA								
Control	10	0.017 ± 0.001	10	0.016 ± 0.001	20	0.017 ± 0.001	0.901	ANOVA
Low	10	0.017 ± 0.001	10	0.015 ± 0.001	20	0.016 ± 0.001		
Mid	10	0.017 ± 0.001	10	0.014 ± 0.001	20	0.016 ± 0.001		
High	10	0.016 ± 0.001	10	0.014 ± 0.001	20	0.015 ± 0.001		
Trend[c]		0.169		0.208		0.059 (−)		
Body Weight								
Control	10	0.59 ± 0.02	10	0.33 ± 0.02	20	0.46 ± 0.02	0.629	ANOVA
Low	10	0.60 ± 0.02	10	0.33 ± 0.02	20	0.46 ± 0.01		
Mid	10	0.60 ± 0.02	10	0.35 ± 0.01	20	0.47 ± 0.01		
High	10	0.57 ± 0.02	10	0.34 ± 0.02	20	0.46 ± 0.01		
Trend[c]		0.551		0.418		0.949		

[a] *P* value for sex × exposure effect. Tests equality of exposure effect across sexes.

[b] Statistical analysis method: ANOVA indicates analysis of variance on raw data; Transformed indicates ANOVA on log-transformed data.

[c] *P* value and direction of linear trend. Trend directions (+ or −) reported for $P \leq 0.1$.

* See Table 2 for units.

APPENDICES AVAILABLE ON THE WEB

Appendix G. Memorandum on Rat Strain Decision

Appendix H. ACES Phase 3B Protocol

Appendix I. ACES Three-Way ANOVA Results at 4 and 13 Weeks

ABOUT THE AUTHORS

Jacob McDonald received a Ph.D. in environmental chemistry with an emphasis in atmospheric chemistry from the University of Nevada, Reno. He is director of the Chemistry and Inhalation Exposure Program, as well as director of the Environmental Respiratory Health Program, both at LRRI. He oversees exposure assessment and exposure atmosphere generation, inhalation toxicology, pharmacokinetics, and analytical/bioanalytical chemistry. He serves as principal investigator for Phase 3 of the ACES program.

Melanie Doyle-Eisele holds a Ph.D. in environmental sciences and engineering. She is a study director (project manager) for various good laboratory practice (GLP) and non-GLP toxicologic studies that evaluate the acute toxicity, deposition, pharmacokinetics, and efficacy using a variety of respiratory disease models. She is the study director for Phase 3 of the ACES program.

Andrew Gigliotti, D.V.M., D.A.C.V.P., holds a Ph.D. in experimental pathobiology and is an associate contract scientist and veterinary pathologist in the LRRI Experimental Toxicology Program. He serves as necropsy/clinical pathology manager and as the associate study pathologist for Phase 3 of ACES, working closely with Rodney Miller to ensure appropriate necropsy, gross observation, and tissue processing and management for the study. He is a board-certified veterinary pathologist who has served as study pathologist for many studies for government and industry sponsors, with an emphasis on the cardiovascular and respiratory systems.

Rodney Miller, D.V.M., D.C.A.V.P., is a board-certified veterinary pathologist at Experimental Pathology Laboratories, Inc. He serves as the study pathologist for Phase 3 of ACES, with responsibility for histopathologic evaluations of all tissues. He works closely with Dr. Gigliotti, the necropsy/clinical pathology manager, to ensure appropriate necropsy, gross observation, and tissue processing and management.

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ABBREVIATIONS AND OTHER TERMS

μTP	micro–total protein
ACES	Advanced Collaborative Emissions Study
ANOVA	analysis of variance
API	American Petroleum Institute
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CARB	California Air Resources Board
CO	carbon monoxide
CO_2	carbon dioxide
Cqs	quasistatic lung compliance
CRC	Coordinating Research Council
DE	diesel exhaust
DL_{CO}	diffusing capacity in the lung for CO
DMM	Dekati Mass Monitor
DOE	U.S. Department of Energy

DPBS	Dulbecco's phosphate buffered saline
DPM	diesel particulate matter
DRI	Desert Research Institute
ELISA	enzyme-linked immunosorbant assay
EMA	Truck & Engine Manufacturers Association
EPA	U.S. Environmental Protection Agency
F	frequency
FEF	forced expiratory flow (at 10%, 25%, 50%, or 75% of total vital capacity)
$FEV_{0.1}$	forced expired volume in 0.1 second
FTP	Federal Test Procedure
FVC	forced vital capacity
GC/MS	gas chromatography/mass spectrometry
GLP	good laboratory practices
HHDD	heavy heavy-duty diesel
HO-1	heme-oxygenase-1
IL-β	interleukin-1β
LRRI	Lovelace Respiratory Research Institute
MECA	Manufacturers of Emission Controls Association
MMEF	Mean Mid-Expiratory Flow
MTD	maximum tolerated dose
NBF	neutral-buffered formalin
Ne	neon
NMHC	nonmethane hydrocarbons
NO	nitrogen monoxide
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
NOAEL	no-observed-adverse-effect level
NRDC	Natural Resources Defense Council
NTP	National Toxicology Program
PAH	polycyclic aromatic hydrocarbon
PASS	Photoacoustic Soot Spectrometer
PBS	phosphate-buffered saline
PEFR	peak expiratory flow rate
PF	peak flow
PLC	programmable logic controller
PM	particulate matter
PMN	polymorphonuclear
P_{tp}	transpulmonary pressure
QC	quality control
RFA	request for applications
RFP	request for proposals
SSA	sulfosalicylic acid
SVC	slow vital capacity
SVOC	semivolatile organic compound
SwRI	Southwest Research Institute
T_{90}	time to reach 90% of target atmosphere
TEAC	Trolox equivalent antioxidant capacity
THC	total hydrocarbons
VOC	volatile organic compound

Part 2. Assessment of Genotoxicity After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 2. Assessment of Genotoxicity After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay

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ABSTRACT

Micronucleus (MN*) formation is a well-established endpoint in genetic toxicology; studies designed to examine MN formation in vivo have been conducted for decades. Conditions that cause double-strand breaks or disrupt the proper segregation of chromosomes during division result in an increase in MN frequency. Thus this endpoint is commonly employed in preclinical studies designed to assess the potential risks of human exposure to a myriad of chemical and physical agents, including inhaled diesel exhaust (DE). As part of the Advanced Collaborative Emissions Study (ACES) this investigation examined the potential of inhaled DE to induce chromosome damage in chronically exposed rodents.

The ACES design included exposure of both rats and mice to DE derived from 2007-compliant heavy-duty engines. The exposure conditions consisted of air control and dilutions of DE resulting in three levels of exposure. At specified times, blood samples were collected, fixed, and shipped by the bioassay staff to Litron Laboratories for further processing and analysis. Significant improvements have been made to MN scoring by using objective, automated methods such as flow cytometry, which allows for the detection of micronucleated reticulocytes (MN-RET), micronucleated normochromatic erythrocytes (MN-NCE), and reticulocytes (RETs) in peripheral blood samples from mice and rats. By using a simple staining procedure coupled with rapid and efficient analysis, many more cells were examined in less time than was possible in traditional, microscopy-based MN assays. Thus, for each sample, 20,000 RETs were scored for the presence of MN. In the chronic-exposure bioassay, blood samples were obtained from independent groups of exposed animals at specific time points throughout the course of the entire study. This automated method is supported by numerous regulatory guidelines and meets the requirements for an Organization of Economic Cooperation and Development (OECD)–compliant assay for genotoxicity. Statistical approaches employed analysis of variance (ANOVA) to compare effects of sex, exposure condition, and duration, as well as their interactions.

This initial assessment of MN was performed on both mouse and rat blood samples from the 1-month and 3-month exposures. The data from mice demonstrate the well established, sex-based difference in MN-RET and MN-NCE frequencies regularly observed in this species, with females exhibiting slightly lower frequencies. There were no sex-based differences observed in rats. An examination of the mean frequencies across the exposure groups and durations of exposure did not show an appreciable induction of MN at the 1- or 3-month exposures in either species. Further statistical analyses did not reveal any significant exposure-related effects.

An examination of the potential genotoxic effects of DE is clearly valuable as part of a large-scale chronic-exposure bioassay. The data and observations from the 1- and 3-month exposure studies will eventually be combined with the results from the 1- and 2-year exposure studies to provide a comprehensive examination of chronic exposure to DE in a rodent model. This examination of chromosome damage serves an important role in the context of the entire ACES bioassay, which was designed to assess the safety of diesel combustion engines.

This Investigators' Report is one part of Health Effects Institute Research Report 166, which also includes a Commentary by the HEI ACES Review Panel and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Jeffrey C. Bemis, Litron Laboratories, 200 Canal View Blvd. Suite 106, Rochester, NY 14623; *jbemis@litronlabs.com*.

J.C.B., D.K.T., and S.D.D. are employees of Litron Laboratories, which holds a patent covering flow cytometric methods for scoring micronuclei as described herein.

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* A list of abbreviations and other terms appears at the end of the Investigators' Report.

INTRODUCTION

BACKGROUND ON GENOTOXICITY ASSESSMENT

Exposure of humans to by-products of combustion engines is an unavoidable fact of modern society. Thus the potential effects of such exposure, both in the environment and the workplace, are of continuing concern for public health agencies and industry alike. The development, characterization, and safety testing of newer heavy-duty diesel engines are important aspects of reducing or eliminating the risks associated with such devices. However, regulatory agencies require that extensive testing of new engines be carried out in order to determine if such designs meet or exceed established standards. As a means to obtain a sufficient assessment of the potential risk to human populations associated with exposure to DE, a large-scale, multi-endpoint assay such as the ACES bioassay must be performed. One important component of this chronic bioassay is the examination of the genotoxicity associated with exposure to DE.

Regulatory guidelines require the implementation of several genetic toxicity tests as part of the routine screening and safety programs that exist in pharmaceutical and industrial chemical manufacturing environments (Zeiger 1998). The in vivo mammalian erythrocyte MN test is one of the more commonly employed tests described in these guidelines (OECD 1997). The MN endpoint indicates the effects of a treatment or exposure on chromosome structure and function within the cell. Any deviation in this strictly regulated process can have both immediate and long-lasting effects on the cell and the organism as a whole. Outcomes such as genomic instability and cancer are often associated with the damage indicated by MN formation. Thus, MN assessment can be considered a short-term indicator of damage that can lead to long-term effects, and has become a well-established part of the safety and risk assessments associated with human exposure to numerous agents, including drugs, cosmetics, food products, and inhaled pollutants (Brown et al. 1997; Kirkland et al. 2005; Soni et al. 2006).

MN are formed after the division of cells that have experienced direct damage to chromosomes (e.g., double-strand breaks) or indirect damage that disrupts proper chromosome handling and segregation (e.g., lagging whole chromosome) (Cimini and Degrassi 2005). The rodent MN test is designed to examine peripheral blood samples from rodents exposed via various routes to the agent(s) of interest. Although some MN assays use bone marrow–derived erythrocytes for analysis, peripheral blood samples have the advantages of ease of collection and of integration into chronic animal studies. Historically, microscopy-based analyses were performed on bone marrow samples collected for MN enumeration. This process is labor-intensive, requires specialized training, and suffers from the subjectivity inherent to the human operator. Litron Laboratories developed an automated, flow cytometry–based method for analyzing MN-RETs that provides greater speed, efficiency, and accuracy when compared with microscopy MN scoring.

The flow cytometry–based method developed by Litron Laboratories (Rochester, NY) is commercially known as In Vivo MicroFlow and has been in use since 1996. It has been considered an indicator of chromosomal damage in mouse blood in support of Food and Drug Administration (FDA) reviews for the registration of new pharmaceutical agents. It is also recognized in the OECD Guideline 474 as a suitable alternative to microscopy-based approaches.

Two of the advantages of the flow cytometry–based method, as compared with traditional techniques, which involved manual scoring, are the greater objectivity afforded by instrument-based analysis and the greater number of cells scored in markedly less time. Thus, while a typical microscopist can take up to 30 minutes to score 2000 cells, the automated flow cytometry–based method routinely counts 20,000 cells in approximately 2 minutes. For rat blood analyses, this methodology has gained the acceptance of expert working groups, such as the one assembled at the Fourth International Workshop for Genotoxicity Testing (Hayashi et al. 2007), and is also recognized as a suitable sample type in the recent International Conference on Harmonisation S2(R1) (ICH 2011). Finally, along with MN-RET frequency, the In Vivo MicroFlow method provides for the enumeration of MN in normochromatic erythrocytes (NCEs). This more mature population of red blood cells represents a pool of cells that can be used to examine long-term or cumulative damage in the mouse model. However, because of the differences in splenic morphology that result in removal of MN-NCEs from circulation, the MN-NCE population is not a reliable indicator of chromosome damage in rats, thus data associated with this population of red blood cells are not shown for samples derived from this species.

RATIONALE FOR MICRONUCLEUS ASSESSMENT AFTER DIESEL EXHAUST EXPOSURE

A large body of literature describes the genotoxic or carcinogenic potential of DE in a number of in vivo and in vitro model systems (examples are described below). Given the frequency with which these tests were done, especially with regard to the rodent MN test, the current study benefits by examining this endpoint in comparison with

historical results. The use of the In Vivo MicroFlow method to assess MN induction in peripheral blood RETs provides the desired information with the added advantages of greater cell numbers examined, improved accuracy and objectivity, and faster turnaround time in comparison with traditional microscopy scoring methods. Additional benefits to using the MicroFlow method include the ease with which it can be integrated into a chronic bioassay. The MicroFlow method requires only a small volume of blood and when used in the context of a multiple-endpoint, long-term study the use of bone marrow for other endpoints of interest is not precluded.

Previous studies designed to investigate the genotoxic effects of DE have yielded equivocal results. Although it is quite common for DE or its derivatives to test positive in bacterial mutation assays (Oh and Chung 2006) and in vitro mammalian cell genotoxicity assays (Dybdahl et al. 2004), in vivo carcinogenicity studies show both positive and negative results (Mauderly et al. 1996). Those studies that demonstrated a positive correlation between DE exposure in vivo and carcinogenicity typically used much older engine designs and considerably higher concentrations of DE (Ishinishi et al. 1986; Mauderly et al. 1994) than those used in the ACES program. Older engine designs can be expected to have considerably different emission profiles than present-day or future engines. Indeed, the characterization studies of the DE constituents associated with this study demonstrated that the 2007-compliant diesel engines yielded significant reductions in parameters such as particulate matter (PM) (Mauderly and McDonald, 2012). Also, many of the previous studies exposed rodents to much higher concentrations of DE than would be achieved under normal ambient or even in extreme occupational conditions (U.S. Environmental Protection Agency [U.S. EPA] 2002). Finally, epidemiologic data on the carcinogenicity of DE in humans provided only weak evidence of effects in occupationally exposed workers (Jarvholm and Silverman 2003).

Perhaps the study that most closely resembled the current ACES design came from experiments conducted by Reed and colleagues (2004) at Lovelace Respiratory Research Institute (LRRI). These researchers exposed mice and rats via whole body inhalation to DE 7 days/wk, 6 hr/day for 6 months. Genotoxic effects were studied in the mice only and the endpoints examined were MN-RET counts and proliferation of adenoma. No significant alterations in either endpoint were observed after the 6-month exposure. This investigation by Reed and colleagues was part of a larger effort organized by the National Environmental Respiratory Center and sought to compare the effects of numerous inhalation exposure conditions to which humans are regularly exposed (Reed et al. 2006).

The specificity of the MN endpoint in reporting on chromosome damage makes it a powerful tool for predicting the potential of a chemical or exposure condition to induce damage that may lead to cancer. However, no single test performed in isolation is an acceptable indicator of carcinogenic potential, especially when the results must be extrapolated from a rodent model to the human condition. Perhaps the greatest advantage associated with using the MicroFlow method for a study such as this is that we were able to evaluate routinely ten times the number of cells examined via the standard microscopy method. This greater number allowed for the reliable detection of even weak responses and also provided increased confidence in the results if a negative outcome was observed.

It should be noted that the peripheral blood MN test as described in the report was a direct indicator of the impact of exposure conditions on the hematopoietic system residing in the bone marrow. Thus any impact of exposure that did not directly target bone marrow should be considered a response to systemic effects of the conditions being investigated. In the case of inhalation exposures to what could be considered a primarily lung-targeted agent there are examples of elevated peripheral blood MN frequencies associated with exposure to cigarette smoke (De Flora et al. 2007; Marchetti et al. 2011). There are also numerous reports of the systemic effects of DE or DE constituents. For example, Burchiel and colleagues (2004) showed systemic immunotoxicity after whole body exposure to diesel in mice. In addition, Song and Ye (1995) demonstrated elevated frequencies of peripheral blood MN-RET after exposure of mice to DE extracts. Arlt and colleagues (2008) examined the genotoxicity of 3-nitrobenzanthrone, a carcinogen found in DE, and its metabolite 3-aminobenzanthrone in a transgenic mouse model. They reported elevated mutation frequencies in liver and bone marrow, but not lung tissue. In terms of hazard identification, the in vivo MN test is a very commonly employed approach across numerous disciplines and industries; it continues to be an important part of regulatory guidelines and risk assessment of myriad types of exposure conditions.

VALIDATION OF FLOW CYTOMETRIC MICRONUCLEUS ASSESSMENT

Litron Laboratories has an extensive history of performing in vivo rodent MN testing and analysis. During the development of the MicroFlow technology, we performed multiple inter-laboratory validation trials that demonstrated the portability and reproducibility of the assay across numerous laboratories (Torous et al. 2001; Torous et al. 2005). The data in Table 1 show an aggregate correlation coefficient of 0.943 within a nine-laboratory trial that examined

Table 1. MN-RET Data and Spearman Correlation Coefficients

Laboratory	Chemical	FCM vs. Microscopy Correlation[a]	Correlation Between FCM Labs[b]
1	Benzo[*a*]pyrene	0.903	0.961
2	5-Fluorouracil	0.529	0.755
3	Cyclophosphamide	0.884	0.902
4	Benzo[*a*]pyrene	0.842	0.903
5	Methotrexate	0.434	0.723
6	Methotrexate	0.818	0.832
7	Cyclophosphamide	0.624	0.876
8	Vincristine	0.945	0.923
9	5-Fluorouracil	0.714	0.690
Aggregate[c]		0.922	0.943

[a] Non-parametric Spearman correlation coefficients (r_s) describing the relationship between microscopy-based and corresponding MN-RET values. FCM = flow cytometry.

[b] Non-parametric Spearman correlation coefficients (r_s) describing the relationship between reference laboratory FCM-based and corresponding laboratory (1–9) FCM-based MN-RET values. For vincristine, Litron sent replicate samples to Laboratory 1 for an independent reading.

[c] These aggregate values were calculated by pooling MN-RET frequencies for the entire set of nine experiments (n = 252 and 248 for FCM vs. microscopy correlation, and correlation between FCM labs, respectively).

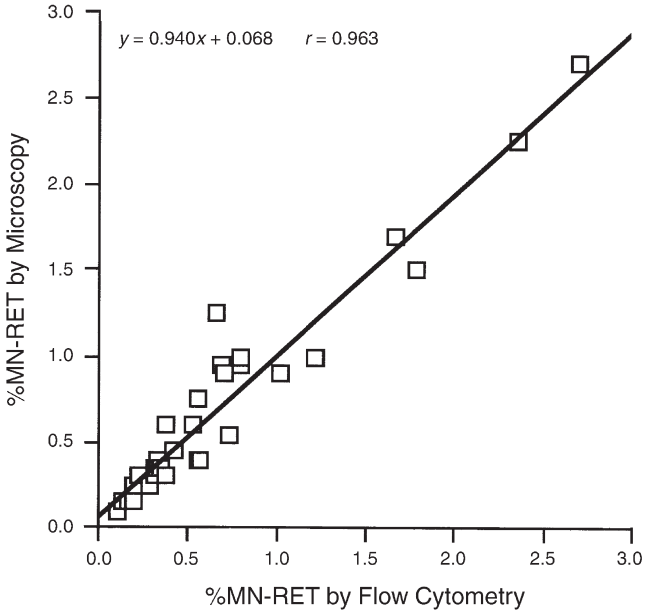

Figure 1. Comparison between microscopic and flow cytometric analyses of mouse blood MN-RET after consecutive 3-day exposures to various doses of CP (r = bivariate Spearman correlation coefficient).

the MN-RET response of mice exposed to various chemical genotoxicants.

In addition to these validation trials, considerable testing was done to compare MN scoring by microscopy and flow cytometry methods (Table 1, Figure 1). Both inter- and intra-laboratory studies have shown an excellent concordance between MN scoring by microscopy and flow cytometry (Dertinger et al. 2006).

The previous studies were performed in mice, however MicroFlow technology can also be used to analyze rat blood samples (Torous et al. 2003). Thus both MacGregor and colleagues (2006) (Figure 2) and Hayashi and colleagues (2007) described the ability to enumerate genotoxicant-induced MN-RETs reliably and accurately in peripheral blood of rats.

As the MicroFlow method has gained greater acceptance, more investigators are employing this technology in their studies. For example, it was used by De Boeck and colleagues (2005), who performed a multiple-sampling time-course study in mice exposed to various mutagens, and by Cammerer and colleagues (2007), who compared the response of mice and rats to aneugenic compounds. In addition, the National Toxicology Program compared microscopy and flow methods to validate the use of the flow-based assay as a replacement for microscopy-based scoring of MN as part of its efforts to evaluate the genotoxicity of substances of public health concern (Witt et al. 2008). With regard to chronic inhalation studies, the above-mentioned studies by Reed and colleagues (2004, 2006) used MicroFlow analysis to investigate the incidence of MN-RETs in peripheral blood of mice exposed to either DE or wood smoke.

In addition to our validation efforts associated with the MicroFlow assay, we conducted a specific, HEI-funded pilot study that demonstrated the ability of the flow cytometry–based method to detect genotoxicant-induced MN responses in older rats (see Appendix B). Blood samples from both male and female rats ranging from 10 to 21 months in age demonstrated cyclophosphamide (CP)-induced elevations in MN-RET frequencies. Thus at ages approximately equivalent to those we will study in the longer-duration exposures (12 and 24 months in ACES), the flow cytometry–based method was able to identify a genotoxic response in peripheral blood cells.

With regard to the statistical power and sensitivity of the in vivo MN test, Kissling and colleagues (2007) performed an assessment of these aspects of the assay across several species and experimental parameters. The authors used a standard experimental design of five animals per group and examined factors such as background MN

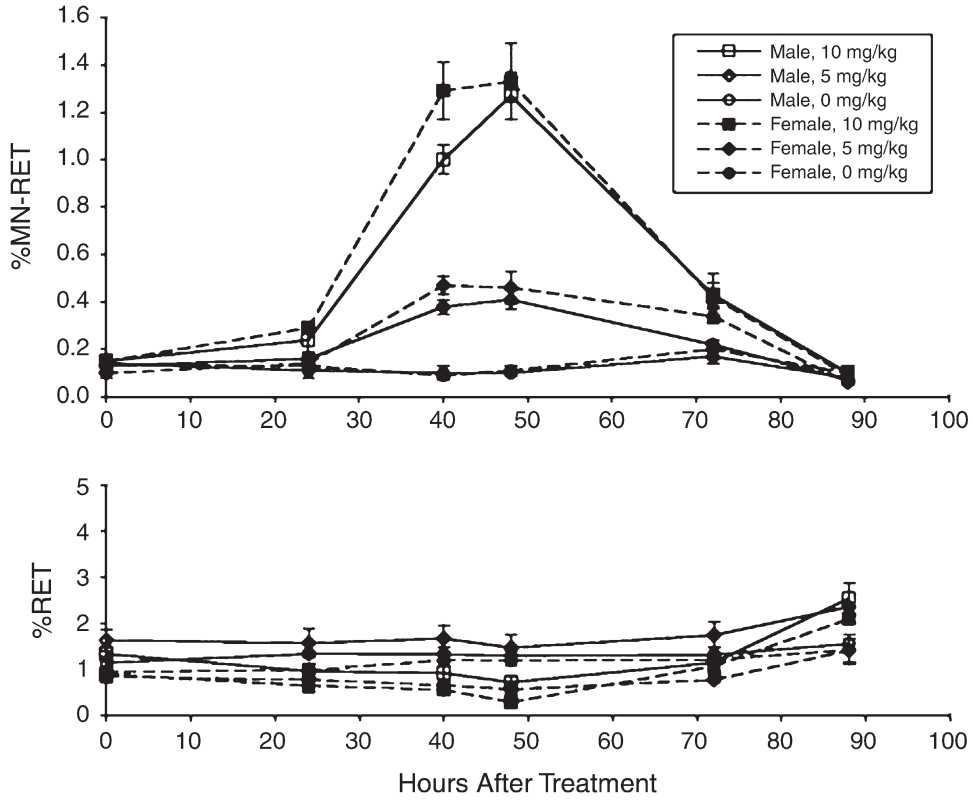

Figure 2. Time course of the frequency of MN-RET in rat blood after a single oral dose of 5 or 10 mg/kg CP. (Reprinted from MacGregor et al. 2006, by permission of the Society of Toxicology.)

frequency, inter- and intra-animal variation, number of cells scored, and associated counting errors in order to determine the statistical power of the assay. As shown in Table 2, for the rat model with a spontaneous frequency of 0.1 (standard deviation [SD] = 0.045), scoring 20,000 reticulocytes can resolve a 2.2- or 3.0-fold increase above baseline at 90% power with a significance level of $P \leq 0.05$ or $P \leq 0.01$, respectively. For this study, the spontaneous frequency of MN-RET in the control rats (pooled across sex and duration, $n = 20$) is 0.11 (SD = 0.037) and for all the rats ($n = 80$) was 0.11 (SD = 0.038).

For the mouse model with a spontaneous frequency of 0.20 (SD = 0.070), scoring 20,000 reticulocytes can resolve a 1.9- to 2.5-fold increase above baseline at 90% power with a significance level of $P \leq 0.05$ or $P \leq 0.01$, respectively. The spontaneous frequencies of MN-RET in the control mice (pooled across duration but not sex) used in this study were 0.35 (SD = 0.044, $n = 12$) for males and 0.22 (SD = 0.054, $n = 8$) for females. These differences in baseline could be attributed to mouse strain because Kissling and colleagues (2007) reported on data obtained from CD-1 mice and the ACES program used C57Bl/6 mice. In any event, the fact that the spontaneous frequencies observed here were greater than those used in the power analysis suggested that similar, if not improved, power was present in the ACES program.

It is generally accepted that for regulatory submission purposes, such as those involving pharmaceuticals for human use, a positive response in genotoxicity tests requires a doubling of the background frequency. Adler and colleagues (1998) argued that a suitable minimum standard for in vivo mutagenicity tests (which include the in vivo MN assay) is "the ability to detect a doubling of the observed endpoint at an α-level of 0.05 and a power of 80%." The authors offered the following as a rationale for this approach: "the increment in mutational events over the spontaneous level to be recognized is determined by biological criteria rather than by purely statistically achievable precision." As described by Kissling and colleagues (2007), the currently employed analytic approach can resolve a two- to threefold elevation from baseline with 90% power at a significance level of 0.05.

Table 2. Minimum Detectable Increases in MN-RET Frequency in Groups of Five Animals as a Function of Spontaneous Frequency and Number of RETs Scored[a,b]

Spontaneous Frequency (%MN-RET)	RETs Scored (*n*)	Minimum Detectable Fold-Increase in Spontaneous Frequency			
		90% Probability		95% Probability	
		$P \leq 0.05$	$P \leq 0.01$	$P \leq 0.05$	$P \leq 0.01$
0.05 (SD = 0.020)	2,000	4.5	6.8	5.6	9.3
	4,000	3.5	5.5	4.0	6.3
	20,000	2.3	3.1	2.4	3.4
	∞	1.8	2.1	1.9	2.2
0.1 (SD = 0.045) (rat peripheral blood)	2,000	3.3	4.8	4.1	6.4
	4,000	2.9	4.2	3.2	4.7
	20,000	2.2	3.0	2.4	3.2
	∞	1.8	2.0	2.1	2.4
0.20 (SD = 0.070) (mouse peripheral blood)	2,000	2.7	3.9	3.0	4.5
	4,000	2.3	3.2	2.4	3.5
	20,000	1.9	2.5	2.2	2.7
	∞	1.7	2.0	1.8	2.1
0.20 (SD = 0.059) (rat bone marrow)	2,000	2.7	3.9	2.9	4.4
	4,000	2.2	3.1	2.4	3.3
	20,000	1.8	2.3	1.9	2.5
	∞	1.6	1.8	1.7	1.9
0.30 (SD = 0.092) (dog peripheral blood)	2,000	2.4	3.4	2.6	3.7
	4,000	2.1	2.8	2.2	3.0
	20,000	1.8	2.3	1.9	2.4
	∞	1.6	1.8	1.7	1.9

[a] Based on data from Kissling et al. 2007.

[b] Values for the cases of infinite cell counts are calculated based on the observed inter-animal variability (SD from Table 1 of Kissling et al. 2007) for the species stated, assuming no counting error; the inter-animal variability for frequency 0.05% is assumed to be 0.02%. The detectable increase depends on the relative magnitudes of the counting error and the inter-animal variability. Although counting error can be reduced by scoring more RETs, the minimum detectable increase cannot go below a bound determined by the inter-animal variability (i.e., the value given in the infinite cell count rows). Species entries correspond to the appropriate spontaneous frequency and associated inter-animal SD in the species specified in Table 1 of Kissling et al. 2007.

In addition to the above information provided by Kissling and colleagues (2007), the OECD Guideline 474 (1997), which defined operating parameters for the in vivo MN assay, stated that five animals per dose group were sufficient. In this report, the majority of experiments met the OECD minimum requirement of five animals per group and in many cases, particularly in the rat studies where data could be pooled across sex, exceeded the requirement by examining up to 10 animals per dose group. The experiments in this report that did not meet this minimum standard of five animals per group are clearly identified.

These studies demonstrated that the MicroFlow method can be used to investigate chromosome damage in mice and rats in a rapid and efficient manner that is superior to other methods of MN detection (microscopy or image analysis, for example). The ease with which this method can be incorporated into a standard chronic bioassay is readily apparent from the numerous and varied examples described above.

SPECIFIC AIMS

The broader goal of the ACES bioassay was to provide information on the safety of the 2007-compliant heavy-duty diesel engine. As part of the ancillary studies associated with ACES bioassay, our investigation sought to evaluate a specific biomarker of chromosome damage as a means to assess the genotoxic potential of DE generated by these engines.

As a biomarker of chromosome damage and an accepted indicator of carcinogenic potential, MN formation

is regularly assessed in animal studies designed to predict human health effects of exposure to various chemical or physical agents. This investigation employed a rapid and efficient flow cytometric method to accurately enumerate MN-RETs in the blood of rodents exposed to diesel engine exhaust via inhalation.

In the current study, assessment of MN was performed in both mice and rats in 1- and 3-month exposure studies to provide a comparison of the potential MN response across species. The results of longer exposure times (i.e., 1 and 2 years) will be assessed in rats only and will be presented in a future report.

METHODS AND STUDY DESIGN

ANIMALS AND EXPOSURE CONDITIONS

All animal exposures, handling, and blood collection and fixation were performed by the bioassay staff at LRRI after approval from the institution's Animal Care and Use Committee. Mauderly and McDonald provided detailed descriptions of the exposure facility, engine specifications, DE characterization, and animal exposure conditions (see the Mauderly and McDonald report, Part 1 of this volume). For the MN data reported here, blood samples from male and female rats and mice were obtained from four DE exposure conditions (classified as air control, low-, mid-, and high-exposure groups). All inhalation exposures were whole body and occurred under the conditions described by Mauderly and McDonald. For the majority of experiments described here each exposure group contained five males and five females. However, there was an error in the exposures performed for the 1-month mouse studies such that the numbers of animals in the control, low-exposure, mid-exposure, and high-exposure groups for males were 7, 4, 6 and 3 and for the females 3, 5, 6, and 6, respectively. Because of the terminal nature of the endpoints associated with the entire study, separate groups of animals were exposed and sampled for the 1- and 3-month time points.

ANALYSIS OF MICRONUCLEATED ERYTHROCYTES BY FLOW CYTOMETRY

All of the solutions, materials, and procedures referenced below are associated with commercially available In Vivo MicroFlow kits (Litron Laboratories, Rochester, NY). The procedures are the same for samples derived from mice and rats unless noted.

Sample Collection and Fixation

Whole blood samples from mice and rats were collected into anticoagulant solution and well mixed. A portion of this diluted blood sample was fixed by rapid introduction to ultracold (−80°C) methanol fixative. All blood samples were processed in this manner and stored at −80°C until further processing. After a minimum of 48 hours in the fixative, the methanol was washed out of the blood samples using the provided wash buffer and transferred to the kit-supplied Long Term Storage Solution (LTSS). These samples in LTSS were maintained at −80°C until they were shipped to Litron Laboratories under controlled cold conditions for further processing and analysis.

Sample Processing and Staining

After receipt of samples at Litron Laboratories, the blood specimens were stored at −80° to −90°C until scheduled for analysis. Samples were first washed to remove the LTSS, and then labeled with anti-CD71-FITC and anti-CD61-PE antibodies in the presence of RNase. DNA staining solution was added to the antibody-labeled blood samples just before flow cytometric analysis.

Flow Cytometer Instrument Set-up and Calibration

Before analyzing experimental samples, the flow cytometer was calibrated with a CD71-negative blood sample and malaria biostandard that was provided with the kit. This process was performed daily and ensured that instrument parameters, such as photomultiplier voltages and compensation settings, were appropriately and consistently applied to all subsequent analyses. Processing of the malaria biostandard and CD71-negative blood samples was performed in parallel with the experimental samples.

ANALYSIS OF EXPERIMENTAL SAMPLES

A more detailed description of sample processing and analysis can be found in a report by Dertinger and colleagues (2004). The analytic scheme is based on the selection and enumeration of specific cells of interest (i.e., RETs and MN-RETs) using flow cytometric software and a specialized template. The fluorescent cell-labeling procedure depends on three main cell markers (CD61, propidium iodide [PI], and CD71). CD61 is expressed on platelets and, in conjunction with light scatter, this marker is used to exclude platelets from analysis. The DNA stain noted above contains PI, which will label both DNA and RNA, but the inclusion of RNase in the sample processing renders the staining DNA-specific. This allows the PI fluorescence to be used as a marker for all nucleated cells, as well as RBCs that contain MN. Based on this staining, nucleated cells exhibit very bright fluorescence, in comparison to the absent or dim staining associated with normal RBCs and those that contain MN. This high fluorescence signal exhibited by the nucleated cells allows them to be excluded from analysis.

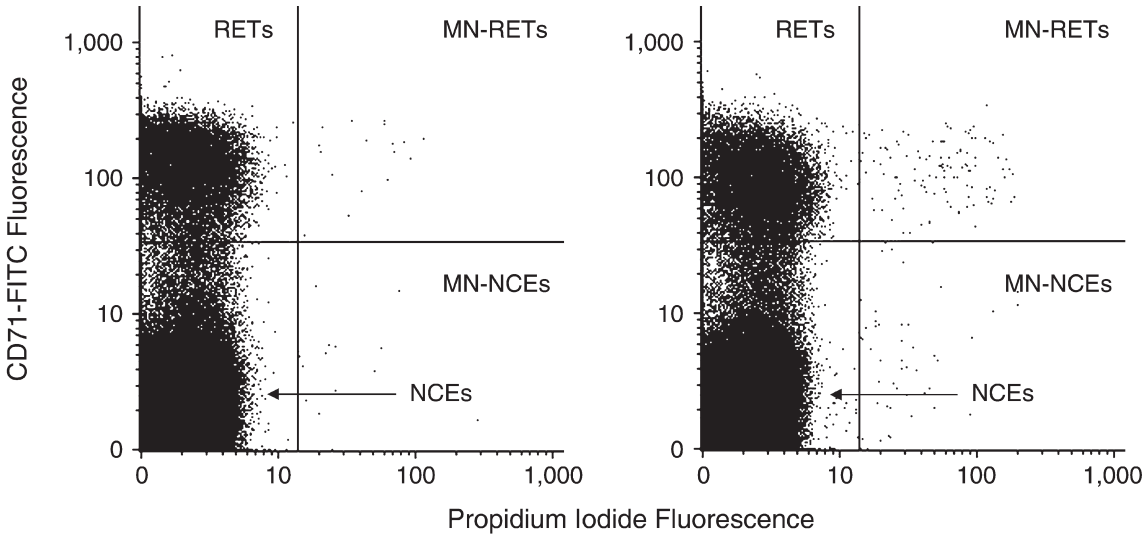

Figure 3. Representative flow cytometry plots of blood samples obtained from a rat treated with vehicle control (left) or a rat treated with a known genotoxic agent (right). In each plot, the upper left quadrant corresponds to RETs; the upper right to MN-RETs; the lower left to NCEs; and the lower right to MN-NCEs.

The third marker, CD71, is expressed on the surface of RETs and is gradually lost as these cells mature, thus the anti-CD71 fluorescent label permits the differentiation of RETs from the mature NCEs. This labeling and selection scheme was accomplished by the assembly of gates and inclusion/exclusion logic present in the software template. The accompanying Figure 3 shows two examples of the final plot in the template used to quantify the number of events in the respective quadrants that correspond to RETs ($CD71^+$, PI^-), MN-RETs ($CD71^+$, PI^+), NCEs ($CD71^-$, PI^-), and MN-NCEs ($CD71^-$, PI^+). The plot on the left is derived from a control-exposed rat and the plot on the right is from a rat exposed to a known genotoxic agent. In the plot on the right, the obvious increase in the frequency of events (MN-RET) in the upper right quadrant is a consequence of MN formation in the bone marrow and indicates chromosome damage has occurred.

Once the flow cytometer was properly set up, all the processed experimental samples were analyzed. Instrument settings and gating were kept consistent across all the samples in a particular analytic run. The typical stop point for each analysis was the collection of 20,000 RETs per sample.

STATISTICAL METHODS AND DATA ANALYSIS

Once all the experimental samples were analyzed, the data were transferred to an Excel spreadsheet (Excel 2008 for Mac; Microsoft, Redmond, WA). These data were used to calculate the percentages of RET, MN-RET, and MN-NCE (%RET, %MN-RET, and %MN-NCE, respectively), based on the number of "events" in each of the defined quadrant regions: upper left (UL), upper right (UR), lower left (LL), lower right (LR):

Mouse and Rat: %RET = (UL+UR)/(UL+UR+LL+LR) × 100
Mouse and Rat: %MN-RET = (UR)/(UR+UL) × 100
Mouse Only: %MN-NCE = (LR)/(LR+LL) × 100

The means and standard errors of the mean (SEM) were then calculated for the respective groups. Once these initial calculations were made, the %MN-RET values were converted to a proportion and an arcsin transformation was performed in Excel using the following equation: new_value = ASIN(SQRT(old_value)). This arcsinSqrt transformation was employed to stabilize group variance and normalize the data, and is based on a consensus opinion generated by the genetic toxicology statistics experts associated with the Pharmaceutical Research and Manufacturers of America (PhRMA) consortium. The arcsinSqrt transformation did not achieve a suitably normal distribution for the MN-NCE or RET data so an alternative strategy was employed. The values for %MN-NCE and %RET were transformed by taking the natural log, if necessary applying a consistent correction factor to shift the data into positivity, then taking the natural log again. This double logarithmic transformation afforded the best approximation of a normal distribution for the respective data with both the mouse and rat data sets. After transformation,

the data were imported into the SAS-based statistics program JMP (v.8 for Mac; SAS, Cary, NC) for further statistical analysis.

For the statistical analyses associated with the ACES program, the ANOVA model was chosen as the preferred method for comparison. This allowed for a full three-way ANOVA that included the factors of sex, exposure condition, and exposure duration within each animal model. As noted below in the Results section, one-, two-, and three-way ANOVA formats were employed. When one-way ANOVA was performed, post-hoc Dunnett *t* test was used to compare control data with other groups as appropriate. Significance was set at the $P \leq 0.05$ level. As described above, efforts were made to maximize the number of animals per exposure level where appropriate, thus the analyses described here employed at least five animals per exposure condition, unless otherwise noted.

RESULTS

Summary tables of the raw and processed data from both rats and mice are included in Appendix A. These tables include both the original and the transformed data as indicated. Although the transformed data were used for the statistical analyses, the original untransformed values are presented in the figures in the main text.

1- AND 3-MONTH EXPOSURE STUDIES IN MICE

There is a well-established sex-based difference in baseline values for %MN-RET and %MN-NCE in the mouse model. Thus, an initial examination of the mouse data included a one-way ANOVA to test for this effect of sex. When comparing males versus females, whether separately (Figure 4) or pooled across the 1- and 3-month exposure durations (not shown), a statistically significant effect of sex on both MN-RET and MN-NCE frequencies was observed. It was observed that female mice consistently demonstrated lower values for both endpoints. No such difference was observed for %RET values (Figure 5). Because sex alone was observed to influence these endpoints of chromosome damage, the remaining comparisons did not employ pooled data for males and females, but rather data for each sex were analyzed separately.

Tables 3–5 show the results from the two-way ANOVA analyses performed on the male mouse data. For %MN-RET values in male mice (Table 3 and Figure 4), there was

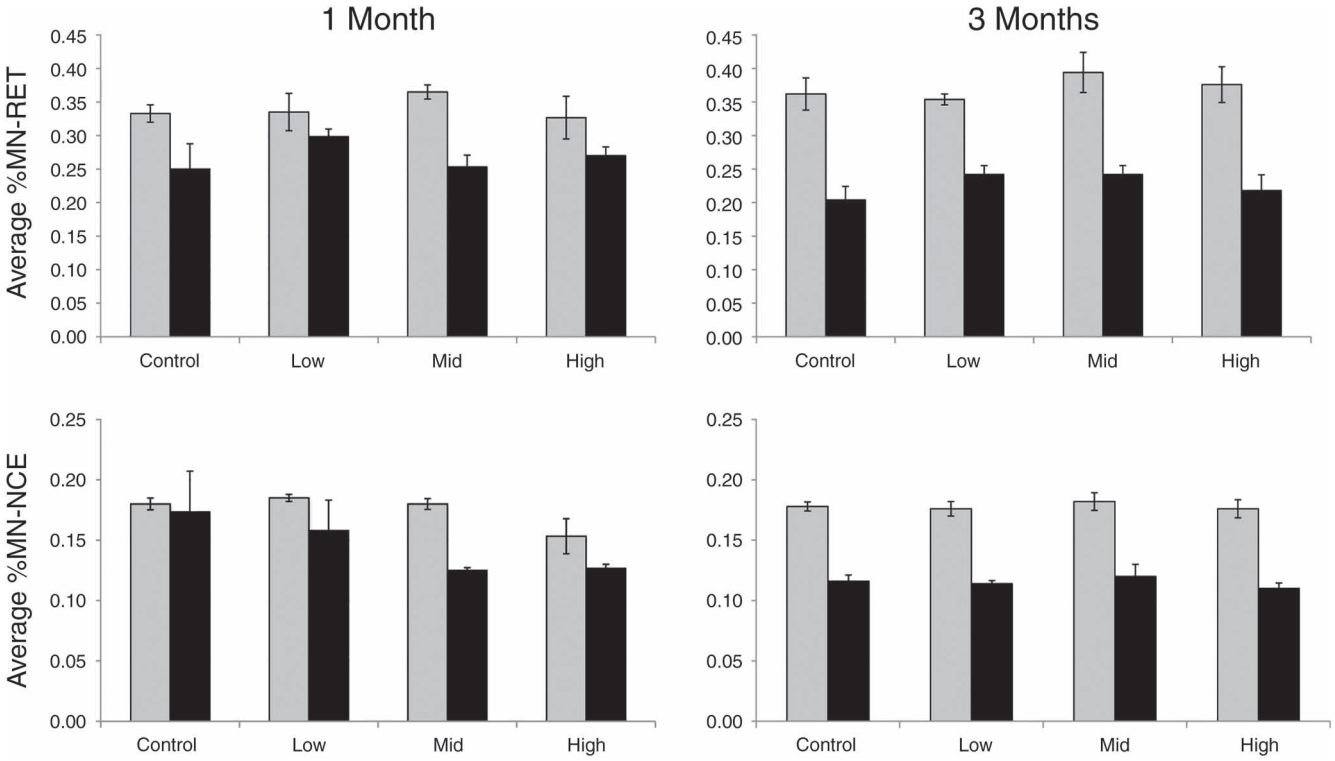

Figure 4. Mean frequencies of MN-RET and MN-NCE in male (gray bar) and female (black bar) mice exposed to DE for 1 or 3 months. Error bars indicate SEM.

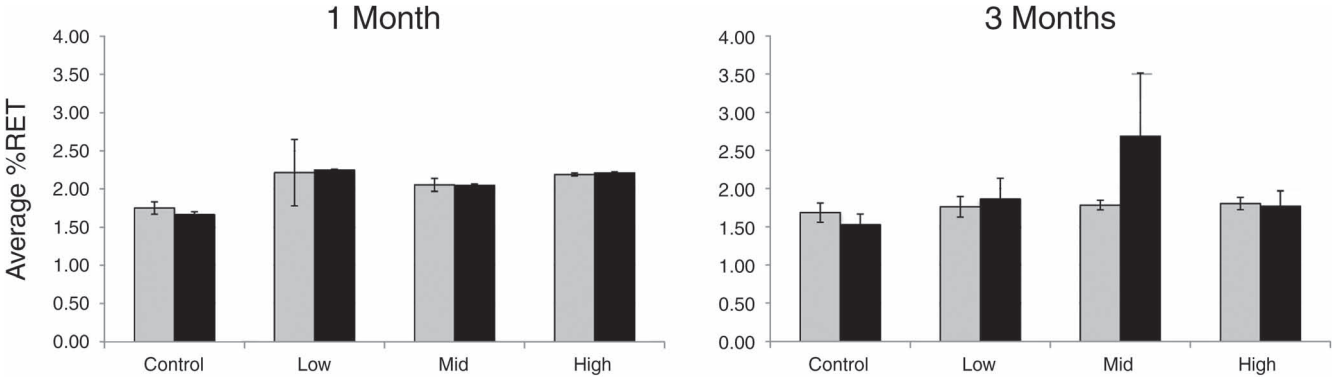

Figure 5. Mean frequency of RET in male (gray bar) and female (black bar) mice exposed to DE for 1 month or 3 months. Error bars indicate SEM.

a statistically significant effect of duration of exposure alone that reflected a reduction in MN-RET at 3 months compared to 1 month. The two-way comparison did not show any interaction with exposure condition, so DE exposure did not appear to influence this observed reduction. A similar trend was observed in reduced %RET values at 3 months that again was not influenced by the exposure conditions (Table 4 and Figure 5).

The statistically significant effect of exposure condition alone for the male mouse %MN-NCE data shown in Table 5 was attributable to the reduction observed at the high exposure group in the 1-month exposed mice (Figure 4, lower left panel). This was confirmed by a one-way ANOVA of the 1-month male mouse MN-NCE data that confirmed a significant *P* value associated with exposure condition ($P \leq 0.0205$); post-hoc Dunnett test revealed that only the high exposure group was significantly different from control ($P \leq 0.0191$).

Tables 6–8 show the results from the two-way ANOVA analyses performed on the female mouse data. Female mouse %MN-RET and %MN-NCE values were reduced at 3 months compared to 1 month, thus statistically significant

Table 3. Two-Way ANOVA for Male Mouse %MN-RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	0.00005753	1.2365	0.3126
Duration of exposure	1	1	0.00006536	4.2139	0.0483[b]
Exposure condition × Duration of exposure	3	3	0.00000641	0.1378	0.9367

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.

[b] Significant at $P \leq 0.05$.

Table 4. Two-Way ANOVA for Male Mouse %RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	0.43937955	2.1075	0.1188
Duration of exposure	1	1	0.49937679	7.1858	0.0115[b]
Exposure condition × Duration of exposure	3	3	0.0498925	0.2393	0.8683

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.

[b] Significant at $P \leq 0.05$.

Table 5. Two-Way ANOVA for Male Mouse %MN-NCE Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	0.04283200	3.3398	0.0314[b]
Duration of exposure	1	1	0.00408770	0.9562	0.3355
Exposure condition × Duration of exposure	3	3	0.03142407	2.4502	0.0815

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

Table 6. Two-Way ANOVA for Female Mouse %MN-RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	0.00005069	1.0133	0.3997
Duration of exposure	1	1	0.00021866	13.1129	0.0010[b]
Exposure condition × Duration of exposure	3	3	0.00006077	1.2148	0.3202

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

Table 7. Two-Way ANOVA for Female Mouse %MN-NCE Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	0.12301566	1.8032	0.1664
Duration of exposure	1	1	0.43334761	19.0567	0.00010[b]
Exposure condition × Duration of exposure	3	3	0.09904181	1.4518	0.2462

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

Table 8. Two-Way ANOVA for Female Mouse %RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Exposure condition	3	3	1.7135839	2.4462	0.0818
Duration of exposure	1	1	0.6058000	2.5945	0.1171[b]
Exposure condition × Duration of exposure	3	3	0.4567746	0.6521	0.5875

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

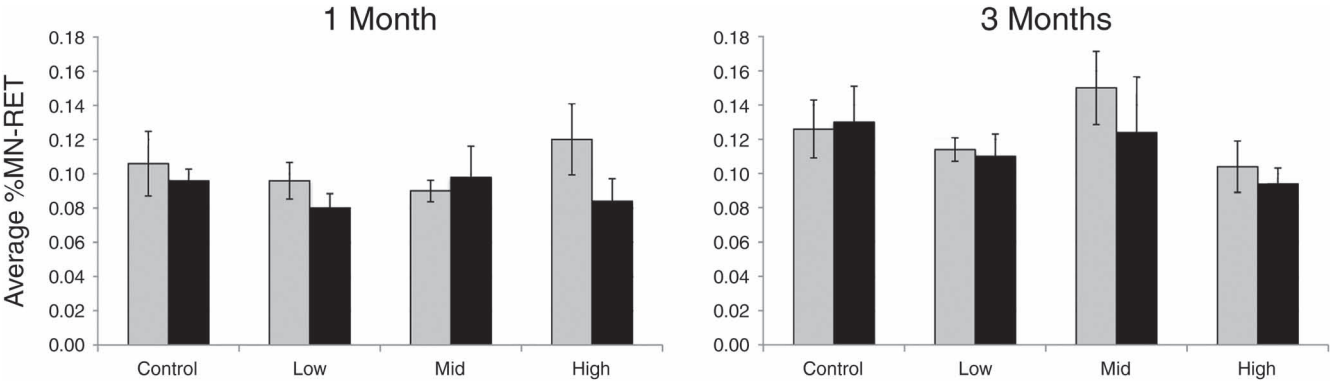

Figure 6. Mean frequency of MN-RET in male (gray bar) and female (black bar) rats exposed to DE for 1 month or 3 months. Error bars indicate SEM.

Table 9. Three-Way ANOVA for Rat %MN-RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Sex	1	1	0.00006552	2.2255	0.1407
Exposure condition	3	3	0.00008284	0.9379	0.4276
Duration of exposure	1	1	0.00022311	7.5781	0.0077[b]
Sex × Exposure condition	3	3	0.00002344	0.2654	0.8501
Sex × Duration of exposure	1	1	0.00000157	0.0533	0.8182
Exposure condition × Duration of exposure	3	3	0.00009227	1.0447	0.3789
Sex × Exposure condition × Duration of exposure	3	3	0.00006124	0.6934	0.5595

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

Table 10. Three-Way ANOVA for Rat %RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Sex	1	1	0.14625421	1.9404	0.1684
Exposure condition	3	3	0.53595581	2.3703	0.0787
Duration of exposure	1	1	0.44588552	5.9159	0.0178[b]
Sex × Exposure condition	3	3	0.15589297	0.6894	0.5618
Sex × Duration of exposure	1	1	0.36789	4.881	0.0307[b]
Exposure condition × Duration of exposure	3	3	0.02379624	0.1052	0.9567
Sex × Exposure condition × Duration of exposure	3	3	0.52502732	2.322	0.0834

[a] Nparm indicates the number of parameters associated with the effect; *F* ratio indicates the *F* statistic for testing that the effect is zero; and Prob > *F* indicates the significance probability for the *F* ratio.
[b] Significant at $P \leq 0.05$.

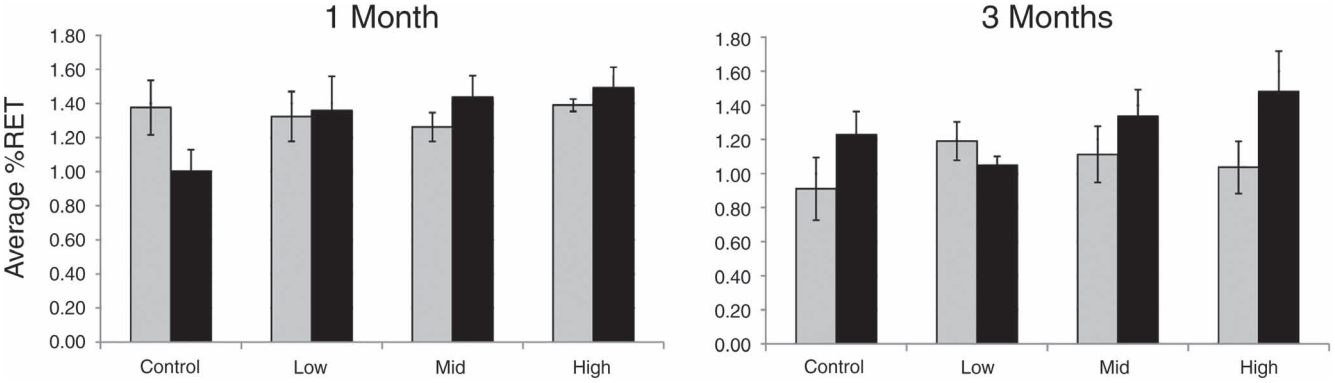

Figure 7. Mean frequency of RET in male (gray bar) and female (black bar) rats exposed to DE for 1 month or 3 months. Error bars indicate SEM.

effects of duration of exposure alone were observed (Tables 6 and 7 and Figure 4). Similar to the male mouse data, the two-way analyses indicated that exposure condition was not a significant factor. No significant effects were observed in %RET values in the female mice (Table 8 and Figure 5).

1- AND 3-MONTH EXPOSURE STUDIES IN RATS

Unlike the results from the studies in mice, there were no statistically significant differences between males and females in the rat %MN-RET data (Figure 6). Thus Tables 9 and 10 show the results of the three-way ANOVA analyses of %MN-RET and %RET values, respectively, using sex, exposure condition, and exposure duration as factors.

There was a statistically significant effect of duration of exposure alone elicited by an increase in %MN-RET at 3 months compared to 1 month (Table 9 and Figure 6). When duration of exposure was combined with exposure condition or exposure condition and sex, this effect was no longer significant. A statistically significant effect of duration of exposure alone was observed for the rat %RET values (Table 10 and Figure 7), again reflecting a reduction in the frequency of these cells at 3 months compared to 1 month. This effect was also observed in the interaction between duration of exposure and sex, as this reduction in %RET value at 3 months was predominantly observed in the male rats. When the influence of exposure condition was examined with either of these reported effects, the significance of the effect was lost.

DISCUSSION AND CONCLUSIONS

This investigation considered the potential for DE to elicit chromosome damage in rodents after 1- and 3-month exposure periods only. A well-established indicator of chromosome damage (i.e., peripheral blood MN-RET) was analyzed by flow cytometric methods. This work was conducted as one of the ancillary studies associated with the core ACES chronic-inhalation study (see the Mauderly and McDonald Investigators' Report, Part 1 of this volume). As such, this investigation will continue with analysis of blood samples from rats exposed to DE for the remaining 1- and 2-year duration periods.

Despite the presence of some statistically significant associations between certain variables within the data for both mice and rats, the overall conclusion was that there was no apparent effect of the specific DE exposure conditions on the endpoints tested. So although there may have been specific comparisons that resulted in statistically significant effects for various factors within the ANOVA, additional comparisons did not support interactions that were related to the exposure conditions or that demonstrated a predictable dose–response relationship. As described above, biologically important responses of these markers of genotoxicity are typically two- to threefold induction above baseline. The data collected for the 1- and 3-month mouse and rat studies did not reveal any effects that approached that magnitude. The lack of any obvious positive induction associated with increasing exposure levels or the longest duration of exposure studied to date (i.e., 3 months) on the chromosome-damage endpoints studied strongly suggested that under the conditions studied there is no discernible risk of genotoxic impact on hematopoietic cells associated with whole-body DE exposure.

This report should be considered within the context of the entire ACES program and these preliminary data examining the earliest time points will require additional analyses when the remaining data become available. Follow-up on some of the observed associations will be possible once the analysis of samples from the 1- and 2-year durations is completed. These additional studies will allow for a more comprehensive examination of the effects noted for

duration of exposure alone in the 1- and 3-month studies reported here. In addition, the comparison of this study's observations with those made by the other investigators, especially those examining related endpoints, will be extremely valuable in the overall assessment of the data and the ultimate risk analysis of health effects associated with DE exposure from the new model engines.

IMPLICATIONS OF FINDINGS

The above data demonstrated an apparent lack of effect of inhalation of DE on MN-RET levels in the blood of either mice or rats after 1- and 3-month exposure periods. This apparent lack of genotoxicity served to strengthen the requirement for similar observations to be made at the longer duration periods of 1 and 2 years in the continuing rat studies. Once the entire data set of all endpoints from the full ACES chronic-inhalation bioassay has been assembled, a complete assessment of the potential impact of DE can be derived.

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APPENDIX A. Raw Data and Calculations from 1- and 3-Month Mouse and Rat Micronucleus Analyses

Table A.1. Raw Data from Male Mice in 1-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
B006	1,128,852	2138	19,925	75
B009	1,205,329	2138	19,931	69
B008	1,260,034	2506	19,936	64
B007	1,012,183	1618	19,934	66
B010	1,298,224	2314	19,934	66
B001	1,116,914	1942	19,947	53
B005	921,420	1638	19,931	69
Low				
B031	547,435	977	19,949	51
B032	1,078,127	2068	19,928	72
B027	1,126,070	1993	19,935	65
B029	1,099,211	2083	19,923	77
Mid				
B051	881,644	1598	19,926	74
B050	878,796	1493	19,924	76
B047	991,032	1646	19,935	65
B054	1,072,569	1928	19,927	73
B046	1,084,443	1957	19,932	68
B052	854,238	1754	19,920	80
High				
B074	910,351	1639	19,946	54
B072	885,317	1296	19,925	75
B071	883,792	1178	19,935	65

Table A.2. Calculated Data from Male Mice in 1-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET			MN-NCE	
	%	LN/LN %	%	Proportion	ArcsinSqrt	%	LN/LN %
Control							
B006	1.74	−0.59	0.3800	0.0038	0.0617	0.1900	0.2921
B009	1.63	−0.72	0.3500	0.0035	0.0592	0.1800	0.2509
B008	1.56	−0.81	0.3200	0.0032	0.0566	0.2000	0.3297
B007	1.93	−0.42	0.3300	0.0033	0.0575	0.1600	0.1548
B010	1.51	−0.89	0.3300	0.0033	0.0575	0.1800	0.2509
B001	1.76	−0.57	0.2700	0.0027	0.0520	0.1700	0.2054
B005	2.12	−0.29	0.3500	0.0035	0.0592	0.1800	0.2509
Mean	1.75	−0.61	0.3329	0.0033	0.0577	0.1800	0.2478
SD	0.22	0.21	0.0340	0.0003	0.0030	0.0129	0.0565
SEM	0.08	0.08	0.0129	0.0001	0.0011	0.0049	0.0214
Low							
B031	3.52	0.23	0.2600	0.0026	0.0510	0.1800	0.2509
B032	1.82	−0.51	0.3600	0.0036	0.0600	0.1900	0.2921
B027	1.74	−0.59	0.3300	0.0033	0.0575	0.1800	0.2509
B029	1.78	−0.55	0.3900	0.0039	0.0625	0.1900	0.2921
Mean	2.22	−0.36	0.3350	0.0034	0.0578	0.1850	0.2715
SD	0.87	0.39	0.0557	0.0006	0.0049	0.0058	0.0238
SEM	0.44	0.20	0.0278	0.0003	0.0025	0.0029	0.0119
Mid							
B051	2.21	−0.23	0.3700	0.0037	0.0609	0.1800	0.2509
B050	2.22	−0.23	0.3800	0.0038	0.0617	0.1700	0.2054
B047	1.97	−0.39	0.3300	0.0033	0.0575	0.1700	0.2054
B054	1.83	−0.50	0.3700	0.0037	0.0609	0.1800	0.2509
B046	1.81	−0.52	0.3400	0.0034	0.0583	0.1800	0.2509
B052	2.28	−0.19	0.4000	0.0040	0.0633	0.2000	0.3297
Mean	2.05	−0.34	0.3650	0.0037	0.0604	0.1800	0.2489
SD	0.21	0.15	0.0259	0.0003	0.0022	0.0110	0.0454
SEM	0.09	0.06	0.0106	0.0001	0.0009	0.0045	0.0185
High							
B074	2.15	−0.27	0.2700	0.0027	0.0520	0.1800	0.2509
B072	2.21	−0.23	0.3800	0.0038	0.0617	0.1500	0.0979
B071	2.21	−0.23	0.3300	0.0033	0.0575	0.1300	−0.0411
Mean	2.19	−0.24	0.3267	0.0033	0.0571	0.1533	0.1026
SD	0.03	0.02	0.0551	0.0006	0.0049	0.0252	0.1460
SEM	0.02	0.01	0.0318	0.0003	0.0028	0.0145	0.0843

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.3. Raw Data from Female Mice in 1-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
B502	1,229,092	2904	19,937	63
B506	973,072	1234	19,963	37
B509	1,423,596	2159	19,953	47
Low				
B533	1,208,541	3069	19,943	57
B527	1,057,293	1301	19,940	60
B530	576,388	817	19,942	58
B525	913,789	1012	19,946	54
B531	852,287	1475	19,932	68
Mid				
B550	845,145	1083	19,953	47
B545	1,091,242	1315	19,952	48
B548	1,191,257	1466	19,949	51
B546	693,999	875	19,936	64
B554	1,095,282	1295	19,962	38
B547	1,016,595	1333	19,946	54
High				
B567	943,054	1243	19,946	54
B572	839,501	1002	19,934	66
B574	724,310	899	19,949	51
B576	1,146,041	1502	19,951	49
B569	1,034,801	1279	19,952	48
B573	748,582	1015	19,946	54

Table A.4. Calculated Data from Female Mice in 1-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET			MN-NCE	
	%	LN/LN %	%	Proportion	ArcsinSqrt	%	LN/LN %
Control							
B502	1.60	−0.76	0.3200	0.0032	0.0566	0.2400	0.4529
B506	2.01	−0.36	0.1900	0.0019	0.0436	0.1300	−0.0411
B509	1.38	−1.13	0.2400	0.0024	0.0490	0.1500	0.0979
Mean	1.66	−0.75	0.2500	0.0025	0.0497	0.1733	0.1699
SD	0.32	0.39	0.0656	0.0007	0.0065	0.0586	0.2547
SEM	0.18	0.22	0.0379	0.0004	0.0038	0.0338	0.1471
Low							
B533	1.62	−0.73	0.2900	0.0029	0.0539	0.2500	0.4785
B527	1.85	−0.49	0.3000	0.0030	0.0548	0.1200	−0.1281
B530	3.35	0.19	0.2900	0.0029	0.0539	0.1400	0.0333
B525	2.14	−0.27	0.2700	0.0027	0.0520	0.1100	−0.2323
B531	2.29	−0.19	0.3400	0.0034	0.0583	0.1700	0.2054
Mean	2.25	−0.30	0.2980	0.0030	0.0546	0.1580	0.0714
SD	0.67	0.34	0.0259	0.0003	0.0023	0.0563	0.2816
SEM	0.30	0.15	0.0116	0.0001	0.0010	0.0252	0.1259
Mid							
B550	2.31	−0.18	0.2400	0.0024	0.0490	0.1300	−0.0411
B545	1.80	−0.53	0.2400	0.0024	0.0490	0.1200	−0.1281
B548	1.65	−0.69	0.2600	0.0026	0.0510	0.1200	−0.1281
B546	2.80	0.03	0.3200	0.0032	0.0566	0.1300	−0.0411
B554	1.79	−0.54	0.1900	0.0019	0.0436	0.1200	−0.1281
B547	1.93	−0.42	0.2700	0.0027	0.0520	0.1300	−0.0411
Mean	2.05	−0.39	0.2533	0.0025	0.0502	0.1250	−0.0846
SD	0.43	0.27	0.0427	0.0004	0.0043	0.0055	0.0477
SEM	0.18	0.11	0.0174	0.0002	0.0017	0.0022	0.0195
High							
B567	2.07	−0.32	0.2700	0.0027	0.0520	0.1300	−0.0411
B572	2.32	−0.17	0.3300	0.0033	0.0575	0.1200	−0.1281
B574	2.68	−0.01	0.2600	0.0026	0.0510	0.1200	−0.1281
B576	1.71	−0.62	0.2500	0.0025	0.0500	0.1300	−0.0411
B569	1.89	−0.45	0.2400	0.0024	0.0490	0.1200	−0.1281
B573	2.60	−0.05	0.2700	0.0027	0.0520	0.1400	0.0333
Mean	2.21	−0.27	0.2700	0.0027	0.0519	0.1267	−0.0722
SD	0.39	0.24	0.0316	0.0003	0.0030	0.0082	0.0670
SEM	0.16	0.10	0.0129	0.0001	0.0012	0.0033	0.0274

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.5. Raw Data from Male Mice in 3-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
B013	955,503	1814	19,920	80
B020	1,149,371	2117	19,929	71
B011	1,553,605	2806	19,922	78
B017	1,205,948	2015	19,923	77
B018	1,109,249	1835	19,946	54
Low				
B034	1,416,903	2349	19,928	72
B039	1,255,535	2356	19,926	74
B043	913,535	1492	19,932	68
B042	1,144,045	2155	19,934	66
B035	978,360	1666	19,926	74
Mid				
B059	1,002,100	1757	19,898	102
B057	1,185,674	2450	19,923	77
B062	1,215,124	2229	19,926	74
B060	1,085,461	1868	19,931	69
B055	1,040,413	1774	19,931	69
High				
B082	1,074,462	1621	19,926	74
B078	974,251	1661	19,913	87
B085	1,299,654	2520	19,915	85
B077	1,055,481	2019	19,933	67
B081	1,074,685	1938	19,941	59

Table A.6. Calculated Data From Male Mice in 3-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET			MN-NCE	
	%	LN/LN %	%	Proportion	ArcsinSqrt	%	LN/LN %
Control							
B013	2.05	−0.33	0.40	0.0040	0.0633	0.19	0.29
B020	1.71	−0.62	0.36	0.0036	0.0600	0.18	0.25
B011	1.27	−1.43	0.39	0.0039	0.0625	0.18	0.25
B017	1.63	−0.72	0.39	0.0039	0.0625	0.17	0.21
B018	1.77	−0.56	0.27	0.0027	0.0520	0.17	0.21
Mean	1.69	−0.73	0.36	0.0036	0.0601	0.18	0.24
SD	0.28	0.42	0.05	0.0005	0.0047	0.01	0.04
SEM	0.13	0.19	0.02	0.0002	0.0021	0.004	0.02
Low							
B034	1.39	−1.11	0.36	0.0036	0.0600	0.17	0.21
B039	1.57	−0.80	0.37	0.0037	0.0609	0.19	0.29
B043	2.14	−0.27	0.34	0.0034	0.0583	0.16	0.15
B042	1.71	−0.62	0.33	0.0033	0.0575	0.19	0.29
B035	2.00	−0.37	0.37	0.0037	0.0609	0.17	0.21
Mean	1.76	−0.63	0.35	0.0035	0.0595	0.18	0.23
SD	0.31	0.34	0.02	0.0002	0.0015	0.01	0.06
SEM	0.14	0.15	0.01	0.0001	0.0007	0.01	0.03
Mid							
B059	1.95	−0.40	0.51	0.0051	0.0715	0.18	0.25
B057	1.66	−0.68	0.39	0.0039	0.0625	0.21	0.36
B062	1.62	−0.73	0.37	0.0037	0.0609	0.18	0.25
B060	1.81	−0.52	0.35	0.0035	0.0592	0.17	0.21
B055	1.88	−0.46	0.35	0.0035	0.0592	0.17	0.21
Mean	1.78	−0.56	0.39	0.0039	0.0627	0.18	0.26
SD	0.14	0.14	0.07	0.0007	0.0051	0.02	0.06
SEM	0.06	0.06	0.03	0.0003	0.0023	0.01	0.03
High							
B082	1.82	−0.51	0.37	0.0037	0.0609	0.15	0.10
B078	2.01	−0.36	0.44	0.0044	0.0664	0.17	0.21
B085	1.51	−0.89	0.43	0.0043	0.0656	0.19	0.29
B077	1.86	−0.48	0.34	0.0034	0.0583	0.19	0.29
B081	1.82	−0.51	0.30	0.0030	0.0548	0.18	0.25
Mean	1.80	−0.55	0.38	0.0038	0.0612	0.18	0.23
SD	0.18	0.20	0.06	0.0006	0.0049	0.02	0.08
SEM	0.08	0.09	0.03	0.0003	0.0022	0.01	0.04

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.7. Raw Data from Female Mice in 3-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
B519	1,335,401	1390	19,962	38
B512	1,855,548	2205	19,949	51
B511	1,092,272	1408	19,952	48
B520	1,313,874	1566	19,964	36
B517	1,083,079	1219	19,970	30
Low				
B534	1,273,193	1492	19,950	50
B537	662,620	778	19,960	40
B538	1,321,606	1492	19,955	45
B543	1,239,199	1393	19,957	43
B542	1,109,816	1208	19,960	40
Mid				
B564	1,221,433	1401	19,952	48
B559	1,193,983	1354	19,946	54
B561	913,630	1002	19,960	40
B562	941,005	998	19,954	46
B566	315,120	507	19,947	53
High				
B548	1,573,565	1566	19,964	36
B580	1,190,875	1308	19,951	49
B583	1,198,061	1226	19,954	46
B587	784,420	966	19,944	56
B578	1,072,200	1271	19,970	30

Table A.8. Calculated Data from Female Mice in 3-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET			MN-NCE	
	%	LN/LN %	%	Proportion	ArcsinSqrt	%	LN/LN %
Control							
B519	1.47	−0.95	0.19	0.0019	0.0436	0.10	−0.36
B512	1.07	−2.69	0.26	0.0026	0.0510	0.12	−0.13
B511	1.80	−0.53	0.24	0.0024	0.0490	0.13	−0.04
B520	1.50	−0.90	0.18	0.0018	0.0424	0.12	−0.13
B517	1.81	−0.52	0.15	0.0015	0.0387	0.11	−0.23
Mean	1.53	−1.12	0.20	0.0020	0.0449	0.12	−0.18
SD	0.30	0.90	0.05	0.0005	0.0050	0.01	0.12
SEM	0.14	0.40	0.02	0.0002	0.0022	0.01	0.05
Low							
B534	1.54	−0.84	0.25	0.0025	0.0500	0.12	−0.13
B537	2.93	0.07	0.20	0.0020	0.0447	0.12	−0.13
B538	1.49	−0.92	0.23	0.0023	0.0480	0.11	−0.23
B543	1.59	−0.77	0.22	0.0022	0.0469	0.11	−0.23
B542	1.77	−0.56	0.20	0.0020	0.0447	0.11	−0.23
Mean	1.86	−0.60	0.22	0.0022	0.0469	0.11	−0.19
SD	0.61	0.40	0.02	0.0002	0.0023	0.01	0.06
SEM	0.27	0.18	0.01	0.0001	0.0010	0.002	0.03
Mid							
B564	1.61	−0.74	0.24	0.0024	0.0490	0.11	−0.23
B559	1.65	−0.69	0.27	0.0027	0.0520	0.11	−0.23
B561	2.14	−0.27	0.20	0.0020	0.0447	0.11	−0.23
B562	2.08	−0.31	0.23	0.0023	0.0480	0.11	−0.23
B566	5.96	0.58	0.27	0.0027	0.0520	0.16	0.15
Mean	2.69	−0.29	0.24	0.0024	0.0491	0.12	−0.15
SD	1.84	0.53	0.03	0.0003	0.0031	0.02	0.17
SEM	0.83	0.24	0.01	0.0001	0.0014	0.01	0.08
High							
B548	1.25	−1.50	0.18	0.0018	0.0424	0.10	−0.36
B580	1.65	−0.69	0.25	0.0025	0.0500	0.11	−0.23
B583	1.64	−0.70	0.23	0.0023	0.0480	0.10	−0.36
B587	2.48	−0.10	0.28	0.0028	0.0529	0.12	−0.13
B578	1.83	−0.50	0.15	0.0015	0.0387	0.12	−0.13
Mean	1.77	−0.70	0.22	0.0022	0.0464	0.11	−0.24
SD	0.45	0.51	0.05	0.0005	0.0058	0.01	0.12
SEM	0.20	0.23	0.02	0.0002	0.0026	0.004	0.05

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.9. Raw Data from Male Rats in 1-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
D008	1,354,198	20	19,972	28
D039	1,177,794	21	19,983	17
E041	2,202,764	42	19,969	31
F041	1,772,462	25	19,985	15
F017	1,138,609	17	19,988	12
Low				
D068	1,237,918	21	19,987	13
D093	1,323,949	18	19,983	17
E074	1,966,410	31	19,978	22
E066	1,228,525	18	19,985	15
F056	2,140,814	27	19,974	26
Mid				
D139	1,400,295	27	19,986	14
D107	1,902,401	21	19,983	17
E114	1,409,824	26	19,983	17
E113	1,841,264	69	19,978	22
F097	1,419,438	16	19,983	17
High				
D160	1,459,906	36	19,978	22
D156	1,352,871	28	19,981	19
E145	1,553,056	31	19,971	29
F182	1,347,572	26	19,988	12
F169	1,395,895	27	19,964	36

Table A.10. Calculated Data from Male Rats in 1-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET		
	%	LN/LN %	%	Proportion	ArcsinSqrt
Control					
D008	1.46	0.32	0.1400	0.0014	0.0374
D039	1.67	0.41	0.0900	0.0009	0.0300
E041	0.90	−0.11	0.1600	0.0016	0.0400
F041	1.12	0.11	0.0800	0.0008	0.0283
F017	1.73	0.44	0.0600	0.0006	0.0245
Mean	1.38	0.23	0.1060	0.0011	0.0320
SD	0.36	0.23	0.0422	0.0004	0.0065
SEM	0.16	0.10	0.0189	0.0002	0.0029
Low					
D068	1.59	0.38	0.0700	0.0007	0.0265
D093	1.49	0.34	0.0900	0.0009	0.0300
E074	1.01	0.01	0.1100	0.0011	0.0332
E066	1.60	0.39	0.0800	0.0008	0.0283
F056	0.93	−0.08	0.1300	0.0013	0.0361
Mean	1.32	0.21	0.0960	0.0010	0.0308
SD	0.33	0.22	0.0241	0.0002	0.0038
SEM	0.15	0.10	0.0108	0.0001	0.0017
Mid					
D139	1.41	0.30	0.0700	0.0007	0.0265
D107	1.04	0.04	0.0900	0.0009	0.0300
E114	1.40	0.29	0.0900	0.0009	0.0300
E113	1.07	0.07	0.1100	0.0011	0.0332
F097	1.39	0.28	0.0900	0.0009	0.0300
Mean	1.26	0.19	0.0900	0.0009	0.0299
SD	0.19	0.13	0.0141	0.0001	0.0024
SEM	0.08	0.06	0.0063	0.0001	0.0011
High					
D160	1.35	0.26	0.1100	0.0011	0.0332
D156	1.46	0.32	0.1000	0.0010	0.0316
E145	1.27	0.21	0.1500	0.0015	0.0387
F182	1.46	0.32	0.0600	0.0006	0.0245
F169	1.41	0.30	0.1800	0.0018	0.0424
Mean	1.39	0.28	0.1200	0.0012	0.0341
SD	0.08	0.05	0.0464	0.0005	0.0069
SEM	0.04	0.02	0.0207	0.0002	0.0031

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.11. Raw Data from Female Rats in 1-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
D542	2,050,703	25	19,980	20
D538	1,992,118	21	19,985	15
E527	2,057,487	47	19,978	22
E505	1,364,464	17	19,979	21
F534	3,077,146	28	19,984	16
Low				
D549	1,238,107	10	19,978	22
D587	1,023,094	21	19,984	16
E559	1,462,846	22	19,984	16
F561	2,832,347	37	19,986	14
F586	1,601,009	24	19,988	12
Mid				
D597	1,140,822	16	19,983	17
D607	1,198,139	16	19,984	16
E637	1,953,106	42	19,967	33
F607	1,318,834	15	19,984	16
F619	1,487,521	33	19,986	14
High				
D645	1,407,330	10	19,992	8
D686	1,351,888	12	19,982	18
E674	1,234,399	26	19,979	21
E646	1,051,450	33	19,978	22
F639	1,728,694	21	19,986	14

Table A.12. Calculated Data from Female Rats in 1-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET		
	%	LN/LN %	%	Proportion	ArcsinSqrt
Control					
D542	0.97	−0.03	0.1000	0.0010	0.0316
D538	0.99	−0.01	0.0800	0.0008	0.0283
E527	0.96	−0.04	0.1100	0.0011	0.0332
E505	1.44	0.31	0.1100	0.0011	0.0332
F534	0.65	−0.56	0.0800	0.0008	0.0283
Mean	1.00	−0.07	0.0960	0.0010	0.0309
SD	0.28	0.31	0.0152	0.0002	0.0025
SEM	0.13	0.14	0.0068	0.0001	0.0011
Low					
D549	1.59	0.38	0.1100	0.0011	0.0332
D587	1.92	0.50	0.0800	0.0008	0.0283
E559	1.35	0.26	0.0800	0.0008	0.0283
F561	0.70	−0.44	0.0700	0.0007	0.0265
F586	1.23	0.19	0.0600	0.0006	0.0245
Mean	1.36	0.18	0.0800	0.0008	0.0282
SD	0.45	0.37	0.0187	0.0002	0.0032
SEM	0.20	0.16	0.0084	0.0001	0.0014
Mid					
D597	1.72	0.43	0.0900	0.0009	0.0300
D607	1.64	0.40	0.0800	0.0008	0.0283
E637	1.01	0.01	0.1700	0.0017	0.0412
F607	1.49	0.34	0.0800	0.0008	0.0283
F619	1.33	0.25	0.0700	0.0007	0.0265
Mean	1.44	0.29	0.0980	0.0010	0.0309
SD	0.28	0.17	0.0409	0.0004	0.0059
SEM	0.13	0.08	0.0183	0.0002	0.0026
High					
D645	1.40	0.29	0.0400	0.0004	0.0200
D686	1.46	0.32	0.0900	0.0009	0.0300
E674	1.59	0.38	0.1100	0.0011	0.0332
E646	1.87	0.49	0.1100	0.0011	0.0332
F639	1.14	0.12	0.0700	0.0007	0.0265
Mean	1.49	0.32	0.0840	0.0008	0.0286
SD	0.27	0.13	0.0297	0.0003	0.0055
SEM	0.12	0.06	0.0133	0.0001	0.0025

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.13. Raw Data from Male Rats in 3-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
D002	4,067,364	53	19,981	19
D040	1,290,854	39	19,980	20
E013	3,308,389	67	19,962	38
F003	2,249,164	50	19,978	22
F036	1,876,642	36	19,974	26
Low				
D081	1,611,816	25	19,976	24
D088	2,430,262	33	19,975	25
E049	1,730,319	61	19,977	23
E071	1,293,937	49	19,983	17
F065	1,593,475	23	19,978	22
Mid				
D135	1,143,139	39	19,973	27
D103	2,091,181	38	19,981	19
E133	1,659,993	36	19,961	39
E141	2,237,776	55	19,960	40
F111	2,461,446	33	19,978	22
High				
D169	1,246,655	35	19,989	11
D185	2,017,543	25	19,981	19
E155	1,774,709	54	19,970	30
F183	2,820,000	26	19,983	17
F157	2,438,850	71	19,976	24

Table A.14. Calculated Data from Male Rats in 3-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET		
	%	LN/LN %	%	Proportion	ArcsinSqrt
Control					
D002	0.49	−1.25	0.1000	0.0010	0.0316
D040	1.53	0.35	0.1000	0.0010	0.0316
E013	0.60	−0.72	0.1900	0.0019	0.0436
F003	0.88	−0.14	0.1100	0.0011	0.0332
F036	1.05	0.05	0.1300	0.0013	0.0361
Mean	0.91	−0.34	0.1260	0.0013	0.0352
SD	0.41	0.64	0.0378	0.0004	0.0050
SEM	0.18	0.29	0.0169	0.0002	0.0023
Low					
D081	1.23	0.19	0.1200	0.0012	0.0346
D088	0.82	−0.22	0.1300	0.0013	0.0361
E049	1.14	0.12	0.1200	0.0012	0.0346
E071	1.52	0.35	0.0900	0.0009	0.0300
F065	1.24	0.19	0.1100	0.0011	0.0332
Mean	1.19	0.13	0.1140	0.0011	0.0337
SD	0.25	0.21	0.0152	0.0002	0.0023
SEM	0.11	0.09	0.0068	0.0001	0.0010
Mid					
D135	1.72	0.43	0.1400	0.0014	0.0374
D103	0.95	−0.05	0.1000	0.0010	0.0316
E133	1.19	0.16	0.2000	0.0020	0.0447
E141	0.89	−0.12	0.2000	0.0020	0.0447
F111	0.81	−0.24	0.1100	0.0011	0.0332
Mean	1.11	0.04	0.1500	0.0015	0.0383
SD	0.37	0.27	0.0480	0.0005	0.0062
SEM	0.16	0.12	0.0214	0.0002	0.0028
High					
D169	1.58	0.38	0.0600	0.0006	0.0245
D185	0.98	−0.02	0.1000	0.0010	0.0316
E155	1.11	0.10	0.1500	0.0015	0.0387
F183	0.70	−0.44	0.0900	0.0009	0.0300
F157	0.81	−0.24	0.1200	0.0012	0.0346
Mean	1.04	−0.04	0.1040	0.0010	0.0319
SD	0.34	0.31	0.0336	0.0003	0.0053
SEM	0.15	0.14	0.0150	0.0002	0.0024

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

Table A.15. Raw Data from Female Rats in 3-Month Exposure Group

Exposure / Animal ID	LL Quadrant NCE (*n*)	LR Quadrant MN-NCE (*n*)	UL Quadrant RET (*n*)	UR Quadrant MN-RET (*n*)
Control				
D527	1,988,543	39	19,964	36
D524	1,179,559	22	19,981	19
E523	1,392,601	16	19,979	21
E509	1,980,310	30	19,965	35
F514	1,893,685	32	19,985	15
Low				
D580	2,084,359	26	19,974	26
D554	1,624,144	24	19,977	23
E596	1,975,676	32	19,978	22
F579	1,759,002	24	19,975	25
F554	2,074,932	15	19,988	12
Mid				
D614	1,384,707	14	19,992	8
D610	2,382,528	26	19,976	24
E606	1,709,903	46	19,953	47
F629	1,160,610	11	19,979	21
F594	1,249,386	18	19,978	22
High				
D682	1,297,692	7	19,985	15
D685	844,934	7	19,987	13
E649	1,286,998	30	19,977	23
E665	1,801,088	23	19,978	22
F680	2,116,042	20	19,983	17

Table A.16. Calculated Data from Female Rats in 3-Month Exposure Group[a]

Exposure / Animal ID	RET		MN-RET		
	%	LN/LN %	%	Proportion	ArcsinSqrt
Control					
D527	1.00	0.00	0.1800	0.0018	0.0424
D524	1.67	0.41	0.1000	0.0010	0.0316
E523	1.42	0.30	0.1100	0.0011	0.0332
E509	1.00	0.00	0.1800	0.0018	0.0424
F514	1.05	0.05	0.0800	0.0008	0.0283
Mean	1.23	0.15	0.1300	0.0013	0.0356
SD	0.30	0.19	0.0469	0.0005	0.0065
SEM	0.14	0.09	0.0210	0.0002	0.0029
Low					
D580	0.95	−0.05	0.1300	0.0013	0.0361
D554	1.22	0.18	0.1200	0.0012	0.0346
E596	1.00	0.00	0.1100	0.0011	0.0332
F579	1.12	0.11	0.1300	0.0013	0.0361
F554	0.95	−0.05	0.0600	0.0006	0.0245
Mean	1.05	0.04	0.1100	0.0011	0.0329
SD	0.12	0.10	0.0292	0.0003	0.0048
SEM	0.05	0.05	0.0130	0.0001	0.0022
Mid					
D614	1.42	0.30	0.0400	0.0004	0.0200
D610	0.83	−0.21	0.1200	0.0012	0.0346
E606	1.16	0.14	0.2400	0.0024	0.0490
F629	1.69	0.42	0.1100	0.0011	0.0332
F594	1.58	0.38	0.1100	0.0011	0.0332
Mean	0.03	0.21	0.1240	0.0012	0.0340
SD	0.01	0.25	0.0723	0.0007	0.0103
SEM	0.005	0.11	0.0323	0.0003	0.0046
High					
D682	1.52	0.35	0.0800	0.0008	0.0283
D685	2.31	0.61	0.0700	0.0007	0.0265
E649	1.53	0.35	0.1200	0.0012	0.0346
E665	1.10	0.09	0.1100	0.0011	0.0332
F680	0.94	−0.06	0.0900	0.0009	0.0300
Mean	1.48	0.27	0.0940	0.0009	0.0305
SD	0.53	0.26	0.0207	0.0002	0.0034
SEM	0.24	0.12	0.0093	0.0001	0.0015

[a] ArcsinSqrt indicates arcsine–square root transformation of the %MN-RET; LN/LN indicates double logarithmic transformation.

APPENDIX B. ACES Pilot Study to Determine Efficacy of Flow Cytometric Assessment of Chromosome Damage in Aged Rats

ABSTRACT

The induction of MN in cells derived from bone marrow and peripheral blood is a well-established indicator of chromosome damage. Traditionally the erythrocyte-based assay is performed in young animals and analysis is conducted via microscopic examination of cells for micronuclei. An alternative method for MN assessment in blood samples has been developed by Litron Laboratories (Rochester, NY) and validated by numerous interlaboratory investigations. This automated methodology is based on flow cytometric detection of MN-RET in peripheral blood and has many benefits over microscopy including objective scoring, increased number of cells scored, and greater reproducibility of data. This pilot study sought to confirm the ability of the flow cytometric methods to identify a chromosome-damage response in older rats.

Male and female Sprague-Dawley rats between the ages of 10 and 21 months were used. Before treatment, blood samples from all rats were obtained to provide baseline information on the desired endpoints. After a 3-week recovery period animals were treated with either CP (15 mg/kg body weight via oral gavage) or vehicle control; 48 hours later blood was collected from all animals. Flow cytometric analysis allowed for the detection of MN-RET and RET frequencies in peripheral blood samples from rats. Using a simple staining procedure coupled with rapid and efficient analysis, we examined many more cells in less time than when using traditional, microscopy-based MN assays. Thus, for each sample up to 20,000 RETs were scored for the presence of MN.

The baseline data established the background frequencies of MN-RET and RET in the blood of laboratory animals. After exposure, both males and females treated with CP displayed a robust induction of MN-RET in peripheral blood and only males demonstrated a commensurate reduction in %RET attributable to bone marrow toxicity. The vehicle-treated animals did not show any significant changes across the study period.

This investigation demonstrated the ability of flow cytometric analysis of MN induction to identify a chromosome-damage response in peripheral blood samples obtained from aged rats exposed to a model genotoxic agent.

INTRODUCTION

In vivo MN studies are an established approach for the detection of chromosome damage in the intact animal after treatment with drugs or other agents (OECD 1997; FDA 2000). Typically these studies involve the microscopic examination of MN-RET derived from bone marrow samples. The development of an automated flow cytometric methodology for the enumeration of MN-RETs in peripheral blood — In Vivo MicroFlow — enables the same assessment of genotoxicity to be performed in a much more readily obtained tissue (i.e., whole blood) (Dertinger et al. 2004). A significant amount of work has been performed to validate this method for use in mouse and rat studies (Torous et al. 2003; Dertinger et al. 2006; Hayashi et al. 2007), such that the FDA accepts mouse In Vivo MicroFlow data for drug safety submission. In general these studies employ young rodents, typically 7 to 9 weeks of age. As there are few data associated with MN induction in aged rodents, this pilot experiment was designed to assess the ability of the In Vivo MicroFlow system to quantify a chemically induced elevation in peripheral blood MN-RET frequency.

SPECIFIC AIMS

The use of flow cytometry–based enumeration of MN-RET as part of the ACES bioassay was proposed for the ACES ancillary studies. Given the lack of information on this endpoint or analytic procedure in aged rats, this study was designed to determine the response of aged rats to genotoxic challenge via an examination of peripheral blood MN induction in erythrocytes after in vivo exposure to CP. This experiment served to validate the use of flow cytometric detection of MN-RET as an indicator of genotoxic damage in an aged rat population.

METHODS AND STUDY DESIGN

Animals

All animal work was performed with the oversight of the University of Rochester's Institutional Animal Care and Use Committee. Water and food were available ad libitum throughout the experimental period. Initially, five male Sprague-Dawley rats (10–16 months of age) and five female Sprague-Dawley rats (13–21 months of age) were obtained from the University of Rochester for this study. Despite the rather broad age range, the collective term "aged" was used throughout this report to characterize the population. Before the study began, the female designated

Table B.1. Individual Rat Information

ID Code	Approximate Age (Months)	Treatment
Females		
F1	14	Vehicle
F3	21	Cyclophosphamide
F4	19	Cyclophosphamide
F5	19	Cyclophosphamide
Males		
M1	10	Vehicle
M2	16	Vehicle
M3	16	Cyclophosphamide
M4	16	Cyclophosphamide
M5	10	Cyclophosphamide

as F2 died of an undetermined cause likely associated with advanced age. Table B.1 lists details of the age of individual animals as well as coding and treatment information.

Reagents and Supplies

Unless otherwise noted, all of the solutions, materials, and procedures mentioned below were associated with In Vivo MicroFlow kits (Litron Laboratories). Cyclophosphamide monohydrate (CP) was obtained from Sigma Aldrich (St. Louis, MO). CP was prepared in water at a concentration of 15 mg/mL.

Animal Treatment and Sample Collection

Initial baseline values for %RETs and %MN-RET were obtained by analyzing a small amount of blood from each animal. Blood was collected by first removing the distal end of the tail with a surgical blade and then expressing approximately 100 µL of whole blood into kit-provided anticoagulant solution. In order to avoid any potential impact of the minimal blood collection on erythropoiesis the animals were maintained for 3 weeks with no further sampling.

After the stated recovery period, three male and two female rats were treated via oral gavage with a single dose of CP (15 mg/kg body weight administered at 1 mL/kg), and the remaining two rats of each sex received vehicle only. The animals were sacrificed 48 hours after treatment and cardiac blood samples were collected. Table B.1 provides information on each animal and the treatment group to which it was assigned. An effort was made to balance the various ages across the treatment groups.

Analysis of Micronucleated Erythrocytes by Flow Cytometry

The reagents and procedures used for the fixation, processing, and analysis of the blood samples associated with this investigation were equivalent to those described in the Investigators' Report.

STATISTICAL METHODS AND DATA ANALYSIS

Given the small number of animals employed for this study and the lack of sex-based differences in MN-RET values in the larger rat data set associated with the bioassay, the pilot study data for males and females were pooled for certain comparisons. For some analyses the baseline data were also included as control values. The MN-RET data were transformed using the arcsin-root function as described in the Investigators' Report. For statistical purposes, ANOVA with post-hoc Dunnett *t* test was used to compare control data with exposed data. In order to provide a simple visual comparison between the baseline data and the post-treatment responses across the experiment, the data represented in Figures B.1 and B.2 and Tables B.1–B.3 are presented on a per animal basis. All raw data are provided in Tables B.2 and B.3 to facilitate additional comparisons.

RESULTS

Figure B.1 shows the MN-RET frequencies in peripheral blood of the male and female rats employed in this study before and after treatment. Using data pooled across sex, aged rats given a bolus dose of CP exhibited a statistically

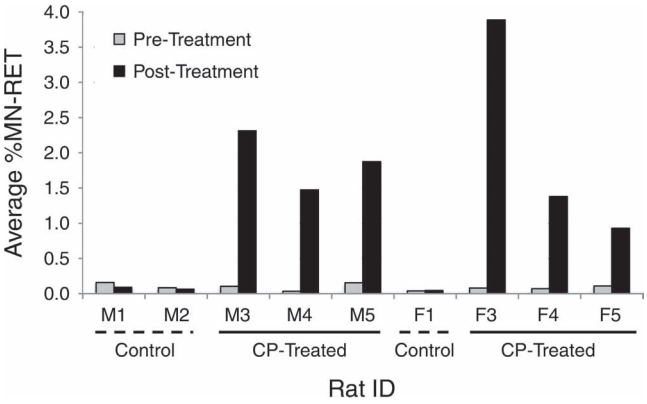

Figure B.1. Pre- and post-treatment frequencies of MN-RET in individual rats. Statistically significant elevations in %MN-RET were observed in rats that received 15 mg/kg CP. Analysis was performed via ANOVA with post-hoc Dunnett *t* test.

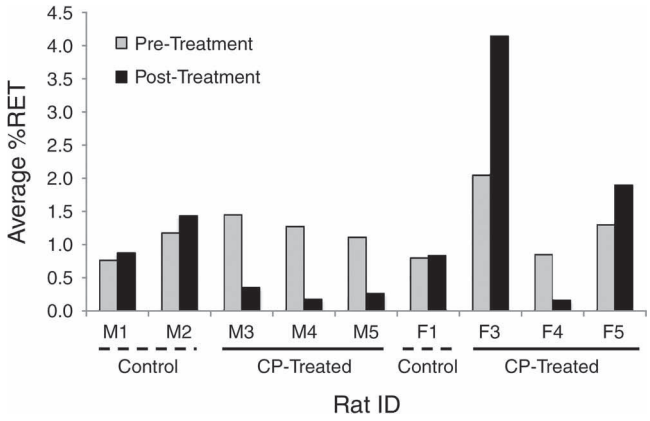

Figure B.2. Pre- and post-treatment frequencies of RET in individual rats. Statistical analysis of male rat data only revealed significant reductions in %RET in animals that received 15 mg/kg CP. Analysis was performed via ANOVA with post-hoc Dunnett *t* test.

significant elevation in MN-RET frequency when compared with the concurrent experimental controls. This statistical significance was maintained when all the baseline data were included as control values. With regard to %RET data, if the analysis was restricted to only the male rats, a statistically significant reduction in %RET was observed. In the exposed females, animal F4 demonstrated a reduction in %RET that was equivalent to that observed in the males. However, the two other females treated with CP did not exhibit the reduction in circulating RETs, but elevations in MN-RET frequency were observed in these individuals.

DISCUSSION AND CONCLUSIONS

The ACES bioassay was designed to examine the long-term effects of chronic exposure to DE. Since this design included the examination of rats that had experienced 1 and 2 years of exposure, the current experiment was performed to verify the ability of the proposed flow cytometric methodology to detect chromosome damage in aged rats. CP was chosen as a model genotoxic agent that readily induces DNA-strand breaks, which lead to the formation of MN in erythroid precursors in the bone marrow (MacGregor et al. 2006). These precursors are ultimately released into circulation as RETs, the target population of this MN analysis.

Across all the animals studied, the MN responses to genotoxicant exposure were as expected. The baseline

Table B.2. Individual Animal Raw Data[a]

Animal ID	Treatment	NCE (*n*)	MN-NCE (*n*)	RET (*n*)	MN-RET (*n*)
Baseline (Pre-Treatment)					
M1	N/A	657,386	51	5,011	8
M2	N/A	1,702,371	109	19,983	17
M3	N/A	1,380,217	328	19,979	21
M4	N/A	1,572,444	230	19,993	7
M5	N/A	1,799,597	389	19,970	31
F1	N/A	1,286,042	33	10,257	4
F3	N/A	977,478	208	19,984	16
F4	N/A	2,359,546	177	19,986	14
F5	N/A	1,540,350	167	19,978	22
Post-Treatment					
M1	Saline	2,286,120	39	19,982	18
M2	Saline	1,395,403	17	19,987	13
M3	15 mg/kg CP p.o.	2,781,308	130	9,774	226
M4	15 mg/kg CP p.o.	2,839,404	47	4,927	73
M5	15 mg/kg CP p.o.	1,871,589	50	4,908	92
F1	Saline	2,402,051	41	19,991	9
F3	15 mg/kg CP p.o.	467,304	64	19,354	752
F4	15 mg/kg CP p.o.	3,100,991	51	4,932	68
F5	15 mg/kg CP p.o.	1,046,031	48	19,816	184

[a] N/A indicates not applicable.

Table B.3. Calculated Frequencies for Individual Animals[a]

Animal ID	Treatment	Calculated Frequencies			MN-RET Proportion	MN-RET ArcsinSqrt
		% RET	% MN-NCE	% MN-RET		
Baseline (Pre-Treatment)						
M1	N/A	0.7623	0.0078	0.1596	0.0016	0.0400
M2	N/A	1.1738	0.0064	0.0851	0.0009	0.0292
M3	N/A	1.4475	0.0238	0.1051	0.0011	0.0324
M4	N/A	1.2715	0.0146	0.0350	0.0004	0.0187
M5	N/A	1.1097	0.0216	0.1552	0.0016	0.0394
F1	N/A	0.7976	0.0026	0.0390	0.0004	0.0197
F3	N/A	2.0444	0.0213	0.0801	0.0008	0.0283
F4	N/A	0.8470	0.0075	0.0700	0.0007	0.0265
F5	N/A	1.2970	0.0108	0.1101	0.0011	0.0332
Post-Treatment						
M1	Saline	0.8741	0.0017	0.0901	0.0009	0.0300
M2	Saline	1.4323	0.0012	0.0650	0.0007	0.0255
M3	15 mg/kg CP p.o.	0.3514	0.0047	2.3123	0.0231	0.1527
M4	15 mg/kg CP p.o.	0.1735	0.0017	1.4816	0.0148	0.1220
M5	15 mg/kg CP p.o.	0.2622	0.0027	1.8745	0.0187	0.1373
F1	Saline	0.8322	0.0017	0.0450	0.0005	0.0212
F3	15 mg/kg CP p.o.	4.1416	0.0137	3.8855	0.0389	0.1984
F4	15 mg/kg CP p.o.	0.1590	0.0016	1.3788	0.0138	0.1177
F5	15 mg/kg CP p.o.	1.8944	0.0046	0.9285	0.0093	0.0965

[a] N/A indicates not applicable; ArcsinSqrt indicates the arcsine–square root transformation of the %MN-RET.

samples were collected from all animals as a means to survey the aged population and establish background values across the various endpoints, which could then be compared to those obtained after exposure. After providing sufficient time for the animals to recover from the initial blood sampling, rats were treated with either a dose of CP known to elicit MN formation or the appropriate vehicle control. The control animals demonstrated similar values to those obtained at baseline, whereas the CP-exposed subjects demonstrated significant elevations in MN-RET frequencies. Examination of the data from only male rats revealed the predictable reduction in %RET values after exposure to CP. This confirmed that the systemic exposure reached the bone marrow and targeted the erythrocyte precursors responsible for generating RETs.

The lack of effect of CP treatment on %RET values in the two female rats was interesting. The general consensus was that sensitivity and expression of cytogenetic damage was not overly influenced by sex. Agents that act directly or indirectly on DNA elicit damage via mechanisms that are essentially the same in males and females. Thus we did not expect to observe differences in the responses of the males and females employed in this study. Indeed the specific marker of chromosome damage examined in this study — MN-RET — showed a significant elevation in these two animals. As mentioned above, the %RET value is not a direct indicator of DNA damage, rather it is a metric of toxicity affecting bone marrow and is typically examined in the context of genotoxicity studies as a means of confirming sufficient exposure of target cells in the tissue of interest.

It may also be of value to note that these two females were burdened with mammary tumors, a pathology frequently observed in this strain (Prejean et al. 1973), especially in older animals. So it is possible that this contributed to the altered response in RET frequency in these animals, but additional definitive testing and necropsy of these subjects was deemed outside of the scope of this investigation. Overall, the females responded with elevated MN-RET values after exposure to a known genotoxicant, thus supporting the use of this endpoint in the aged females that will be used in the long-term exposure (12 and 24 months) bioassay.

This investigation provided two important observations that served to validate the use of flow-cytometric

analysis of MN as an indicator of chromosome damage in the chronic-inhalation bioassay: (1) that aged rats, even when experiencing conditions related to advanced age, were still able to respond to genotoxicant challenge by producing MN-RET, and (2) that flow cytometric methods for detecting MN-RET in peripheral blood from aged rats was a suitable approach for genotoxicity assessment. Thus, the MN data generated as part of the ACES DE study can be interpreted with regard to the potential impact of treatment and not to methodologic deficiencies.

IMPLICATIONS OF FINDINGS

This report justifies the use of flow cytometric assessment of peripheral blood MN-RET as a valid methodology for investigation of the effects of DE in aged rats that is part of the ACES chronic-inhalation bioassay.

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Jeffrey Bemis received his Ph.D. in Environmental Health and Toxicology from the University at Albany School of Public Health in Albany, New York. His thesis focused on the effects of combined exposure to environmental contaminants such as polychlorinated biphenyls (PCBs) and methyl mercury on central neurotransmitter systems. Bemis then spent three years as a postdoctoral fellow in the Department of Environmental Medicine at the University of Rochester School of Medicine and Dentistry in Rochester, New York. He studied the effects of PCBs and dioxin on the nervous and immune systems, as well as identifying target tissues in the developing fetus. Bemis joined Litron Laboratories in August of 2006 as a research scientist and is currently director of clinical studies.

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ABBREVIATIONS AND OTHER TERMS

5-FU	5-fluorouracil
ACES	Advanced Collaborative Emissions Study
ANOVA	analysis of variance
BP	benzo[*a*]pyrene
CP	cyclophosphamide
FCM	flow cytometry
ICH	International Conference on Harmonisation
LTSS	Long Term Storage Solution
LRRI	Lovelace Respiratory Research Institute
MTX	methotrexate
MN	micronucleus; micronuclei
MN-RET	micronucleated reticulocyte
MN-NCE	micronucleated normochromatic erythrocyte
NCE	normochromatic erythrocyte
OECD	Organization of Economic Cooperation and Development
PhRMA	Pharmaceutical Research and Manufacturers of America
PM	particulate matter
RET	reticulocyte
SEM	standard error of the mean
U.S. EPA	United States Environmental Protection Agency

Part 3. Assessment of Genotoxicity and Oxidative Stress After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 3. Assessment of Genotoxicity and Oxidative Stress After Exposure to Diesel Exhaust from U.S. 2007-Compliant Diesel Engines: Report on 1- and 3-Month Exposures in the ACES Bioassay

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ABSTRACT

Human health hazards due to diesel exhaust (DE*) exposure have been associated with both solvent and combustion components. In the past, diesel engine exhaust components have been linked to increased mutagenicity in cultures of *Salmonella typhimurium* and mammalian cells (Tokiwa and Ohnishi 1986). In addition, DE has been shown to increase both the incidence of tumors and the induction of 8-hydroxy-deoxyguanosine adducts (8-OHdG) in ICR mice (Ichinose et al. 1997). Furthermore, DE is composed of a complex mixture of polycyclic aromatic hydrocarbons (PAHs) and particulates. One such PAH, 3-nitrobenzanthrone (3-NBA), has been identified in DE and found in urban air. 3-NBA has been observed to induce micronucleus formation in DNA of human hepatoma cells (Lamy et al. 2004).

The purpose of the current research, which is part of the Advanced Collaborative Emissions Study (ACES), a multidisciplinary program being carried out by the Health Effects Institute and the Coordinating Research Council, is to determine whether improvements in the engineering of heavy-duty diesel engines reduce the oxidative stress and genotoxic risk associated with exposure to DE components. To this end, the genotoxicity and oxidative stress of DE from an improved diesel engine was evaluated in bioassays of tissues from Wistar Han rats and C57BL/6 mice exposed to DE. Genotoxicity was measured as strand breaks using an alkaline-modified comet assay. To correlate possible DNA damage found by the comet assay, measurement of DNA-adduct formation was evaluated by a competitive enzyme-linked immunosorbent assay (ELISA) to determine the levels of free 8-OHdG found in the serum of the animals exposed to DE. 8-OHdG is a specific modified base indicating an oxidative type of DNA damage to DNA nucleotides. In addition, a thiobarbituric acid reactive substances (TBARS) assay was used to assess oxidative stress and damage in the form of lipid peroxidation in the hippocampus region of the brains of DE-exposed animals.

Results from the comet assay showed no significant differences in rats between the control and exposed groups ($P = 0.53$, low exposure; $P = 0.92$, medium exposure; $P = 0.77$, high exposure) after 1 month of DE exposure. There were no differences between sexes in the responses of rats to these exposures. Likewise, there were no significant differences found after 3 months of exposure. Similarly, no significant differences were found between the mice exposed for 1 and 3 months to DE, nor were any differences found between sexes.

Measurements of 8-OHdG in both mice and rats showed no significant difference among DE exposure groups ($P = 0.46$, mice; $P = 0.86$, rats). In mice, measured 8-OHdG was lower in the 3-month group than the 1-month group. In rats, the inverse was true.

This Investigators' Report is one part of Health Effects Institute Research Report 166, which also includes a Commentary by the HEI ACES Review Panel and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. L.M. Hallberg, Environmental Toxicology, Department of Preventive Medicine and Community Health, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1110.

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* A list of abbreviations and other terms appears at the end of the Investigators' Report.

In mice, no significant differences in the levels of lipid peroxidation, as measured by TBARS, were found between the controls and DE exposure groups ($P = 0.92$), nor were there any differences between sexes. In rats, comparisons between the control and low-exposure groups approached significance, but no significant differences were found between the other DE exposure groups. Additionally, in rats, there were no significant differences between the 1- and 3-month DE exposure groups.

INTRODUCTION

Historically, diesel emissions have been a human health concern because of human exposure to the volatile organic compound (VOC), PAH, divalent metal, and particulate matter (PM) components of DE. Exposure to DE is suspected of contributing to lung cancer and cardiopulmonary diseases (Pope et al. 2002). Induction of mutagenic and/or tumorigenic events is likely involved in carcinogenesis. Therefore, to be carcinogenic, these DE components would have to be linked to increased mutagenicity. Such a link has been observed in both bacterial and mammalian cell systems (Tokiwa and Ohnishi 1986). Studies of human–hamster hybrid (A/L) cells have demonstrated that diesel exhaust particulates (DEP; National Institute of Standards and Technology [NIST] SRM2975) could be transported inside cells through phagocytic action, which resulted in a greater than twofold increase in mutation frequency. DE is also composed of a complex mixture of PAHs, including 3-NBA, which has been identified in both DE and urban air and has been found to induce micronucleus formation in human hepatoma cells (Lamy et al. 2004). Additionally, studies of ICR mice have shown increases in both the incidence of tumor formation and the formation of 8-OHdG adducts with exposure to DEP (Ichinose et al. 1997). Similarly, other studies of mice exposed to DEP have also demonstrated increased 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-Oxo-dG) adduct formation in lung tissue after a single DEP exposure. In these experiments, the formation of DNA single-strand breaks also increased in bronchoalveolar lavage cells after repeated DEP exposure (Risom et al. 2003). Observations in animal models, cell culture experiments, and cell-free systems have suggested that exposure to DE initiates oxidative DNA damage through generation of reactive oxygen species (ROS) and/or induction of inflammatory responses with resultant recruitment of macrophages, which in turn can generate ROS and oxidative damage (Tokiwa et al. 1999, 2005). Because macrophage recruitment can induce cellular oxidative stress, it has been implicated as one of the underlying mechanisms behind the genotoxic effects of DE. This oxidative potential can result in oxidized DNA nucleotides such as guanine. Exposure to PM with an aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$) and to smaller ultrafine particulates (UFP; PM with an aerodynamic diameter < 0.1 μm) has been correlated with guanine oxidation in DNA (Risom et al. 2005). This evidence has prompted the use of biomarkers of oxidative stress and DNA damage as a means of biomonitoring humans for exposure to DE.

It has been hypothesized that improvements in the engineering of 2007 diesel engines and diesel fuel preparations would result in reduced emissions. This reduction could result in emissions that were less genotoxic than those of older diesel engines, assuming an exposure–response relationship exists at these lower, 2007-compliant levels. If advances in diesel engine design effectively reduced total emissions, as anticipated, then we would be likely to also see a reduction in the levels of oxidized nucleotides and adduct formation. This, in turn, would result in a reduction in the number of mutagenic lesions, apurinic/apyrimidinic sites, and resultant strand breaks. While new engineering standards might reduce emissions of DE/DEP, it is possible that these lower levels would still be significantly genotoxic and mutagenic. It is also possible that chronic exposure to these lower levels might continue to result in a cumulative genotoxic effect. Either of these scenarios would indicate that these lower levels could still pose a significant genotoxic risk.

The exact mechanism by which DE induces or enhances mutagenic and carcinogenic events is not well understood. However, evidence points to the generation of oxidative stress either directly, by the production of ROS, or indirectly, through the induction of the inflammatory response as a major underlying mechanism (Donaldson et al. 2002; Terzano et al. 2010; Riedl and Diaz-Sanchez 2005). Thus, the use of endpoints that measure oxidative-type DNA damage, as was done in earlier research, should be effective in determining the potential, or lack thereof, of newly engineered diesel engines to induce genetic damage in exposed populations. If exhaust from these new engines could be shown to have a reduced ability to damage DNA, this would point to a reduced negative impact on health. The guidelines for 2007 and later diesel engines require a reduction in total particulate emissions. However, if this reduction is limited to larger particulates, then UFP could potentially continue to have a health impact. For example, it has been observed that UFP are able to be inhaled deeper into the lungs, where such nanoparticles can pass into the circulation (Mühlfeld et al. 2008). Additionally, these particles can also pass through the blood–brain barrier, inducing a neurological inflammatory response (Chen et al. 2007;

Peters et al. 2006). Research has shown that fine particles can induce damage directly or indirectly (Risom et al. 2005; Kenyon and Liu 2011; Dybdahl et al. 2004). For example, one direct mechanism is the induction of ROS by the redox-reactive metal-containing surface. Ultrafine particles have been shown to be phagocytized by alveolar macrophage. This can result in elicitation of a macrophage-induced inflammatory response resulting in ROS production, oxidative stress, and resultant DNA damage (Beck-Speier et al. 2005; Lundborg et al. 2007). Since the majority of health impacts caused by ultrafine PM are through an apparent increase in oxidative stress, the endpoints we have proposed should be suitable for investigating this impact.

SPECIFIC AIMS

Numerous studies have shown associations between air pollutants and environmental health risks. Among the components of concern are the criteria pollutants sulfur dioxide (SO_2), nitrogen oxides (NO_x), and particulates. These pollutants are also combustion products of diesel engines. Improvements in diesel engine engineering and fuel formulations have led to reductions in many combustion products of concern. However, the formation of particulates and NO_x species continues to be a concern. As diesel engine design continues to improve, we need to monitor the effects of diesel combustion products on health outcomes for the general population. One way to do this is to evaluate the effects of long-term testing of newly engineered diesel engines, as in the current project. Induction of oxidative stress in the human body can result from exposure to various components of DE, including organic and inorganic gases, metals, and particulates. A result of this induction could be the production of oxidative damage in DNA, proteins, and lipids. This project was designed to answer the following questions:

1. Does exposure of a primary target tissue, such as lung, to DE from 2007-compliant diesel engines lead to oxidative damage to DNA, resulting in the formation of strand breaks, adducts, or abasic sites in an exposure-dependent manner?
2. Does exposure to DE from a 2007-compliant diesel engine lead to oxidative damage to lipids in the brain, particularly in the region of the hippocampus, yielding lipid peroxidation and the formation of TBARS?

Specific Aim 1: Determine Whether Exposure to DE Results in Induction of DNA damage. The aim is to use the comet assay to determine whether oxidative damage has occurred in the DNA of exposed animals. We use the alkaline-modified comet assays to detect the presence of DNA strand breaks in lung tissue. To support the finding of the comet assay, we will perform an 8-OHdG assay to measure free 8-OHdG residues in serum.

Specific Aim 2: Determine Whether Exposure to DE Results in Induction of Lipid Damage. The aim is to use the TBARS assay to detect the presence of TBARS, which result from lipid peroxidation. This assay involves testing tissues from the sensitive hippocampus region of the brain.

METHODS AND STUDY DESIGN

DE GENERATION

Generation of the exposure atmosphere was conducted at Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico. Once the characterization of the emissions of four engines during Phase 1 of this study was completed, HEI randomly selected one engine for these health studies.

Exhaust Dilutions

Four chamber-exposure levels were targeted for the rodent bioassays: low, medium, and high DE dilutions, and clean air. The ACES Oversight Committee based dilutions of DE on predetermined nitrogen dioxide (NO_2) concentrations. NO_2 is the pollutant with the highest concentrations in the exhaust of 2007-compliant engines and is known to be associated with noncancer effects similar to those that have been observed with whole DE from older engines. Because of the overall low level of exhaust emissions, an additional issue considered in setting the highest chamber concentration was the chamber temperature. The acceptable range of temperatures when the animals were in the chamber was set at 75 ± 3°F. The NO_2 concentrations selected and the rationales for selecting these concentrations were as follows:

1. The highest concentration of NO_2 was 4.2 ppm. This concentration was derived from a prior study of chronic NO_2 exposures (Mauderly 2000) in which animals were exposed to 9.5 ppm NO_2 for 6 hours. This concentration served as the maximum tolerated dose; the equivalent concentration for ACES, with a 16-hour exposure duration, would be 4.15 ppm. Given that actual concentrations could vary during the 16-hour cycle and might have ended up slightly below or above the target, the ACES Oversight Committee recommended targeting 4.2 ppm, with the understanding

that concentrations should not go below 4.0 ppm. We thought it was possible at this concentration to maintain the exposure chamber temperature within the specified range.

2. The intermediate concentration of NO_2 was 0.8 ppm. Based on the highest and lowest concentrations, using a factor of 5.5, the intermediate concentration was between 0.7 and 0.8 ppm. The ACES Oversight Committee recommended targeting 0.8 ppm (but not exceeding it) and going no lower than 0.7 ppm.
3. The lowest concentration of NO_2 was 0.1 ppm, or as close as possible to that concentration, to provide a likely no-observed-adverse-effect level. This concentration was near the ambient NO_2 air quality standard of 0.053 ppm. Concentrations in this range would be expected to be quite variable due to the high dilution ratio.

SELECTION OF THE RAT STRAIN

The criteria considered in selecting a rat strain were longevity, whether a strain had been used previously in studies of chronic inhalation (particularly of DE), whether there existed a historical database of cancer incidence in the strain (based on control groups from studies of chronic exposure using inhalation or other methods of administering toxic compounds), and the maximum body weight reached by males (because it affects housing in inhalation chambers with limited available space). The final recommendation by the ACES Oversight Committee was to use Wistar Han rats.

SELECTION OF THE MOUSE STRAIN

Similar criteria (i.e., longevity, background tumor incidence, and use in previous inhalation studies) were used to identify a suitable mouse strain for ACES. Based on longevity and lower incidence of lung tumors than other strains under consideration, the C57BL/6 strain was proposed by the LRRI team and approved by the ACES Oversight Committee.

NUMBER OF ANIMALS

We determined that four animals would be sufficient to observe differences between exposure levels (Table 1), but we increased the number to five animals of each sex to look for sex differences. All assays were performed blindly. The findings showed that our assays were unable to detect any differences by sex associated with DE exposure. Therefore, we pooled the male and female data, giving us an $n = 10$, and repeated the analysis.

MODIFIED COMET ASSAY

The neutral comet assay was slightly modified to incorporate an alkaline nucleus treatment prior to electrophoresis. The standard neutral comet assay does not incorporate this modification and is capable of primarily detecting only genomic double-strand breaks. The alkaline modification used in the analysis of lung tissues from both rodent species exposed to defined dilutions of DE for 1 or 3 months theoretically increases the sensitivity of the assay, allowing for increased resolution of both double- and single-strand breaks. In addition, some fraction of alkali-labile sites may also be resolved using this modified procedure. Cell suspensions were generated by manually mincing lung tissue in cold phosphate-buffered saline (PBS) containing calcium and magnesium ions on a clean glass surface with a clean razor blade. Initially, cell counts on a hemocytometer, after the mincing of different size cell blocks using frozen lung tissue from a laboratory mouse (not one from the experimental groups), were used to optimize the amount of starting material necessary for generating an adequate cell suspension. The manufacturer's suggested procedure for the neutral comet assay was followed, with the exception of an additional step in which agarose-immobilized nuclei on microscope slides were immersed in an alkaline solution (pH > 13) for 60 minutes in the dark at room temperature. Slides were then rinsed twice in distilled water and placed in a neutral electrophoresis buffer for 5 minutes at room temperature. The remaining neutral comet assay procedure was then followed.

Nuclei were imaged and captured using a Nikon Eclipse 90i (Nikon Instruments, Inc., Melville, NY) microscope at 10× magnification fitted with a SensiCam QE camera (PCO, Kelheim, Germany) using IP Labs version 3.7 image capturing software (IP Labs, Bonn, Germany). All images were autoexposed prior to capture, to objectively minimize variable background staining among images.

Comet profiles were fit to captured images, and associated metrics were estimated using the CASP version 1.2.2 software (Comet Assay Software Project; *http://casplab.com/*). To verify that this freely available software was suitable, five randomly selected rat lung samples were analyzed using the CASP software and a commercially available platform, TriTek CometScore (TriTek Corp., Sumerduck, VA). Correlation coefficients were > 95% for tail moment, tail length, and olive tail moment metrics for the two programs. Preparation of samples, imaging, capture, and analysis were conducted using a double-blind approach.

BRADFORD PROTEIN ASSAY

To normalize data for the 8-OHdG assay, DNA quantification was considered. A small pilot study was conducted

to determine the efficiency of DNA isolation from serum samples. With the available serum, we were not able to isolate sufficient DNA to allow for duplicate samples when assaying for 8-OHdG. Therefore, the decision was made to utilize whole serum and use protein content to correct the results. Since the TBARS assay would need to be normalized based on protein content, we decided to use protein as the normalizing factor for both the 8-OHdG and TBARS assays. A microassay method was used, per the manufacturer's instructions, with a few modifications (Cell BioLabs Inc., San Diego, CA).

Preparation of Standards

Bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used to produce a standard curve for protein content analysis. The following procedure was used to prepare these standards. BSA was weighed and then diluted with molecular grade water to prepare a concentrated solution of greater than 10 mg/mL. The actual protein concentration of the stock was determined spectrophotometrically at a wavelength of 280 nm. Once the actual concentration was determined, the solution was further diluted to give a final concentration of 10 mg/mL based on the 280-nm reading. Standards of 1600, 800, 400, 200, 100, and 0 µg/mL were prepared by diluting the stock standard (10 mg/mL) to a working solution concentration of 1.6 mg/mL (1600 µg/mL) using molecular grade water. The remaining standards were prepared by serially diluting the 1.6 mg/mL standard 1:1 with water. Twenty-five µL of these standards were aliquoted into tubes in which they were mixed and incubated for 5 minutes at room temperature with 6.25 µL of Bradford Dye Reagent Concentrate (Bio-Rad, Hercules, CA) before being read in a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) in the Bradford mode (595 nm). Triplicate samples of the standards were read to establish the standard curve.

Preparation of Samples for Assay

A 5-µL aliquot of serum or cell lysate from each sample was transferred to a 1-mL eppendorf centrifuge tube and diluted 1:10 with DNase-RNase-free molecular grade water to a final volume of 25 µL. Small batches of samples were assayed to prevent long-term incubation. Bradford Dye Reagent Concentrate (6.25 µL) was added to each batch as in the standard preparation protocol, mixed, and allowed to incubate for 5 minutes at room temperature. Three-µL aliquots were placed onto the Nanodrop platform, and protein concentrations were measured spectrophotometrically in duplicate at a wavelength of 595 nm.

8-OHdG ELISA ASSAY

DNA oxidative damage occurs physiologically through endogenous as well as exogenous exposure to ROS, including carcinogenic chemicals. The formation of 8-OHdG, a by-product of the removal of oxidative DNA damage by exonucleases, serves as a pervasive biologic marker. Upon excision, 8-OHdG is excreted without being metabolized further. We used a competitive enzyme immunoassay, the OxiSelect Oxidative DNA Damage ELISA Kit (Cell BioLabs Inc., San Diego, CA), for the detection and quantitation of 8-OHdG in serum samples. An 8-OHdG standard curve, measured at 450 nm, was used to quantify 8-OHdG in unknown samples. The sensitivity range of the kit was 100 to 2000 pg/mL The 8-OHdG ELISA assay was conducted according to the manufacturer's recommendations. Levels of 8-OHdG in samples were expressed as pg/mL/µg protein.

TBARS ASSAY

Cellular damage, as a result of lipid peroxidation, is well-defined in animal cells and is an indication of oxidative stress. As a result of lipid peroxidation, naturally occurring lipid peroxides are formed but are unstable, decomposing to form more complex and reactive compounds, such as malondialdehyde (MDA). Measurement of lipid peroxide by-products is one of the most widely accepted assays for oxidative damage. We used the OxiSelect TBARS Assay (Cell BioLabs Inc.) to monitor lipid peroxidation by the direct quantitative measurement of MDA in brain tissue. MDA-containing samples were reacted with thiobarbituric acid and then read spectrophotometrically at the wavelength of 532 nm. The MDA content in samples was determined by comparison with an MDA standard curve. TBARS concentrations in samples were expressed as µM MDA/mg protein in tissue.

Preparation of Samples for Assay

Whole-brain tissues from rats and mice were rapidly extracted and snap frozen at –80° C. After all the tissue was available, whole or half brains were dissected in chilled PBS supplemented with 1× butylated hydroxytoluene (BHT) (to inhibit further oxidation of the tissue during excision and assay) to isolate hippocampi. Hippocampi were then processed for the TBARS assay by homogenizing tissue on ice in PBS containing 1× BHT (75 mg/mL). The homogenate was then centrifuged at 10,000*g* for 5 minutes and the supernatant transferred to a new tube for protein and TBARS analysis.

STATISTICAL METHODS AND DATA ANALYSIS

POWER ANALYSIS

A power analysis was performed based on the comet assay measurement of olive tail moment with the assumption of differences by exposure level from data in mice ($n = 4$). The standard deviation (SD) was set to 0.85, which is similar to that observed with human lymphocytes, with a control olive tail moment of 1 (also observed in human lymphocytes). The power was set to be greater than 0.92 with the effect size set to 0.99. Table 1 shows the expected differences in olive tail moment. Sex and exposure level and duration were analyzed, and the results were compared to those for the controls.

COMET ASSAY

We examined the distribution of the following four sets of data: (1) rat 1-month exposure, modified comet tail length; (2) rat 1-month, modified comet tail moment; (3) mouse 1-month, modified comet tail length; and (4) mouse 1-month, modified comet tail moment. Descriptive statistics were used to examine the mean and standard error (SE) of the outcomes for rats and mice (modified comet tail length and modified comet tail moment) by sex and exposure groups (low, mid, and high exposure vs. control). Since the data were normally distributed and the outcomes were repeatedly measured for each subject, a mixed linear regression model was used to examine the exposure effects, controlling for sex. The sex and exposure interaction was also examined in the regression model. For data for which a significant exposure effect was detected (rat 1-month exposure, modified comet tail length; mouse 1-month, modified comet tail length; and mouse 1-month, modified comet tail moment), multiple comparisons with Tukey adjustment were used for pair-wise comparisons among exposure groups. Analyses were conducted using SAS 9.2. A follow-up analysis of the percentage of DNA in tail was also conducted using both parametric and nonparametric methods. Students' *t* tests, analysis of variance (ANOVA), and Kruskal-Wallis tests were used to analyze the effects of sex, exposure duration, and exposure level as well as any interactions between these factors. Post-hoc mean comparisons following the ANOVA were Bonferroni-corrected. These analyses were conducted with SPSS 16.1 (IBM Corp., Armonk, NY). A P value of less than 0.05 was used as our cutoff for statistical significance.

Table 1. Power Analysis of Olive Tail Moment (Comet Assay) to Determine the Number of Mice Necessary to See a Difference Between Exposure Levels[a]

Exposure Level	Olive Tail Moment (pixels)
Control	1[b]
Low	1.75
Mid	2.5
High	3.25

[a] SD = 0.85, $n = 4$, effect size = 0.99, $P < 0.05$, power > 0.92.

[b] Similar to that observed in otherwise unchallenged human lymphocytes.

8-OHdG STATISTICAL ANALYSIS

We first examined the distribution of the two sets of data: (1) mouse 8-OHdG data at 1 and 3 months and (2) rat 8-OHdG data at 1 and 3 months. Descriptive statistics were used to examine the mean and SE of the outcomes by sex, exposure groups (low, mid, and high exposure vs. control), and duration (1 and 3 months). Since the data were not normally distributed, nonparametric regression analysis was used to examine the exposure effects, controlling for sex and stratifying by duration (1 and 3 months). The sex and exposure interactions were also examined in the regression model. Since the overall exposure effects were not statistically significant for both sets of data, no multiple comparisons were conducted for pair-wise comparisons among exposure groups. The duration effect was evaluated using the mixed model of rank-based regression analysis because of the (1) nonnormal distribution of the data and (2) measurement of outcomes at 1 and 3 months for each subject. Levels of 8-OHdG (pg/mL/µg protein) were analyzed using general linear models in SPSS 16.0 for Windows.

TBARS STATISTICAL ANALYSIS

The distribution of the mouse and rat TBARS data was examined. Descriptive statistics were used to examine the mean and SD of the outcomes (TBARS corrected for protein, µM MDA/mg tissue) by sex, exposure groups (low, mid, and high exposure vs. control), and duration (1 and 3 months). Since the data were close to normal distribution and the outcome was measured at both 1 and 3 months, the mixed model for repeated measures was used to examine the exposure effects, controlling for sex and duration. All possible interactions were examined (the full model) and found to be insignificant. A model with only sex and duration (the reduced model) was also examined. For the mouse data, the overall exposure effects were not statistically significant for either the full model or the reduced model, so no multiple comparisons were conducted for

pair-wise comparisons among exposure groups. However, multiple comparisons among exposure groups were conducted with the rat data. The *P* values from the multiple comparisons were adjusted using the Tukey adjustment. All the analyses were conducted using SAS 9.2.

RESULTS

DNA DAMAGE: MODIFIED COMET ASSAY

Analysis by Modified Comet Assay

The comet assay was performed on the lung tissue from a total of 75 rats and 80 mice, as shown in Tables 2 and 3. Analysis of the data from the comet assay was conducted on tail length (comet head diameter subtracted from comet length), tail moment (percentage of DNA in tail × tail length), and percentage of DNA in tail. Analyses of exposure groups, exposure duration, and sex effects were performed.

Table 2. Number of Animals Used in the Comet Assay (Rats)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	5	4	9	5	5	10
Low	5	4	9	5	5	10
Mid	5	4	9	5	5	10
High	4	4	8	5	5	10
All	19	16	35	20	20	40

Table 3. Number of Animals Used in the Comet Assay (Mice)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	3	7	10	5	5	10
Low	6	5	11	5	5	10
Mid	4	4	8	5	5	10
High	7	4	11	5	5	10
All	20	20	40	20	20	40

Mouse Data

Table 3 presents the distribution of mice used in the comet assay. After 1 month of exposure, no significant differences were noted in any comparisons of those exposed to different levels of DE and the control ($P = 0.56$, low; $P = 0.200$, mid; $P = 0.61$, high; $P = 0.79$ for sex effects) using tail moment (Tables 4 and 5, Figure 1) or tail length ($P = 0.60$, low; $P = 0.21$, mid; $P = 0.63$, high; $P = 0.73$ for sex effects) (Tables 6 and 7). In mice exposed 3 months, there were no significant differences using tail moment (control vs. exposed, $P = 0.15$ for group) (Tables 8 and 9, Figure 2) or tail length ($P = 0.16$ for group) (Tables 10 and 11). No sex differences were perceived (for sex: tail moment, $P = 0.93$; tail length, $P = 0.99$). No significant difference with respect to sex or exposure level relative to the controls was noted for the percentage of DNA in the tail. There was no significant difference between the 1- and 3-month

Table 4. Analysis of Mouse Lung Tissue by Modified Comet Tail Moment Assay After 1 Month of Exposure[a]

	Male	Female	All
Control	41.6 ± 6.6	32.7 ± 4.3	35.4 ± 3.6
Low	37.9 ± 4.7	46.7 ± 5.1	41.9 ± 3.4
Mid	26.0 ± 5.8	23.8 ± 5.7	24.9 ± 4.0
High	30.0 ± 4.3	28.2 ± 5.7	29.4 ± 3.4
All	33.3 ± 2.9	33.5 ± 2.9	

[a] Data are mean ± SE.

Table 5. Comparison of the Effects on Mice Exposed 1 Month (Comet Tail Moment Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.004[a]
Control vs. Low	0.560
Control vs. Mid	0.200
Control vs. High	0.610
Low vs. Mid	0.007
Low vs. High	0.047
Mid vs. High	0.830
Sex	
Male vs. Female	0.790
Exposure level × Sex interaction	0.400

[a] *P* value represents overall comparison among all groups.

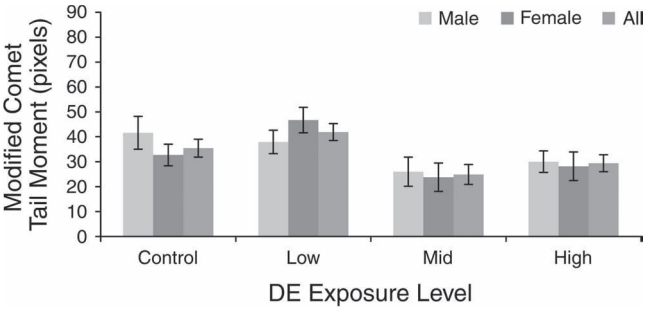

Figure 1. Results of modified comet assay using tail moment data in mice (1-month DE exposure). Significant differences were noted between low- and mid-exposure levels ($P = 0.007$) and between low and high levels ($P = 0.047$). No sex effects were observed ($P = 0.79$).

Table 6. Analysis of Mouse Lung Tissue by Modified Comet Tail Length Assay After 1 Month of Exposure[a]

	Male	Female	All
Control	73.0 ± 8.8	61.9 ± 5.8	65.3 ± 4.8
Low	68.4 ± 6.3	80.2 ± 6.9	73.8 ± 4.6
Mid	54.2 ± 7.7	49.0 ± 7.7	51.6 ± 5.4
High	58.5 ± 5.8	56.0 ± 7.7	57.6 ± 4.6
All	62.8 ± 3.8	62.7 ± 3.8	

[a] Data are mean ± SE.

Table 7. Comparison of the Effects on Mice Exposed 1 Month (Comet Tail Length Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.01[a]
Control vs. Low	0.60
Control vs. Mid	0.21
Control vs. High	0.63
Low vs. Mid	0.01
Low vs. High	0.05
Mid vs. High	0.84
Sex	
Male vs. Female	0.73
Exposure level × Sex interaction	0.40

[a] *P* value represents overall comparison among all groups.

Table 8. Analysis of Mouse Lung Tissue by Modified Comet Tail Moment Assay After 3 Months of Exposure[a]

	Male	Female	All
Control	31.1 ± 5.0	25.8 ± 5.0	28.5 ± 3.4
Low	27.0 ± 5.0	30.2 ± 5.1	28.6 ± 3.4
Mid	34.5 ± 5.0	34.4 ± 5.0	34.4 ± 3.4
High	36.4 ± 5.0	39.8 ± 5.2	38.1 ± 3.4
All	32.2 ± 2.5	32.6 ± 2.5	

[a] Data are mean ± SE.

Table 9. Comparison of the Effects on Mice Exposed 3 Months (Comet Tail Moment Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.15[a]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.93
Exposure level × Sex interaction	0.81

[a] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

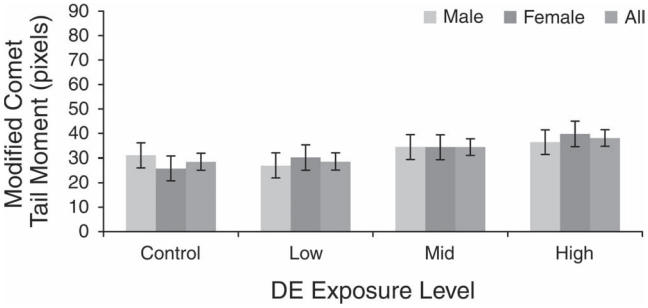

Figure 2. Results of modified comet assay using tail moment data in mice (3-month DE exposure). No significant differences were noted between exposure level ($P = 0.15$) or sex ($P = 0.93$).

Table 10. Analysis of Mouse Lung Tissue by Modified Comet Tail Length Assay After 3 Months of Exposure[a]

	Male	Female	All
Control	58.9 ± 6.4	52.5 ± 6.5	55.7 ± 4.4
Low	54.2 ± 6.5	58.0 ± 6.5	56.1 ± 4.4
Mid	64.2 ± 6.5	63.3 ± 6.5	63.8 ± 4.4
High	66.2 ± 6.5	69.9 ± 6.5	68.1 ± 4.4
All	60.9 ± 3.2	60.9 ± 3.2	

[a] Data are mean ± SE.

Table 11. Comparison of the Effects on Mice Exposed 3 Months (Comet Tail Length Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.16[a]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.99
Exposure level × Sex interaction	0.84

[a] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

samples and no significant interactions among the factors. A single sample (1-month group, 1 mid exposure) had cells with pycnotic nuclei. Analyses of the percentage of DNA in the tail were done with and without this sample. Results were unchanged (Tables 12 and 13, Figure 3). Mice in the low-exposure group at 1 month had a significantly higher percentage of tail DNA than mice in the mid-exposure group ($F = 3.47$, df = 3, $P < 0.03$; chi-square = 8.96, df = 3, $P < 0.03$). Mice had significantly higher percentages of DNA in the tail than rats (data not shown).

Rat Data

After 1 month of exposure in rats, no significant differences were noted in any comparisons of groups exposed to different levels of DE and the control animals ($P = 0.53$, low; $P = 0.92$, mid; $P = 0.77$, high), nor were there any statistically significant effects by sex ($P = 0.43$) when tail moment data was used (Tables 14 and 15, Figure 4). Similar results were observed using tail length (control vs. exposed: $P = 0.59$, low; $P = 0.90$, mid; $P = 0.42$, high; $P = 0.16$ for sex effects) (Tables 16 and 17, Figure 5). All samples

Table 13. Analysis of Percentage of DNA in Comet Tail of Mice Exposed 3 Months[a,b]

	All Samples			
	Control	Low	Mid	High
Female	46.0 ± 3.9	45.9 ± 5.8	51.1 ± 3.0	55.2 ± 1.8
Male	50.2 ± 1.8	45.9 ± 4.6	49.9 ± 4.2	52.7 ± 2.9
Both	48.1 ± 2.1	45.9 ± 3.5	50.5 ± 2.4	54.0 ± 1.6

[a] Data are average percentage of DNA ± SEM.
[b] No pycnotic cells were identified in this group.

Table 12. Analysis of the Effects of Pycnotic Cells on the Percentage of DNA in Comet Tail of Mice Exposed 1 Month[a]

	All Samples				Samples with Pycnotic Cells Removed			
	Control	Low	Mid	High	Control	Low	Mid	High
Female	49.2 ± 2.7	57.2 ± 0.8	43.9 ± 6.4	45.6 ± 3.9	49.2 ± 2.7	57.2 ± 0.8	50.1 ± 2.3	45.6 ± 3.9
Male	56.2 ± 2.1	52.4 ± 2.8	45.0 ± 4.6	49.0 ± 2.1	56.2 ± 2.1	52.4 ± 2.8	45.0 ± 4.6	49.0 ± 2.1
Both	51.3 ± 2.2	54.6 ± 1.7	44.4 ± 3.7	47.8 ± 2.0	51.3 ± 2.2	54.6 ± 1.7	47.2 ± 2.8	47.8 ± 2.0

[a] Data are average percentage of DNA ± SEM.

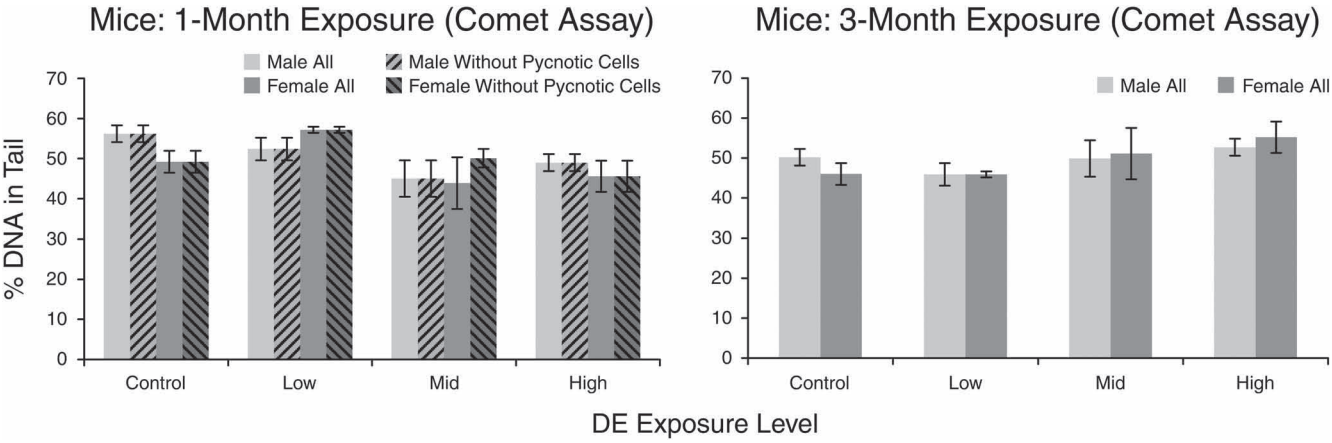

Figure 3. Results of modified comet assay using mouse data on the percentage of DNA in tail (1- or 3-month DE exposure). The data were analyzed with or without pycnotic cells. No significant differences were noted between any DE exposure level and control.

Table 14. Analysis of Rat Lung Tissue by Modified Comet Tail Moment Assay After 1 Month of Exposure[a]

	Male	Female	All
Control	15.3 ± 6.7	26.0 ± 8.2	19.6 ± 5.5
Low	20.4 ± 7.4	42.7 ± 8.3	30.3 ± 5.8
Mid	31.6 ± 8.2	15.7 ± 8.2	24.6 ± 8.8
High	11.6 ± 8.2	12.0 ± 8.3	11.8 ± 6.1
All	19.9 ± 4.0	24.1 ± 4.5	

[a] Data are mean ± SE.

Table 15. Comparison of the Effects on Rats Exposed 1 Month (Comet Tail Moment Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.10[a]
Control vs. Low	0.53
Control vs. Mid	0.92
Control vs. High	0.77
Low vs. Mid	0.90
Low vs. High	0.12
Mid vs. High	0.43
Sex	
Male vs. Female	0.43
Exposure level × Sex interaction	0.09

[a] *P* value represents overall comparison among all groups.

Table 16. Analysis of Rat Lung Tissue by Modified Comet Tail Length Assay After 1 Month of Exposure[a]

	Male	Female	All
Control	37.8 ± 9.2	52.4 ± 11.3	43.6 ± 7.3
Low	44.3 ± 10.1	73.7 ± 11.3	57.3 ± 7.7
Mid	56.4 ± 10.1	45.2 ± 11.3	51.4 ± 7.7
High	23.1 ± 11.3	32.9 ± 11.3	28.0 ± 8.2
All	41.1 ± 5.5	51.0 ± 6.1	

[a] Data are mean ± SE.

Table 17. Comparison of the Effects on Rats Exposed 1 Month (Comet Tail Length Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.04[a]
Control vs. Low	0.59
Control vs. Mid	0.90
Control vs. High	0.42
Low vs. Mid	0.95
Low vs. High	0.04
Mid vs. High	0.13
Sex	
Male vs. Female	0.16
Exposure level × Sex interaction	0.30

[a] *P* value represents overall comparison among all groups.

were visually screened for the presence of pycnotic cells. Pycnotic cells were scored along with nonpycnotic cells because of the blind manner in which data were collected. Analyses of comet measures were conducted both with and without pycnotic cells. No significant difference with respect to sex or exposure level relative to the controls was noted for the percentage of DNA in the tail in the 1-month group (Table 18, Figure 6). After 3 months of exposure in rats, no significant differences were noted using either tail moment (Tables 19 and 20, Figure 7) or tail length (Tables 21 and 22). No significant difference with respect to sex or exposure level relative to the controls was noted for the percentage of DNA in the tail in the 3-month group (Table 23). Our results showed no significant difference between the 1- and 3-month samples and no significant interactions among the factors. A few samples (four subjects total in the 1-month group, with 1 control and 3 high-exposure subjects [Table 18] and five subjects total in the 3-month group, with 1 low-exposure, 3 mid-exposure, and 1 high-exposure subject [Table 23]) had cells with pycnotic nuclei, but these were not restricted to any exposure group, sex, exposure duration, or sample processing batch. Analyses of the percentage of DNA in the tail were done with and without these samples. Results were unchanged (Figure 8).

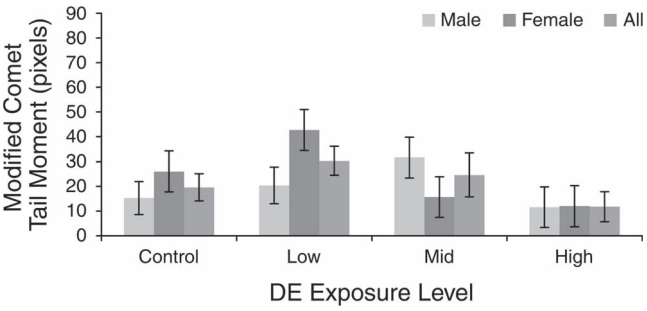

Figure 4. Results of modified comet assay using tail moment data in rats (1-month DE exposure). No significant differences were noted between exposure level ($P = 0.10$) or sex ($P = 0.43$).

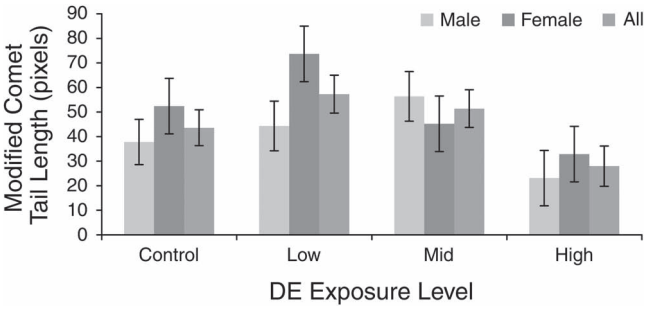

Figure 5. Results of modified comet assay using tail length data in rats (1-month DE exposure). A significant difference was noted between the low- and high-exposure levels ($P = 0.04$); no differences based on sex ($P = 0.16$) were observed.

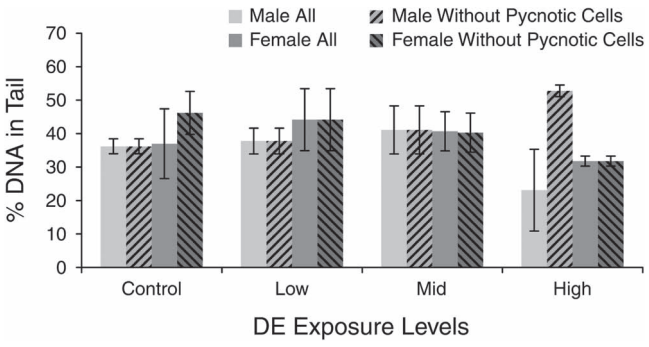

Figure 6. Results of modified comet assay using data in rats on the percentage of DNA in tail (1-month DE exposure). The data were analyzed with and without pycnotic cells. No significant differences were noted between any DE exposure level and control.

Table 18. Analysis of the Effects of Pycnotic Cells on the Percentage of DNA in Comet Tail of Rats Exposed 1 Month[a]

	All Samples				Samples with Pycnotic Cells Removed			
	Control	Low	Mid	High	Control	Low	Mid	High
Female	37.0 ± 10.4	44.2 ± 9.26	40.7 ± 5.86	31.8 ± 1.51	46.2 ± 6.44	44.2 ± 9.26	40.3 ± 5.86	31.8 ± 1.51
Male	36.2 ± 2.22	37.8 ± 3.85	41.1 ± 7.17	23.1 ± 12.19	36.2 ± 2.22	37.8 ± 3.85	41.1 ± 7.17	52.8 ± 1.73
Both	36.6 ± 5.02	41.0 ± 4.84	40.9 ± 4.37	27.5 ± 5.97	40.6 ± 3.37	41.0 ± 4.84	40.9 ± 4.37	37.8 ± 4.01

[a] Data are average percentage of DNA ± SEM.

Table 19. Analysis of Rat Lung Tissue by Modified Comet Tail Moment Assay After 3 Months of Exposure[a]

	Male	Female	All
Control	27.9 ± 9.4	33.0 ± 9.4	30.4 ± 6.3
Low	32.6 ± 9.4	29.6 ± 9.4	31.1 ± 6.3
Mid	18.0 ± 9.4	16.0 ± 9.4	17.0 ± 6.3
High	36.4 ± 9.4	45.5 ± 9.4	40.9 ± 6.3
All	28.7 ± 4.8	31.0 ± 4.8	

[a] Data are mean ± SE.

Table 20. Comparison of the Effects on Rats Exposed 3 Months (Comet Tail Moment Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.09[a]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.73
Exposure level × Sex interaction	0.90

[a] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

Table 21. Analysis of Rat Lung Tissue by Modified Comet Tail Length Assay After 3 Months of Exposure[a]

	Male	Female	All
Control	51.8 ± 12.9	59.8 ± 12.9	55.8 ± 8.6
Low	58.5 ± 12.9	53.3 ± 12.9	55.9 ± 8.6
Mid	35.5 ± 12.9	32.1 ± 12.9	33.8 ± 8.7
High	61.7 ± 12.9	57.0 ± 12.9	59.4 ± 8.7
All	51.9 ± 6.4	50.6 ± 6.4	

[a] Data are mean ± SE.

Table 22. Comparison of the Effects on Rats Exposed 3 Months (Comet Tail Length Assay)

Type of Group / Comparison	*P* Value
All exposure-level groups	0.18[a]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.89
Exposure level × Sex interaction	0.95

[a] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

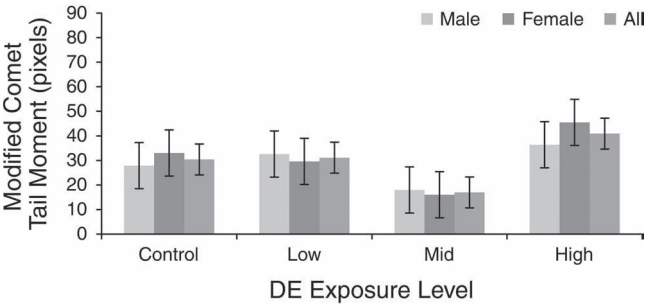

Figure 7. Results of modified comet assay using tail moment data in rats (3-month DE exposure). No significant differences were noted between exposure level ($P = 0.09$) or sex ($P = 0.73$).

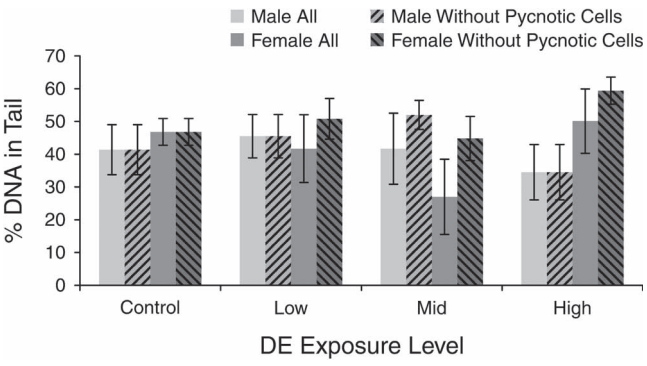

Figure 8. Results of modified comet assay using data in rats on the percentage of DNA in tail (3-month DE exposure). The data were analyzed with or without pycnotic cells. No significant differences were noted between DE exposure level and control.

Table 23. Analysis of the Effects of Pycnotic Cells on the Percentage of DNA in Comet Tail of Rats Exposed 3 Months[a]

	All Samples				Samples with Pycnotic Cells Removed			
	Control	Low	Mid	High	Control	Low	Mid	High
Female	46.8 ± 4.07	41.7 ± 10.33	27.0 ± 11.49	50.1 ± 9.81	46.8 ± 4.07	50.8 ± 6.20	44.8 ± 6.75	59.4 ± 4.14
Male	41.4 ± 7.64	45.5 ± 6.63	41.7 ± 10.84	34.5 ± 8.46	41.4 ± 7.64	45.5 ± 6.63	52.0 ± 4.44	34.5 ± 8.46
Both	44.1 ± 4.18	43.6 ± 5.82	34.4 ± 7.84	42.3 ± 6.64	44.1 ± 4.18	47.9 ± 4.41	48.9 ± 3.77	45.5 ± 6.47

[a] Data are average percentage of DNA ± SEM.

DNA DAMAGE: 8-OHdG/SERUM ELISA ASSAY

Comparisons among rodent species were first made to determine if there were any species-related differences. Rats in all exposure groups at both time points (1 month and 3 months) had significantly higher levels of 8-OHdG than mice ($F = 48.5$, $df = 1$, $P < 0.001$).

Mouse Data

Table 24 shows the distribution of mice within the exposure-level and duration categories used in the analysis of the 8-OHdG assay. In the 1- and 3-month groups, there were no significant differences among exposure groups in outcome measurements ($P = 0.46$; Tables 25 and 26, Figure 9). The outcome measurements for 3-month exposure were significantly lower than those for 1-month exposure ($P = 0.007$).

Table 24. Number of Animals Used in the 8-OHdG ELISA Assay (Mice)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	3	7	10	5	5	10
Low	6	5	11	5	5	10
Mid	4	4	8	5	5	10
High	7	3	10	5	5	10
All	20	19	39	20	20	40

Table 25. Mouse ACES Data, Descriptive Statistics[a]

	1-Month Exposure		3-Month Exposure	
	Male	Female	Male	Female
Control	4.2 ± 1.60	1.9 ± 0.5	1.8 ± 0.9	1.5 ± 0.8
Low	2.2 ± 0.03	1.4 ± 0.3	1.0 ± 0.2	2.1 ± 1.0
Mid	2.3 ± 0.15	2.0 ± 0.4	3.2 ± 1.5	1.8 ± 1.0
High	2.3 ± 0.34	3.2 ± 1.1	3.2 ± 1.3	1.7 ± 0.6

[a] Data are mean ± SE.

Table 26. Mouse ACES 1-Month Data: Mixed Model for Repeated Measures[a]

Type of Group / Comparison	*P* Value
All exposure-level groups	0.46[b]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.180
Duration (1 month vs. 3 months)	0.007
Exposure level × Sex interaction	0.700
Exposure level × Duration interaction	0.890
Sex × Duration interaction	0.620
Exposure level × Sex × Duration interacton	0.380

[a] Outcome is log-transformed.

[b] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

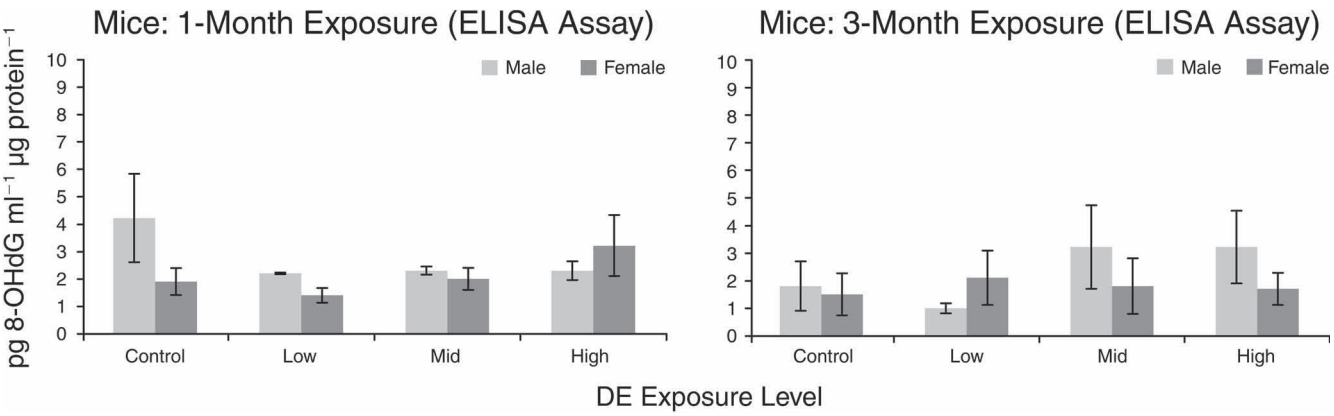

Figure 9. Results of 8-OHdG ELISA assay of mouse serum (1- or 3-month DE exposure). No significant differences were seen between controls and exposure levels ($P = 0.46$). The outcome measurements for 3 months were significantly lower than those for 1 month ($P = 0.007$).

Rat Data

Table 27 shows the distribution of rats in the duration and exposure-level categories used in the analysis of the 8-OHdG assay. There were no significant differences among exposure groups in outcome measurements ($P = 0.86$, Tables 28 and 29, Figure 10). The outcome measurements for 3-month exposure were significantly higher than those for 1-month exposure ($P < 0.0001$).

The lack of significance for an exposure effect holds in both the full model (the model with all the interactions) and the reduced model (the model with only exposure level, sex, and duration as main effects).

Table 27. Number of Animals Used in the 8-OHdG ELISA Assay (Rats)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	5	5	10	5	5	10
Low	5	5	10	5	5	10
Mid	5	5	10	5	5	10
High	5	5	10	5	5	10
All	20	20	40	20	20	40

Table 28. Rat ACES Data, Descriptive Statistics[a]

	1-Month Exposure		3-Month Exposure	
	Male	Female	Male	Female
Control	36.9 ± 8.8	49.6 ± 8.9	156.1 ± 41.4	99.1 ± 25.2
Low	39.8 ± 15.7	43.1 ± 21.2	105.7 ± 30.7	123.1 ± 36.6
Mid	32.0 ± 10.0	35.9 ± 8.3	141.6 ± 32.1	110.7 ± 33.4
High	32.2 ± 8.9	63.4 ± 19.1	108.8 ± 26.8	105.3 ± 20.7

[a] Data are mean ± SE.

Table 29. Rat ACES 1-Month Data: Mixed Model for Repeated Measures[a]

Type of Group / Comparison	*P* Value
All exposure-level groups	0.86[b]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.66
Duration	
1 Month vs. 3 Months	<0.0001
Exposure level × Sex interaction	0.87
Exposure level × Duration interaction	0.86
Sex × Duration interaction	0.16
Exposure level × Sex × Duration interaction	0.90

[a] Outcome is log-transformed.

[b] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

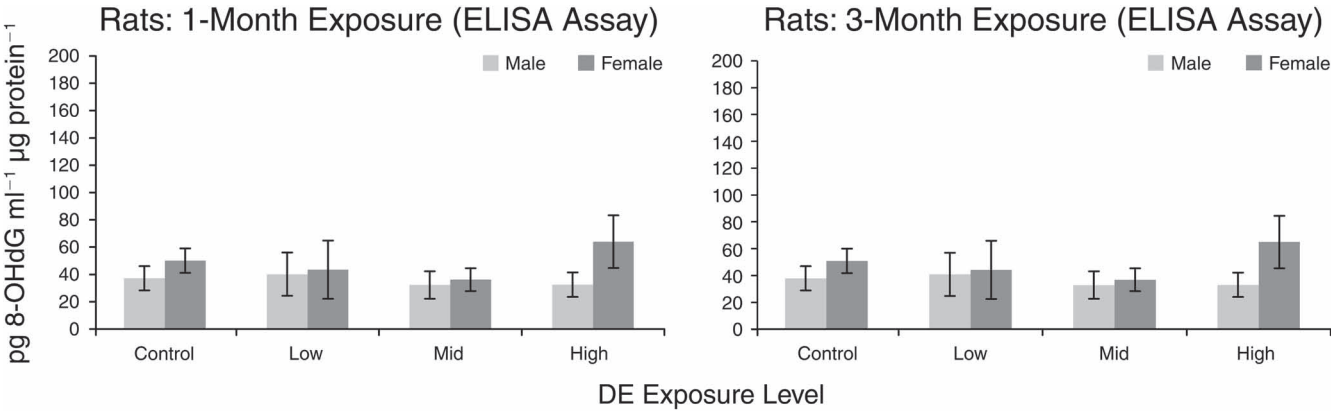

Figure 10. Results of 8-OHdG ELISA assay of rat serum (1- or 3-month DE exposure). No significant differences were seen between controls and exposure levels ($P = 0.86$). The 8-OHdG levels of the 3-month group were significantly higher than those of the 1-month group ($P < 0.001$).

LIPID PEROXIDATION: TBARS ASSAY

Mouse Data

Table 30 describes the distribution of mice in their duration and exposure-level categories used in the analysis of the TBARS assay. The groups (low, mid, and high vs. control) were not significantly different in the measures of TBARS normalized for protein (µM MDA/mg tissue) ($P = 0.92$). Male and female mice were not significantly different in these TBARS measures. After normalizing for protein concentration, TBARS at 1 month were significantly higher than those at 3 months ($P < 0.0001$). However, none of the interaction terms was statistically significant (Tables 31 and 32, Figure 11).

Table 30. Number of Animals Used in the TBARS Assay (Mice)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	3	6	9	5	5	10
Low	6	4	10	5	5	10
Mid	4	3	7	5	5	10
High	6	3	9	5	5	10
All	19	16	35	20	20	40

Table 31. Mouse TBARS Data, Descriptive Statistics[a]

	1-Month Exposure		3-Month Exposure	
	Male	Female	Male	Female
Control	0.73 ± 0.06	0.66 ± 0.06	0.40 ± 0.04	0.44 ± 0.08
Low	0.85 ± 0.12	0.59 ± 0.05	0.47 ± 0.14	0.51 ± 0.11
Mid	0.68 ± 0.10	0.84 ± 0.11	0.37 ± 0.05	0.36 ± 0.01
High	0.75 ± 0.13	0.85 ± 0.27	0.34 ± 0.02	0.39 ± 0.09

[a] Data are mean ± SE.

Table 32. Mouse TBARS Data: Mixed Model for Repeated Measures[a]

Type of Group / Comparison	*P* Value
All exposure-level groups	0.92[b]
Control vs. Low	—
Control vs. Mid	—
Control vs. High	—
Low vs. Mid	—
Low vs. High	—
Mid vs. High	—
Sex	
Male vs. Female	0.89
Duration	
1 Month vs. 3 Months	<0.0001
Exposure level × Sex interaction	0.53
Exposure level × Duration interaction	0.45
Sex × Duration interaction	0.63
Exposure level × Sex × Duration interaction	0.4

[a] Values are corrected for protein.

[b] *P* value represents overall comparison among all groups. If the overall interaction between exposure level and sex did not show statistical significance, no further analyses were made (indicated by —).

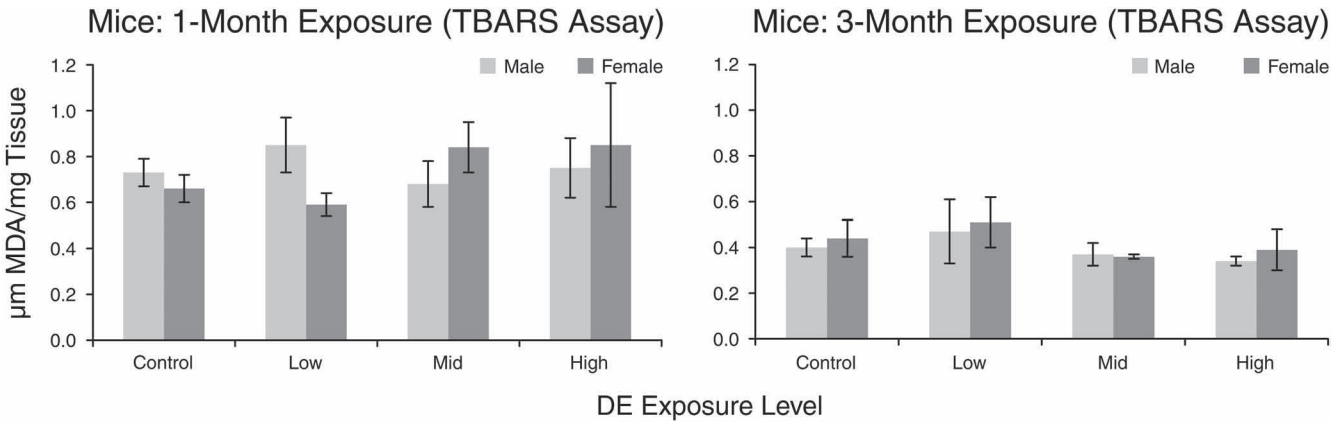

Figure 11. TBARS analysis of mouse hippocampus brain tissue (1- or 3-month DE exposure). No significant differences were seen between controls and those exposed to DE at different levels ($P = 0.92$). A significant difference in TBARS levels between the 1-month and 3-month groups was noted ($P < 0.0001$).

Rat Data

Table 33 describes the distribution of rats in their duration and exposure-level categories used in the analysis of the TBARS assay. In rats, there was a significant group difference ($P = 0.02$) and a significant difference between the low and mid groups ($P = 0.03$). The difference between the low and control groups was nearly significant ($P = 0.06$). There was no significant difference between other groups. The measures of TBARS normalized for protein show an almost-significant sex difference ($P = 0.06$). TBARS normalized for protein measures at 1 month were not significantly different from those at 3 months ($P = 0.11$). A significant difference in the levels of TBARS was found only between the low-exposure and mid-exposure groups. None of the other interaction terms was statistically significant (Tables 34 and 35, Figure 12).

Table 33. Number of Animals Used in the TBARS Assay (Rats)

	1-Month Exposure			3-Month Exposure		
	Male	Female	All	Male	Female	All
Control	5	5	10	4	5	9
Low	5	5	10	5	5	10
Mid	5	5	10	4	5	9
High	5	5	10	5	5	10
All	20	20	40	18	20	38

Table 34. Rat TBARS Data, Descriptive Statistics[a]

	1-Month Exposure		3-Month Exposure	
	Male	Female	Male	Female
Control	0.29 ± 0.03	0.19 ± 0.08	0.39 ± 0.07	0.31 ± 0.04
Low	0.45 ± 0.13	0.25 ± 0.04	0.49 ± 0.05	0.56 ± 0.14
Mid	0.26 ± 0.03	0.27 ± 0.05	0.29 ± 0.01	0.30 ± 0.03
High	0.54 ± 0.15	0.27 ± 0.03	0.34 ± 0.04	0.32 ± 0.05

[a] Data are mean ± SE.

Table 35. Rat TBARS Data: Mixed Model for Repeated Measures

Type of Group / Comparison	*P* Value
All exposure-level groups	0.02[a]
Control vs. Low	0.06
Control vs. Mid	0.99
Control vs. High	0.58
Low vs. Mid	0.03
Low vs. High	0.55
Mid vs. High	0.40
Sex	
Male vs. Female	0.06
Duration	
1 Month vs. 3 Months	0.11
Exposure level × Sex interaction	0.53
Exposure level × Duration interaction	0.11
Sex × Duration interaction	0.09
Exposure level × Sex × Duration interaction	0.45

[a] *P* value represents overall comparison among all groups.

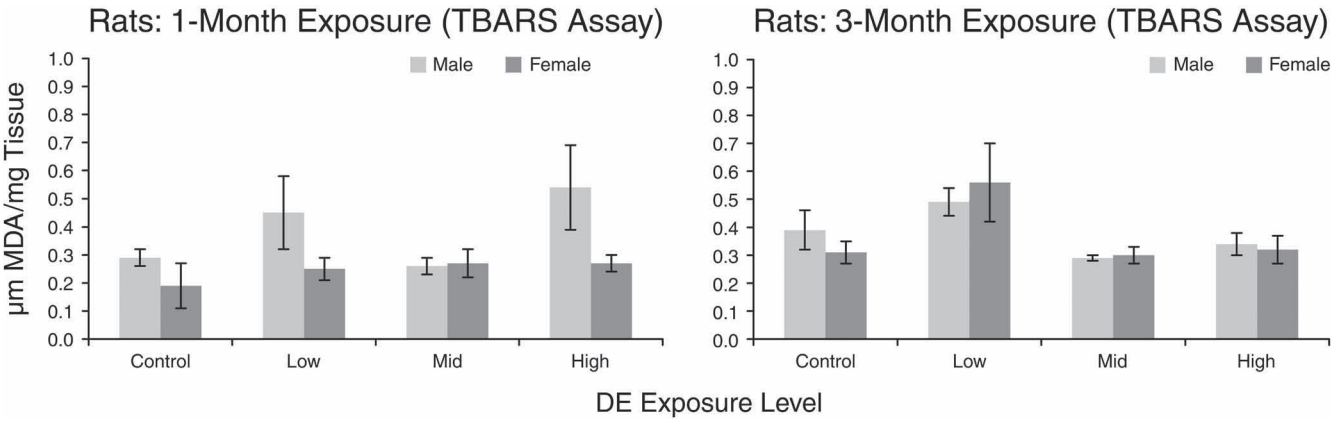

Figure 12. TBARS analysis of rat hippocampus brain tissue (1- or 3-month DE exposure). Significant differences were seen between low- and mid-exposure levels ($P = 0.03$). No significant difference in TBARS levels between the 1-month and 3-month groups was noted ($P = 0.11$).

DISCUSSION AND CONCLUSIONS

GENOTOXIC COMPONENTS OF DE

The adverse health effects of air pollution are generally attributed to oxidative stress (Ayres et al. 2008; Lai et al. 2005). For the past several decades, adverse effects have also been attributed to the genotoxicity of DE caused, to a great extent, by the particulate fraction, especially UFP. This is based on the supposition that particulates can exert their effects either directly, by producing oxidative damage to lipids, proteins, or DNA, or indirectly, by the induction of an inflammatory response and the recruitment of inflammatory cells, which in turn induce oxidative damage. UFP tend to deposit deeper into the lungs and have easier access to the blood stream and other tissues than do larger particles. Furthermore, DEP have hundreds of chemicals adsorbed to them (Lai et al. 2005) including PAHs, which are thought to be a substantial contributor to the health effects of PM in areas of traffic (Lewtas 2007).

VOCs, including benzene and 1,3-butadiene, are also components of DE. These are gaseous in nature and are usually inhaled into the respiratory tract. Other gaseous components include NO_x and carbon monoxide (CO). The main sources of anthropogenic emissions of NO_x into the air include heating, power generation, and vehicles. The NO_x can be transformed into NO_2 in the presence of ozone (WHO 2001). Both in vitro and in vivo studies in humans and animals demonstrate that NO_2 can activate oxidant pathways (Bayram et al. 2001).

Past research into the adverse effects of DE on human health has revealed that DNA damage and lipid peroxidation can be induced in the presence of this environmental toxicant. In our current investigation, we wanted to determine whether DNA damage and lipid peroxidation would continue to be problematic with 2007-compliant engines.

Our approach to answer this question was to determine the extent of DNA damage as a function of strand breaks and adduct formation in male and female rats and mice exposed to DE. Additionally, we wanted to confirm these findings by looking for stable DNA-adduct excision products in serum in the form of 8-OHdG, a ubiquitous marker of oxidative stress. 8-OHdG is physiologically formed and can be enhanced by the exposure of DNA to toxicants. It is formed during the repair of damaged DNA in vivo by exonucleases and can then be eliminated without being further metabolized. 8-OHdG can be found circulating in the blood and in the urine, where it is finally excreted.

We also wanted to investigate whether DE-induced oxidative stress could occur in the brain. Previous studies have indicated that toxicants might be able to find their way into the brain, by passing through the blood–brain barrier or by passing along the olfactory bulb and migrating into the hippocampus. Therefore, we investigated whether lipid peroxidation, a well-defined mechanism of cellular damage in animals, was taking place within the hippocampus region. The lipid peroxides, formed by peroxidation, are unstable indicators of oxidative stress that decompose to form more complex and more reactive compounds, such as MDA and 4-hydroxynonenal, natural by-products of lipid peroxidation.

When this investigation was first proposed, it was hypothesized that the induction of oxidative damage would be the result of exposure to DE gases and especially DEP. As the characterization of the test engine proceeded, it was determined that the design of the newer engine had

reduced DEP to almost ambient levels. With this information, we realized that the possibility of particulate-induced oxidative stress would likely decrease, since it was DEP and in particular the PAH components that were most often associated with the observed health effects. However, NO_x is still present in DE. Since NO_x can also induce oxidative stress, it remains a concern for adverse health effects.

GENOTOXICITY OF DE IN RATS, MICE, AND HUMANS

The comet assay is an appropriate test for DNA damage, as it examines a broad range of oxidized purines and pyrimidines in DNA (Moller 2006). Using the comet assay, a study of bus drivers and garage workers in Prague, Czech Republic, found the level of double-strand breaks in lymphocyte DNA higher among these workers than the controls (Bagryantseva et al. 2010). Similarly, in a Danish study using the comet assay, increased DNA damage was detected in persons who bicycled in traffic. The results were attributed to UFP in the air. UFP concentrations were associated with measurements of ambient temperature and CO levels, as well as temperature and NO_2 levels, as measured at fixed stations (Vizents et al. 2005). In a study by Muller and colleagues (2004), Big Blue rats exhibited higher levels of strand breaks in their lungs after oral ingestion of DEP.

The results of the current study do not show any significant exposure-related increases in DNA damage in either rodent species, as measured by the comet assay, compared to controls. Further, we found no difference in DNA damage between sexes or in comparisons of the 1-month and 3-month exposure groups. We found that mice had a significantly higher percentage of DNA in comet tails compared to rats.

Variation has been noted in epidemiologic studies using 8-OHdG level as an endpoint measurement in populations exposed to DE. In a recent study (Lee et al. 2011), it was observed that an increase in urinary 8-OHdG in DE emissions inspectors exposed to DE was correlated with levels of PAHs, but in a study by Harri and colleagues (2005), no differences in urinary 8-OHdG between garage workers, waste collectors, and controls were observed. Our findings using the 8-OHdG assay support the lack of response (with no sex effects) seen with the comet assay in both the 1- and 3-month exposure groups at three exposure levels.

LIPID PEROXIDATION IN BRAIN TISSUE

Acute, subchronic, and chronic exposures to PM and pollutant gases can affect people. Furthermore, respiratory-tract inflammation can produce mediators that are capable of reaching the brain. Systemic circulation of PM and disruption of the nasal respiratory and olfactory barriers also are possible in exposed populations. In a study by Calderón-Garcidueñas and colleagues (2003), DNA damage in healthy dogs exposed to urban air in Mexico City was evaluated. They found that nasal, respiratory, and olfactory epithelium were early targets of air pollution. Both the olfactory bulbs and the hippocampi of exposed dogs were observed to have significantly higher apurinic/apyrimidinic sites than those of the control animals. Additionally, increased inflammatory-distress proteins were found in the brain of the exposed dogs, providing evidence that respiratory tract inflammation and deterioration of the olfactory barrier may play an important role in this neuropathy. Another study found that autopsied human brain tissue from cognitively and neurologically intact individuals exposed to high levels of air pollution had significantly higher COX2 expression in the frontal cortex and hippocampus compared to the brain tissue of control residents from cities with low air pollution. These findings suggest that exposure to severe air pollution is associated with brain inflammation (Calderón-Garcidueñas et al. 2004). Additionally, Mexico City residents exposed to severe air pollution exhibited olfactory bulb inflammation and endothelial hyperplasia. In that study, UFP were observed in olfactory endothelial cytoplasm and basement membranes (Calderón-Garcidueñas et al. 2010). Further, Gerlofs-Nijland and colleagues (2010) investigated the effects of exposure to DE in rats. These animals were exposed to DE in a nose-only exposure chamber for 6 hours a day, 5 days a week, for 4 weeks. After the final exposure, the brain was dissected into cerebellum, frontal cortex, hippocampus, olfactory bulb and tubercles, and striatum. Baseline levels of proinflammatory cytokines, tumor necrosis factor-alpha, and interleukin-1 alpha were dependent on the region analyzed and were found to increase in the striatum after exposure to DE. These results indicated that different brain regions might respond differently to changes induced by exposure to DE. Our study of the inflammatory effects of DE on the hippocampus in both rats and mice revealed no significant difference between exposed groups. A nearly significant difference between sexes in rats was observed.

STUDY LIMITATIONS

One critical limitation of all the studies we conducted was the lack of a positive control. One approach could have been to use as a positive control samples from a group exposed to an older type of diesel engine. Running these samples with the current samples would have given us a better feel for the effects of the engineering changes.

Nonetheless, the use of very similar cumulative NO_2 exposures in this study and the older studies by Mauderly and colleagues (Mauderly et al. 1987; 1989) was a key feature that facilitated comparisons between the studies.

As stated earlier, our original hypothesis was that these newer engines would continue to emit UFP, leaving open the possibility of adverse effects as a result of oxidative stress. After the initiation of this study, we were informed that the amount of particulates emitted by the study engine was very low, to the point of being close to ambient levels. Had we known this as we designed our experiments, it might have made a difference in our approach. However, because the DE was not aged, as might be normally encountered, any particulates released by the study engine would be more reactive and therefore more potent in this study. Thus, the lack of oxidative-type damage as depicted by our study provides evidence that these engines appear to be running cleaner.

One of our concerns was the amount of time between the end of the exposure and the harvesting of tissues. It was pointed out that a possible confounder, and a possible explanation for the low levels of oxidative damage noted in this study, was that DNA was being repaired as the animals waited to be processed. Therefore, we analyzed the amount of time that passed between the end of exposure and the end of processing. In mice exposed 1 month, this averaged 1 hour and 23 minutes in the female animals and 1 hour and 37 minutes in the males. In mice exposed 3 months, female processing averaged 2 hours and 22 minutes; male processing, 1 hour and 42 minutes. In rats exposed 1 month, female processing averaged 2 hours and 31 minutes; male processing, 3 hours and 41 minutes. In the 3-month exposure rat group, female processing averaged 4 hours and 11 minutes. The average for this group was higher than the others because one female was processed earlier in the day than the rest of the group. In male rats exposed for 3 months, processing averaged 3 hours and 21 minutes. In summary, processing time took a minimum of 1 hour and 23 minutes and a maximum of 4 hours and 11 minutes for the females, and a minimum of 1 hour and 37 minutes and a maximum of 3 hours and 42 minutes for the males. It is true that DNA repair mechanisms would continue to decrease damage caused by the exposure to DE during this period. However, our contention is that any damage induced by the exposure that could be repaired in an efficient, error-free manner in such a short amount of time may be considered biologically irrelevant. The more important concern would involve DNA damage that was persistent. This type of damage would have several biological fates: (1) It could induce cell cycle delay as damage was repaired, in which case there would be no change in phenotype, or (2) damaged cells would continue through the cell cycle and fix the damage as a mutation. This could lead to an increased risk of the cells developing DNA instability, invoking a change in cellular phenotype, which could lead to some adverse health effects.

IMPLICATIONS OF FINDINGS

In our experimental design, we anticipated the presence of particulates, SO_2, and NO_x. These have been implicated in the induction of oxidative stress resulting in DNA damage. To determine whether exposure to DE from 2007-compliant diesel engines increased DNA damage in lung tissue, we measured strand breaks using the comet assay. Also, since DNA strand breakage can result from oxidative stress, we used the 8-OHdG ELISA assay to support our findings from the comet assay. An initial power analysis was performed and was based on our experience with human lymphocytes. No statistically significant differences between controls and exposure levels were observed in either species. However, in mice, we did see a statistically significant difference between the low-exposure and the mid-exposure groups exposed for 1 month; but we maintain that because neither of these groups differed from the controls, there were no statistically significant differences in any DE exposure group we tested as compared to groups exposed to clean air. Thus, the difference observed is a statistically interesting observation, but not likely to be biologically important, from the perspective of DEP. Therefore, based on the endpoints we evaluated, we conclude that these 2007-compliant engines did not produce any significant, persistent oxidatively induced DNA damage to rats or mice at the exposures we tested.

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ABBREVIATIONS AND OTHER TERMS

3-NBA	3-nitrobenzanthrone
8-OHdG	8-hydroxy-deoxyguanosine
ACES	Advanced Collaborative Emissions Study
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CO	carbon monoxide
DE	diesel exhaust
DEP	diesel exhaust particulates
ELISA	enzyme-linked immunosorbent assay
LRRI	Lovelace Respiratory Research Institute
MDA	malondialdehyde
NO_2	nitrogen dioxide
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PM	particulate matter
$PM_{2.5}$	PM with an aerodynamic diameter $\leq$ 2.5 μm
ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SO_2	sulfur dioxide
TBARS	thiobarbituric acid reactive substances
UFP	ultrafine particulates
VOC	volatile organic compound

Part 4. Effects of Subchronic Diesel Engine Emissions Exposure on Plasma Markers in Rodents: Report on 1- and 3-Month Exposures in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 4. Effects of Subchronic Diesel Engine Emissions Exposure on Plasma Markers in Rodents: Report on 1- and 3-Month Exposures in the ACES Bioassay

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ABSTRACT

Although epidemiologic and experimental studies suggest that exposure to diesel exhaust (DE*) emissions causes adverse cardiovascular effects, neither the specific components of DE nor the mechanisms by which DE exposure could induce cardiovascular dysfunction and exacerbate cardiovascular disease (CVD) are known. Moreover, because the advance of new technologies has resulted in cleaner fuels and decreased engine emissions, there is even more uncertainty about the relationship between DE exposure and cardiovascular health effects. To address this ever-changing baseline of engine emissions, we tested for exposure-, sex- and duration-dependent alterations in plasma markers following subchronic exposure of mice and rats to DE emissions from a 2007-compliant diesel engine. Many plasma markers — several recognized as known human CVD risk factors — were measured in the plasma of rodents exposed to 1 or 3 months of air (the control) or DE emissions. Few changes in plasma markers resulted from exposure to DE, although significant exposure-level-dependent increases in total cholesterol and high-density lipoprotein (HDL) cholesterol were observed in male rats after 1 month of DE exposure, an effect that was neither sustained nor observed in any other group. These data indicate that DE emissions from a 2007-compliant diesel engine as tested in this study had little adverse effect on CVD markers in rodents.

INTRODUCTION

Air pollution exposure is associated with increased cardiovascular morbidity and mortality, and is especially associated with ischemic heart disease (Pope et al. 2006; Brook et al. 2010). Although most air pollution studies have focused on the association between airborne $PM_{2.5}$ (particulate matter $\leq$ 2.5 μm in aerodynamic diameter) and cardiovascular outcomes, the specific contribution of traffic pollutants to this relationship has not been fully elucidated (Brunekreef et al. 2009; Rosenbloom et al. 2012). Moreover, because of the recent introduction of new engine technologies and fuel reformulations, the contribution of any given mobile-source pollutant to human cardiovascular risk must be constantly reassessed. Studies have shown that acute and chronic DE exposures lead to alterations in plasma cholesterol in rodents (Reed et al. 2004, 2006), perhaps indicating the activation of an acute phase response (APR) as observed in sepsis (Harris et al. 2000). Moreover, changes in cholesterol level and lipoprotein oxidation state can be complex. For example, cholesterol levels may be increased or decreased and changes may be restricted to one lipoprotein class (e.g., very-low-density lipoprotein, low-density lipoprotein [LDL, oxidized LDL], and HDL) that may remain unidentified. Additionally, in normolipidemic rodents, the majority of circulating cholesterol is carried by HDL not by LDL, as in humans; thus, a decreased total cholesterol level in rodents could very well be the result of a loss of HDL (or "good

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* A list of abbreviations and other terms appears at the end of the Investigators' Report.

cholesterol") as proposed in studies of acrolein exposure in mice (Shao et al. 2005 a,b). Because a decreased cholesterol level has been reported in rodent studies of DE exposure, these results should be interpreted carefully.

The mechanism by which air pollution increases human CVD risk is unknown, although several hypotheses have been proposed (Brook et al. 2010). Time series analyses of epidemiologic data have shown that CVD risk increases longer term and also acutely within two discrete time lags following exposure to peak air pollution. These peaks in cardiovascular response occur within 1 to 2 hours and at approximately 24 hours after peak air pollution, which suggests that posttranslational events could have been initiated in the shorter time frame and transcriptional events in the longer (Brook et al. 2010). Moreover, changes in plasma markers may precipitate frank cardiovascular changes and, thus, further support the mechanistic contribution of initiation, transcription, and secretion events. Changes in plasma markers indicate that three general and overlapping mechanisms likely participate in the precipitation of cardiovascular morbidity and mortality associated with exposure to air pollutants. These include (1) increased thrombogenicity, (2) increased vascular inflammation, and (3) increased APR (systemic inflammation) (Brook et al. 2010). The overlap between these pathways is incontrovertible, and yet studying the initiation and sequence of events that govern the relationship between these pathways is critical, in order to therapeutically intervene (Esmon et al. 1999; Hukkanen et al. 2009).

Epidemiologic studies have associated increased plasma APR reactants (e.g., C-reactive protein [CRP], fibrinogen) with exposure to PM pollution in humans (Peters et al. 2001; Pope et al. 2004). Certain markers reflect a distinct biological response — as in an APR (e.g., serum amyloid A [SAA]) (Cabana et al. 1999; Kindy et al. 2000), vascular inflammation (e.g., soluble intercellular adhesion molecule-1 [sICAM-1]) (Kitagawa et al. 2002), or thrombosis (e.g., platelet factor 4 [PF4]) — whereas some markers can participate in more than one pathway (e.g., fibrinogen is a marker of both an APR and thrombosis) (Reinhart 2003).

Many cytokines and chemokines have been associated with pulmonary injury following exposure to pollutants such as DE (Saito et al. 2002; Hiramatsu et al. 2003; Seagrave et al. 2005; Fujimaki et al. 2006; Inoue et al. 2006; Saber et al. 2006; Arimoto et al. 2007; Li et al. 2007). Some circulating cytokines and chemokines (tumor necrosis factor-α [TNF-α]; interleukin-6 [IL-6]; monocyte chemotactic protein-1 [MCP-1]; and keratinocyte-derived chemoattractant [KC], also called CXCL1) are implicated in lung injury in rodents in response to pollutant exposure. The association of some with rodent lung injury is not well established in the literature, and for some others the relationship with systemic inflammation needs to be investigated (Esmon et al. 1999; Erdely et al. 2011). For example, vascular endothelial growth factor (VEGF) is a chemokine marker of sepsis that also reflects acute endothelium injury as well as hypoxia and increases vascular permeability (Grad et al. 1998; Nolan et al. 2004; Pickkers et al. 2005; Yano et al. 2006; Bateman et al. 2007). Similarly, leptin is an important regulator of feeding behavior and metabolism but is also considered a positive APR reactant (Faggioni et al. 2000). Vascular injury and activation are reflected in increased plasma levels (secreted molecules) of VEGF and sICAM-1. Areas of the vasculature (e.g., resistance arteries and arterioles) may be affected during DE exposure (Campen et al. 2005; Cherng et al. 2011) and could contribute to increased plasma levels of endothelin-1 (ET-1). However, not all pollutants necessarily increase these factors; our recent study showed that acrolein, an environmental pollutant, decreased plasma levels of sICAM-1 (Conklin et al. 2011b) as a result of the inhibition of intercellular adhesion molecule-1 (ICAM-1) shedding from endothelial cells (Tsakadze et al. 2004, 2006).

Well-established biomarkers reflect different aspects of thrombosis and various coagulation pathways. For example, PF4 is a platelet-specific marker, and thus, represents a very specific marker of platelet-dependent thrombosis (Lorenz and Brauer 1988; Pitsilos et al. 2003; Slungaard 2004; Kowalska et al. 2007). On the other hand, fibrinogen, a soluble plasma protein, is converted to fibrin when the coagulation cascade is activated and represents a more general marker of thrombosis. Fibrinogen is also an APR reactant as its level of expression is increased during the APR via transcriptional upregulation in the liver (Gervois et al. 2004; Zambon et al. 2006). Increased systemic or pulmonary oxidative stress has been reported in a number of pollutant-exposure models (Ikeda et al. 1995; Hirano et al. 2003; Reed et al. 2004; Seagrave et al. 2005). Moreover, DE exposure in rodents could lead to extrapulmonary organ toxicity. Thus, the purpose of this study is to analyze the effects of DE on the APR, vascular inflammation, and thrombosis pathways. It will provide the basis for subsequent studies assessing how real-world DE exposures contribute to an increased human CVD risk associated with exposure to air pollution (Brook et al. 2010; Channell et al. 2012).

Table 1. Plasma Levels (Mean ± Standard Error [SE]) of Markers Measured in Male and Female Mice Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a,b]

	CHOL (n = 156) (mg/dL)	HDL (n = 143) (mg/dL)	LDL (n = 143) (mg/dL)	HDL/LDL (n = 141)	TRIG (n = 147) (mg/dL)	ALB (n = 159) (g/dL)
Mice: 1-Month Exposure						
Male						
Air	93.67 ± 2.83 (9)	70.57 ± 2.5 (7)	12.06 ± 0.43 (7)	5.9 ± 0.22 (7)	39.82 ± 8.56 (7)	3.10 ± 0.04
Low	95.54 ± 2.52	70.64 ± 2.96	12.08 ± 1.04	6.35 ± 0.66	35.72 ± 6.2	3.10 ± 0.04
Mid	97.65 ± 3.83	73.98 ± 2.5	11.8 ± 0.89	6.54 ± 0.43	33.42 ± 7.12	3.10 ± 0.04
High	96.27 ± 2.43	71.88 ± 1.79 (9)	11.24 ± 0.75 (9)	6.69 ± 0.6 (9)	38.12 ± 6.82 (9)	3.17 ± 0.04
Female						
Air	82.6 ± 2.38 (9)	56.64 ± 1.54	16.39 ± 0.85	3.43 ± 0.14 (9)	26.18 ± 4.28	3.20 ± 0.05
Low	87.1 ± 1.19 (9)	58.23 ± 1.36 (7)	16.93 ± 0.82 (7)	3.51 ± 0.23 (7)	39.66 ± 6.85 (9)	3.28 ± 0.05 (9)
Mid	86.31 ± 2.22 (9)	59.95 ± 1.3 (5)	17.39 ± 1.36 (5)	3.67 ± 0.17 (4)	34.25 ± 10.04 (6)	3.34 ± 0.06
High	88.58 ± 2.62	59.74 ± 1.87 (6)	17.02 ± 1.63 (6)	3.62 ± 0.24 (6)	20.85 ± 4.4 (6)	3.35 ± 0.02
Mice: 3-Month Exposure						
Male						
Air	88.6 ± 2.54	67.54 ± 2.32 (9)	8.05 ± 0.65 (9)	8.62 ± 0.37 (9)	24.79 ± 2.27	3.03 ± 0.03
Low	88.59 ± 3.11	67.69 ± 3.06	7.38 ± 0.44	9.34 ± 0.45	23.89 ± 1.7	3.01 ± 0.04
Mid	89.34 ± 4.5	68.2 ± 3.8	7.97 ± 0.26	8.6 ± 0.49	35.1 ± 4.5	3.09 ± 0.05
High	94.29 ± 1.97	72.5 ± 1.79	8.81 ± 0.4	8.33 ± 0.32	24.32 ± 1.41	3.03 ± 0.05
Female						
Air	86.28 ± 3.59	60.19 ± 2.51	11.99 ± 0.83	5.21 ± 0.35	23.79 ± 3.78	3.27 ± 0.05
Low	84.58 ± 2.11	61.31 ± 1.93	12.68 ± 0.62	4.94 ± 0.29	22.39 ± 2.89	3.14 ± 0.05
Mid	87.37 ± 2.17	61.71 ± 1.87	14.15 ± 0.51	4.41 ± 0.18	19.47 ± 2.77	3.26 ± 0.03
High	89.09 ± 3.73	62.5 ± 2.77	14.8 ± 0.9	4.31 ± 0.18	16.44 ± 1.64	3.23 ± 0.02

(*Table continues on next page*)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

[b] CHOL indicates total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; HDL/LDL ratio; TRIG, triglycerides; ALB, albumin; TP, total protein; NAP, nonalbumin protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CREA, creatinine; LDH, lactate dehydrogenase; PF4, platelet factor 4; IgE, immunoglobulin E; IgG, immunoglobulin G; and IgM, immunoglobulin M.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Core Study

The experimental time course and levels of exposure to 2007-compliant diesel engine exhaust followed the procedure carried out at the Lovelace Respiratory Research Institute (LRRI) for the DE exposure Chronic Bioassay Study as described elsewhere in this report (see the report by McDonald et al., Part 1 of this volume). In brief, young, healthy male and female Wistar Han rats and C57BL/6 mice (12 weeks old) were used for all exposures. Up to 14 rats and 30 mice were assigned into one of four exposure groups: those exposed to filtered air (the control), or low (0.1 ppm nitrogen dioxide [NO_2]; 2 μg/m^3 PM), medium (mid; 0.8 ppm NO_2; 3 μg/m^3 PM), or high (4.2 ppm NO_2; 9 μg/m^3 PM) levels of DE. Rats and mice were exposed for 1 or 3 months. Groups of 10 mice or rats were used for all measurements of lipids, albumin (ALB), total protein (TP), cytokines and chemokines, and systemic markers of toxicity except where indicated (see Tables 1, 2, 3, and 4). Plasma samples were shipped overnight on dry ice from LRRI to the University of Louisville.

Table 1 (*Continued*). Plasma Levels (Mean ± Standard Error [SE]) of Markers Measured in Male and Female Mice Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a,b]

	TP (n = 152) (g/dL)	NAP (n = 152) (g/dL)	ALT (n = 150) (U/L)	AST (n = 138) (U/L)	CK (n = 134) (U/L)	CREA (n = 90) (mg/dL)
Mice: 1-Month Exposure						
Male						
Air	4.57 ± 0.05 (9)	1.49 ± 0.03 (9)	29.25 ± 1.87 (9)	70.59 ± 10.21 (9)	235.06 ± 22.95 (9)	0.20 ± 0.02 (5)
Low	4.65 ± 0.12	1.54 ± 0.09	31.82 ± 4.02 (9)	64.98 ± 6.43 (8)	225.43 ± 27.27 (8)	0.20 ± 0.01 (4)
Mid	4.64 ± 0.12	1.53 ± 0.08	33.77 ± 4.01	82.47 ± 14.11 (9)	305.46 ± 62.17 (9)	0.25 ± 0.02 (5)
High	4.76 ± 0.11 (9)	1.59 ± 0.07 (9)	45.61 ± 10.47 (9)	85.74 ± 10.39 (9)	249.24 ± 58.27 (9)	0.19 ± 0.03 (5)
Female						
Air	4.51 ± 0.08 (9)	1.3 ± 0.04 (9)	26.98 ± 2.73 (9)	73.56 ± 5.49 (9)	210.65 ± 20.07 (9)	0.15 ± 0.02 (4)
Low	4.54 ± 0.09 (8)	1.28 ± 0.06 (8)	25.74 ± 2.35 (8)	57.98 ± 4.25 (7)	137.62 ± 21.64 (7)	0.16 ± 0.01 (4)
Mid	4.6 ± 0.12 (8)	1.29 ± 0.05 (8)	25.16 ± 2.51 (8)	66.07 ± 3.02 (7)	143.76 ± 16.95 (5)	0.19 ± 0.01
High	4.77 ± 0.07	1.42 ± 0.06	31.3 ± 3.47	74.52 ± 7.53 (9)	187.78 ± 29.74 (8)	0.22 ± 0.02 (4)
Mice: 3-Month Exposure						
Male						
Air	4.64 ± 0.06 (9)	1.61 ± 0.05 (9)	61.77 ± 10.13 (9)	116.22 ± 14.18 (7)	306.56 ± 36.5 (7)	0.19 ± 0.01 (5)
Low	4.74 ± 0.07	1.73 ± 0.04	61.77 ± 9.19 (9)	116.45 ± 21.64 (9)	330.5 ± 101.8	0.18 ± 0.01 (8)
Mid	4.85 ± 0.1	1.75 ± 0.06	45.42 ± 1.4	86.92 ± 7.08 (9)	176.63 ± 18.27 (8)	0.17 ± 0.01 (6)
High	4.75 ± 0.12	1.72 ± 0.09	74.08 ± 15.86	117.46 ± 17.87 (9)	208.79 ± 14.77 (9)	0.19 ± 0.01 (8)
Female						
Air	4.9 ± 0.11	1.63 ± 0.08	43.79 ± 1.03	97.98 ± 5.87 (9)	180.63 ± 22.79 (9)	0.16 ± 0.01 (8)
Low	4.75 ± 0.07	1.6 ± 0.04	42.6 ± 0.87	107.84 ± 8.94	346.7 ± 48.7	0.17 ± 0.01 (5)
Mid	4.9 ± 0.04	1.64 ± 0.03	47.67 ± 6.73	95.24 ± 6.89 (9)	281.15 ± 37.7 (9)	0.18 ± 0.01 (8)
High	4.92 ± 0.08	1.69 ± 0.08	41.42 ± 1.25	92.45 ± 4.85 (9)	229.45 ± 30.72 (8)	0.22 ± NA (1)

(*Table continues on next page*)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

[b] CHOL indicates total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; HDL/LDL ratio; TRIG, triglycerides; ALB, albumin; TP, total protein; NAP, nonalbumin protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CREA, creatinine; LDH, lactate dehydrogenase; PF4, platelet factor 4; IgE, immunoglobulin E; IgG, immunoglobulin G; and IgM, immunoglobulin M.

Table 1 (*Continued*). Plasma Levels (Mean ± Standard Error [SE]) of Markers Measured in Male and Female Mice Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a,b]

	LDH (n = 126) (U/L)	PF4 (n = 148) (ng/mL)	IgE (n = 79) (ng/mL)	IgG (n = 80) (mg/mL)	IgM (n = 79) (µg/mL)
Mice: 1-Month Exposure					
Male					
Air	250.85 ± 25.78 (9)	46.61 ± 6.58	69.6 ± 15.52 (5)	0.72 ± 0.19 (5)	93.23 ± 14.71 (5)
Low	229.86 ± 24.84 (7)	55.89 ± 10.63 (9)	31.25 ± 10.18 (4)	0.48 ± 0.14 (4)	82.58 ± 11.96 (4)
Mid	240.28 ± 20.12 (9)	60.48 ± 9.35	21.83 ± 3.26 (6)	0.53 ± 0.13 (6)	99.68 ± 11.61 (6)
High	246.56 ± 16.02 (8)	54.75 ± 10.42	18.1 ± 1.58 (5)	0.39 ± 0.06 (5)	75.48 ± 3.63 (5)
Female					
Air	216.8 ± 18.27 (9)	54.19 ± 8.96 (9)	37.75 ± 6.34 (5)	0.5 ± 0.05 (5)	119.65 ± 29.42 (5)
Low	198.17 ± 13.35 (7)	98.63 ± 54.91 (9)	24.8 ± 3.82 (5)	0.59 ± 0.06 (5)	111.25 ± 11.36 (5)
Mid	200.72 ± 19.13 (5)	53.52 ± 8.36	24.35 ± 4.71 (5)	0.47 ± 0.02 (5)	103.0 ± 17.02 (5)
High	247.0 ± 25.13 (6)	108.43 ± 62.71 (9)	50.93 ± 10.67 (5)	0.76 ± 0.18 (5)	101.14 ± 11.45 (5)
Mice: 3-Month Exposure					
Male					
Air	306.16 ± 14.35 (6)	195.51 ± 67.31 (8)	19.91 ± 5.87 (5)	0.66 ± 0.11 (5)	133.85 ± 9.13 (5)
Low	404.35 ± 60.43	173.4 ± 65.5	46.46 ± 10.3 (4)	0.88 ± 0.24 (5)	198.67 ± 25.13 (5)
Mid	289.44 ± 21.51 (8)	126.39 ± 31.37	47.34 ± 5.75 (5)	1.01 ± 0.3 (5)	190.51 ± 51.77 (5)
High	286.48 ± 28.57 (9)	138.67 ± 63.69	60.25 ± 8.47 (5)	1.22 ± 0.38 (5)	230.35 ± 70.84 (4)
Female					
Air	337.97 ± 53.35 (9)	203.38 ± 92.02 (8)	102.69 ± 17.15 (5)	1.1 ± 0.24 (5)	238.63 ± 76.64 (5)
Low	278.88 ± 11.01 (9)	260.8 ± 111.76 (7)	137.82 ± 29.2 (5)	1.04 ± 0.21 (5)	259.51 ± 76.88 (5)
Mid	255.44 ± 25.03 (8)	164.6 ± 47.36	83.13 ± 22.8 (5)	1.29 ± 0.24 (5)	306.97 ± 41.67 (5)
High	248.5 ± 21.27 (7)	222.22 ± 83.37 (9)	60.05 ± 16.27 (5)	1.41 ± 0.28 (5)	365.14 ± 65.04 (5)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

[b] CHOL indicates total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; HDL/LDL ratio; TRIG, triglycerides; ALB, albumin; TP, total protein; NAP, nonalbumin protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CREA, creatinine; LDH, lactate dehydrogenase; PF4, platelet factor 4; IgE, immunoglobulin E; IgG, immunoglobulin G; and IgM, immunoglobulin M.

Table 2. Plasma Levels (Mean ± SE) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Mice Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a,b]

	GM-CSF (n = 160) (pg/mL)	MCP-1 (n = 159) (pg/mL)	IL-1β (n = 159) (pg/mL)	IL-6 (n = 159) (pg/mL)	IL-10 (n = 159) (pg/mL)	IFN-γ (n = 160) (pg/mL)	KC (n = 160) (pg/mL)
Mice: 1-Month Exposure							
Male							
Air	41.17 ± 7.19	24.63 ± 1.57	23.73 ± 1.35	16.01 ± 2.56	22.44 ± 2.33	18.92 ± 1.22	132.30 ± 22.62
Low	81.90 ± 34.4	42.48 ± 12.21	28.49 ± 5.98	48.0 ± 27.85	21.99 ± 3.03	14.56 ± 0.81	214.12 ± 29.04
Mid	49.40 ± 5.56	30.36 ± 7.48	27.41 ± 7.25	25.76 ± 4.46	17.63 ± 2.65	18.63 ± 2.75	185.11 ± 23.81
High	39.63 ± 8.34	30.76 ± 6.86	20.97 ± 1.06	19.0 ± 2.75	16.40 ± 2.81	17.3 ± 3.3	183.73 ± 36.30
Female							
Air	33.64 ± 5.82	23.94 ± 1.04	23.34 ± 1.21	20.25 ± 3.61	20.75 ± 1.26	14.85 ± 0.95	106.87 ± 15.22
Low	33.51 ± 9.11	28.97 ± 6.11	21.24 ± 1.84	16.30 ± 4.29	17.76 ± 2.85	10.88 ± 1.30	127.34 ± 18.26
Mid	51.77 ± 7.80	33.70 ± 6.29	29.01 ± 5.36	20.70 ± 2.74	23.16 ± 2.02	18.53 ± 2.72	136.47 ± 34.93
High	38.93 ± 9.92	44.79 ± 11.91	37.7 ± 12.14	16.19 ± 2.63	15.75 ± 3.41	14.98 ± 2.05	123.69 ± 19.87
Mice: 3-Month Exposure							
Male							
Air	37.92 ± 10.36	25.05 ± 3.65	24.2 ± 2.82	16.73 ± 3.13	13.03 ± 3.22	10.13 ± 1.27	207.15 ± 45.0
Low	95.7 ± 34.59	50.04 ± 15.45	35.22 ± 9.11	24.83 ± 9.89	14.17 ± 3.4	9.64 ± 1.99	292.68 ± 73.23
Mid	54.98 ± 19.01	58.2 ± 15.79	43.57 ± 16.46	15.68 ± 3.0	13.85 ± 2.79	7.53 ± 1.23	110.59 ± 31.88
High	126.4 ± 21.77	75.08 ± 20.51	69.34 ± 27.36	20.67 ± 3.85	13.96 ± 3.64	9.16 ± 1.29	173.32 ± 29.14
Female							
Air	115.33 ± 38.4	85.9 ± 24.7 (9)	88.1 ± 39.9 (9)	18.26 ± 4.87 (9)	37.54 ± 15.33 (9)	9.31 ± 1.77	117.57 ± 30.11
Low	53.89 ± 23.43	56.28 ± 12.5	32.98 ± 7.96	16.38 ± 2.86	13.41 ± 2.97	7.27 ± 1.39	228.11 ± 31.14
Mid	66.55 ± 21.08	43.09 ± 8.7	33.05 ± 5.69	12.08 ± 2.53	14.13 ± 3.15	8.45 ± 1.42	116.22 ± 15.31
High	86.77 ± 32.64	144.5 ± 70.14	139.64 ± 80.6	26.34 ± 9.38	26.8 ± 10.8	14.24 ± 4.24	129.89 ± 21.61

(*Table continues on next page*)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

[b] GM-CSF indicates granulocyte/macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IFN-γ, interferon-γ; KC, keratinocyte-derived chemoattractant; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; sICAM-1, soluble intercellular adhesion molecule-1; SAA, serum amyloid A; and CRP, C-reactive protein.

Table 2 (*Continued*). Plasma Levels (Mean ± SE) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Mice Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a,b]

	TNF-α (*n* = 159) (pg/mL)	VEGF (*n* = 160) (pg/mL)	sICAM-1 (*n* = 160) (pg/mL)	Fibrinogen (*n* = 160) (pg/mL)	Leptin (*n* = 159) (pg/mL)	SAA (*n* = 157) (μg/mL)	CRP (*n* = 160) (ng/mL)
Mice: 1-Month Exposure							
Male							
Air	19.63 ± 3.91	8.73 ± 1.54	37,854.0 ± 1,753.0	1814.3 ± 384.4	45.76 ± 6.45 (9)	3.13 ± 1.49	557.58 ± 15.94
Low	26.98 ± 2.64	7.3 ± 0.42	37,418.0 ± 2,310.0	2026.4 ± 337.3	73.33 ± 9.32	2.89 ± 1.09 (9)	534.44 ± 19.71
Mid	16.48 ± 4.7	10.02 ± 1.48	38,102.0 ± 1,658.0	1932.29 ± 313.0	46.61 ± 7.61	1.02 ± 0.02 (9)	548.88 ± 19.76
High	13.97 ± 3.49	10.54 ± 3.7	35,107.0 ± 1,323.0	1701.65 ± 357.0	55.24 ± 10.0	1.72 ± 0.72	544.91 ± 16.76
Female							
Air	16.51 ± 3.77	6.19 ± 0.74	36,186.0 ± 1,587.0	2208.9 ± 244.7	15.14 ± 2.85	1.01 ± 0.01	587.48 ± 15.11
Low	12.52 ± 3.89	5.29 ± 0.8	36,356.0 ± 951.2	2319.65 ± 71.9	23.12 ± 1.57	1.22 ± 0.12	561.44 ± 10.66
Mid	25.27 ± 3.38	7.02 ± 1.01	36,695.0 ± 1,338.0	2017.5 ± 332.6	18.25 ± 2.92	2.9 ± 1.35	566.39 ± 14.99
High	18.86 ± 5.27	6.33 ± 0.78	34,781.0 ± 827.8	1897.2 ± 309.4	29.19 ± 6.66	1.57 ± 0.55	567.35 ± 22.32
Mice: 3-Month Exposure							
Male							
Air	15.14 ± 2.69	4.78 ± 0.74	34,000.0 ± 1,486.0	2107.9 ± 234.6	66.86 ± 17.68	6.14 ± 3.82	518.72 ± 18.05
Low	13.49 ± 4.08	6.81 ± 1.55	39,801.0 ± 1,580.0	1869.0 ± 302.1	63.51 ± 13.28	1.7 ± 0.66	465.65 ± 22.95
Mid	15.17 ± 2.37	3.97 ± 0.74	40,251.0 ± 2,075.0	973.2 ± 367.8	95.18 ± 17.66	6.13 ± 4.22	440.79 ± 9.36
High	16.08 ± 3.08	4.25 ± 0.43	40,648.0 ± 2,888.0	1154.3 ± 361.6	55.14 ± 9.08	8.42 ± 4.83	482.73 ± 16.68
Female							
Air	20.49 ± 3.65 (9)	3.84 ± 0.36	40,423.0 ± 2,876.0	1704.4 ± 352.4	33.25 ± 5.23	1.40 ± 0.29 (9)	466.12 ± 10.93
Low	12.94 ± 3.2	3.84 ± 0.64	37,991.0 ± 1,782.0	2128.9 ± 235.3	24.04 ± 4.19	1.52 ± 0.35	448.4 ± 11.41
Mid	12.12 ± 2.18	3.59 ± 0.53	37,724.0 ± 1,749.5	1453.2 ± 376.8	18.89 ± 4.19	1.33 ± 0.14	467.12 ± 13.48
High	38.2 ± 18.4	3.92 ± 0.92	35,801.0 ± 2,022.9	1369.2 ± 357.4	13.87 ± 1.85	1.2 ± 0.13	465.64 ± 12.93

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (*n*) after the value.

[b] GM-CSF indicates granulocyte/macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein-1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IFN-γ, interferon-γ; KC, keratinocyte-derived chemoattractant; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; sICAM-1, soluble intercellular adhesion molecule-1; SAA, serum amyloid A; and CRP, C-reactive protein.

Table 3. Plasma Levels (Mean ± Standard Error [SE]) of Markers Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a]

	CHOL (n = 160) (mg/dL)	HDL (n = 160) (mg/dL)	LDL (n = 160) (mg/dL)	HDL/LDL (n = 160)	TRIG (n = 160) (mg/dL)	ALB (n = 160) (g/dL)	TP (n = 160) (g/dL)	NAP (n = 160) (g/dL)
Rats: 1-Month Exposure								
Male								
Air	78.13 ± 5.18	47.06 ± 3.64	14.64 ± 1.13	3.24 ± 0.21	92.95 ± 18.51	3.93 ± 0.06	6.07 ± 0.09	2.13 ± 0.05
Low	82.43 ± 3.39	48.96 ± 2.45	14.15 ± 1.17	3.62 ± 0.3	132.2 ± 13.59	3.92 ± 0.04	5.99 ± 0.07	2.07 ± 0.04
Mid	94.05 ± 4.47	56.98 ± 2.93	17.73 ± 1.94	3.43 ± 0.25	116.9 ± 19.47	4.13 ± 0.09	6.37 ± 0.17	2.25 ± 0.09
High	94.95 ± 6.5	59.17 ± 3.88	17.71 ± 2.39	3.7 ± 0.34	107.9 ± 20.25	4.05 ± 0.08	6.18 ± 0.15	2.12 ± 0.08
Female								
Air	78.32 ± 3.62	48.29 ± 2.08	14.99 ± 0.74	3.24 ± 0.16	56.79 ± 13.67	4.67 ± 0.08	6.82 ± 0.16	2.15 ± 0.08
Low	86.74 ± 4.68	54.82 ± 2.63	15.93 ± 1.16	3.52 ± 0.18	55.87 ± 10.82	4.78 ± 0.09	7.11 ± 0.18	2.34 ± 0.09
Mid	85.17 ± 3.25	53.85 ± 2.2	15.42 ± 0.99	3.57 ± 0.19	73.34 ± 14.51	4.62 ± 0.09	6.78 ± 0.18	2.17 ± 0.1
High	73.62 ± 3.00	46.75 ± 2.51	12.36 ± 1.15	4.08 ± 0.44	58.64 ± 15.04	4.56 ± 0.09	6.7 ± 0.17	2.14 ± 0.09
Rats: 3-Month Exposure								
Male								
Air	83.47 ± 3.15	51.56 ± 1.87	13.64 ± 1.11	4.05 ± 0.46	114.79 ± 15.0	4.09 ± 0.04	6.11 ± 0.12	2.02 ± 0.08
Low	90.85 ± 3.76	56.87 ± 2.87	14.71 ± 0.76	3.94 ± 0.19	116.4 ± 14.49	4.15 ± 0.04	6.17 ± 0.07	2.02 ± 0.06
Mid	83.04 ± 3.64	49.8 ± 2.06	15.4 ± 2.04	3.72 ± 0.44	70.22 ± 11.52	4.12 ± 0.04	6.2 ± 0.15	2.08 ± 0.12
High	83.55 ± 3.86	53.44 ± 2.3	14.02 ± 1.16	3.94 ± 0.22	98.05 ± 16.56	4.04 ± 0.04	6.05 ± 0.11	2.01 ± 0.08
Female								
Air	92.11 ± 5.54	61.14 ± 3.71	14.57 ± 1.21	4.35 ± 0.34	57.38 ± 16.57	4.79 ± 0.11	6.89 ± 0.19	2.09 ± 0.09
Low	81.15 ± 2.88	53.69 ± 2.32	13.05 ± 0.89	4.34 ± 0.44	53.04 ± 9.49	4.69 ± 0.07	6.75 ± 0.12	2.07 ± 0.07
Mid	74.84 ± 3.60	49.6 ± 2.81	12.29 ± 1.36	4.35 ± 0.43	78.98 ± 22.91	4.69 ± 0.09	6.68 ± 0.15	1.99 ± 0.09
High	87.78 ± 3.78	58.46 ± 2.81	13.48 ± 0.95	4.52 ± 0.35	67.09 ± 21.92	4.62 ± 0.11	6.67 ± 0.2	2.05 ± 0.10

(*Table continues on next page*)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

Positive Controls

Stored plasma samples (from mice and rats) from previously conducted studies at the University of Louisville were used for internal validation of plasma markers but not for immunoglobulins. That is, plasma samples were initially collected 24 hours after 12- to 16-week-old male C57BL/6 mice (Conklin et al. 2010) and Sprague-Dawley rats (Conklin et al. 2011a) were gavage fed either water (n = 3) or acrolein (5 mg/kg [mice] or 0.5 mg/kg [rat]; n = 3). These samples were stored frozen at –80°C, thawed, and then run in parallel in assays at the same time as plasma samples from the current ACES study. Many plasma markers, e.g., fibrinogen, leptin, and VEGF, were measured for the first time in the thawed samples. Control values and those of acrolein-treated rodents were compared using the Student t test (unequal variance), and a P value < 0.05 was considered statistically significant.

PLASMA MARKERS

Plasma Lipids and Proteins

Lipids and proteins in 150 µL of frozen plasma from a group of 10 animals were measured using a Cobas Mira Plus clinical chemistry autoanalyzer (Roche, Indianapolis, IN) with calibrated standards and clinical quality reagents (Wako; Randox; ThermoElectron) (Conklin et al. 2010). CHOL (total cholesterol), HDL cholesterol, LDL cholesterol, and TRIG (triglycerides) were measured in mg/dL. Based on Wako's insert, HDL cholesterol was measured using the precipitating antibody specific to apolipoprotein B, and LDL cholesterol was measured by first oxidizing all non-LDL cholesterol. TP and ALB (g/dL) were measured using Bradford and Bromocresol Green reagents, respectively (Randox) (Conklin et al. 2010).

Table 3 (*Continued*). Plasma Levels (Mean ± Standard Error [SE]) of Markers Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a]

	ALT (n = 159) (U/L)	AST (n = 160) (U/L)	CK (n = 158) (U/L)	CREA (n = 159) (mg/dL)	LDH (n = 159) (U/L)	IgE (n = 158) (ng/mL)	IgG (n = 160) (mg/mL)	IgM (n = 160) (µg/ml)
Rats: 1-Month Exposure								
Male								
Air	35.99 ± 2.24	67.3 ± 6.95	283.4 ± 40.23	0.40 ± 0.01	204.1 ± 25.1	6.33 ± 3.88 (9)	2.15 ± 0.26	98.39 ± 11.01
Low	37.09 ± 1.89	62.75 ± 4.39	384.2 ± 178.3 (9)	0.39 ± 0.01	232.1 ± 30.1	2.13 ± 0.3	1.55 ± 0.24	75.13 ± 12.51
Mid	42.92 ± 3.83	73.19 ± 5.93	281.8 ± 36.75	0.38 ± 0.02	249.3 ± 50.8	3.89 ± 0.99	1.71 ± 0.16	56.84 ± 8.19
High	35.23 ± 2.17	69.44 ± 5.64	245.9 ± 16.66 (9)	0.42 ± 0.04	235.3 ± 38.0	3.06 ± 1.11	1.48 ± 0.18	79.88 ± 11.13
Female								
Air	32.19 ± 1.28 (9)	69.27 ± 9.36	277.7 ± 35.02	0.42 ± 0.02	223.0 ± 12.7 (9)	3.92 ± 1.32	2.75 ± 0.36	70.47 ± 6.48
Low	41.41 ± 4.4	74.17 ± 7.07	244.8 ± 23.08	0.39 ± 0.01	253.5 ± 24.5	10.38 ± 3.9 (9)	2.30 ± 0.43	70.99 ± 12.59
Mid	38.37 ± 2.49	66.53 ± 4.44	188.5 ± 12.87	0.4 ± 0.01	199.0 ± 16.7	7.44 ± 4.61	1.91 ± 0.38	58.99 ± 10.13
High	38.54 ± 3.44	83.0 ± 9.46	438.3 ± 115.4	0.42 ± 0.02	241.4 ± 24.6	2.39 ± 0.44	2.93 ± 0.57	82.36 ± 8.26
Rats: 3-Month Exposure								
Male								
Air	39.56 ± 1.94	81.9 ± 8.29	307.25 ± 58.5	0.56 ± 0.03	216.2 ± 25.2	7.58 ± 3.02	4.82 ± 0.68	145.8 ± 16.97
Low	48.97 ± 8.87	80.49 ± 6.55	268.45 ± 26.3	0.52 ± 0.01	214.4 ± 13.1	2.71 ± 0.73	3.5 ± 0.46	149.3 ± 12.22
Mid	41.53 ± 4.71	91.96 ± 13.32	429.8 ± 117.2	0.52 ± 0.02	379.0 ± 173	6.94 ± 2.87	4.26 ± 0.85	151.8 ± 15.43
High	38.35 ± 1.10	69.52 ± 4.29	231.3 ± 27.1	0.51 ± 0.04	195.3 ± 10.5	3.25 ± 0.97	5.78 ± 0.95	165.6 ± 24.99
Female								
Air	65.77 ± 11.4	117.5 ± 17.34	255.5 ± 30.35	0.46 ± 0.02	242.6 ± 22.6	10.21 ± 4.93	5.41 ± 0.33	130.26 ± 7.69
Low	66.85 ± 9.25	105.3 ± 14.59	220.9 ± 24.1	0.48 ± 0.02	232.6 ± 18.1	7.86 ± 2.55	5.22 ± 0.61	111.3 ± 13.27
Mid	46.92 ± 5.26	91.3 ± 10.54	279.78 ± 44.1	0.46 ± 0.02 (9)	227.9 ± 20.2	3.98 ± 1.32	6.89 ± 1.16	138.25 ± 9.35
High	46.82 ± 5.25	103.1 ± 15.84	390.1 ± 148.0	0.47 ± 0.02	265.3 ± 35.0	3.81 ± 1.54	7.29 ± 0.88	149.7 ± 17.23

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

Cytokines, Chemokines, and Other Plasma Proteins

For measuring chemokines, cytokines, and growth factors (Saber et al. 2005, 2006), plasma samples (100 µL) were shipped overnight on dry ice from the University of Louisville to AssayGate (Ijamsville, MD), which used a bead-based antibody technology and a custom-designed array to measure protein levels of the following rat and mouse plasma markers: granulocyte-macrophage colony-stimulating factor (GM-CSF), MCP-1, interleukin-1β (IL-1β), IL-6, interleukin-10 (IL-10), interferon-γ (IFN-γ), KC, TNF-α, VEGF, sICAM-1, fibrinogen, leptin, SAA, and CRP.

Thrombosis Markers

PF4 was measured using a murine enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Fibrinogen was measured as part of the commercial cytokine and chemokine array (see the previous section). sICAM-1 was also measured by commercial array (AssayGate). Array values of sICAM-1 for positive controls (those treated with acrolein) were compared with data generated using an in-house sICAM-1 ELISA system (Sithu et al. 2007, 2010). Notably, significant concordance was found between the values measured by commercial array and those by manual ELISA, confirming the analytical integrity of these marker values.

Immunoglobulins

Plasma immunoglobulin levels of immunoglobulin E (IgE), immunoglobulin G (IgG), and immunoglobulin M (IgM) were measured in duplicate rat (n = 10/group) and murine (n = 4–6/group) samples using commercial ELISA kits according to the manufacturer's instructions, which included recommended dilutions (GenWay Biotech, San Diego, CA). Absorbance was measured at 450 nm using an automated plate reader (BioTek Synergy Mx; BioTek Instruments, Winooski, VT), and a four-parameter logistic model equation was fitted to standard-curve absorbance values ($r^2 > 0.99$) using the plate reader software. A sample concentration was calculated from the model equation and exported to Excel.

Table 4. Plasma Levels (Mean ± SE) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a]

	GM-CSF (n = 160) (pg/mL)	MCP-1 (n = 160) (pg/mL)	IL-1β (n = 160) (pg/mL)	IL-6 (n = 160) (pg/mL)	IL-10 (n = 160) (pg/mL)	IFN-γ (n = 160) (pg/mL)	KC (n = 160) (pg/mL)
Rats: 1-Month Exposure							
Male							
Air	112.7 ± 19.6	110.0 ± 26.6	24.8 ± 5.88	3190.0 ± 2370.0	113.5 ± 32.5	310.0 ± 222.0	182.0 ± 14.1
Low	122.27 ± 33.2	95.7 ± 10.4	37.26 ± 8.0	1559.0 ± 783.0	123.3 ± 48.4	109.0 ± 29.0	208.0 ± 30.4
Mid	111.0 ± 28.65	91.0 ± 8.75	24.6 ± 7.07	769.8 ± 385.0	206.9 ± 59.3	102.0 ± 24.3	190.0 ± 35.8
High	138.9 ± 20.8	99.6 ± 7.73	31.4 ± 13.6	8958.0 ± 5845.0	199.6 ± 70.9	874.0 ± 727.0	189.0 ± 37.7
Female							
Air	102.2 ± 27.4	51.4 ± 7.1	37.5 ± 15.8	305.4 ± 76.0	141.2 ± 59.1	61.8 ± 9.6	237.0 ± 46.3
Low	190.0 ± 131.8	58.4 ± 8.3	17.2 ± 3.74	6516.0 ± 4139.0	125.4 ± 21.2	405.8 ± 239	249.0 ± 38.4
Mid	93.91 ± 18.8	66.6 ± 8.91	28.2 ± 9.6	1845.0 ± 836.0	116.4 ± 24.9	143.0 ± 51.6	186.0 ± 26.1
High	115.4 ± 23.8	47.7 ± 7.26	22.0 ± 7.26	2733.0 ± 1244.0	110.2 ± 35.9	147.0 ± 56.3	212.0 ± 31.8
Rats: 3-Month Exposure							
Male							
Air	123.96 ± 29.0	81.5 ± 12.1	36.3 ± 13.6	1186.0 ± 548.0	123.5 ± 32.1	114.0 ± 36.2	247.0 ± 34.5
Low	84.22 ± 17.6	73.73 ± 6.17	50.1 ± 16.1	3433.0 ± 1899.0	194.8 ± 34.3	148.0 ± 41.4	318.0 ± 65.2
Mid	85.7 ± 17.7	75.2 ± 9.4	41.2 ± 15.8	3142.0 ± 1316.0	113.0 ± 31.1	252.0 ± 99.2	237.0 ± 37.7
High	191.1 ± 41.4	76.4 ± 9.95	31.8 ± 9.4	2498.0 ± 1369.0	169.0 ± 34.7	163.6 ± 57.9	287.0 ± 55.7
Female							
Air	105.8 ± 21.5	53.7 ± 13.9	32.4 ± 9.8	1087.0 ± 387.0	104.0 ± 28.7	104.9 ± 21.8	349.0 ± 78.6
Low	121.2 ± 22.6	44.54 ± 6.1	26.97 ± 7.6	5687.0 ± 4590.0	101.0 ± 24.6	352.0 ± 264.5	272.0 ± 45.0
Mid	84.1 ± 21.38	49.7 ± 8.85	30.3 ± 11.2	1247.0 ± 604.0	105.9 ± 33.4	128.0 ± 52.2	259.0 ± 49.8
High	77.7 ± 20.01	40.4 ± 16.7	11.8 ± 3.3	7410.0 ± 5928.0	180.0 ± 104.0	489.0 ± 413.7	282.0 ± 46.28

(*Table continues on next page*)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

Systemic Organ Toxicity Markers

To discern whether DE exposure stimulated systemic organ toxicity, multiple plasma markers were measured simultaneously during lipid- and protein-panel measurements (see the section Plasma Lipids and Proteins) using a semiautomated Cobas Mira Plus analyzer. As indicators of general or specific organ toxicity, the following were measured: plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST), associated with the liver (Infinity, Thermo Electron); creatine kinase (CK), associated with striated muscle (CK, Thermo Scientific); lactate dehydrogenase (LDH), associated with nonspecific cell toxicity (LDH-L, Pointe Scientific); and creatinine (CREA), associated with the kidneys (Infinity, Thermo Scientific).

STATISTICAL ANALYSES

General toxicity markers, cytokines, and chemokines were measured in the plasma of male and female mice and rats exposed to air or low, mid, or high levels of DE. Exposure durations were 1 month and 3 months. In general, the sample size was 10 for each exposure level for each sex and for each exposure duration, which resulted in 160 readings for each marker.

Data Quality Control

To test for outliers (i.e., values out of the likely range of the response variables), we calculated the median of the observations at each exposure level (stratified by sex and exposure duration) and the differences (i.e., residuals) of the observations and the medians at each exposure level. We calculated the standard deviation (SD) of all the residuals. Based on the Bonferroni method for multiple tests (Faraway 2005), the observations with the residuals outside the range of 4 SDs were identified (i.e., flagged) for examination of their plausibility and methodological validity. Among these flagged values, we removed only those

Table 4 (*Continued*). Plasma Levels (Mean ± SE) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1 Month or 3 Months[a]

	TNF-α (n = 160) (pg/mL)	VEGF (n = 159) (pg/mL)	sICAM-1 (n = 160) (pg/mL)	Fibrinogen (n = 160) (pg/mL)	Leptin (n = 159) (pg/mL)	CRP (n = 160) (ng/mL)
Rats: 1-Month Exposure						
Male						
Air	15.79 ± 8.8	13.9 ± 2.55	7620.0 ± 1081.4	537.0 ± 16.8	546.0 ± 110.6	221.0 ± 14.0
Low	6.58 ± 0.71	15.4 ± 3.85	7328.0 ± 558.9	556.0 ± 18.9	1004.0 ± 267.0	224.0 ± 13.9
Mid	6.36 ± 0.9	7.04 ± 1.19	6031.0 ± 480.4	556.0 ± 18.1	530.0 ± 92.5	230.0 ± 22.9
High	9.24 ± 1.18	8.51 ± 1.87	6371.0 ± 409.1	562.0 ± 16.5	564.0 ± 64.4 (9)	235.0 ± 14.4
Female						
Air	6.66 ± 0.81	8.48 ± 1.55	3530.0 ± 378.0	535.0 ± 23.7	194.0 ± 27.2	262.0 ± 18.6
Low	10.12 ± 3.5	9.7 ± 2.46 (9)	3677.0 ± 236.0	588.0 ± 19.5	313.0 ± 61.8	285.0 ± 19.3
Mid	7.35 ± 0.78	12.9 ± 4.1	4012.0 ± 284.0	537.0 ± 25.5	249.6 ± 53.3	255.0 ± 19.59
High	7.15 ± 1.15	12.6 ± 2.78	4228.0 ± 351.0	545.0 ± 23.6	277.0 ± 42.4	290.0 ± 12.87
Rats: 3-Month Exposure						
Male						
Air	6.16 ± 0.83	7.79 ± 1.95	5174.0 ± 472.0	556.0 ± 19.2	915.0 ± 169.5	232.0 ± 11.76
Low	4.99 ± 0.38	7.19 ± 2.16	5204.0 ± 205.0	559.6 ± 16.6	1087.0 ± 186.0	252.0 ± 16.03
Mid	5.81 ± 0.71	9.83 ± 2.5	5710.0 ± 439.0	549.5 ± 20.7	557.0 ± 78.34	252.0 ± 13.06
High	5.97 ± 0.98	6.59 ± 1.23	4848.0 ± 258.0	552.2 ± 21.3	939.0 ± 101.9	232.0 ± 10.52
Female						
Air	4.95 ± 0.46	7.96 ± 2.05	3082.0 ± 219.0	517.4 ± 11.1	321.0 ± 48.7	267.0 ± 15.3
Low	5.11 ± 0.45	10.6 ± 4.01	3416.0 ± 617.0	577.5 ± 20.2	318.0 ± 46.8	252.0 ± 13.3
Mid	5.36 ± 0.5	10.0 ± 2.66	2541.0 ± 164.0	557.0 ± 15.9	325.0 ± 30.6	268.0 ± 11.0
High	8.15 ± 2.98	8.15 ± 3.35	3664.0 ± 910.0	584.7 ± 21.7	377.0 ± 49.5	249.0 ± 7.2

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value.

values that were both improbable (based on our experience) and that exceeded 4 × SD. For murine samples, we removed only 14 values (≈ 0.28% of the total values). These included removal of values for ALT (1), AST (1), CREA (1), MCP-1 (1), IL-1β (1), IL-6 (1), IL-10 (1), TNF-α (1), leptin (1), SAA (3), IgE (1), and IgM (1). For rat samples, we removed only six readings (≈ 0.13% of the total), which included CK (2), VEGF (1), leptin (1), and IgE (2). The resulting data set excluding these values was used for subsequent statistical analyses. The mean and standard error (SE) for each group based on these data sets are reported in Tables 1, 2, 3, and 4.

Statistical Analysis Methods

For each marker, assumptions of normality and equal variance among different groups were determined, and data were transformed as appropriate. For each marker or its transformation, we first carried out three-way ANOVA, with exposure level, sex, and exposure duration as factors and included all two-way and three-way interactions. If the three-way interaction was not significant, we excluded it and explored whether we could further remove insignificant two-way interactions by using the Akaike information criterion (AIC) (Venables and Ripley 2002). The mean squares for each factor and their interactions (if any) in the final model and the P values for these significance tests are reported in tables described in the Results section.

Additionally, we determined whether the results for each exposure level were significantly different from those for the control for each combination of exposure duration and sex. The significant results for exposure-level effects are reported in tables described in the Results section.

All analyses were carried out using the statistics software R (*www.r-project.org*). The major functions used were for linear model (lm), ANOVA (anova), and the AIC-based model selection (step). A P value smaller than 0.05 was considered statistically significant. For a more detailed account of our statistical analyses, see Appendix A.

RESULTS

POSITIVE CONTROLS

Mice

Where possible, assay values of positive controls obtained in the present study were compared with previously acquired values using the same subset of samples (Conklin et al. 2010) as quality control for assay reproducibility. For stable analytes (i.e., CHOL, HDL, LDL, TRIG, ALB, TP, and CREA), excellent concordance with previous measures was found (96.9 ± 3.2% of previous control values; 101.2 ± 3.4% of previous values for acrolein-treated mice). For enzymatic activity measurements (i.e., ALT, AST, CK, and LDH), we expected activity to be lower in samples after the freeze–thaw cycle. Instead, values in the present study were higher than the original readings (167.9 ± 10.4% of previous values for controls; 164.2 ± 10.6% of previous values for acrolein-treated mice), although the within-group variation was similar. The mean values (± SE) of the murine positive controls are shown in Table 5. As expected, acrolein-treated samples had dramatically increased SAA compared with controls (353 × control), yet acrolein also significantly increased levels of VEGF ($P = 0.026$) and significantly decreased levels of sICAM-1 ($P = 0.047$) and leptin ($P = 0.01$) (Conklin et al. 2011b). Similarly, acrolein nearly quadrupled levels of IL-6 ($P = 0.061$).

Table 5. Plasma Levels (Mean ± SE) of Markers Measured in Mice Exposed to Acrolein as Positive Controls

	Control ($n = 3$)	Acrolein ($n = 3$)	P value
CHOL (mg/dL)	77.78 ± 3.57	112.8 ± 4.24	0.004[a]
HDL (mg/dL)	55.81 ± 3.71	55.9 ± 0.83	0.984
LDL (mg/dL)	10.76 ± 0.67	34.48 ± 1.78	0.002[a]
HDL/LDL (mg/dL)	5.23 ± 0.43	1.63 ± 0.09	0.011[a]
TRIG (mg/dL)	29.56 ± 4.67	144.04 ± 32.51	0.069
ALB (g/dL)	3.25 ± 0.12	2.02 ± 0.1	0.002[a]
TP (g/dL)	4.77 ± 0.2	3.5 ± 0.14	0.009[a]
NAP (g/dL)	1.52 ± 0.09	1.48 ± 0.07	0.73
ALT (U/L)	40.98 ± 4.55	45.6 ± 3.79	0.48
AST (U/L)	94.84 ± 21.05	107.62 ± 10.74	0.627
CK (U/L)	327.62 ± 132.67	383.97 ± 54.66	0.724
CREA (mg/dL)	0.25 ± 0.04	0.28 ± 0.0	0.525
LDH (U/L)	217.2 ± 82.03	247.86 ± 59.7	0.779
PF4 (ng/mL)	656.44 ± 234.33	901.61 ± 353.74	0.599
GM-CSF (pg/mL)	31.3 ± 26.1	79.0 ± 2.38	0.208
MCP-1 (pg/mL)	32.53 ± 13.59	20.1 ± 1.68	0.457
IL-1ß (pg/mL)	19.6 ± 4.44	22.87 ± 5.56	0.671
IL-6 (pg/mL)	5.87 ± 1.31	20.57 ± 4.18	0.061
IL-10 (pg/mL)	8.73 ± 4.79	3.93 ± 0.07	0.422
IFN-γ (pg/mL)	6.0 ± 1.32	8.03 ± 1.6	0.384
KC (pg/mL)	32.87 ± 15.93	180.93 ± 94.56	0.256
TNF-α (pg/mL)	9.77 ± 3.43	11.1 ± 1.6	0.749
VEGF (pg/mL)	2.07 ± 0.23	3.37 ± 0.28	0.026[a]
sICAM-1 (pg/mL)	30,713.0 ± 1,342.0	20,580.0 ± 2,745.0	0.047[a]
Fibrinogen (pg/mL)	2,317.47 ± 38.98	1,310.23 ± 541.83	0.204
Leptin (pg/mL)	74.9 ± 8.3	21.9 ± 8.0	0.01[a]
SAA (µg/mL)	1.0 ± 0.0	353.1 ± 166.81	0.169
CRP (ng/mL)	448.73 ± 13.29	438.0 ± 19.1	0.671

[a] P value < 0.05 (Student t test).

Rats

Where possible, levels of plasma markers were compared in frozen and thawed samples with the originally obtained values (Conklin et al. 2011a). Excellent reproducibility was observed between the two sets of values, although the values were more variable than those for the murine samples (controls: 112.2 ± 26.8% of previous values; acrolein: 107.9 ± 2.3% of previous values). Mean values (± SE) of the rat positive controls are shown in Table 6. Although acrolein significantly increased plasma cholesterol and HDL in rats, it neither increased TRIG nor induced hypoalbuminemia. This could have been a function of the lower acrolein dose used in rats versus mice (i.e., 0.5 mg/kg vs. 5 mg/kg). Acrolein treatment significantly increased the levels of IL-1β (P = 0.006) and CRP (P = 0.033), which are novel observations. In contrast to the response observed in mice, acrolein gavage in rats nearly increased sICAM-1 to a significant level (P = 0.051), yet did not significantly alter SAA or VEGF levels. These data indicate the likelihood of differences in the regulatory processes controlling acute phase and inflammatory responses in mice and rats.

EFFECTS OF DE EXPOSURE LEVEL ON PLASMA MARKERS IN MICE

Only a few markers were changed significantly by DE exposure independent of sex or exposure duration. The plasma marker values for the three DE-exposure levels (see the rows labeled "Exposure level" in Tables 7 and 8) for HDL, fibrinogen, and CRP were significantly different from those of the controls (air). The ANOVA results

Table 6. Plasma Levels (Mean ± SE) of Markers Measured in Rats Exposed to Acrolein as Positive Controls

	Control (n = 3)	Acrolein (n = 3)	P value
CHOL (mg/dL)	61.95 ± 1.17	93.67 ± 1.02	<0.001[a]
HDL (mg/dL)	30.28 ± 0.04	54.79 ± 1.26	0.003[a]
LDL (mg/dL)	21.2 ± 0.8	26.83 ± 1.78	0.07
HDL/LDL (mg/dL)	1.43 ± 0.03	2.03 ± 0.12	0.03[a]
TRIG (mg/dL)	39.59 ± 14.88	22.25 ± 3.69	0.364
ALB (g/dL)	3.09 ± 0.11	3.58 ± 0.07	0.028[a]
TP (g/dL)	4.2 ± 0.19	5.4 ± 0.17	0.009[a]
NAP (g/dL)	1.12 ± 0.08	1.82 ± 0.11	0.008[a]
ALT (U/L)	49.23 ± 0.41 (2)[b]	43.27 ± 0.93	0.014[a]
AST (U/L)	74.21 ± 18.07	67.72 ± 2.94	0.755
CK (U/L)	431.32 ± 237.1	351.92 ± 13.42	0.77
CREA (mg/dL)	0.45 ± 0.05 (2)[b]	0.5 ± 0.01	0.519
LDH (U/L)	222.53 ± 70.41 (2)[b]	176.28 ± 16.84	0.629
GM-CSF (pg/mL)	71.77 ± 54.22	20.2 ± 8.34	0.443
MCP-1 (pg/mL)	76.7 ± 10.94	85.5 ± 29.58	0.801
IL-1ß (pg/mL)	21.4 ± 9.07	102.77 ± 11.2	0.006[a]
IL-6 (pg/mL)	3141.0 ± 1755.55	713.87 ± 256.1	0.3
IL-10 (pg/mL)	142.03 ± 35.18	209.33 ± 63.73	0.421
IFN-γ (pg/mL)	184.37 ± 103.4	110.97 ± 15.45	0.553
KC (pg/mL)	258.93 ± 32.78	291.63 ± 41.89	0.574
TNF-α (pg/mL)	4.77 ± 0.47	4.3 ± 0.00	0.423
VEGF (pg/mL)	4.8 ± 0.0	11.57 ± 6.77	0.423
sICAM-1 (pg/mL)	4226.33 ± 522.97	6197.63 ± 182.55	0.051
Fibrinogen (pg/mL)	536.7 ± 31.15	556.17 ± 34.67	0.698
Leptin (pg/mL)	37.1 ± 12.08	65.87 ± 3.0	0.133
SAA (µg/mL)	3.6 ± 0.0	3.6 ± 0.0	1.0
CRP (ng/mL)	144.8 ± 5.3	235.97 ± 18.95	0.033[a]

[a] P value < 0.05 (Student t test).
[b] For this group, n = 2.

Table 7. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Each Plasma Marker Measured in Male and Female Mice Exposed to Air or DE for 1 Month or 3 Months[a]

	CHOL Rank Transformation		HDL Rank Transformation		LDL Original Scale	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	3,095.0	0.150	2,936.0	0.038[b]	2.68	0.683
Sex (*df* = 1)	40,578.0	<0.001[b]	89,605.0	<0.001[b]	888.8	<0.001[b]
Duration (*df* = 1)	2,823.0	0.202	100.1	0.754	462.8	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—
Sex:Duration (*df* = 1)	7,389.0	0.040[b]	6,599.0	0.012[b]	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—
Residuals	1,719.9	NA	1,019.0	NA	5.37	NA
	Estimate		Estimate		Estimate	
Parameters						
Sex (female = 1)	−46.28	0.0[b]	−65.92	<0.001[b]	5.28	<0.001[b]
Duration (3 months = 1)	−22.10	0.019[b]	−11.16	0.133	−3.63	<0.001[b]
Sex:Duration	27.54	0.040[b]	27.55	0.012[b]	—	—

	HDL/LDL Rank Transformation		TRIG Box–Cox Transformation		ALB Original Scale	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	442.3	0.352	0.039	0.415	0.045	0.073
Sex (*df* = 1)	139,736.0	<0.001[b]	0.607	<0.001[b]	1.249	<0.001[b]
Duration (*df* = 1)	36,342.0	<0.001[b]	0.42	0.002[b]	0.207	0.001[b]
Exposure level:Sex (*df* = 3)	—		—	—	—	—
Exposure level:Duration (*df* = 3)	974.4	0.069	—	—	0.037	0.121
Sex:Duration (*df* = 1)	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—
Residuals	402.48	NA	0.041	NA	0.019	NA
	Estimate		Estimate		Estimate	
Parameters						
Sex (female = 1)	−65.72	<0.001[b]	−0.12	<0.001[b]	0.18	<0.001[b]
Duration (3 months = 1)	32.20	<0.001[b]	−0.11	<0.001[b]	−0.07	0.001[b]
Sex:Duration	—	—	—	—	—	—

(*Table continues on next page*)

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 7 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Each Plasma Marker Measured in Male and Female Mice Exposed to Air or DE for 1 Month or 3 Months[a]

Sources of Variation	TP Original Scale		NAP Rank Transformation		ALT Rank Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.164	0.10	2,491.0	0.111	1,936.0	0.101
Sex (*df* = 1)	0.073	0.334	21,954.0	<0.001[b]	20,833.0	<0.001[b]
Duration (*df* = 1)	1.140	<0.001[b]	77,806.0	<0.001[b]	122,591.0	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—
Sex:Duration (*df* = 1)	0.259	0.069	8,026.0	0.011[b]	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—
Residuals	0.077	NA	1,223.0	NA	916.7	NA
	Estimate		Estimate		Estimate	
Parameters						
Sex (female = 1)	−0.05	0.476	−40.46	<0.001[b]	−25.13	<0.001[b]
Duration (3 months = 1)	0.09	0.136	30.97	<0.001[b]	57.25	<0.001[b]
Sex:Duration	0.17	0.061	29.12	0.012[b]	—	—

	AST Box–Cox Transformation		CK Box–Cox Transformation		CREA Original Scale	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.006	0.644	0.010	0.749	0.003	0.052
Sex (*df* = 1)	0.003	0.592	0.074	0.083	0.006	0.016[b]
Duration (*df* = 1)	0.514	<0.001[b]	0.115	0.031[b]	0.007	0.007[b]
Exposure level:Sex (*df* = 3)	—	—	0.015	0.604	0.003	0.051
Exposure level:Duration (*df* = 3)	—	—	0.054	0.087	0.003	0.041[b]
Sex:Duration (*df* = 1)	—	—	0.114	0.031[b]	0.007	0.01[b]
Exposure level:Sex:Duration (*df* = 3)	—	—	0.099	0.008[b]	—	—
Residuals	0.01	NA	0.024	NA	0.001	NA
	Estimate		Estimate		Estimate	
Parameters						
Sex (female = 1)	−0.01	0.618	−0.12	0.003[b]	−0.03	0.004[b]
Duration (3 months = 1)	0.12	<0.001[b]	0.0	1.0	−0.03	0.004[b]
Sex:Duration	—	—	0.13	0.011[b]	0.04	0.0002[b]

(*Table continues on next page*)

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 7 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Each Plasma Marker Measured in Male and Female Mice Exposed to Air or DE for 1 Month or 3 Months[a]

	LDH Box–Cox Transformation		PF4 Original Scale		IgE Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.003	0.618	612.681	0.707	1.020	0.375
Sex (*df* = 1)	0.024	0.022[b]	5.855	0.947	14.879	<0.001[b]
Duration (*df* = 1)	0.126	<0.001[b]	81,244.5	<0.001[b]	28.884	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	0.442	0.713
Exposure level:Duration (*df* = 3)	0.01	0.078	—	—	5.523	0.002[b]
Sex:Duration (*df* = 1)	—	—	—	—	9.578	0.003[b]
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	9.129	<0.001[b]
Residuals	0.004	NA	1,317.24	NA	0.967	NA
	Estimate		Estimate		Estimate	
Parameters						
Sex (female = 1)	−0.03	0.003[b]	0.29	0.961	0.145	0.644
Duration (3 months = 1)	0.06	<0.001[b]	46.91	<0.001[b]	0.487	0.130
Sex:Duration	—	—	—	—	0.432	0.0002[b]

	IgG Box–Cox Transformation		IgM Box–Cox Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.065	0.876	0.006	0.747
Sex (*df* = 1)	1.582	0.021[b]	0.148	0.001[b]
Duration (*df* = 1)	8.419	<0.001[b]	1.030	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—
Residuals	0.283	NA	0.013	NA
	Estimate		Estimate	
Parameters				
Sex (female = 1)	0.282	0.02[b]	0.084	0.002[b]
Duration (3 months = 1)	0.649	<0.001[b]	0.229	<0.001[b]
Sex:Duration	—	—	—	—

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 8. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Models for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Mice Exposed to Air or DE for 1 Month or 3 Months[a]

	GM-CSF Box–Cox Transformation		MCP-1 Box–Cox Transformation		IL-1β Rank Transformation		IL-6 Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	2.123	0.769	0.100	0.263	606.901	0.833	0.140	0.891
Sex (*df* = 1)	7.652	0.245	0.092	0.268	577.997	0.600	0.737	0.297
Duration (*df* = 1)	3.453	0.434	0.773	0.002[b]	11,534.1[b]	0.020[b]	2.194	0.073
Exposure level:Sex (*df* = 3)	16.19	0.038[b]	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	10.98	0.123	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	5.608	NA	0.074	NA	2,096.0	NA	0.674	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−0.44	0.236	0.05	0.213	3.92	0.59	−0.14	0.283
Duration (3 months = 1)	0.29	0.434	0.14	0.001[b]	17.04[b]	0.02[b]	−0.23	0.079
Sex:Duration	—	—	—	—	—	—	—	—

	IL-10 Rank Transformation		IFN-γ Rank Transformation		KC Rank Transformation		TNF-α Box–Cox Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	1,965.7	0.402	2726.8	0.121	7,958.0	0.007[b]	0.174	0.793
Sex (*df* = 1)	0.6	0.986	3591.0	0.109	17,098.0	0.003[b]	0.008	0.899
Duration (*df* = 1)	23,484.0	0.001[b]	114,704.0	<0.001[b]	403.0	0.646	0.131	0.612
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	0.885	0.16
Exposure level:Duration (*df* = 3)	—	—	—	—	4,335.0	0.082	0.771	0.211
Sex:Duration (*df* = 1)	—	—	2839.0	0.154	—	—	0.256	0.478
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	1.455	0.038[b]
Residuals	1,996.0	NA	1385.0	NA	1,900.2	NA	0.506	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−0.03	0.997	−17.9	0.033[b]	−20.68	0.003[b]	−0.09	0.575
Duration (3 months = 1)	−24.31	0.001[b]	−61.98	<0.001[b]	3.18	0.645	−0.14	0.383
Sex:Duration	—	—	16.85	0.154	—	—	0.16	0.488

(*Table continues on next page*)

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 8 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Models for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Mice Exposed to Air or DE for 1 Month or 3 Months[a]

	VEGF Box–Cox Transformation		sICAM-1 Box–Cox Transformation		Fibrinogen Rank Transformation		Leptin Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.011	0.992	0.00010	0.449	6,081.0	0.028[b]	0.357	0.294
Sex (*df* = 1)	4.109	<0.001[b]	0.00009	0.366	1,294.0	0.417	45.08	<0.001[b]
Duration (*df* = 1)	16.298	<0.001[b]	0.00031	0.100	21,414.0	0.001[b]	0.459	0.207
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	0.231	0.491
Exposure level:Duration (*df* = 3)	0.671	0.1	—	—	—	—	1.550	0.001[b]
Sex:Duration (*df* = 1)	—	—	—	—	—	—	0.277	0.327
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	0.893	0.028[b]
Residuals	0.317	NA	0.00012	NA	1,950.0	NA	0.286	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−0.32	0.001[b]	0.0015	0.366	5.69	0.416	−0.98	<0.001[b]
Duration (3 months = 1)	−0.64	<0.001[b]	0.0014	0.100	−23.14	0.001[b]	0.19	0.116
Sex:Duration	—	—	—	—	—	—	−0.16	0.348

	SAA Rank Transformation		CRP Original Scale	
	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	909.1	0.59	7,235.2	0.043[b]
Sex (*df* = 1)	3226.3	0.134	820.8	0.575
Duration (*df* = 1)	2643.2	0.174	317,998.0	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—
Exposure level:Duration (*df* = 3)	2806.7	0.12	—	—
Sex:Duration (*df* = 1)	—	—	15,496.0	0.016[b]
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—
Residuals	1419.7	NA	2,596.0	NA
	Estimate		Estimate	
Parameters				
Sex (female = 1)	−9.12	0.129	24.21	0.035[b]
Duration (3 months = 1)	7.99	0.184	−69.48	<0.001[b]
Sex:Duration	—	—	−39.37	0.0157[b]

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

indicate that the four groups (three exposures and one control) were significantly different, while the ANOVA post-hoc comparisons were used to examine which exposure group was different from the control (Table 9). Also, the fibrinogen level at the highest DE exposure level was significantly lower than that of the air-exposure group, and CRP levels for both the low- and mid-level DE exposure groups were significantly lower than those of air-exposed mice (Table 10). The final model for KC included a significant effect of exposure levels; however, it also included an insignificant interaction between exposure level and duration (Table 8) indicating that the exposure-level effect on KC may depend on exposure duration. A post-hoc comparison indicated that the 3-month mean value for KC for the low-DE-exposure group was significantly greater than the mean value for the air-exposure group (Table 10).

Several plasma markers had at least one significant interaction between exposure level and sex or exposure level and duration (or a three-way interaction): CK, CREA, IgE, GM-CSF, TNF-α, and leptin (Tables 7 and 8). ANOVA post-hoc testing indicated that (1) the CK measurements for female mice exposed 3 months to low- and mid-level DE were significantly higher than those for their matched air controls (Table 9); (2) the CREA measurements for male mice exposed 1 month to mid-level DE, for female mice exposed 1 month at mid- and high-level DE, and for female mice exposed 3 months to high-level DE were significantly higher than those for the respective air controls (Table 9); (3) the IgE measurements for male mice exposed 1 month to low-, mid-, and high-level DE were significantly lower than those for the matched air controls, whereas the IgE measurements for male mice exposed 3 months to

Table 9. Mice: Comparisons of DE Exposure Groups and Controls for the Markers Whose Final ANOVA Models Included Significant Exposure Effect or Significant Interaction of Exposure with Sex or Duration[a]

	HDL[b]		CK		CREA		IgE	
	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
Mice: 1-Month Exposure								
Male								
Low vs. Control	2.238	0.766	−0.019	0.801	−0.003	0.857	−1.589	0.019[c]
Mid vs. Control	9.604	0.211	0.046	0.532	0.036	0.036[c]	−2.005	0.001[c]
High vs. Control	13.402	0.081	−0.033	0.651	−0.01	0.576	−2.264	0.001[c]
Overall *P* value		0.259		0.726		0.044[c]		0.002[c]
Female								
Low vs. Control	2.238	0.766	−0.165	0.037[c]	0.013	0.489	−0.731	0.244
Mid vs. Control	9.604	0.211	−0.133	0.125	0.062	0.001[c]	−0.806	0.200
High vs. Control	13.402	0.081	−0.062	0.408	0.067	0.001[c]	0.460	0.463
Overall *P* value		0.259		0.163		0.001[c]		0.138
Mice: 3-Month Exposure								
Male								
Low vs. Control	2.238	0.766	−0.048	0.533	−0.004	0.814	1.739	0.011[c]
Mid vs. Control	9.604	0.211	−0.184	0.023[c]	−0.011	0.501	1.872	0.004[c]
High vs. Control	13.402	0.081	−0.12	0.128	−0.003	0.875	2.317	<0.001[c]
Overall *P* value		0.259		0.100		0.913		0.002[c]
Female								
Low vs. Control	2.238	0.766	0.204	0.005[c]	0.012	0.443	0.561	0.371
Mid vs. Control	9.604	0.211	0.149	0.043[c]	0.015	0.292	−0.65	0.300
High vs. Control	13.402	0.081	0.084	0.267	0.075	0.001[c]	−1.36	0.033[c]
Overall *P* value		0.259		0.033[c]		0.014[c]		0.019[c]

[a] Shown are overall *P* values for the exposure effects. Also shown are the estimated differences and *P* values based on comparing the exposure groups to the control for each sex and duration.

[b] Because the final ANOVA for HDL did not include an interaction either between exposure level and duration or between exposure level and sex, the estimation of the difference between each exposure level and the control is the same across different exposure durations and sexes.

[c] *P* value < 0.05.

Table 10. Mice: Comparisons with Controls of Cytokines, Chemokines, and APR Reactants in Each DE Exposure Group[a]

	KC[b]		Fibrinogen[c]		Leptin		CRP[c]	
	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
Mice: 1-Month Exposure								
Male								
Low vs. Control	23.8	0.086	−5.49	0.579	0.46	0.063	−29.99	0.009[d]
Mid vs. Control	17.95	0.195	−15.32	0.123	−0.037	0.879	−26.68	0.020[d]
High vs. Control	14.2	0.305	−28.09	0.005[d]	0.135	0.583	−17.32	0.131
Overall *P* value		0.36		0.028[d]		0.156		0.043[d]
Female								
Low vs. Control	23.8	0.086	−5.49	0.579	0.560	0.021[d]	−29.99	0.009[d]
Mid vs. Control	17.95	0.195	−15.33	0.123	0.218	0.364	−26.68	0.020[d]
High vs. Control	14.2	0.305	−28.09	0.005[d]	0.585	0.016[d]	−17.32	0.131
Overall *P* value		0.36		0.028[d]		0.043[d]		0.043[d]
Mice: 3-Month Exposure								
Male								
Low vs. Control	35.35	0.011[d]	−5.49	0.579	−0.043	0.856	−29.99	0.009[d]
Mid vs. Control	−19.0	0.17	−15.33	0.123	0.387	0.108	−26.68	0.020[d]
High vs. Control	1.0	0.942	−28.09	0.005[d]	−0.108	0.653	−17.32	0.131
Overall *P* value		0.001[d]		0.028[d]		0.161		0.043[d]
Female								
Low vs. Control	35.35	0.011[d]	−5.49	0.579	−0.324	0.177	−29.99	0.009[d]
Mid vs. Control	−19.0	0.17	−15.33	0.123	−0.591	0.015[d]	−26.68	0.020[d]
High vs. Control	1.0	0.942	−28.09	0.005[d]	−0.814	0.001[d]	−17.32	0.131
Overall *P* value		0.001[d]		0.028[d]		0.006[d]		0.043[d]

[a] Shown are overal *P* values for the exposure effects. Also shown are the estimated differences and *P* values based on comparing the exposure groups to the control for each sex and duration.

[b] Although the final ANOVA for KC did not include an interaction between exposure level and sex, it did indicate an interaction between exposure level and duration. Therefore, the estimation of the difference between each exposure level and the control is the same for both sexes, but not the same for 1- and 3-month durations.

[c] Because the final ANOVA for fibrinogen and CRP did not indicate an interaction between exposure level and duration or between exposure level and sex, the estimates of the contrasts between exposure level and the control are the same across different exposure durations and sexes.

[d] *P* value < 0.05.

low-, mid-, and high-level DE were significantly greater than those for the air controls, and the IgE level for female mice exposed 3 months to high-level DE was significantly lower than that for the matched air control (Table 9); and (4) leptin levels for female mice exposed 1 month to low- and high-level DE were significantly greater than those for the air controls, whereas the leptin levels for female mice exposed 3 months to mid- and high-level DE were significantly lower than those for the matched air controls (Table 10).

EFFECTS OF SEX AND EXPOSURE DURATION ON PLASMA MARKERS IN MICE

The values of several markers changed independently, either as a function of sex or of exposure. That is, the differences between male and female values did not depend on exposure duration, and the differences between 1- and 3-month exposure values did not depend on sex. As shown in Tables 7 and 8, the mean values of the following markers were significantly lower in female than in male mice: the HDL/LDL ratio, TRIG, ALT, LDH, KC, and VEGF [see the rows labeled "Sex (female = 1)"]. Female mice also had significantly higher levels of LDL, ALB, IgG, and IgM than male mice [see the rows labeled "Sex (female = 1)"]. Mice exposed for 3 months had significantly lower levels of LDL, TRIG, ALB, IL-10, VEGF, and fibrinogen than mice exposed for 1 month [see the rows labeled "Duration (3 months = 1)"]. Also, mice exposed for 3 months compared with mice exposed for 1 month had significantly

higher HDL/LDL ratios and higher levels of ALT, AST, LDH, PF4, IgG, IgM, MCP-1, and IL-1β [see the rows labeled "Duration (3 months = 1)"].

Some plasma marker levels varied between females and males, with different patterns at 1-month and 3-month exposures. As shown in Tables 7 and 8, the mean values of the following were significantly lower in female mice exposed 1 month than in male mice exposed 1 month: CHOL, HDL, nonalbumin protein (NAP), CK, CREA, IFN-γ, and leptin [see the rows labeled "Sex (female = 1)"]. Also, female mice exposed for 1 month had significantly higher levels of CRP than did male mice exposed 1 month [Table 8; see the row labeled "Sex (female = 1)"]. Male mice exposed 3 months compared with male mice exposed 1 month had significantly lower CHOL, CREA, IFN-γ, and CRP [Tables 7 and 8; see the rows labeled "Duration (3 months = 1)"]. Male mice exposed 3 months had significantly higher levels of NAP than male mice exposed 1 month [Table 7; see the row labeled "Duration (3 months = 1)"]. The delta change for CRP in female mice between 3- and 1-month exposures was significantly lower than in male mice over the same duration (Table 8; see the *P* value in the row labeled "Sex:Duration"). In addition, the delta change for CHOL, HDL, NAP, CK, CREA, and IgE in female mice between 3- and 1-month exposures was significantly greater than in male mice over the same period (Table 7; see the row labeled "Sex:Duration").

EFFECTS OF DE EXPOSURE LEVEL ON PLASMA MARKERS IN RATS

Only two plasma markers, IgG (Table 11) and leptin (Table 12), were significantly altered by DE exposure independent of sex or exposure duration (see the rows labeled "Exposure level"). ANOVA post-hoc tests indicated that IgG values at both the low and mid levels of DE exposure were significantly lower than those of the matched air-exposure groups (Table 13), and that the leptin value at the low level of DE exposure level was significantly greater than that of the matched air-exposure group (Table 14).

The effects of DE exposure levels on CHOL and HDL were different across sex and exposure duration, which is reflected by a three-way interaction (i.e., exposure level, sex, and duration; see Table 11). The ANOVA post-hoc tests indicated that (1) mean CHOL values of male rats exposed 1 month to both mid- and high-level DE were significantly greater than those of the matched air controls (Tables 3 and 13; Figure 1, top-left panel), and the CHOL level of female rats exposed 3 months to mid-level DE was significantly lower than that of the matched control (Tables 3 and 13; Figure 1, bottom-right panel); and (2) the mean HDL values for male rats exposed 1 month to both mid- and high-level DE were significantly higher than those of the matched controls (Tables 3 and 13; Figure 1, top-left panel), and the mean HDL value for female rats exposed 3 months to mid-level DE was significantly lower than that of the matched air control (Tables 3 and 13; Figure 1, bottom-right panel). These data indicate that DE emissions exposure altered total cholesterol levels (driven by changes in HDL) in male rats (increased levels, 1-month exposure) and female rats (decreased levels, 3-month exposure) yet in opposite directions and at different durations (Figure 1).

EFFECTS OF SEX AND EXPOSURE DURATION ON PLASMA MARKERS IN RATS

Several markers were changed either as a function of sex or of exposure duration but not by an interaction of these factors, because for these markers no interaction between exposure duration and sex occurred. As shown in Tables 11 and 12 [see the rows labeled "Sex (female = 1)"], female rats had significantly lower levels of CHOL, TRIG, IgM, MCP-1, IL-10, sICAM-1, and leptin than did male rats; and female rats had significantly higher levels of TP, AST, LDH, and IgG than male rats. Rats exposed 3 months had significantly lower levels of LDL, NAP, MCP-1, TNF-α, VEGF, and sICAM-1 than rats exposed 1 month; and rats exposed 3 months had significantly higher HDL/LDL ratios and significantly higher levels of AST, IgG, IgM, KC, and leptin than rats exposed 1 month [Tables 11 and 12; see the rows labeled "Duration (3 months = 1)"].

Some plasma marker levels varied between females and males, with different patterns at 1-month and 3-month exposures, indicated by the interaction between exposure duration and sex. As shown in Tables 11 and 12 [see the rows labeled "Sex (female = 1)"], after a 1-month exposure female rats had significantly lower CHOL than male rats; and female rats had significantly higher levels of ALB and CRP than male rats exposed 1 month. Male rats exposed 3 months had significantly higher levels of ALB and CREA than male rats exposed 1 month [Tables 11 and 12; see the rows labeled "Duration (3 months = 1)"]. The delta change for CREA in female rats between 3- and 1-month exposures was significantly lower than that for male rats; likewise, the delta change for ALT in female rats between 3- and 1-month exposures was significantly higher than the change score in male rats (Table 11; see the row under Parameters labeled "Sex:Duration"). However, ALT and CREA levels in female rats exposed 1 month were not significantly different from those of male rats exposed 1 month [Table 11; see the row labeled "Sex (female = 1)"], yet male rats exposed 3 months had significantly greater CREA (but not ALT) readings than male rats exposed 1 month [Table 11; see row labeled "Duration (3 months = 1)"]. Because these changes were independent of exposure level and modest, the biological significance of these changes is uncertain.

Table 11. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Each Plasma Marker Measured in Male and Female Rats Exposed to Air or DE for 1 Month or 3 Months[a]

	CHOL Original Scale		HDL Original Scale		LDL Box–Cox Transformation		HDL/LDL Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	41.1	0.868	47.1	0.603	0.022	0.867	0.061	0.370
Sex (*df* = 1)	590.6	0.065	4.76	0.803	0.225	0.118	0.210	0.057
Duration (*df* = 1)	7.225	0.837	218.2	0.092	0.405	0.037[b]	0.757	<0.001[b]
Exposure level:Sex (*df* = 3)	379.5	0.088	156.8	0.107	—	—	—	—
Exposure level:Duration (*df* = 3)	695.2	0.008[b]	355.2	0.004[b]	—	—	—	—
Sex:Duration (*df* = 1)	267.2	0.213	242.0	0.076	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	679.4	0.009[b]	305.8	0.009[b]	—	—	—	—
Residuals	170.9	NA	75.9	NA	0.091	NA	0.057	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−6.43	0.029[b]	−2.11	0.28	−0.07	0.118	0.07	0.057
Duration (3 months = 1)	−2.16	0.461	−0.12	0.949	−0.10	0.037[b]	0.14	<0.001[b]
Sex:Duration	5.17	0.213	4.92	0.076	—	—	—	—

	TRIG Box–Cox Transformation		ALB Rank Transformation		TP Rank Transformation		NAP Box–Cox Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	1.458	0.734	774.3	0.36	873.1	0.59	0.003	0.86
Sex (*df* = 1)	133.1	<0.001[b]	221,787.0	<0.001[b]	128,199.0	<0.001[b]	0.007	0.419
Duration (*df* = 1)	1.205	0.553	3,339.8	0.033[b]	483.03	0.553	0.113	0.001[b]
Exposure level:Sex (*df* = 3)	—	—	1,552.8	0.095	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	1,404.2	0.164	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	3.415	NA	718.3	NA	1,363.4	NA	0.01	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−1.82	<0.001[b]	80.39	<0.001[b]	56.61	<0.001[b]	0.013	0.419
Duration (3 months = 1)	−0.17	0.553	15.06	0.013[b]	−3.48	0.553	−0.053	0.001[b]
Sex:Duration	—	—	−11.85	0.164	—	—	—	—

(*Table continues on next page*)

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 11 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Each Plasma Marker Measured in Male and Female Rats Exposed to Air or DE for 1 Month or 3 Months[a]

	ALT Box–Cox Transformation		AST Rank Transformation		CK Rank Transformation		CREA Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	0.02	0.245	194.2	0.958	509.3	0.869	344.2	0.829
Sex (*df* = 1)	0.073	0.023[b]	10,677.0	0.018[b]	2694.9	0.263	3,737.6	0.076
Duration (*df* = 1)	0.237	<0.001[b]	42,380.0	<0.001[b]	45.86	0.884	139,385.0	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	0.028	0.117	—	—	—	—	—	—
Sex:Duration (*df* = 1)	0.085	0.015[b]	—	—	—	—	12,458.0	0.001[b]
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	0.014	NA	1,868.0	NA	2134.3	NA	1,168.5	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−0.004	0.867	16.34	0.018[b]	−8.25	0.264	8.28	0.281
Duration (3 months = 1)	0.032	0.234	32.55	<0.001[b]	1.08	0.884	76.81	<0.001[b]
Sex:Duration	0.093	0.015[b]	—	—	—	—	−35.41	0.001[b]

	LDH Rank Transformation		IgE Box–Cox Transformation		IgG Rank Transformation		IgM Original Scale	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	661.5	0.816	6.915	0.082	3,154.0	0.015[b]	2,958.2	0.163
Sex (*df* = 1)	8842.6	0.042[b]	3.510	0.285	20,048.0	<0.001[b]	7,617.6	0.036[b]
Duration (*df* = 1)	995.0	0.494	0.284	0.761	176,757.0	<0.001[b]	188,335.0	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	2112.0	NA	3.04	NA	875.7	NA	1,707.6	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	14.88	0.043[b]	0.298	0.285	22.39	<0.001[b]	−13.8	0.036[b]
Duration (3 months = 1)	5.00	0.494	−0.085	0.761	66.48	<0.001[b]	68.62	<0.001[b]
Sex:Duration	—	—	—	—	—	—	—	—

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.

[b] *P* value < 0.05.

Table 12. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Models for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or DE for 1 Month or 3 Months[a]

	GM-CSF Rank Transformation		MCP-1 Rank Transformation		IL-1β Box–Cox Transformation		IL-6 Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	3037.9	0.233	251.9	0.92	1.174	0.58	1639.7	0.517
Sex (*df* = 1)	7631.4	0.059	92,016.0	<0.001[b]	4.873	0.101	2504.3	0.283
Duration (*df* = 1)	30.6	0.904	12,058.0	0.006[b]	0.001	0.999	1606.6	0.389
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	2107.1	NA	1,531.1	NA	1.788	NA	2153.3	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−13.81	0.059	−48.0	<0.001[b]	−0.35	0.101	−7.91	0.283
Duration (3 months = 1)	−0.88	0.904	−17.36	0.006[b]	−0.0002	0.999	6.34	0.389
Sex:Duration	—	—	—	—	—	—	—	—

	IL-10 Rank Transformation		IFN-γ Rank Transformation		KC Rank Transformation		TNF-α Rank Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	996.89	0.706	446.61	0.891	1,378.1	0.559	600.7	0.791
Sex (*df* = 1)	8658.3	0.046[b]	6477.03	0.085	2,681.4	0.248	558.8	0.571
Duration (*df* = 1)	1.81	0.977	66.31	0.861	27,563.0	<0.001[b]	34,663.0	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	2139.6	NA	2156.4	NA	1,992.9	NA	1,729.0	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−14.71	0.046[b]	−12.73	0.085	8.19	0.248	−3.74	0.571
Duration (3 months = 1)	−0.21	0.977	−1.29	0.861	26.25	<0.001[b]	−29.44	<0.001[b]
Sex:Duration	—	—	—	—	—	—	—	—

(*Table continues on next page*)

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.
[b] *P* value < 0.05.

Table 12 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Models for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or DE for 1 Month or 3 Months[a]

Sources of Variation	VEGF Rank Transformation		sICAM-1 Rank Transformation		Fibrinogen Original Scale		Leptin Box–Cox Transformation	
	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level (*df* = 3)	452.4	0.845	444.1	0.667	8557.1	0.082	1.404	0.009[b]
Sex (*df* = 1)	186.98	0.737	179,627.0	<0.001[b]	129.42	0.853	46.93	<0.001[b]
Duration (*df* = 1)	17,260.8	0.002[b]	29,322.2	<0.001[b]	956.0	0.615	6.059	<0.001[b]
Exposure level:Sex (*df* = 3)	—	—	—	—	—	—	—	—
Exposure level:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Sex:Duration (*df* = 1)	—	—	—	—	—	—	—	—
Exposure level:Sex:Duration (*df* = 3)	—	—	—	—	—	—	—	—
Residuals	1,655.5	NA	849.8	NA	3767.4	NA	0.35	NA
	Estimate		Estimate		Estimate		Estimate	
Parameters								
Sex (female = 1)	−2.03	0.753	−67.01	<0.001[b]	1.799	0.853	−1.08	<0.001[b]
Duration (3 months = 1)	−20.84	0.002[b]	−27.08	<0.001[b]	4.89	0.615	0.390	<0.001[b]
Sex:Duration	—	—	—	—	—	—	—	—

Sources of Variation	CRP Box–Cox Transformation	
	Mean Square	*P* Value
Exposure level (*df* = 3)	0.101	0.881
Sex (*df* = 1)	8.14	<0.001[b]
Duration (*df* = 1)	0.053	0.734
Exposure level:Sex (*df* = 3)	—	—
Exposure level:Duration (*df* = 3)	—	—
Sex:Duration (*df* = 1)	1.65	0.058
Exposure level:Sex:Duration (*df* = 3)	—	—
Residuals	0.454	NA
	Estimate	
Parameters		
Sex (female = 1)	0.654	<0.001[b]
Duration (3 months = 1)	0.239	0.114
Sex:Duration	−0.406	0.0584

[a] *df* indicates degrees of freedom; dash (—) indicates no interaction of factors; NA indicates not available.

[b] *P* value < 0.05.

Table 13. Rats: Comparisons of DE Exposure Groups and Controls for the Markers Whose Final ANOVA Models Included Significant Exposure Effect or Significant Interaction of Exposure with Sex or Duration[a]

	CHOL		HDL		IgG[b]	
	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
Rats: 1-Month Exposure						
Male						
Low vs. Control	4.30	0.463	1.902	0.626	−17.7	0.008[c]
Mid vs. Control	15.92	0.007[c]	9.927	0.012[c]	−14.238	0.033[c]
High vs. Control	16.83	0.005[c]	12.11	0.002[c]	−1.663	0.802
Overall *P* value		0.008[c]		0.004[c]		0.015[c]
Female						
Low vs. Control	8.419	0.152	6.526	0.096	−17.7	0.008[c]
Mid vs. Control	6.854	0.243	5.565	0.155	−14.238	0.033[c]
High vs. Control	−4.70	0.423	−1.539	0.693	−1.663	0.802
Overall *P* value		0.092		0.101		0.015[c]
Rats: 3-Month Exposure						
Male						
Low vs. Control	7.389	0.208	5.313	0.175	−17.7	0.008[c]
Mid vs. Control	−0.42	0.943	−1.751	0.654	−14.238	0.033[c]
High vs. Control	0.089	0.988	1.887	0.629	−1.663	0.802
Overall *P* value		0.482		0.310		0.015[c]
Female						
Low vs. Control	−10.97	0.063	−7.446	0.058	−17.7	0.008[c]
Mid vs. Control	−17.3	0.004[c]	−11.54	0.004[c]	−14.238	0.033[c]
High vs. Control	−4.33	0.460	−2.683	0.492	−1.663	0.802
Overall *P* value		0.021[c]		0.018[c]		0.015[c]

[a] Shown are overall *P* values for the exposure effects. Also shown are the estimated differences and *P* values based on comparing the exposure groups to the control for each sex and duration.

[b] Because the final ANOVA for IgG did not indicate either an interaction between exposure level and duration or an interaction between exposure level and sex, the estimation of the difference between each exposure level and the control is the same across different exposure durations and sexes.

[c] *P* value < 0.05.

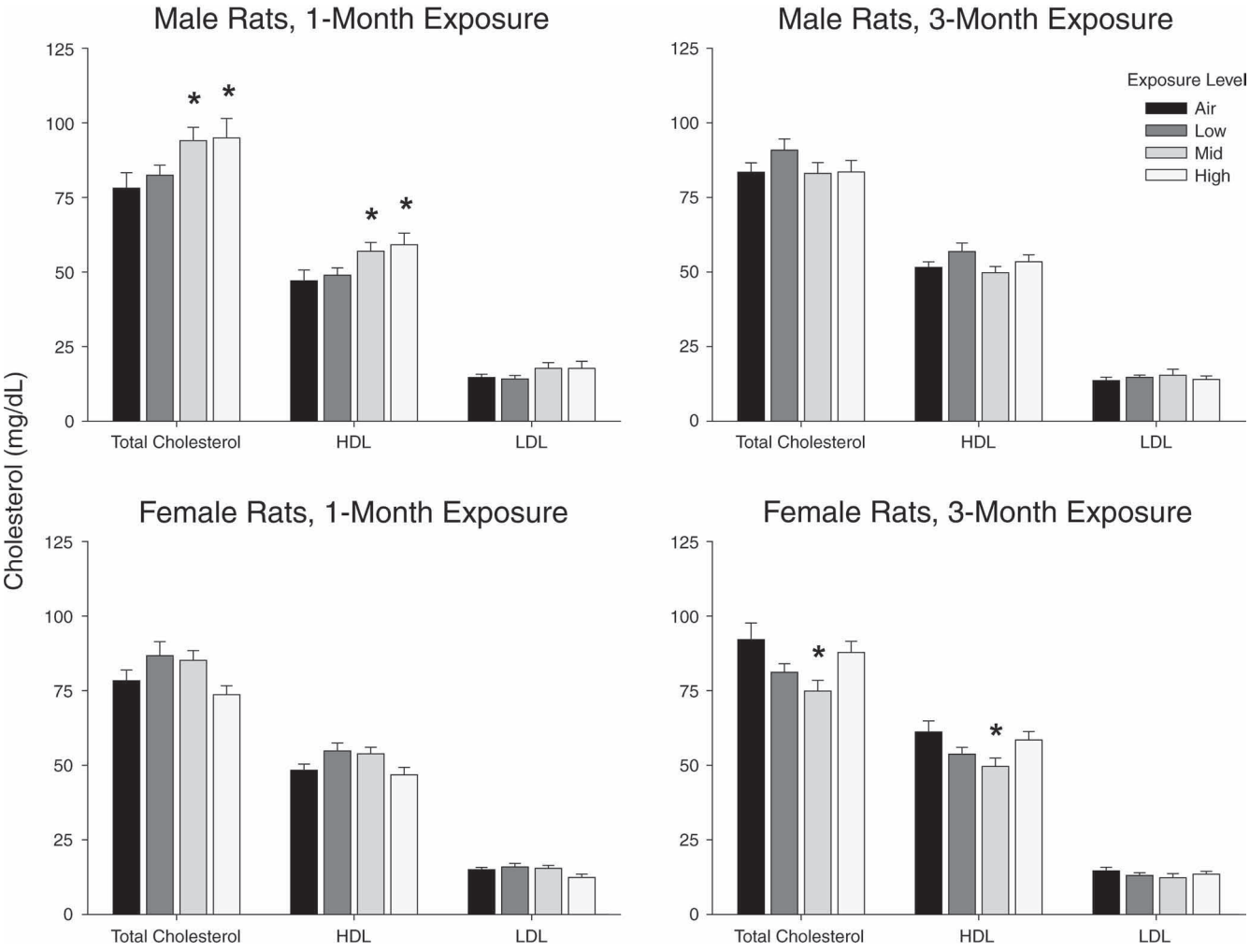

Figure 1. Effects of short-term DE emission exposure on plasma cholesterol in rats. Top-left panel: Total cholesterol and HDL but not LDL cholesterol levels of male rats were significantly increased by exposure to mid- and high-level DE after 1 month of exposure. **Top-right panel:** No changes in total cholesterol or HDL or LDL cholesterol levels of male rats were measured after 3 months of DE exposure. **Bottom-left panel:** Total cholesterol and HDL and LDL cholesterol levels of female rats were unaltered by DE exposure after 1 month of exposure. **Bottom-right panel:** Total cholesterol and HDL but not LDL cholesterol levels of female rats were significantly decreased by mid-level exposure to DE after 3 months of exposure. Asterisk (*) indicates $P < 0.05$ based on the comparison analyses under the framework of the three-way ANOVA; $n = 9–10$ rats per group.

Table 14. Rats: Comparisons with Controls of Leptin in Each DE Exposure Group[a]

	Leptin[b]	
	Estimate	*P* value
Rats: 1-Month Exposure		
Male		
Low vs. Control	0.358	0.008[c]
Mid vs. Control	−0.039	0.769
High vs. Control	0.229	0.088
Overall *P* value		0.008[c]
Female		
Low vs. Control	0.358	0.008[c]
Mid vs. Control	−0.039	0.769
High vs. Control	0.229	0.088
Overall *P* value		0.008[c]
Rats: 3-Month Exposure		
Male		
Low vs. Control	0.358	0.008[c]
Mid vs. Control	−0.039	0.769
High vs. Control	0.229	0.088
Overall *P* value		0.008[c]
Female		
Low vs. Control	0.358	0.008[c]
Mid vs. Control	−0.039	0.769
High vs. Control	0.229	0.088
Overall *P* value		0.008[c]

[a] Shown are overall *P* values for the exposure effects. Also shown are the estimated differences and *P* values based on comparing the exposure groups to the control for each sex and duration.

[b] Because the final ANOVA for leptin did not indicate either an interaction between exposure level and duration or an interaction between exposure level and sex, the estimation of the difference between each exposure level and the control is the same across different exposure durations and sexes.

[c] *P* value < 0.05.

DISCUSSION

To better understand how air pollution, and specifically diesel engine emissions, affects CVD risk, the potentially causative (primary) events must be disentangled from secondary (pathological) or compensatory responses. To this end, we measured 29 plasma markers representative of inflammation (vascular and systemic), immune function, and general toxicity that either reflect injury to a specific organ (e.g., ALT released from injured liver into the blood) or are associated with a stress-response pathway (e.g., increased SAA is indicative of an APR). The primary objective was to identify a sensitive marker (or subset of markers) that is altered both very early after the onset of DE exposure and at a low level of DE. Because a marker that is altered early and is sensitive to DE level could be causally related to subsequent pathology or increased CVD risk associated with chronic DE exposure, it is imperative to identify such a biomarker. Moreover, we expect that a useful biomarker would be conserved across species and that mice, rats, and humans would possess this marker. Likewise, we also expect the molecular regulatory signals to be conserved across species. By these standards, the plasma markers measured in this study were appropriately chosen as potential markers. Moreover, previous studies have examined some of the same endpoints (e.g., CHOL) using female and male rats exposed subchronically (as in the present study) or chronically to DE (Reed et al. 2004, 2005, 2006; Seagrave et al. 2005).

According to the criteria indicated above, we observed changes in the peak and pattern of plasma total cholesterol and HDL levels in male rats exposed to mid- and high-level DE for 1 month (see Table 3; Figure 1, top-left panel) that were consistent with cholesterol being a biomarker of DE exposure. Moreover, the absolute change in cholesterol was similar to that induced by acrolein gavage (0.5 mg/kg) in rats (compare the values in Table 3 with those in Table 6) (Conklin et al., 2011a). However, the change in cholesterol was not sustained in male rats after 3 months of DE exposure, nor was the increased cholesterol observed in female rats or in mice at either exposure duration. Thus, although there is evidence that 1-month DE exposure increased total and HDL cholesterol levels in male rats (see Table 3), the biological significance and mechanism of this change are unknown. For example, we found little corroborating evidence that this change was part of an overall APR. Moreover, previous reports show that DE exposure induces a decreased cholesterol level in rats (Reed et al. 2004, 2005, 2006), a change that was observed only in female rats exposed to mid-level DE for 3 months in the present ACES study (see Table 3; see McDonald et al. 2012). Whether circulating cholesterol is a sensitive marker of DE exposure in rats is unclear and warrants further study in rodents.

Although stimulation of the APR is one possible response to DE-induced toxicity, we also investigated markers of vascular inflammation and thrombosis. Activated endothelium and platelets secrete chemokines and growth factors (e.g., VEGF, PDGF, PF4) and increase expression of adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), after injury. Subsequent shedding of the extracellular receptor portion of adhesion molecules leads to increased circulating levels of sICAM and soluble VCAM-1 (Nakashima et al. 1998). Acrolein gavage significantly decreases the sICAM-1 level in mice and increases it in rats (Conklin et al. 2011a,b), an effect

that is not observed in any DE-exposed group of mice or rats in the present ACES study (Tables 2 and 4). Moreover, the plasma level of PF4, which is increased by acrolein inhalation (Sithu et al. 2010), is unaltered by DE exposures in mice (see Table 1). Unfortunately, no commercial assay is available for the measurement of PF4 in rats. Collectively, these data indicate little evidence of activation of the endothelium or platelets in mice or rats by subchronic DE exposure at the levels of NO_2 and the concentrations of PM (highest level PM < 10 $\mu g/m^3$) used in this ACES study (see the study by McDonald et al., Part 1 of this volume). These levels were significantly lower than those used in previous studies of DE emissions (Campen et al. 2010; lowest level PM ≈ 109 $\mu g/m^3$) (Reed et al. 2004, 2005, 2006).

To measure 29 plasma markers of inflammation, immune function, and general toxicity, assays were performed at the University of Louisville or via a commercial vendor using well-established assays, calibration standards, and positive controls for quality control and assurance. Although many analytes are stable while frozen at −80°C, analyte values can vary over time because of decay and alterations in assay protocol, sensitivity, or accuracy. To reduce variation, all rat and mice samples were processed together, and freezing and thawing was thus minimized. For lipid and protein analyses performed at the University of Louisville, samples were thawed only once before analysis, and a separate aliquot was sent on dry ice to the commercial vendor for cytokine and chemokine array analysis. Excellent concordance was observed between the analytical values for plasma lipids and proteins measured in the current study and those obtained previously using these same positive control samples (Conklin et al. 2010, 2011a). Moreover, commercial vendor analyses of plasma sICAM-1 and SAA levels in our murine positive controls corresponded well with our previous measurements of those analytes using commercially available ELISA kits (i.e., for acrolein-treated mice) (Conklin et al. 2011b). Although the data presented here and those provided by McDonald and colleagues (see Part 1 of this volume) on rat sera chemistry (only for rats exposed 3 months) provided assay validation for some analytes, uncertainty remained about the quality and robustness of assays for many other analytes, especially some of those measured via commercial vendor (e.g., murine fibrinogen, rat SAA) (Marhaug and Dowton 1994). We conclude that assay conditions and protocols were appropriate for the majority of endpoints measured but not for all.

Although methodological approaches can be a source of variation, especially between different studies, some variation in the current study was not due to methodology because all samples were processed uniformly within a 72-hour period in batches using the same reagents and methods (in both in-house and commercial-vendor assays). One source of biological variation could be that the rodents were not fasted prior to euthanasia and collection of blood plasma. Because circulating lipid levels are dependent on the fed state, we expected plasma CHOL and TRIG levels to be more highly variable, and although this was observed for some markers, most endpoints were relatively uniform and in the expected normal range (albeit high normal); for CHOL, compare Tables 1 (mice) and 3 (rats) with Tables 5 (mice) and 6 (rats). A shift in the baseline because the rodents were unfasted could obfuscate more subtle changes resulting from DE exposure. For example, air-exposed male rats at 3 months had a baseline TRIG level (i.e., 115 ± 15 mg/dL) that was +22 mg/dL higher than that of male rats at 1 month of air exposure (Table 3). Although this increased baseline at 3 months was not statistically significant, it illustrates a potential problem with interpreting statistically significant changes observed in lipid markers, some of which are important risk factors in human CVD. For example, total cholesterol and HDL cholesterol were significantly decreased in female rats exposed for 3 months to mid-level DE compared with the air controls; however, the total cholesterol level in female air controls exposed for 3 months was 14 mg/dL higher than that in the 1-month controls (see Tables 13 and 3). Other unexplained and dramatic increases in baseline levels of cytokines and chemokines were also present in air-exposed female mice. For example, at 3 months of exposure, the levels of the following were two to three times the levels at 1 month: GM-CSF, MCP-1, IL-1β, and IL-10 (see Table 2). These changes are not easily explained by an unfasted state. The source of variation in these baselines and its biological significance are unclear at this time, and, perhaps, this variation reflects the effects of an unidentified condition associated with longer-term exposures (e.g., age, noise, and single housing) (Peng et al. 1989; Perkins and Lipman 1996; Perez et al. 1997).

Despite the variation in endpoint levels, we used a multilayered statistical approach that teased out DE-level effects from those dependent on exposure duration (as discussed above) or on sex only by testing for interactions using linear models and three-way ANOVA. We systematically identified only six markers that were altered by DE level independent of sex or exposure duration. These markers could be useful because of this independence; yet of the ones identified (mice: HDL, KC, fibrinogen, and CRP; rats: leptin and IgG) none had convincing changes that were dependent on exposure level, uniform, and consistent. Moreover, DE exposure decreased fibrinogen and CRP levels in mice and IgG levels in rats, and such changes do not fit with current paradigms suggesting

increased levels of fibrinogen in an APR or in CRP in immune activation (Giffen et al. 2003; Peisajovich et al. 2008). The DE-induced change in HDL in mice was marginal, and the change in leptin (an appetite suppressant) level was confounded by the unfasted state, so these findings appear of limited significance.

Injurious stimuli, including lipopolysaccharide from gram-negative bacteria, turpentine, burn trauma, cyclophosphamide, and acrolein, trigger an APR that induces changes in APR reactants such as lipids (increased CHOL, HDL, LDL, and TRIG) and proteins (hypoalbuminemia, increased SAA) that are in part transcriptionally regulated (Loudet et al. 1984; Kitagawa et al. 1992; Kindy et al. 2000; Conklin et al. 2010). The APR is a coordinated and rapid response to either local or systemic injury, and we included plasma samples from acrolein-gavaged mice and rats as examples of an "APR-like" state or condition with a robust increase in lipids relative to water-fed controls (see Tables 5 and 6). Although we did not observe a similar magnitude of lipid change in mice exposed to DE in the present study as that evoked by acute acrolein gavage (Conklin et al. 2010, 2011b), this was not wholly unexpected given the levels of dilute DE used in the present study (see also McDonald et al., Part 1 of this volume).

CONCLUSIONS

Despite measuring a large number of important plasma indicators of CVD risk and general toxicity, we found little evidence of any sustained injury that would especially reflect organ- or cardiovascular-system-level toxicity. In this ACES study, data analyses indicate that DE emissions exposure likely triggers an exposure-level-dependent, yet modest and short-lived, increase in cholesterol in male rats that is not accompanied by systemic inflammation (e.g., no changes in SAA or CRP). Moreover, there does not appear to be a concomitant or robust endothelium injury related to DE exposure level (e.g., no change in sICAM-1 or VEGF). Thrombosis did not appear stimulated in mice, but we tested only one marker of thrombosis (fibrinogen) in rats, so we cannot draw a conclusion about this arm of our hypothesis. It appears that although changes in cholesterol level may be an early marker of DE-induced effects, these changes did not persist after 3 months of DE exposure. To fully evaluate the CVD risk associated with DE emission exposure, additional work is required to identify bona fide sensitive and sustained markers associated with DE exposure. It is important to note that new studies evaluating new technologies (e.g., engines, fuel) are required, as it is difficult to compare current and past studies of DE exposure performed under very different conditions (in particular, different PM concentrations) (Campen et al. 2005, 2010; Cherng et al. 2009, 2011; Maresh et al. 2011) (see also McDonald et al., Part 1 of this volume).

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APPENDIX A. Statistical Analysis Methods

For each marker, we started with a three-way ANOVA (with exposure level, sex, and exposure duration as factors) as well as two-way interactions of factors (exposure level by sex, exposure level by exposure duration, and sex by exposure duration) and a three-way interaction

(exposure level by sex by exposure duration). To examine the validity of the model assumption in terms of normality and equal variance among different groups, a residual plot and the Shapiro–Wilk test were applied to the residuals of the three-way ANOVA model (Faraway 2005). If the Shapiro–Wilk test indicated that the residuals from the three-way ANOVA model were not normally distributed, the Box–Cox transformation was considered for the marker (Box and Cox 1964). If normality was still lacking after the Box–Cox transformation, then the ranks of observations for the marker were applied (Christensen 2002). Whether a transformation was applied and what type are indicated by the column heads "Rank Transformation" or Box-Cox Transformation" in Tables 7, 8, 11, and 12 (where "Original Scale" indicates that no transformation was applied). For each marker or its transformation (where appropriate), we first carried out three-way ANOVA, with exposure level, sex, and exposure duration as factors, and included all two-way and three-way interactions of the factors. If the three-way interaction was not significant, we excluded it and explored whether we could further remove insignificant two-way interactions by using the AIC (Venables and Ripley 2002). We retained the exposure level, sex, and exposure duration in the final model whether or not they were significant.

The mean squares for each factor and their interactions (if any) in the final model and the *P* values for these significance tests are reported in Tables 7, 8, 11, and 12. Based on the final model, we performed ANOVA post-hoc tests to examine the differences between females and males, between 1- and 3-month exposures, and for the interaction between sex and exposure duration, which are also reported in Tables 7, 8, 11, and 12. If an interaction between sex and exposure duration existed, the estimates in the rows labeled "Sex (female = 1)" are the differences between the values for females and males with 1 month of exposure. The estimates in the rows labeled "Duration (3 months = 1)" are the differences between the values for male 3- and 1-month exposures. The estimates in the rows labeled "Sex:Duration" are the delta change of females between 3- and 1-month exposures versus the delta change of males between 3- and 1-month exposures. If there was no interaction between sex and exposure duration, the differences between female and male values were independent of exposure duration, and the differences between the values for 1- and 3-month exposures were independent of sex. In these cases, the estimates in the rows labeled "Sex (female = 1)" are the differences between the values for females and males, and the estimates in the rows labeled "Duration (3 months = 1)" are the differences between the values for male 3- and 1-month exposures.

In addition, we performed ANOVA post-hoc testing to determine whether the results for each exposure level were significantly different from those for the control, for each combination of exposure duration and sex. The significant results for exposure-level effects are reported in Tables 9, 10, 13, and 14. When the final ANOVA model included a three-way interaction, or a two-way interaction between exposure level and exposure duration as well as a two-way interaction between exposure level and sex, the estimation of the differences between each exposure level and the control varied by exposure duration and sex (see CK, CREA, and IgE in Table 9; leptin in Table 10; and CHOL and HDL in Table 13). However, if the final ANOVA model indicated only a two-way interaction between exposure level and exposure duration, the difference between exposure level and the control was dependent on exposure duration but not sex; this resulted in the same estimated values for females and males (see KC in Table 10). If the final ANOVA model had neither a two-way interaction between exposure level and sex nor a two-way interaction between exposure level and exposure duration, the difference between exposure level and the control did not depend on sex or exposure duration. This resulted in equivalent estimated differences by sex and exposure duration (these conditions apply for HDL, Table 9; fibrinogen and CRP, Table 10; IgG, Table 13; and leptin, Table 14).

All analyses were carried out using the statistics software R (*www.r-project.org*). The major functions we used were for linear model (lm), ANOVA (anova), and the AIC-based model selection (step). A *P* value smaller than 0.05 was considered statistically significant.

ABOUT THE AUTHORS

Daniel J. Conklin, Ph.D., received his doctorate from the University of Notre Dame in 1995 and was a National Institute of Environmental Health Sciences toxicology postdoctoral fellow at the University of Texas Medical Branch at Galveston from 1996 to 1998. He has published over 50 peer-reviewed manuscripts, book chapters, and editorials on cardiovascular physiology and toxicology. He is a full member of the Society of Toxicology (SOT) and vice-president of the Cardiovascular Toxicology Specialty Section of SOT. He is cochairman of an American Heart Association grant review panel on vascular and endothelium biology and has been a member of the editorial board of the journal *Toxicology and Applied Pharmacology* since 2007. His laboratory, funded by the National Institutes of Health, investigates the cardiovascular toxicity

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ABBREVIATIONS AND OTHER TERMS

AIC	Akaike information criterion
ALB	albumin
ALT	alanine aminotransferase
ANOVA	analysis of variance
APR	acute phase response
AST	aspartate aminotransferase
CHOL	cholesterol
CK	creatine kinase
CREA	creatinine
CRP	C-reactive protein
CVD	cardiovascular disease
DE	diesel exhaust
DEP	diesel exhaust particles
ELISA	enzyme-linked immunosorbent assay
ET-1	endothelin-1
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDL	high-density lipoprotein
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-10	interleukin-10
KC	keratinocyte-derived chemoattractant (also called CXCL1)
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LRRI	Lovelace Respiratory Research Institute
MCP-1	monocyte chemotactic protein-1
NAP	nonalbumin protein
NO_2	nitrogen dioxide
PM	particulate matter
$PM_{2.5}$	particulate matter ≤ 2.5 μm in aerodynamic diameter
PF4	platelet factor 4
SAA	serum amyloid A
SE	standard error
sICAM-1	soluble intercellular adhesion molecule-1
TNF-α	tumor necrosis factor-α
TP	total protein
TRIG	triglycerides
SD	standard deviation
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

Research Report 166, *Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity*, J.D. McDonald et al., J.C. Bemis et al., L.M. Hallberg et al., and D.J. Conklin and M. Kong

INTRODUCTION

This Commentary summarizes HEI's independent evaluation of four studies conducted as one phase — Phase 3B — of the multipart Advanced Collaborative Emissions Study (ACES*) program. These studies investigated the health effects of subchronic exposures of mice and rats to diesel exhaust (DE) emissions from a heavy-duty diesel-engine system compliant with 2007 regulations. The studies were led by Dr. Jacob McDonald, of Lovelace Respiratory Research Institute (LRRI), Albuquerque, New Mexico; Dr. Jeffrey Bemis, of Litron Laboratories, Rochester, New York; Dr. Lance Hallberg, of the University of Texas Medical Branch, Galveston, Texas; and Dr. Daniel Conklin, of the University of Louisville, Louisville, Kentucky.

HEI's evaluation of the ACES Phase 3B studies was conducted by a specially convened ACES Review Panel, which reviewed the Investigators' Reports submitted by the principal investigators.

The reports included in this volume by Bemis, Hallberg, and Conklin and their colleagues include the 1- to 3-month results from their studies. The report by McDonald and colleagues includes some 12-month data as well as the 1- and 3-month results.

All four investigator teams will submit reports that cover assessments of the health effects of chronic exposure to DE, for 24 months or even longer. Publication in 2014 of those final ACES Phase 3B reports will be accompanied by a commentary that summarizes the ACES Review Panel's assessment of the reports.

This Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of these studies and by placing these Investigators' Reports (IRs) into scientific and regulatory perspective.

BACKGROUND TO THE ACES PROGRAM

In 2001, in light of concerns identified by scientific research over many decades about the potential health effects of diesel emissions, the U.S. Environmental Protection Agency (U.S. EPA) and California Air Resources Board (CARB) adopted stringent new standards for DE emissions and fuel for light- and heavy-duty highway diesel engines. Based on technological advances by industry, these standards set goals for reducing concentrations of major components of DE over the next several years. Engines needed to meet a new standard for particulate matter (PM) (light-duty engines by 2006 and heavy-duty engines by 2007) and for nitrogen oxides (NO_x, primarily nitrogen monoxide [NO] and nitrogen dioxide [NO_2] by 2010). The engine manufacturers and other industries developed advanced engine technology and exhaust aftertreatment systems (in particular, diesel particle filters to reduce PM emissions and selective catalytic reduction devices for reducing NO_x emissions). The regulatory agencies also mandated that sulfur in fuel be reduced substantially; such ultra-low-sulfur fuels (< 15 ppm) were essential for proper functioning of the aftertreatment systems.

Dr. McDonald's study, "Development of a Diesel Exhaust Exposure Facility and Conduct of a Chronic Inhalation Bioassay in Rats and Mice," began in February 2010. The draft Investigators' Report from Dr. McDonald and colleagues was received for review in July 2011. A revised report, received in December 2011, was accepted for publication in January 2012.

Dr. Bemis's study, "Genotoxicity of Inhaled Diesel Exhaust: Examination of Rodent Blood for Micronucleus Formation," began in February 2010. The draft Investigators' Report from Dr. Bemis and colleagues was received for review in June 2011. A revised report, received in December 2011, was accepted for publication in January 2012.

Dr. Hallberg's study, "Assessment of the Genotoxicity of Diesel Exhaust/Diesel Exhaust Particulates from Improved Diesel Engines," began in May 2010. The draft Investigators' Report from Dr. Hallberg and colleagues was received for review in August 2011. A revised report, received in December 2011, was accepted for publication in January 2012.

Dr. Conklin's study, "Effects of Diesel Emissions on Vascular Inflammation and Thrombosis," began in February 2010. The draft Investigators' Report from Conklin and Kong was received for review in August 2011. A revised report, received in December 2011, was accepted for publication in January 2012.

During the review process, the HEI ACES Review Panel and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Reports and the Review Panel's Commentary.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

* Lists of abbreviations and other terms appear at the end of the Investigators' Reports.

Introduction of these changes was expected to result in substantially reduced emissions of PM and NO_x, as well as other exhaust constituents such as air toxics.

The U.S. EPA and CARB projected that the reduction in pollutant emissions that would result from the introduction of new technologies and fuels during the past decade — about a 90% reduction compared with pre-2007 engine systems — would have substantial public health benefits. HEI's industrial sponsors and government officials at the U.S. EPA, CARB, and the U.S. Department of Energy (U.S. DOE) expressed strong interest in research into the emissions and health effects of the new diesel technology, with the expectation that reduced emissions would also lead to reduced adverse health effects. After planning workshops with multiple stakeholders in 2003 and 2004, HEI — working in collaboration with the Coordinating Research Council (CRC), a nonprofit organization with expertise in emissions characterization — launched ACES.

The overall goal of ACES was to characterize the exhaust emissions of these new advanced heavy-duty diesel (Class 8) engines with aftertreatment systems and using ultra-low-sulfur fuels and to assess the possible adverse health effects of exposure to these emissions.

SELECTION OF ACES PHASE 3B CORE AND ANCILLARY STUDY INVESTIGATORS

As described in the Preface to this ACES report, ACES was designed in three phases. Phases 1 and 2 focused on emissions characterization; Phase 3 focused on an evaluation of health effects. In view of its expertise in emissions characterization and its existing expert committee and oversight structure to plan and monitor such activities, CRC took a leading role in the Phase 1 and 2 studies, whereas HEI took the leading role in the Phase 3 studies. The overall effort was led by HEI and guided by an ACES Steering Committee consisting of representatives of CRC, the U.S. DOE, engine manufacturers, the U.S. EPA, the petroleum industry, CARB, manufacturers of emission-control devices, the National Resources Defense Council, and others. At the inception of ACES, the first step taken jointly by the CRC and HEI committees was the development of a detailed project plan that formed the basis for the subsequent requests for applications issued by CRC and HEI.

The design and implementation of Phase 3 and reporting of results were the responsibility of HEI and were overseen by the ACES Oversight Committee (a subset of the HEI Research Committee augmented by other independent experts from several disciplines), with advice from a Health Advisory Committee of ACES stakeholder experts. In 2006 HEI issued Request for Proposals (RFP) 06-1, *Exposure Facility and Conduct of a Chronic Inhalation Bioassay,* to solicit proposals for Phase 3 of the ACES study, a chronic inhalation study assessing cancer and noncancer effects in rats and mice exposed to emissions from a 2007-compliant engine system. The study would be conducted in two subphases: *Phase 3A,* the establishment of an exposure facility, as well as characterization and optimization of engine exposure conditions before the start of the inhalation study, and *Phase 3B*, the evaluation of health outcomes in animals exposed to DE for up to 24 months or longer, depending on the number of animals surviving.

In response, Dr. Joe L. Mauderly and a team at the Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico, submitted a proposal, "Development of a Diesel Exhaust Exposure Facility and Conduct of a Chronic Inhalation Bioassay in Rats and Mice." After extensive discussions among HEI, the ACES Oversight Committee, and additional experts, Mauderly's proposal was selected, and the study was approved by the HEI Board of Directors in February 2007. Dr. Mauderly retired in 2010, and HEI agreed that Dr. Jacob D. McDonald, who had been responsible for the exposure generation and characterization in the study, would become the principal investigator.

Phase 3B, the chronic inhalation study, was designed to be similar to the standard National Toxicology Program (NTP) bioassays for assessment of the carcinogenicity of chemical compounds (NTP 2011), albeit with a more extensive daily exposure regime than most NTP studies. To maximize the information that would be provided by the Phase 3B exposures, HEI issued Request for Applications (RFA) 06-2, "Additional Health Effects Measurements During the Chronic Bioassay and Short-Term Study." This RFA solicited applications from respondents to RFP 06-1 as well as other scientists interested in measuring additional endpoints — such as genotoxicity, vascular markers of inflammation and damage, and immune responses — that were not included in the core chronic inhalation bioassay funded under RFP 06-1. HEI selected the five investigator teams described in the Preface to conduct these ACES Phase 3B "ancillary studies."

REVIEW OF THE ACES PHASE 3B REPORTS

ACES Phase 3B was designed to examine the effects of chronic exposure to DE in rats for up to 24 months or longer and in mice for up to 3 months. The ACES protocol stipulated that the investigators would evaluate blood and tissue samples collected at intermediate exposure time points. Each investigator team prepared a draft Investigators' Report with results from the interim 1- and 3-month exposures of rats and mice and submitted it to HEI. To

review the reports from the ACES Phase 3B core and ancillary studies, HEI set up a special ACES Review Panel. The panel comprised experts in the fields of cardiovascular effects, pathology, genotoxicity, and biostatistics and was supplemented by members of HEI's Review Committee, a body of experts that reviews completed HEI studies and reports.

As the review of these reports was beginning, the panel recognized that McDonald and colleagues had already collected samples from rats that had been exposed for 12 months and had analyzed data from some important endpoints. Thus, McDonald and colleagues were requested to include a selection of these results in their report, which can be found in Appendix F to McDonald et al. (part 1 of this volume). Because the panel considered these results likely to improve understanding of the longer-term effects of diesel exposure, they are discussed at some length in this Commentary.

COMMENTARY ON ACES PHASE 3B CORE STUDY OF BIOLOGIC EFFECTS BY McDONALD ET AL.: EFFECTS OF EXPOSURE TO DE AFTER 1, 3, AND 12 MONTHS IN RATS AND MICE

SCIENTIFIC BACKGROUND

Long-Term Inhalation Exposure to "Traditional Diesel" in Rats and Mice

Before the current study of DE emissions from a 2007-compliant engine, several studies had investigated the effects of long-term inhalation exposure in multiple animal species to what has been called "traditional diesel" (Hesterberg et al. 2005) — emissions derived from diesel engines before the new technologies were introduced and new standards enforced. Many chronic inhalation studies in rats (lasting 24 months or longer) had found elevated incidence of lung tumors in animals exposed to high DE concentrations (> 2 mg/m^3) as compared with incidence in rats in a control group (Brightwell et al. 1986; Heinrich et al. 1986, 1995; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1987a; Nikula et al. 1995). In contrast to the effects in rats, few of the studies conducted in mice (or hamsters) found excess tumors resulting from long-term inhalation of DE as compared with incidence in control animals. For example, the studies of Heinrich and colleagues (1995) and Mauderly and colleagues (1996) found no tumors in mice exposed to DE concentrations that induced tumors in rats.

The importance of the particulate fraction of DE in causing tumors in rats in these early studies was supported by studies in which DE particles had been removed by filters; in the absence of DE particles, the gaseous components alone produced no change in the number of lung tumors in exposed rats as compared with controls (Brightwell et al. 1986; Heinrich et al. 1986; Iwai et al. 1986, 1997).

Pathways of Induction of Tumors in Rats Exposed to DE Several early studies identified organic components of diesel emissions as carcinogenic when applied to the skin or implanted in the lungs of rodents (Nesnow et al. 1982; Grimmer et al. 1987) and as mutagenic in the in vitro Ames test (Huisingh et al. 1978). Of the organic components, polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs were shown to be a large fraction of the mass of an organic extract of DE (Gallagher et al. 1994), and some organic components — especially nitropyrenes — had been shown to be carcinogenic in laboratory animals and mutagenic in vitro (reviewed in IARC 1998). Thus, a major hypothesis that emerged from these studies to explain the carcinogenicity of DE was that organic components of DE damaged deoxyribonucleic acid (DNA) — for example, by inducing the formation of adducts — and induced mutations (see Beland 1995).

However, long-term inhalation studies in rats that compared the effects of exposure to high concentrations of DE (2.5 mg/m^3) with the effects of exposure to similar or higher concentrations of other insoluble particles that had been previously considered to lack toxic properties found that all these particles induced lung tumors in rats. In particular, carbon black (Nikula et al. 1995) — chemically similar to the core of a diesel particle, but without adsorbed components — and titanium dioxide (Heinrich et al. 1995) induced lung tumors in rats but *not* in mice (Heinrich et al. 1995; Nikula et al. 1995; Mauderly et al. 1996).

These findings suggested that the development of lung tumors in rats after exposure to diesel and other insoluble particles was specific to rats and did not depend on the presence of organic, mutagenic components. Rather, the development of lung tumors in rats exposed to high concentrations of insoluble particles was attributed to a rat-specific "particle overload" mechanism, originally proposed by Vostal (1986), in which airway macrophages were unable to effectively clear high concentrations of insoluble diesel particles (Nikula et al. 1995; Mauderly and McCunney 1996). Particle overload was hypothesized to result in the generation of reactive oxygen species (ROS) and oxidative damage — for example, to DNA — that would lead to airway inflammation and ultimately to tumor development (as reviewed in Hesterberg et al. 2005).

Studies have evaluated whether long-term exposure of rodents to DE enhances damage to DNA, such as DNA-adduct formation, or oxidative damage. Bond and colleagues (1990), in a 3-month exposure of rats, and Randerath and colleagues (1995), who examined samples from the 23-month exposure in the study by Nikula and colleagues (1995), did not find an increase in DNA adducts. On the other hand, Iwai and colleagues (2000) found progressive morphologic changes in rat lungs over the course of the exposure (at concentrations of 3.5 mg/m^3 for up to 30 months) and increases in DNA adducts and in 8-hydroxy-deoxyguanosine (8-OHdG), a marker of oxidative damage, in lung tissue. Reed and colleagues (2004) at LRRI exposed male and female A/J mice and male and female F344 rats to different concentrations of DE (up to 1 mg/m^3) for 7 days per week for 6 months. Multiple endpoints were examined, but genotoxic effects were studied only in mice. Exposure to DE did not affect the numbers of micronucleated reticulocytes (MN-RETs) or the incidence of lung tumors (adenomas) 6 months after the end of the exposure.

Discussing the possible mode of action of DE in its Health Assessment Document for Diesel Engine Exhaust (U.S. EPA 2002), the U.S. EPA again considered the hypothesis that tumor induction in rats exposed to high DE concentrations was related to the overloading of normal lung clearance mechanisms, the accumulation of particles, lung inflammation, and cell damage. Other mechanisms may predominate at lower DE concentrations; for example, production of ROS generated from organic compounds may cause DNA damage at concentrations lower than those required to produce particle overload. Moreover, organic compounds, especially PAHs, can bind DNA and may be involved in the induction of tumors. Based in part on these toxicology results, the International Agency for Research on Cancer (IARC) (1989) and the U.S. EPA (2002) deemed diesel exhaust to be a "probable human carcinogen."

All the studies cited in this section exposed animals to "traditional diesel" — that is, to DE from engines manufactured before 2007 (and even before required reductions in diesel emissions at various stages in the 1990s and 2000s). The current report by McDonald and colleagues is the first study to report comprehensively on the biologic effects of the "new diesel" emissions — emissions from an engine compliant with 2007 regulations that has very low levels of particle emissions (even as compared with 2004 engines), as described in the ACES Phase 1 reports (Khalek et al. 2009, 2011). The "new diesel" particle emissions were also very different from "traditional diesel" emissions in the composition and size distribution of the particles (Hesterberg 2011).

Long-Term Exposure to NO_2

NO_2 is a major gaseous component of both traditional and new DE. Thus, all the studies of "traditional diesel" cited above had some level of NO_2 and other types of NO_x; for example, the study by Heinrich and colleagues (1986) used an NO_2 concentration between 1.2 and 1.5 ppm and a NO_x concentration between 10 and 11 ppm. However, because the PM level in DE emitted by 2007-compliant engines is substantially reduced (Khalek et al. 2009, 2011), the HEI ACES Oversight Committee recognized that it would be problematic, in calibrating the DE exposures in the ACES program, to base the dilution ratios for animal exposures on these very low PM concentrations. Because the ratio of NO_2 to PM in the current study is much higher than those of earlier studies, NO_2 was therefore considered as an alternative to set the dilution ratios for DE. Thus, as explained in the Preface to this Research Report, the highest DE exposure level in the current study was set in terms of the NO_2 rather than the PM concentration. As a result, prior studies that have evaluated health effects associated with exposure to NO_2 alone are relevant to interpreting and understanding the results of the current study.

In epidemiologic studies, long-term exposure of children to NO_2 has been associated with respiratory effects in a variety of locations; in particular, it has been associated with decreased lung growth and concomitant decrease in lung function in California (Gauderman et al. 2004, 2007), Mexico City, Mexico (Rojas-Martinez et al. 2007), and Oslo, Norway (Oftedal et al. 2008). In addition, early (pre-1993) studies of laboratory animal exposures to NO_2 showed what were described in the U.S. EPA's most recent evaluation of the health effects of NO_2 (U.S. EPA 2008) as "morphological changes to the respiratory tract . . . that may provide further biological plausibility for the decrements in lung function growth observed in epidemiologic studies."

Two long-term NO_2 exposure studies funded by HEI are of particular relevance (Mauderly et al. 1987a, 1989). In the first, Mauderly and colleagues (1987a) exposed two sets of male F344 rats to 9.5 ppm NO_2 (7 hr/dy, 5 dy/wk) for 6 months — either from conception to 6 months of age or from 6 to 12 months of age. The investigators found no histologic effects in the lung and no changes in pulmonary function in either set of rats exposed for 6 months. Both sets of animals had decreased body weight compared with controls. In the exposed adult rats, some small increases were detected in biochemical markers in bronchoalveolar lavage fluid (BALF): lactate dehydrogenase and glutathione peroxidase levels were increased, as were levels of acid and alkaline phosphatases. These changes were suggestive of mild cytotoxicity and oxidative stress.

In their second study, Mauderly and colleagues (1989) used NO_2 exposure conditions identical to those used in their earlier study (Mauderly et al. 1987b) to compare the effects of exposure for up to 24 months of NO_2 in rats with or without evidence of emphysema. Animals were examined at intermediate exposure time points (12 and 18 months). After 12 months of exposure to NO_2, the rats had mild airway epithelial hyperplasia with thickening of the walls of terminal bronchioles and inflammation in proximal alveoli, with small changes in some markers of respiratory function and in biochemical markers in BALF. After 24 months, histologic changes persisted, and lung volume and lung weight were increased, as were indicators of cell damage and oxidant protective mechanisms (levels of glutathione [GSH]) in BALF.

As noted in the Preface, the NO_2 exposure conditions used in these two earlier studies by Mauderly and colleagues (7 hr/dy, 5 dy/wk to 9.5 ppm NO_2 for a total of 332.5 ppm · hr/wk) were the basis for the decision in the current study to limit the concentration of NO_2 in the group exposed to the highest level of DE to 4.2 ppm, using a 16-hour exposure cycle for 5 dy/wk (for a total of 336 ppm · hour/week). (This is discussed further in the subsection "Technical Approach" later in this Commentary.) Thus, based on the findings of these earlier studies by Mauderly and colleagues, the concentration of NO_2 in the current study was expected to cause some biologic changes but was generally considered safe for long-term exposures.

Effects of longer-term NO_2 exposure on markers of oxidative stress have also been found in other studies; for example, rats exposed to 0.4 ppm NO_2 for 9 months or longer and to 4.0 ppm NO_2 for 6 months had increases in lipid peroxidation as measured by an increase in thiobarbituric acid reactive substances (TBARS) (Ichinose et al. 1983). Long-term (18-month) exposure to 0.4 or 4.0 ppm NO_2 has also been shown to decrease the activity of enzymes that regulate levels of the antioxidant GSH (Sagai et al. 1984).

The U.S. EPA's 2008 NO_2 assessment concluded that it has not been established how long-term exposure to NO_2 affects the airways, but the EPA considered that acute exposure studies have shed light on potential mechanisms. NO_2 is an oxidant and, after inhalation, is believed to interact rapidly with components of the epithelial fluid — such as surfactants and antioxidants — that line the airways. Controlled exposure of healthy adult humans to $\leq$ 2 ppm NO_2 has resulted in acute inflammatory effects in the airways — such as increased numbers of polymorphonuclear (PMN) leukocytes in BALF — within hours after exposure (see, e.g., Solomon et al. 2000; Frampton et al. 2002). In animal studies, NO_2 has also been shown to induce changes in the membrane of cells lining the airways — in particular, to induce lipid peroxidation, which can result in altered phospholipid composition of the membrane and may adversely affect membrane fluidity.

Long-term exposure to NO_2 has not been linked to carcinogenicity in animal studies (US EPA 2008). Some — but not all (e.g., Brunekreef et al. 2009) — epidemiologic studies in Europe have found associations between estimated concentrations of NO_2 and a small increase in incidence of lung cancer (Nyberg et al. 2000; Nafstad et al. 2003; Vineis et al. 2006). However, the exposures in these epidemiologic studies are to all pollutants in the ambient air, and not solely to NO_2.

TECHNICAL APPROACH

As discussed in the Preface, before the ACES 3B studies began, the ACES Oversight Committee and the ACES investigators at LRRI decided on several key design features. These were as follows:

- Of four engines tested in ACES Phase 1, one engine would be selected for health effects testing. The engine would be a heavy heavy-duty diesel engine (i.e., gross vehicle weight higher than 33,000 lb — hereinafter referred to as a "heavy-duty" engine) with control systems designed to meet the 2007 standards;
- Wistar Han rats and C57BL/6 mice would be evaluated;
- The duration of exposure would be 24 months (or 30 months, depending on survival) for rats and 3 months for mice, with evaluations at intermediate time points; and
- Three concentrations of diesel exhaust and a control atmosphere (filtered air) would be evaluated. The DE concentrations would be based on NO_2, rather than PM, concentrations because levels of NO_2 are much higher than those of PM in the exhaust emissions from the new engines. The three DE exposure concentrations would be 4.2 ppm (high), 0.8 ppm (mid), and 0.1 ppm (low) NO_2. The highest concentration in the current study (336 ppm · hours) was selected in order to allow comparisons with the cumulative weekly exposure (332.5 ppm · hours) used in previous HEI-funded long-term rat exposure studies by Mauderly and colleagues (1987a, 1989).

Objectives and Hypothesis

The goal of the ACES Phase 3B subchronic exposure studies was to compare responses of rats and mice exposed for 1 or 3 months by inhalation to DE from a 2007-compliant engine.

The investigators tested the primary (null) hypothesis for ACES stated in RFA 06-1: "Emissions from combined new heavy-duty diesel engine, aftertreatment, lubrication and fuel technologies designed to meet the 2007 NO_x and PM emission standards will have very low pollutant levels and will not cause an increase in tumor formation or substantial toxic health effects in rats and mice at the highest concentration of exhaust that can be used (based on temperature and NO_2 or CO levels) compared to animals exposed to 'clean air', although some biologic effects may occur."

Study Design

Generation and Characterization of DE Comprehensive information about the set-up of emissions-system and exposure chambers, as well as optimization of exposure conditions, has been published in HEI Communication 17 (Mauderly and McDonald 2012). The key features are described here. Detailed descriptions are also provided in Appendix B of the report by McDonald and colleagues.

Generation McDonald and colleagues generated exhaust from a 2007-compliant heavy-duty diesel engine equipped with emission controls (selected from four candidate engines tested in ACES Phase 1 according to specific selection criteria described in the Preface). The engine was fueled with ultra-low-sulfur diesel fuel meeting current on-road specifications and was operated with a dynamometer. The engine and associated systems were maintained as recommended by the engine manufacturer. The crankcase lubricating oil, changed every 250 hours, was a proprietary blend provided by Lubrizol, and was also used in Phase 1 of the ACES program. The engine was run on a unique and strenuous 16-hour cycle specifically designed for the ACES program (as described in the Preface) to represent a combination of highway and urban driving conditions rather than the steady-state operation of such engines, which had been used in past studies.

Engine exhaust and gases from the crankcase that had passed through the aftertreatment system entered a primary dilution tunnel where they were mixed and diluted with filtered air. The dilution ratio was 5:1. The diluted exhaust then passed into transit lines connected to each exposure chamber, where it was further diluted before entering each chamber. Three exposure levels were targeted based on the NO_2 concentrations. Additional dilution air was added in the chambers as needed to maintain the average NO_2 concentration (integrated over 16 hours) and keep it close to concentration targets, namely, 4.2 ppm (high), 0.8 ppm (mid), and 0.1 ppm (low) NO_2. The overall total dilution ratios at these concentrations were approximately 25:1, 115:1, and 840:1, respectively. The residence time of DE in the dilution tunnel and transit lines was less than 5 seconds. After the exhaust reached the exposure chamber, the residence time was approximately 4 minutes. The control exposure consisted of filtered air.

Daily average temperatures in the chambers were maintained within a target range of 18 to 26°C. The investigators noted that the actual temperatures in the chamber fluctuated, typically increasing during engine operation, with the highest temperatures generally found at the highest DE exposure level (1.5–2°C higher than lower exposure levels and control).

Exposure Characterization Concentrations of NO, NO_2, and NO_x were measured continuously for each exposure level throughout each exposure day. NO_x were also measured from the primary dilution tunnel for the purpose of calculating the dilution ratio between the primary tunnel and the chambers. Concentrations of carbon monoxide (CO), carbon dioxide (CO_2), nonmethane hydrocarbons, particle mass (using a Dekati Mass Monitor) and particle size distribution (using an aerodynamic or fast-mobility particle sizer), and black carbon (with a photoacoustic spectrometer) were also measured continuously in the high-exposure chamber on a daily basis. During periodic intensive characterizations (at 1.5 months into the mouse exposure, and at 2.5 and 11.5 months during the rat exposures), these measurements were made at the other exposure levels, but not in the high-level exposure chamber.

A more detailed measurement of particle size was conducted once per week at each exposure level using a fast-mobility particle sizer, which measures particles between 5 and 500 nm in diameter. Integrated PM mass concentration was measured once per week at each exposure level by gravimetric analysis of Teflon-membrane filters placed at both the inlet of the chamber and inside the exposure chamber. Taking measurements at both locations allowed the investigators to determine how much of the PM in the exposure chamber was contributed by the animals themselves (e.g., from their dander or food supply).

Animal Exposure Groups and Biologic Sample Collection A total of 180 male and 180 female Wistar Han rats per exposure level and a total of 66 males and 66 C57BL/6 female mice per exposure level were entered in the study and exposed to filtered air as a control or one of three exhaust dilutions — 4.2 (high), 0.8 (mid), or 0.1 (low) ppm NO_2 — of whole DE from the 2007-compliant engine. Exposures were conducted 16 hr/dy (plus the few minutes taken to reach 90% of the target concentration) from

approximately 1600 to 0800 hours for 5 days per week (Sunday through Thursday). Exposures of the mice were completed before the start of the rat exposures in order to ensure that the engine and exposure system were operating as intended for several weeks without technical problems that could jeopardize the chronic bioassay.

Groups of 10 male and 10 female rats were euthanized after 1, 3, and 12 months of exposure, and 10 male and 10 female mice were euthanized after 1 and 3 months. An additional group of 10 male and 10 female rats will be euthanized after 24 months of exposure. The remaining rats were assigned to the chronic inhalation groups for assessment of cancer endpoints and will continue to be exposed for 24 months or 30 months (depending on survival rates). Results from these longer-term exposures will be presented in a separate final report after completion of the exposures and subsequent analyses of the collected samples.

After euthanizing sets of mice and rats at different time points, LRRI investigators harvested blood and tissues. They retained samples for their analyses and sent aliquots or tissue samples to the ACES Phase 3B ancillary studies investigators.

Biologic Assays Table 2 of the report by McDonald and colleagues shows all the endpoints examined. A selection of endpoints measured after 12 months of exposure in rats was also included in this report (see Appendix F); a full set of results at 12 months will be published in a future report.

The endpoints fell into the following broad categories:

- *Hematology* (rats and mice, 3-month exposures) — counts of multiple cell types, plus coagulation endpoints;
- *Serum chemistry* (rats and mice, 3-month exposures) — including triglyceride and protein components;
- *Lung lavage endpoints* (rats and mice, 1- and 3-month exposures) — numbers of cells, cytotoxicity (level of total protein), multiple cytokines, and markers of oxidative stress;
- *Pulmonary function* (rats only, conducted 1 week before euthanization at the 3- and 12-month exposure time points), and
- *Other clinical observations* (rats 1-, 3-, and 12-month exposures, and mice, 1- and 3-month exposures) — mortality, tissue histopathology, and body and individual organ weights.

Histopathology The evaluation of hematoxylin- and eosin-stained slides made from 4 µm paraffin tissue sections is described in detail in the Methods section of the report, as well as in Appendix H of the report by McDonald and colleagues, which is available only on the Web at *www.healtheffects.org*). In summary, the tissue evaluation process and the sequence of slide reading were consistent with the procedures used by contractors to the NTP (Morton et al. 2010). Slides from rats and mice were read by two pathologists — Drs. Andrew Gigliotti, from LRRI, and Rodney Miller, from Experimental Pathology Laboratories — who evaluated multiple indicators of inflammation, cytotoxicity, and parenchymal changes, and scored them on a scale in which *minimal* (1) was considered difficult to observe, *mild* (2) quite easy to find, *moderate* (3) quite extensive, and *marked* (4) involving most of an organ. They then calculated the average of the scores in each category for each animal and generated an average score ± standard error of the mean (SEM) for each exposure group. As described in Appendix A of the report by McDonald et al., their diagnoses and evaluations of exposure effects or lack thereof were confirmed by two independent peer-review pathologists, Drs. Ernest McConnell (ToxPath, Inc., and a member of the ACES Oversight Committee) and Ronald Herbert (NIEHS, NTP).

Statistical Approaches McDonald and colleagues used both one-way and two-way analysis of variance (ANOVA) to evaluate exposure-related effects at each exposure time point. One-way ANOVA was used to assess diesel exposure effects for each sex; two-way ANOVA models contained terms for exposure group (control, low-, mid-, and high-level diesel exposures), sex, and sex × exposure group interaction effects. The investigators also used a two-way ANOVA with no interaction term to assess effects across sexes under the condition that there was no substantial evidence of sex-based differences in exposure-related response. If the one-way or two-way ANOVA indicated a significant difference among exposure group responses, however, McDonald and colleagues used the Dunnett multiple comparison procedure (Dunnett 1955, 1980) to compare diesel-exposed group means with those of controls. They used the linear term of the ANOVA to assess exposure-related trends in response across control and exposed groups. Endpoints in which the variances of the means in different groups were highly variable were logarithmically transformed before using the ANOVA regression model. Three-way ANOVA analyses were also done (see Appendix I) for all results found to be positive in the above analyses.

The data are summarized using the standard plots to describe both the averages of groups and level of variations within these groups. In analyses across sexes, the

reported mean values are the averages of the means by sex in the four exposure groups, and the reported standard errors are based on pooled sample variance estimates, after adjustment for differences by sex (rather than variances calculated across all animals without regard for differences between sexes). All *P* values are two-sided, and statistical significance was assessed at $P < 0.05$ and $P < 0.01$. Statistical calculations were performed using the SAS software system, version 9.2. Some examples of the statistical approaches taken for some selected endpoints are provided in Appendix C of the report.

THE ACES REVIEW PANEL'S EVALUATION OF THE REPORT BY MCDONALD ET AL.

General Comments

The HEI ACES Review Panel, which conducted an independent review of the study, thought that the study addressed an important issue: the effects of inhalation of DE generated by a heavy-duty engine compliant with 2007 regulations that significantly reduced particulate emissions.

The review highlighted several strengths of this carefully designed and executed study: This is the first and so far only in vivo evaluation of the subchronic effects (up to 12 months to date) of a range of exposures from the new generation of heavy-duty diesel engines. On a daily basis, these exposures were much longer (16 hours per day) than traditional NTP exposures (typically 6 hours per day) and were created from a unique and strenuous engine cycle that included regeneration of the particle filters in order to best represent actual driving conditions. The investigators provided a comprehensive analysis of the physical and chemical composition of the emissions and exposure atmospheres. Furthermore, over the 12-month exposure period McDonald and colleagues maintained the exposure levels (high, mid, and low) at close to the target levels, which were predetermined based on levels of NO_2.

The standardized toxicity endpoints that were measured were also appropriate for evaluating the hypothesis for the study. As described in detail below, the panel generally agreed with the investigators that exposure of rats to DE from the 2007-compliant engine for up to 12 months induced few biologic effects, and those effects that were detected were mild; exposure of mice for 3 months had even fewer effects. Some limitations to the interpretation were also noted; in particular, the lack of internal controls for some assays and the use of some of the statistical approaches taken. In addition, the panel thought that the histopathology data could have been expanded by including more detailed quantitative measures.

Key Results

Exposure Characterization The key exposure characterization findings are summarized here:

- The most abundant pollutants by mass in the DE were CO_2, CO, NO, and NO_2.
- Particle concentrations in the DE atmospheres were very low over the course of the exposures; thus, the ratios of NO_2 to NO_x and of PM mass to NO_2 in this study are substantially different from those found in "traditional diesel" — much higher and much lower, respectively.
- PM levels rose only during regeneration of the diesel filter, which occurred once or twice in a 16-hour exposure period.
- Concentrations of sulfur dioxide (SO_2) and semivolatile and volatile organic compounds (SVOCs and VOCs) were also very low.
- Particles found in the exhaust were in the ultrafine range — median particle size approximately 20 nm (based on number) and 40 nm (based on mass) — which is consistent with measurements made in the ACES Phase 3A study (Mauderly and McDonald 2012).
- Approximately 50% of the mass of the particles measured in the chamber when the animals were present was carbon — the proportion of organic carbon in the total carbon varied slightly at different DE exposure levels. The majority of the remainder of the mass consisted of nitrate, ammonium, and sulfate ions. Some PM appears to have been formed in the chamber from reactions between exhaust gases and ammonia from the animals; this may explain why PM composition was slightly different at each of the exposure levels.
- The animals in the exposure chambers also generated particles in the fine and coarse range (data not shown) that were major contributors to the particle mass. Additionally, the animals contributed a significant portion of the VOCs measured. Finally, chemical reactions occurred between components of the exposure atmosphere in the presence of the animals, and these changed somewhat the proportions of the levels of components of the emissions from what was expected in one or other of the exposure chambers.
- The actual average exposure concentrations exposures of rats and mice — 16-hour averages for NO_2 were 3.6, 0.95, and 0.11 ppm for the rats and 4.3, 0.8, and 0.1 ppm for the mice for high-, mid-, and low-level DE exposures, respectively. This was close to — but not identical to — the target concentrations;

the differences can be attributed to the difficulty in maintaining specific NO_2 concentrations averaged of 16 hours given the variable 16-hour cycle.

More detailed descriptions of the chamber exhaust characterization, including comparisons with characterization results from older diesel bioassays and ACES Phase 1 and 3A, are provided in the Commentary Appendix (found on the HEI Web site at *www.healtheffects.org*).

Biologic Effects The great majority of the biologic tests performed by the investigators showed no effect. The few statistically significant changes reported by the authors are summarized in the Commentary Table on the next page. (Note that *only* histopathology and pulmonary function evaluated in rats exposed for 12 months are included in this report; effects on other endpoints will be included in the final report submitted after the terminal euthanization.) The DE-associated effects were as follows:

Mild Histologic Changes in Rats in the Respiratory Tract after 3- and 12-Month Exposures to DE No changes were detected in any animal in any tissue after 1 month, and no changes were detected *outside* the respiratory tract at 3 months.

In the respiratory tract of the rats exposed to a high-level of DE at 3 months, the investigators reported the following: in the lung (Table 7 and Figure 7 in the report by McDonald et al.), a small increase in the number of basophilic epithelial cells lining distal terminal bronchioles, alveolar ducts, and alveoli that were close to the terminal bronchial and alveolar ducts. The distribution was uniform and focused at the central acinus (the junction of the conducting airways and the gas exchange region of the lung). In addition, there were small increases in alveolar macrophages, concentrated and centered on periacinar areas. Scattered mixed inflammatory cells were also present in those areas. In some males and females, there was also a minimal increase in interstitial fibrosis — fibrous connective tissue along and around alveolar ducts and the junctions of the alveolar ducts with alveolar walls in periacinar areas. No increase in mucous cell metaplasia was detected. No changes were reported in the animals in the two lower-level exposure groups.

By 12 months, the changes observed at 3 months in the lungs had progressed somewhat in that they were detectable in more animals at the highest DE exposure, but they were still of mostly minimal severity (see Table F.1 in Appendix F, and Appendix A in the report by McDonald et al.). Alveolar epithelial hyperplasia of the proximal alveolar duct, macrophage infiltration of the alveolar space, and interstitial fibrosis were detected in most male and female animals in the high-level DE exposure groups. However, at 12 months, there was now evidence of "bronchiolization" (that is, that the normally flat epithelial cells had changed to a cuboidal shape, similar to cells lining the terminal bronchioles) — a metaplastic change of the alveolar epithelium in the centriacinar region (the proximal alveolar duct/alveoli, and the junction of the conducting airways and the gas exchange region of the lung). No exposure-related changes were detectable in the lungs at the mid and low DE exposure levels.

In addition, the nose and turbinate of a small number of rats showed scattered changes. At 3 months, squamous cell metaplasia was detected in a few male and female animals (Table 8 in the report by McDonald et al.). However, McDonald and colleagues consider that these findings were not associated with DE exposure because a small number of females in the control group also showed changes in the same endpoint. Rare male and female animals (1 in 10) also showed mild degeneration of the olfactory epithelium at different exposure levels. At 12 months, this generally mild degeneration of the olfactory epithelium was detected in a few more male and female rats at low-, mid-, and high-level DE exposures (Table F.2).

Small Decreases in Some Respiratory Function Measures in Female Rats at 3 Months, But Not at 12 Months, Except for a Trend in DL_{CO} At 3 months, compared with control animals, small decreases in peak expiratory flow (PEF), forced expiratory flow between 25% and 50% of forced vital capacity ($FEF_{25–50}$) and maximal mid-expiratory flow (MMEF) were detected in female rats in the high-level DE exposure group (Tables 6 and E.10 in the report by McDonald et al.). The investigators reported a statistically significant reduction in diffusing capacity of CO (DL_{CO}, a measure of alveolar-capillary gas exchange) when they combined results from both sexes, but the changes were not significant in either sex alone. At 12 months, the only pulmonary function endpoint in any DE-exposed group that differed from the control (see Table F.3) was DL_{CO}, when data from both sexes were combined.

Small Changes in Rats in Levels of Some Markers in Lung Tissue and/or BALF after 1- or 3-Month Exposures to DE Of the many endpoints measured, some small changes were noted in a few associated with inflammation (IL-1β in lung), lung cytotoxicity (micro–total protein in BALF), and changes in oxidation status (increases in heme-oxygenase-1 [HO-1], decreases in Trolox equivalent antioxidant capacity [TEAC]); nearly all these changes were detected after exposure to the highest level of DE. These changes were not observed consistently at 1 and 3 months and were not generally observed in both sexes; for example, IL-1β levels

Endpoints from McDonald et al. Showing a Significant Change After 1, 3, or 12 Months' Exposure in Rats and 1 and 3 Months' Exposure in Mice[a]

		Sex	
Endpoint	Tissue	Male	Female
Rats (evaluated at 1, 3, or 12 months)			
Histology[b]	Lung	3 months: mild changes, with high DE only, and in some animals 12 months: continued mild changes, including mild bronchiolization, and moderate interalveolar septal fibrosis with increased frequency in high DE only	3 months: mild changes, with high DE only, in a few animals 12 months: continued mild changes, including mild bronchiolization, and moderate interalveolar septal fibrosis with increased frequency in high DE only
	Nose/turbinate	3 months: mild changes 12 months: mild changes	3 months: mild changes, but also detected in controls 12 months: mild changes
Pulmonary function[c]			
PEF		=	3 months: ↓, high only 12 months: =
$FEF_{25–50}$		=	3 months: ↓, high only 12 months: =
MMEF		=	3 months: ↓, high only 12 months: =
DL_{CO}		3 months: ↓ when combing results from both sexes; not significant in either sex alone 12 months: ↓ trend, when combing results from both sexes; not significant in either sex alone	
Lung, biochemical[d]			
μTP	BALF	3 months: ↑, high only	3 months: ↑, high only
Albumin	BALF	3 months: ↑, high only	3 months: =
TEAC	BALF	1 month: = 3 months: ↓ at all levels	1 month: ↓ at low and mid 3 months: ↓ at all levels
IL-1β	Lung tissue	1 month: All ↓ 3 months: =	3 months: ↑, high only
IL-6	Lung	1 and 3 months: =	3 months: ↑, high only
GSH	Lung	1 and 3 months: =	1 month: ↑, high only
HO-1	Lung	3 months: ↑, high only	3 months: ↑, high only
Total GSH	Lung	1 and 3 months: =	1 month: ↑, high only
Mice (evaluated at 1 and 3 months)			
Cells			
PMN absolute count	BALF	Trend to ↑ at 3 months, when sexes combined	
PMN differential count	BALF	Trend to ↑ at 3 months, when sexes combined	
IL-6	BALF	1 month: ↑, high only	=
Albumin	BALF	3 months: ↑, high only	=

[a] Low, mid, high refer to DE exposure levels; ↑, ↓ indicate statistical significance either up or down vs. control; = indicates no change compared with control.

[b] Evaluated at 1, 3, and 12 months.

[c] Measured at 3 and 12 months.

[d] Measured *only* at 1 and 3 months in this report.

decreased in males after 1 month but not after 3 months, but increased in females at 3 months. In addition, endpoints associated with one particular pathway, in particular oxidative stress, which might have been expected to change in concert, did not change consistently; for example, at 3 months TEAC decreased in both sexes at all DE levels, HO-1 increased in both sexes at only the high level exposure, and reduced GSH levels were not affected.

Summary of Effects in Mice Mouse exposures lasted only 3 months, and respiratory function was not evaluated. No histologic changes were observed in the lungs and nose after 1 or 3 months in either sex. Of the many lung cell and biochemical endpoints examined, there were small changes in just a few, and these were found in BALF: increased IL-6 (at 1 month in males exposed to high levels of DE only) and increased albumin (only in males after 3 months of exposure to high levels of DE) (see Table 9 and D.5 in the report by McDonald et al.), and a trend to an increase in PMN numbers at 3 months.

Summary of Results The panel agreed with McDonald and colleagues that only a few and relatively mild changes were observed after 1, 3, and 12 months of exposure to DE in rats, and after 1 or 3 months of exposure in mice. Comparing results at 1 and 3 months across the species, more changes in endpoints were reported in rats than in mice, and those changes occurred mostly after exposure to the high level of DE.

The investigators observed mild histologic changes in the respiratory tract in rats. Changes were detected in the lung after 3 months' exposure to high-level DE and had progressed at the 12-month exposure time point, in that changes that were noted were more widespread within the lung and were seen in more animals. Nonetheless, the histologic changes were still mild as defined by the investigators' scoring system. In addition, the nose and turbinate in a very small number of rats showed scattered changes after 3 months' exposure, and these generally mild changes were detected in a few more male and female rats at all DE exposure levels at 12 months.

Some small changes in respiratory function were noted at 3 months, but of these only a decrement in DL_{CO} may have persisted at 12 months. A decrease in DL_{CO} suggests the possibility of effects on pulmonary gas transfer or the pulmonary circulation, which would be consistent with the observed histologic changes in the gas exchange region of the lung. Some small changes in biochemical endpoints, particularly those related to oxidant stress pathways, were also noted in BALF and lung tissue at 1 and 3 months. (Results of biochemical assessments in samples from rats exposed for 12 months will be included in the final report from the investigators.) Overall though, these changes were small, and there was a lack of coherence among the endpoints; that is, those endpoints that might have been expected to change in concert because they are a part of the same pathway did not do so. These discrepancies among endpoints in the same pathway may reflect the different sensitivities of the individual assays used to measure changes, or they may mean that the results were anomalous.

Limitations to the Interpretation of Results

Although the panel generally agreed with the investigators' interpretations of the data, it noted some limitations to the data and interpretations presented in the report by McDonald and colleagues, as described below.

Statistical Approaches For the respiratory function and biochemical data, McDonald and colleagues used a two-way ANOVA (time and exposure level) and a trend test to identify whether the results for each sex were significant at $P < 0.5$. As explained in Appendix C, the investigators then combined data for both sexes to provide a combined value.

However, although the panel recognized that the investigators were conducting their statistical analyses in line with the recommendations of the HEI ACES Oversight Committee, they had some concerns regarding trend analysis and joint analysis of the data on the two sexes and the use of a two-way ANOVA rather than a three-way ANOVA. First, the small sample sizes and large variances found in some groups complicated the analysis and reduced the power of the *t* test performed to detect any signal. Second, the investigators did not calculate the variances for the combined sexes using raw data; instead, they were estimated by using the equal variance assumption with some unconventional methods for adjustment. Overall, the panel thought that the use of these approaches was unnecessarily complicated and that the validity of results obtained with them was therefore open to alternative interpretations.

During its review of the report, the panel recommended to the investigators that they use the standard three-way ANOVA approach to analyze the exposure effects of experiments involving three factors (sex, time, and exposure). In response, McDonald and colleagues provided the results of a three-way ANOVA for all the endpoints that showed a significant change in the two-way ANOVA (see Appendix I of the report by McDonald et al.). This analysis found that results from the three-way ANOVA did not appear to differ much from those obtained using the two-way ANOVA. It is reassuring that the interpretation of positive data in the report on the basis of the two-way ANOVA approach

was not affected by this additional analysis, although some uncertainty in interpretation remains because the three-way ANOVA was not applied to the full data set.

Assay Controls Most of the biochemical assays did not show an effect of exposure. As with any negative study, however, there is always the question of whether the results are truly negative or that the lack of effects may be attributed to design and methodologic issues, including the sensitivity of the assays employed. The panel considered that adding positive "technical" controls — for example, by using "spiked" samples, containing agents known to affect a particular endpoint — would have provided a standard by which to judge the potential exposure-related changes and might have alleviated concerns about the sensitivity of the particular assay to measure changes. In particular, the lack of assay sensitivity may have been an issue in the measurements of GSH levels, in view of the fact that GSH levels were much lower than those reported in other animal studies (Weldy et al. 2011; da Cunha et al. 2011).

Histopathologic Evaluation The panel considered the histopathologic evaluation to have been handled appropriately, in accordance with accepted NTP procedures, and verified by a panel of independent experts (see Appendix A of the report by McDonald et al.). Although the report provides quantitative severity scores for each group, the panel thought including a more precise quantitative approach to the pathologic analysis, such as morphometric measures of the interalveolar septal and capillary surface area of the lung, would have been more informative.

Uncertainties Introduced by the Exposure Conditions Over the course of the exposures, the investigators kept to within the range specified for the target NO_2 concentrations (± 20%). Owing to the variable 16-hour cycle, pollutant concentrations in the exhaust varied widely over the course of the exposure period, and it was difficult to maintain a precise target concentration. The investigators made weekly adjustments to keep close to the target. For example, if after 1 week the concentration was slightly below target (e.g., 4.0 instead of 4.2 ppm), they would change the dilution so that the next week the exposure concentrations would be slightly above target to compensate for the difference.

For some exposure groups, particularly the high-level DE group in rats, the average concentration was lower than the target (3.6 ppm NO_2 rather than 4.2 ppm), although still within the ± 20% margin established in the protocol. It does not seem likely that the slightly lower concentration of NO_2 would have had a major impact on the outcome of the study. As noted earlier, there were complex interactions among emission constituents and compounds produced by the animals in the chambers, which may have led to some difficulties in achieving the higher NO_2 levels.

Another issue that was identified during the study design phase was the potential for high temperatures in the exposure chambers. Heavy-duty engines have more power and produce significantly more heat when they operate compared with light- and medium-duty engines. In addition, owing to the relatively low pollutant concentrations in the exhaust of these new engines, the dilution levels were kept relatively low to reach the desired exposure concentrations, which meant that the hot exhaust was not diluted with cool air to the same extent as in previous studies. The investigators tried to compensate for the additional heat by adding extra cooling in the chamber, and they were thus able to maintain the temperature in the exposure chambers within the specified range of 18 to 26°C. Because of these special circumstances, this range was wider than normally specified for chronic inhalation studies, which are usually conducted in the temperature range 23 ± 2°C. As a result, animals in the high-level DE exposure group in the current study were more often exposed to higher temperatures than those in other groups. How these differences in temperature among the exposure groups might have affected the results is not clear.

Lack of a Positive Control Engine The study design did not include a side-by-side comparison with an older pre-2007 model-year engine. In their design of the ACES program, the ACES Oversight Committee had weighed the desirability of such a comparison but decided that such a "positive control" could not be included as it would have substantially increased the complexity and cost of the study and would have posed enormous logistical challenges. While acknowledging these problems, the panel thought that such a side-by-side comparison could have enhanced the study.

Other Key Considerations

Comparison with Results of Prior Long-Term Inhalation Studies Although most studies of long-term exposures of rats to "traditional diesel" emissions have focused on evaluating the induction of tumors at the final exposure time point (24 months or longer) and did not include assessments at interim time points (reviewed in Hesterberg et al. 2005), some studies have done so. In particular, the 1989 study by Mauderly and colleagues evaluated the effects of exposure to DE in male F344 rats after 12, 18, and

24 months. (As described below, this study also compared the effects of exposure to NO_2 in rats). After 12 months' exposure of the animals to DE, the investigators did not find lung tumors but did find some small changes in lung markers in BALF (specifically, increased levels of lactate dehydrogenase, beta-glucuronidase, and acid proteinase, and decreased levels of sialic acid), as well as in some respiratory function measures (such as decreased lung compliance). As in the current study by McDonald et al., no increase in the induction of tumors was found after 12 months' exposure.

Findings from the chronic (24-month) exposure study in rats by Heinrich and colleagues (1986), in which particles from DE were removed by filtration, are also relevant to the current study. PM emissions were also very low in that study, and females of the same rat strain were used. Multiple comparisons were made at 12 months; body and lung weights, levels of proteins in lung lavage fluid, and cytologic data did not differ between control rats and rats exposed to filtered DE. In the 1986 study, the gaseous DE emissions had a lower NO_2 level (1.1 ppm) and a lower NO_2:NO_x ratio than in the DE at the high-level of exposure in the current study, but the ratio of PM to NO_2 in the filtered DE was very low, as was found in the current study.

Scattered changes similar to those described in the current study were noted in a study by Mauderly and colleagues (1989) after exposure of F344 rats for 12 months to 9.5 ppm NO_2. They found no significant change in body weight, lung weight, or any lung morphometric measures (external lung volume, mean linear intercept, or internal surface area) or in the activity of the lung enzymes glutathione peroxidase and reductase (involved in oxidant balance). However, they did find small changes in biochemical markers in BALF (specifically, decreases in total protein and sialic acid) and in pulmonary function (specifically, increases in DL_{CO}, total lung capacity, and vital capacity). However, the histopathologic findings in the lungs after 12 months in that 1989 study were remarkably similar to those reported in the current study: "mild hyperplasia of epithelium in terminal bronchioles and an extension of bronchiolar epithelial cell types into proximal alveoli, giving the appearance of 'respiratory bronchioles.' Terminal bronchiolar walls were slightly thickened and eosinophilic. A slight inflammatory infiltrate of mixed cell type was occasionally found in alveoli adjacent to thickened bronchioles" (Mauderly et al. 1989). The report also indicated that "lesions at 18 and 24 months . . . were very similar to those observed at 12 months. The epithelialization of proximal alveoli appeared to progress slightly with time, but the inflammatory responses remained minimal."

In light of these comparisons with prior studies, the panel agreed with McDonald and colleagues that the histopathologic and other changes noted in the rat lung in the current study are consistent with exposure to NO_2 present in the DE. However, effects mediated by other gaseous components of DE cannot be ruled out.

Choice of Rat and Mouse Strains The Preface to this Research Report explains the reasons behind selecting the single inbred strain of each species for the current exposure studies (see also Appendix G of the report by McDonald et al., available only on the Web at *www.healtheffects.org*). The ACES Oversight Committee, after extensive discussions, selected the Wistar Han rat for several reasons, including the strain's longevity, its previous use in chronic inhalation studies of DE (particularly in Europe; for example, in the studies of Heinrich and colleagues [1986, 1995]), the existence of a historical database of information on cancer incidence in this strain, the relatively low rate of spontaneous background tumors compared with some other species, and an acceptable maximum bodyweight that would be reached by males during the study (affecting housing in inhalation chambers). One possible concern that was discussed during the study design phase was that a proportion of Wistar Han rats have a mutation in the aryl hydrocarbon receptor (AhR) gene, which may alter their responses to dioxin and PAHs that also bind to the AhR. Because PAHs are present in DE and some have known carcinogenic properties, some scientists have argued that the mutation might make the Wistar Han strain more resistant than strains with a non-mutated AhR gene to the potential toxic and carcinogenic effects of DE. However, the AhR mutation in the Wistar Han rat does not render it unresponsive to dioxin — the strain is highly resistant to dioxin's lethality, but not to other dioxin-mediated effects (Okey et al. 2005). Responses to PAHs in this strain are thus likely to be similar to the responses to dioxin. More important, there is no evidence that the AhR mutation affects the development of cancer in this strain; tumors develop in Wistar Han rats with long-term exposure (20 or more months) to high concentrations of "traditional" DE, as well as to other particles (Karagianes et al. 1981; Heinrich et al. 1986, 1995).

The C57BL/6 mouse strain was a straightforward choice for the current study; it has a relatively low incidence of spontaneous lung tumors and has been used in previous diesel bioassays. However, the C57BL/6 mouse expresses a version of the AhR gene product that binds dioxin — and likely PAHs — 10 times more sensitively than the AhR found in other inbred mouse strains, such as DBA/2 (Okey et al. 2005). Thus, it is possible that the C57BL/6 strain used by McDonald et al. may be more sensitive than other mouse strains to the effects of PAHs. Nonetheless, the investigators observed few, if any, effects in C57BL/6 mice exposed to DE in the current study.

Sensitivity of Rats Versus Mice to Irritants Although mild, more changes were detected in rats than in mice exposed for the same time period (3 months). Mice have a greater ability to reduce the amount of an inhaled irritant such as formaldehyde by decreasing their minute volume (Alarie 1966; Chang et al. 1981). This may at least partially explain why fewer changes were noted in mice than rats exposed to DE. On the other hand, this may reflect a greater sensitivity of the rat strain to DE, despite the issue of the mutation in the AhR gene discussed earlier.

Summary and Conclusions

This comprehensive and well-executed study from McDonald and colleagues reports findings from a subchronic exposure study in rats (1, 3, and 12 months), and mice (1 and 3 months), to assess the effects of inhalation of a range of DE levels from a heavy-duty engine compliant with 2007 emissions regulations, in accordance with the hypothesis developed at the beginning of the study, namely, that the emissions would not cause an increase in tumor formation or substantial toxic health effects in rats and mice, although some biologic effects might occur.

The HEI ACES Review Panel conducted an independent review of the study and highlighted several strong points: The study is the first to conduct a careful and comprehensive evaluation of the subchronic effects in rodents of inhalation of a range of DE levels from a heavy-duty 2007-compliant engine. The investigators successfully maintained, even while applying a unique and strenuous 16-hour engine operating cycle, the continuous operation for 12 months of a facility in which engine exhaust was generated and transported to rodent exposure chambers. The investigators also attained DE levels within 20% of the designated target over the exposure period. Because PM levels in the engine exhaust were greatly reduced compared with emissions from older engines, the exposure concentrations were based on levels of NO_2 (4.2, 0.8, and 0.1 ppm) rather than PM. These NO_2 levels were chosen to provide a comparison with the same cumulative exposure to NO_2 (the product of concentration and exposure duration) used in a prior HEI-funded rat inhalation study by Mauderly and colleagues (1989), in which minor biologic changes were observed in the respiratory tract.

McDonald and colleagues in the current study provided an extensive analysis of the physical and chemical composition of the emissions and exposure atmospheres. They found that in contrast to the levels in "traditional diesel," the levels of PM, SO_2, and SVOCs and VOCs in the DE from 2007-compliant engines were very low. The most abundant pollutants were CO_2, CO, NO, and NO_2.

The panel concluded that the multiple standardized toxicity endpoints evaluated in this study — including histology, serum chemistry, and respiratory function — were appropriate for evaluating the hypothesis. The panel agreed with McDonald and colleagues that there were no DE-exposure associated changes in the majority of biologic endpoints measured in rats and mice. Comparing results at 1 and 3 months across the species, the few changes observed in endpoints were reported more often in rats than mice and were associated almost exclusively with exposure to high-level DE.

The investigators found mild histologic changes in the respiratory tract of rats after exposure to DE, but no changes were found outside the respiratory tract. In the lung, changes were detected after 3 months of exposure to high-level DE, and progressed at the 12-month exposure time point, in that changes were more widespread within the lung and found in more animals. Nonetheless, the histologic changes were still mild as defined by the investigators' scoring system. In addition, the nose and turbinate of a very small number of rats showed scattered changes after 3 months of exposure, and these generally mild changes were detected in a few more male and female rats at all DE exposure levels at 12 months.

Histologic changes in the lung — in particular, a mild thickening of the central acinus — were generally consistent with findings from previous studies of long-term exposure to NO_2. Thus, the panel agreed with the investigators' suggestion that the histopathologic changes in the lung in the current study are consistent with responses to NO_2. However, the effects of other gaseous components of DE cannot be ruled out.

Some small changes in respiratory function were noted at 3 months, but of these only a decrement in DL_{CO} may have persisted at 12 months. A decrease in DL_{CO} suggests the possibility of effects on pulmonary gas transfer or the pulmonary circulation, which would be consistent with the observed histologic changes in the gas exchange region of the lung. Some small changes in biochemical endpoints, particularly related to oxidant stress pathways, were also noted in BALF and lung tissue at 1 and 3 months. (The results of the 12-month biochemical assessments will be reported in the final report from the investigators.) Overall though, these changes were small, and there was a lack of coherence among the endpoints; that is, the endpoints that might have been expected to have changed in concert — because they share a common pathway — did not do so. These discrepancies among endpoints in the same pathway may reflect the different sensitivities of the individual assays used to measure changes, or they may be just anomalous observations.

The panel noted some limitations to the interpretation of the study: the study design did not include a simultaneous comparison with emissions from an older engine built before the 2007 regulations. While recognizing that including such a "positive control" would have increased the complexity and cost of the study very substantially and would have been logistically impossible, the panel thought that a side-by-side evaluation of emissions from an older engine could have enhanced the study. The panel also identified some other limitations to the study — the lack of some positive assay controls (to identify that an assay was sensitive enough to detect changes) and the use of certain statistical approaches (combining sexes and performing a trend analysis rather than conducting a standard three-way ANOVA). In addition, the panel thought that the histopathology data could have been expanded by more detailed quantitative measures.

Overall, however, these results indicate that rats exposed to one of three levels of DE from a 2007-compliant engine for up to 12 months, for 16 hours per day, 5 days a week, and using a strenuous operating cycle that was more realistic than cycles used in previous studies, show few exposure-related effects. The effects that were observed were limited to the respiratory tract, were mild, and were consistent with previous findings in lungs after long-term exposure to NO_2 — a major component of the exposure atmosphere. Rats will continue to be exposed for at least 24 months in this study, possibly longer, depending on their survival rates. McDonald and colleagues will submit a future report with complete results from the 12- and 24-month exposures, which will provide a further comprehensive review of the cancer and noncancer effects of long-term exposure to DE emitted by a 2007-compliant engine.

COMMENTARY ON ACES PHASE 3B ANCILLARY STUDIES: EFFECTS ON GENOTOXICITY (BEMIS ET AL. AND HALLBERG ET AL.) AND VASCULAR MARKERS (CONKLIN AND KONG) AFTER 1 AND 3 MONTHS OF EXPOSURE

INTRODUCTION

As noted above, ACES Phase 3B, the chronic inhalation study funded under RFP 06-1 was designed to be similar to the standard NTP bioassay for assessment of carcinogenicity of chemical compounds (NTP 2011). However, there was interest in measuring additional health outcomes not covered in the NTP bioassay, such as genotoxicity, vascular markers of inflammation and damage, and immune responses. Consequently, as described in the Preface, HEI funded several additional studies, of which three are included in the current Research Report. They were led by

- Dr. Jeffrey C. Bemis, Litron Laboratories, Rochester, New York: "Genotoxicity of inhaled diesel exhaust: Examination of rodent blood for micronucleus formation," to evaluate MN development in the blood of DE-exposed animals;
- Dr. Lance M. Hallberg, University of Texas Medical Branch, Galveston, Texas: "Assessment of the genotoxicity of diesel exhaust/diesel exhaust particulates from improved diesel engines," to evaluate DNA damage and oxidative stress in tissues and blood from DE-exposed animals; and
- Dr. Daniel Conklin, University of Louisville, Louisville, Kentucky: "Effects of diesel engine emissions on vascular inflammation and thrombosis," to measure effects on multiple vascular markers.

Exposure to DE in the Genotoxicity Studies

As described earlier, McDonald and colleagues generated exhaust from a 2007-compliant heavy-duty diesel engine equipped with emission controls (selected from four candidate engines in Phase 1 of the ACES program). They exposed groups of male and female Wistar Han rats and male and female C57BL/6 mice by inhalation to filtered air as a control or one of three dilutions — 4.2 (high), 0.8 (mid), or 0.1 (low) ppm NO_2 — of whole DE from a 2007-compliant system. Exposures were conducted 16 hours per day from approximately 1600 to 0800 hours, 5 days per week (Sunday through Thursday). Animals were fed ad libitum during the entire study.

After euthanizing sets of mice and rats at 1 and 3 months, LRRI investigators harvested blood and tissues and sent aliquots to Drs. Bemis, Hallberg, and Conklin. Rat blood and tissues were taken from the same animals assessed in the McDonald and colleagues' study; mouse blood and tissues were taken from groups of animals that were set up specifically for the ancillary studies. More rats will continue to be exposed to DE for 24 months, or even longer depending on survival rates. Results from 12-month and final time point exposures in the ACES ancillary studies, together with further results from the core McDonald study, will be presented in a final report, to be published in 2014.

FEATURES UNIQUE TO THE STUDY BY BEMIS ET AL.

Aims

The goal of the study by Bemis and colleagues was to evaluate in the blood of DE-exposed animals the number

of immature red blood cells (RETs) that contain an MN. Conditions that cause double-strand breaks or disrupt the proper segregation of chromosomes during division result in an increase in MN frequency.

Samples

Peripheral blood (100 µL) from 5 male and 5 female rats per exposure group and an identical number of mice euthanized after the 1- and 3-month exposures was collected at LRRI in a special anticoagulant and then fixed according to established procedures. Samples were shipped frozen to Dr. Bemis.

Methods

Pilot Study Before the main study started, Bemis and colleagues conducted a pilot study (see Appendix B of the report by Bemis et al.) to determine whether MN could be detected in older rats after exposure to a known genotoxic agent, cyclophosphamide. After first establishing a baseline level of micronucleated and RETs for each animal by collecting a sample from the tail, the investigators treated 3 males and 2 female rats aged between 10 and 21 months with cyclophosphamide (15 mg/kg, 1 mL/kg) by gavage, and two rats of each sex with vehicle only. Animals were killed 48 hours later and cardiac blood collected.

MN Assay The investigators used flow cytometry to differentially identify and count in each blood sample four types of red blood cells: RET without or with MN (RET and MN-RET), and mature normochromatic erythrocytes (NCE) without or with MN (NCEs and MN-NCEs). Figure 3 of the report by Bemis et al. shows that by using fluorescent antibody specific for CD71 (expressed on RET) and propidium iodide (with ribonuclease) as a fluorescent DNA probe, each type of red blood cell could be grouped into and assessed quantitatively in one of the four quadrants of the readout — RET in the upper left, and MN-RET in the upper right; NCEs in the lower left, and MN-NCEs in the lower right.

Statistical Approaches

The means and SEM were calculated for each group of mice or rats (at least 5 animals per treatment condition). Once these initial calculations were made, the %MN-RET values were converted to a proportion, and an arcsin–square root transformation was performed to stabilize group variance and normalize the data. However, this arcsin transformation did not achieve a suitably normal distribution for the MN-NCE or RET data, so an alternative strategy was employed: The values for %MN-NCE and %RET were transformed by taking the natural log, if necessary applying a consistent correction factor to shift the data into positivity, and then taking the natural log again. This double logarithmic transformation afforded the best approximation of a normal distribution for the respective data with both the mouse and rat data sets.

One-, two-, and three-way ANOVA formats were employed. When one-way ANOVA was performed, a post-hoc Dunnett *t* test was used to compare control data with other groups as appropriate. Significance was set at the $P \leq 0.05$ level. A three-way ANOVA included the factors sex, exposure condition, and exposure duration.

Key Results

1. Exposure of mice and rats for 1 or 3 months to DE from a 2007-compliant engine did *not* increase the frequency of MN-RET in peripheral blood.
2. Treatment with cyclophosphamide increased the frequency of MN detected in peripheral blood RETs from elderly rats — 1% to 4%, compared with < 0.2% in the blood of vehicle controls.
3. Male and female mice had different background frequencies of MN-RET and MN-NCE (control females were almost 50% lower than control males at the 3-month exposure time point). Rats did not show sex-based differences in MN-RET frequency.

The sex-based differences in background MN frequencies in mice confirm previous findings (Witt et al. 2000). The investigators indicated that they did not show MN-NCE data from rats because these cells are removed from the circulation by the action of the spleen; thus, levels of MN-NCEs in rat blood are not reliable indicators of chromosomal damage.

THE ACES REVIEW PANEL'S EVALUATION OF THE STUDY BY BEMIS ET AL.: RESULTS AND INTERPRETATION

The panel considered that Bemis and colleagues had assessed a well-accepted endpoint — the presence of MN in red blood cell populations — to determine whether exposure to DE from a 2007-compliant engine had genotoxic effects. The data were analyzed with appropriate statistical approaches, and the conclusions drawn were generally appropriate — in particular, that there were no exposure-related changes in the numbers of MN detected in RETs measured in the blood of mice or rats exposed for 1 or 3 months to DE. Data from a pilot study indicated that an increase in MN- in RETs *could* be detected after treating rats with cyclophosphamide, establishing that changes in the endpoint could be detected by the assay used.

One limitation specific to the Bemis study was noted: the small numbers of RETs counted. The pilot study data indicated that the proportion of MN-RET in total RET detected after acute treatment with a known genotoxic agent, cyclophosphamide, was below 5%. The proportion of MN-RET in RET in animals exposed to DE was likely to be even smaller. However, in both treatment scenarios, the investigators counted only 20,000 total cells per assay run. The investigators indicate that counting this number is standard in the field and close to optimal sensitivity (Kissling et al. 2007). Nonetheless, the panel believed that counting more cells (for example, 100,000) might have increased the chances of detecting any small but statistically significant increase in the number of MN-RET over background.

FEATURES UNIQUE TO THE STUDY BY HALLBERG ET AL.

Aims

The goal of the ancillary study by Hallberg and colleagues was to assess oxidative damage to DNA in the lung and blood — measured as DNA strand breaks and 8-OHdG adducts, respectively — and damage to lipids in brain tissue (specifically, the hippocampus) — measured as TBARS.

Samples

Samples from animals euthanized after the 1- and 3-month exposures were collected at LRRI and shipped frozen to Hallberg and colleagues. The samples were taken from the following: from each of 5 male and 5 female rats — 50 µL plasma, lung right accessory lobe, and half the brain (with olfactory bulb); and from each of 5 male and 5 female mice — 50 µL (or as much as feasible) plasma, lung right accessory lobe, and the brain with olfactory bulb.

Methods

Comet Assay This assay detects damage to DNA by immobilizing cells on a slide containing agarose gel, lysing the cells, and then having the lysed cells undergo electrophoresis. In the electric current, intact double-stranded DNA remains in the nucleus, whereas damaged DNA moves out of the cell and can be detected as a tail or comet. DNA damage is quantified by measuring the displacement between DNA in the nucleus ("comet head") and the resulting "tail." Measurements included *tail length* (the distance of DNA migration in the tail) and *tail moment* (the product of the tail length and the fraction of total DNA in the tail). The investigators also used *olive tail moment*, which equals tail %DNA × tail moment length (that is, the distance from the center of the head to the center of the tail).

The investigators used an alkali modification (treatment at pH > 13) of the comet assay on lung tissue samples. This alkali modification detects single and double DNA strand breaks, as well as additional DNA structures, such as abasic sites (missing a nucleotide) and sites at which excision repair is occurring.

Hallberg and colleagues indicated that they would also assess DNA damage by the FLARE (fragment length analysis using repair enzymes) assay. Similar to the comet assay, the FLARE assay assesses DNA damage in lysed cells immobilized in an agarose gel after electrophoresis. However, they did not present data from these experiments in the report.

8-OHdG Adducts 8-OHdG is produced as a consequence of oxidative damage to DNA; specifically, it is secreted from cells during repair of damaged DNA. Hallberg and colleagues measured 8-OHdG in serum using an ELISA method and a commercial kit with sensitivity in the range of 100 pg/mL to 2 ng/mL. Results were normalized across blood samples for protein content and expressed as pg/mL/µg protein.

TBARS Lipid peroxidation is another mechanism of oxidative damage to cells resulting from oxidative stress. Lipid peroxides are unstable and decompose to form a mixture of more complex and reactive compounds, including hydroperoxides and aldehydes. One aldehyde that can be formed is malondialdehyde (MDA), which in vitro reacts with thiobarbituric acid to form a byproduct, TBARS, that can be measured colorimetrically or fluorometrically. In the current study, the investigators measured TBARS in hippocampal samples as MDA normalized for protein content of the tissue (µM MDA/mg).

Statistical Approaches

Comet Assay Hallberg and colleagues used descriptive statistics to examine the mean and SE of comet tail length and tail moment for rats and mice by sex and exposure groups (low, mid, high vs. control). They then used a mixed linear regression model to examine the exposure effects, controlling for sex, after assessing that the data were normally distributed. Sex and exposure interactions were also examined in the regression model.

For data with significant exposure effects (e.g., in rats and mice, 1-month tail length; and in mice, 1-month tail moment), multiple comparisons with Tukey adjustment were used for pair-wise comparisons among exposure

groups. Analyses were conducted using SAS, version 9.2. A follow-up analysis of %DNA in the tail was also conducted using both parametric and nonparametric methods. Student *t* tests, ANOVA, and Kruskal-Wallis tests were used to analyze sex, duration, and exposure-level effects, as well as any interactions between these factors. Post-hoc mean comparisons after the ANOVA were Bonferroni-corrected. These analyses were conducted with SPSS, version 16.1. A P value < 0.05 was used as the cutoff for statistical significance.

8-OHdG Hallberg and colleagues used descriptive statistics to examine the mean and SE of mouse and rat 8-OHdG levels (pg/mL/µg protein) by sex, exposure-level groups (low, mid, and high vs. control), and duration (1 and 3 months). Because the data were not normally distributed, they used nonparametric regression analysis to examine the exposure effects, controlling for sex, and stratified by duration (1 and 3 months). Sex and exposure-level interactions were also examined in this model. A duration effect was evaluated using the mixed model of rank-based regression analysis.

TBARS Descriptive statistics were used to examine the mean and SD of TBARS (MDA/mg tissue protein) by sex, exposure-level groups (low, mid, and high vs. control), and duration (1 and 3 months). The data were close to normal distribution, and so the investigators used a mixed model for repeated measures to examine the exposure effects, controlling for sex and duration. All possible interactions were examined in this full model. Results were found to be insignificant in the model. The investigators also used a reduced model with only sex and duration. No multiple comparisons were conducted for pair-wise comparisons among exposure groups for mouse data, because the overall treatment effects were not statistically significant in either the full or reduced model. Multiple comparisons among exposure groups were conducted with rat data. P values from the multiple comparisons were adjusted using the Tukey adjustment. All analyses were conducted using SAS, version 9.2.

Key Findings

Exposure of mice and rats for 1 or 3 months to DE from a 2007-compliant engine did *not* increase the following:

- DNA damage in lung cells, as measured by tail moment and tail length in the alkali modification of the comet assay; or
- Oxidative damage, as measured by 8-OHdG levels in serum or lipid peroxidation in the hippocampus (TBARS).

THE ACES REVIEW PANEL'S EVALUATION OF THE STUDY BY HALLBERG ET AL.: RESULTS AND INTERPRETATION

The panel considered that Hallberg and colleagues assessed well-accepted endpoints to determine whether exposure to DE from a 2007-compliant engine had genotoxic effects. The data were analyzed with generally appropriate statistical approaches. The report also provided a fair and balanced discussion of the study's limitations (discussed further below). The panel agreed with the investigators' overall conclusions that there were no exposure-related changes in DNA damage in lung cells, as measured by the alkali modification of the comet assay, in serum (by 8-OHdG levels), or by lipid peroxidation in the hippocampus (TBARS, as measured by MDA equivalents).

Some limitations to the interpretation of the findings were identified. First, the TBARS assay is not a particularly sensitive or specific assay for oxidative stress, and the assay used by Hallberg and colleagues measured only MDA, just one lipid peroxidation product. Thus, a full spectrum of possible changes in lipid peroxidation products was not observed in the hippocampus. Some uncertainties also remain about the statistical analysis given that the actual models used could have been better described. Thus, it was not clear whether model assumptions were valid for these analyses. This could have affected the interpretation of the results. Finally, the investigators did not use a "positive control" (e.g., the use of cyclophosphamide, as in the Bemis study), which might have provided additional confidence that changes in the genotoxic endpoints evaluated could have been detected by the assays used.

THE STUDIES BY BEMIS ET AL. AND HALLBERG ET AL.: JOINT ASSESSMENT BY THE ACES REVIEW PANEL

Items Common to the ACES Ancillary Genotoxicity Studies

In addition to the points made in the previous sections that were unique to each ancillary study, the panel made the following points about both studies.

Comparison with Previous Studies The lack of changes in rat and mouse genotoxic endpoints after 1 and 3 months of exposure to DE in the Bemis et al. and Hallberg et al. studies is consistent with previous studies that have looked at genotoxic endpoints in even longer exposures. These include Randerath and colleagues (1995), who did not find an increase in DNA adducts in samples from rats exposed for 23 months to DE, and Reed and colleagues (2004), who exposed male and female A/J mice at LRRI to

concentrations of DE up to 1 mg/m^3 for 7 days per week for 6 months. Exposure to DE did not affect the numbers of MN-RET 6 months after the end of the exposure. Ichinose and colleagues (1997) did find increases in tumors and 8-OHdG in rat lung tissue, and Iwai and colleagues (2000) found progressive changes in rat lung morphology and increases in lung tissue of DNA adducts and 8-OHdG over the course of the exposure (3.5 mg/m^3 for up to 30 months). Thus, evaluation of genotoxic endpoints at later exposure time points is expected to be informative.

Evaluation of Genotoxic Effects Outside the Airways The initial effects of inhaled DE are found in the airways, but the studies by Bemis et al. and Hallberg et al. both evaluated genotoxic effects in cells outside the airways: in addition to examining the lung, Bemis et al. assessed the incidence of MN in red blood cells, and Hallberg et al. assessed changes in blood and brain cells. How relevant to the effects of DE exposure are changes in non-airway cells? Several studies, reviewed in Riedl et al. 2012, have indicated that inhaled DE or other particles initially target the airways but that effects extend beyond the lungs and can be detected in blood and in other tissues and organs, including the brain (Crüt et al. 2008). How the effects of inhaled particles are mediated outside the lungs is not completely resolved and was not the object of these studies; effects may result from the direct transfer of smaller-sized particles out of the lung, or via indirect mechanisms involving inflammatory or neurologic signaling in the lung, which releases mediators that act outside the lung (Brook et al. 2010).

The panel recognized that the ACES genotoxic ancillary studies investigators anticipated finding effects attributable to inhaled particles, and thus agreed that evaluating the potential genotoxic effects of DE in blood cells was justifiable. Bemis et al. studied MN in peripheral blood red blood cells because they are an easily available target reporting on conditions leading to the development of MN in red blood cell precursors, which develop in the bone marrow and move into the circulation. Similarly the assessment of Hallberg and colleagues of one of their genotoxic endpoints — 8-OHdG — in blood was reasonable. The panel understood that Hallberg and colleagues chose to study lipid peroxidation in the brain because particles might affect the function of this organ, but the panel thought that evaluating this same endpoint in blood might have been equally useful.

Lack of a Positive Control Engine The study design did not include a side-by-side comparison with an older pre-2007 model-year engine. In their design of the ACES program, the ACES Oversight Committee had weighed the desirability of such a comparison but decided that such a "positive control" could not be included as it would have substantially increased the complexity and cost of the study and would have posed enormous logistical challenges. While acknowledging these problems, and recognizing that the ancillary studies investigators were not involved in this decision, the panel thought that such a side-by-side comparison could have enhanced the study.

Small Group Size The Bemis et al. and Hallberg et al. studies both evaluated samples from only 5 animals of each sex per each exposure group. Although each investigator justified the choice of 5 samples through the use of a power calculation, the evaluation of such a small number of samples raises issues about the reliability of the data. The power calculation provided only a minimum number of samples to be evaluated. The sample size may still be too small for the ANOVA approach that was used, since the estimate for the variations in these groups might not be accurate.

Use of Short-Term Assays Both the Bemis and Hallberg teams used short-term assays to assess genotoxicity. In the study by Bemis et al., the MN-RET assay assessed the effects of DE exposure on cells in the bone marrow, which traffic to the periphery within 24 to 36 hours after development in the bone marrow. Thus, the assay measures events that have occurred within the last 24 to 36 hours, rather than over the whole exposure period. In mice, however, MN-NCEs have a lifespan of approximately 1 month (MacGregor et al. 1990; Hayashi et al. 2000) and so can be considered a surrogate marker for DNA damage that has occurred over that time period. In the study by Hallberg et al. the comet assay measured DNA damage, which is rapidly repaired; thus, this endpoint also reflects a short-term effect. In addition, Hallberg and colleagues measured 8-OHdG, which has a rapid turnover in blood, and TBARS, which has a similarly short half-life.

The panel recognized that the endpoints assessed in the Bemis et al. and Hallberg et al. studies are accepted markers of genotoxic effects and on the critical path to the development of tumors. Nonetheless, they considered that the use of assays of short-term effects was uncertain in assessing effects of long-term exposure to DE. Assays to assess mutations — for example, in the *hprt* gene in peripheral blood cells — might have provided valuable information on the longer-lasting effects of DE exposures.

Summary and Conclusions

In its independent review of the ancillary studies led by Bemis and Hallberg, the ACES Review Panel concluded

that the investigators assessed generally well-accepted markers of genotoxicity — MN formation in RETs (in the report by Bemis et al.) and DNA damage and lipid peroxidation (in the report by Hallberg et al.) — and that the studies were valuable extensions to the ACES core study. The panel agreed with the investigators' conclusions that no genotoxic effects associated with exposure for up to 3 months to any level of DE (0.1, 0.8, or 4.2 ppm NO_2) from the 2007-compliant engine could be detected. The small group size (only 5 animals of each sex in each exposure group) and the assessment of genotoxic endpoints that were relatively short term (lasting 1 month or less) slightly reduced confidence in the utility of these negative findings.

FEATURES UNIQUE TO THE STUDY BY CONKLIN AND KONG

Aims

The goal of the study by Conklin and Kong was to evaluate changes in blood lipids and protein markers of inflammation and thrombosis after exposure to DE. The authors' primary goal was to determine whether exposure to DE would induce systemic inflammation and a low-grade acute phase response (APR) that would result in abnormal activation of platelets and endothelial cells. Such thrombotic changes might be an indicator of downstream acute or chronic cardiovascular injury. To complement evaluation of the proposed blood markers, the investigators agreed to HEI's request to provide some measures of immunotoxicity by evaluating levels of selected immunoglobulin (Ig) classes in blood.

As a positive control for the assay of many of these markers, the investigators compared levels found in the current study with levels of the same markers in rats and mice that they had previously exposed to acrolein.

Scientific Background

Cardiovascular Effects of Exposure to DE Several studies have shown that long-term inhalation exposure to traditional DE enhances the development of lung cancer in rodents, particularly rats (see Scientific Background in the section "Commentary on ACES Phase 3B Core Study of Biologic Effects by McDonald et al.," which provides the core study background earlier in this Commentary). In addition, studies in humans and animals have shown that exposure to traditional DE or its components can affect *cardiovascular* responses (reviewed in the Commentary accompanying Riedl et al. 2012). Thus, although the major focus of ACES Phase 3B was to evaluate effects of "new" DE on carcinogenicity and associated endpoints such as genotoxicity, the study also provided an excellent opportunity to examine possible changes in cardiovascular-associated endpoints.

Several studies have found that short-term inhalation (1–2 hours) of "traditional" DE (100–300 $\mu g/m^3$ PM) by healthy human volunteers results in rapid changes in airway markers of the inflammatory response (e.g., Stenfors et al. 2004; Behndig et al. 2006, 2011). In addition, inhalation of DE (300 $\mu g/m^3$ PM for 1 hour) can cause vascular dysfunction in healthy men (Mills et al. 2005), and can alter subclinical markers of cardiac ischemia and thrombosis in men with coronary heart disease (Mills et al. 2007).

Short-term inhalation exposure of rats and mice to traditional DE has also been found to change markers of inflammation, vascular function, and thrombosis, particularly in models of cardiovascular conditions such as the spontaneously hypertensive rat (Campen et al. 2003) or the apolipoprotein E knockout mouse, which develops atherosclerosis (Hansen et al. 2007). Investigators at LRRI have conducted studies of the effects of short-term, subchronic, and lifetime exposures to traditional DE on inflammatory endpoints in "normal" rats and mice (F344 and A/J strains, respectively). After 3 months' exposure of F344 rats to 2.4 or 6.3 mg/m^3 PM in DE, Mauderly and colleagues (1994) found morphologic changes in the lungs associated with an inflammatory response — alveolar macrophage and epithelial cell hyperplasia. In more recent studies, Reed and colleagues (2004) found few changes in serum endpoints of F344 rats other than small decreases in cholesterol levels in both males and females exposed to up to 1000 $\mu g/m^3$ PM in DE — circulating levels decreased by approximately 20% after 1 week of exposure in both male and females and by approximately 14% in males only after 6 months. Serum cholesterol levels were not changed in similarly exposed A/J mice. In addition, Reed and colleagues (2006) found small decreases in serum cholesterol levels in rats exposed for 9 to 10 weeks to DE containing 500 but not 250 $\mu g/m^3$ PM.

Immunologic Effects of Exposure to DE Several early rodent studies showed that diesel exhaust particles (DEP) in the absence of gaseous components act as an *adjuvant* — that is, coadministration of DEP with antigen to rodents enhanced the antigen-specific antibody response, and in particular, the allergic-type response mediated by immunoglobulin E (IgE) (Muranaka et al. 1986; Takafuji et al. 1987, 1989; Fujimaki et al. 1997; Takano et al. 1997). Using intranasal administration of DEP, Diaz-Sanchez and colleagues (1994, 1997, 1999, 2000a,b) confirmed and extended these findings in both healthy and allergic humans, showing that DEP could enhance IgE-mediated responses

in the nose and an acute systemic inflammatory response. In addition, inhalation exposure to whole DE (i.e., containing both DEP and gases) (100 μg/m^3 PM for 2 hours) slightly enhanced levels of mediators associated with the allergic response and decreased viral clearance in the nasal lavage fluid of healthy volunteers exposed to attenuated flu virus (Noah et al. 2012). However, Diaz-Sanchez and colleagues were unable to find either systemic effects on the allergic response or effects on the lower airways of inhalation of whole DE (100 μg/m^3 PM for 2 hours) in human volunteers who were asthmatic and allergic to cat dander (Riedl et al. 2012). Thus, it remains unclear whether effects of exposure to DE in humans differ between the nose on the one hand and the lower airways and the circulation on the other.

Conklin and Colleagues' Studies of Acrolein Effects in Rodents Conklin and colleagues have previously examined the short-term cardiovascular effects in rodents of exposure to the toxic aldehyde acrolein — a combustion product present in cigarette smoke and engine exhaust — with a particular focus on changes in plasma lipid and lipoprotein levels and effects in liver after exposure via the gastrointestinal tract. (The liver is the site of the production of many APR reactants — see the accompanying sidebar.) They found that adult C57BL/6 and apolipoprotein E knockout mice fed 0.1 to 5 mg/kg acrolein by gavage had increased plasma cholesterol and triglyceride levels, with maximum levels 24 hours after exposure (Conklin et al. 2010). Acrolein-fed C57BL/6 mice also had modified plasma and hepatic proteins and increased plasma triglycerides within 15 minutes of exposure (Conklin et al. 2011). Sprague-Dawley rats fed 2 mg/kg acrolein showed an increase in plasma cholesterol lasting up to 5 days and elevated levels of phospholipids and triglycerides (Conklin et al. 2010). As described in the sections below, the data obtained after acrolein exposure provided useful comparisons with the data obtained after DE exposure in the current study.

Methods

Plasma Sample Collection

DE-Exposed Animals Peripheral blood from 10 male and 10 female rats (500 μL) and 10 male and 10 female mice (100 μL or more) killed after the 1- and 3-month exposures was collected at LRRI. Samples were shipped frozen to Dr. Conklin.

Acrolein-Exposed Animals Plasma samples from mice and rats exposed via gavage to acrolein (or water as the control) — referred to as "internal validation" or "positive controls" — were analyzed at the same time as the samples from the current study. These positive control samples, stored frozen, were from Conklin and colleagues' previous studies (2010, 2011), in which many of the same endpoints were measured.

Endpoints Measured The investigators measured plasma levels of lipids (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides) as well as total protein and albumin. Unless otherwise indicated in the report, all measurements were made using 10 samples per sex per exposure group for rats and 10 samples per sex per exposure group for mice. They also measured plasma concentrations of three immunoglobulin classes — IgE, IgG and IgM — by enzyme-linked immunosorbent assay (10 samples per sex per exposure group for rats, 4 to 6 samples per sex per exposure group for mice).

A commercial vendor measured levels of several cytokines, chemokines, and other soluble factors in plasma: granulocyte-macrophage colony-stimulating factor, monocyte chemotactic protein-1, IL-1β, IL-6, IL-10, interferon-γ, keratinocyte-derived chemoattractant, tumor necrosis factor-α, vascular endothelial growth factor, soluble intercellular adhesion molecule-1, fibrinogen, leptin, serum amyloid A, and C-reactive protein.

THE ACUTE PHASE RESPONSE

One key aspect of the early inflammatory response to an external agent (such as particles or an infectious pathogen) is the induction of an APR — cytokines such as interleukin (IL)-1, IL-6 and IL-8, and tumor necrosis factor-α entering the circulation and within hours stimulating the liver to synthesize APR reactants that include C-reactive protein, fibrinogen, and serum amyloid A. Levels of APR reactants such as albumin are decreased. The APR is also characterized by alterations in lipid metabolism and in the lipoprotein profile. In addition to their role in the acute response to external agents, APR reactants can also be detected in several chronic conditions, such as obesity, cardiovascular disease, and inflammatory arthritis.

Statistical Analyses The investigators used a standard 3-way analysis of variance with exposure level, sex, and exposure duration as factors and explored all possible interactions to make sure that the estimates for main effects were accurate. They also performed tests to ensure that any transformations of the data were appropriate.

Results

Exposure to DE at any concentration for 1 or 3 months had no effect on most of the blood markers measured in rats or mice. Some scattered changes were detected in mice and rats in one or more DE-exposed groups. However, of these changes, most were found at only one exposure time point and in one sex; some of these changes were inconsistent in direction, that is, they increased at 1 month but decreased at 3 months compared with the control, or vice versa.

For example, fibrinogen levels in mice were decreased compared with controls in males exposed to mid- and high-concentration DE only at 3 months. In addition, at all DE exposure levels, IgE levels were decreased in male mice at 1 month, but increased at 3 months. IgE levels in DE-exposed females decreased at 3 months compared with controls. In male rats, leptin levels increased after exposure to the low concentration of DE at 1 month, but decreased at the mid concentration of DE at 3 months.

In rats, plasma lipids and lipoproteins showed consistent changes at one time point and in one sex. Compared with controls, cholesterol and HDL levels increased approximately 20% in males at 1 month after exposure to the mid or high concentration of DE, and decreased approximately 20% in females at 3 months after exposure to the mid concentration of DE.

Exposure of mice and rats (albeit a different rat strain than was used in the current study) to acrolein via gavage resulted in increases in multiple plasma markers 24 hours after exposure, confirming that many of the plasma marker assays were sensitive enough to detect changes in the current study.

THE ACES REVIEW PANEL'S EVALUATION OF THE STUDY BY CONKLIN AND KONG: RESULTS AND INTERPRETATION

General Comments

In its independent review of the ancillary study by Conklin and Kong, the ACES Review Panel concluded that the major strengths of the study were that the investigators used appropriate statistical techniques to assess a large number of plasma markers associated with thrombosis and inflammation — including the APR — with the goal of identifying a sensitive marker or set of markers in blood that might reflect cardiovascular effects of exposure to DE from a 2007-compliant engine. They also measured multiple plasma markers of organ toxicity (such as of the liver and kidney). An additional strength of the study was that the investigators made side-by-side measurements of many of the same endpoints in the blood of rats and mice that they had treated with acrolein in separate studies. These measurements served as a positive control to verify that the assays used in the current study were sensitive enough to detect changes in response to another pollutant.

The Panel agreed with the investigators that DE exposure for 1 or 3 months had no effect on most of the blood markers that were measured in rats or mice. The lack of meaningful changes suggested that 16-hours-per-day exposure to DE for 1 or 3 months was associated with very few effects on thrombotic or inflammatory pathways, including the APR. They also agreed that the few changes in plasma markers that were detected were difficult to interpret, either because the changes were seen at only one time point or in one sex, or were inconsistent in terms of the direction of the effect at the two exposure time points. Similarly, some inconsistent changes in levels of Ig classes were also detected. These changes in Ig levels did not support the interpretation that new-technology diesel emissions acted as an adjuvant — an enhancer of specific immune responses — of the allergic response. This was not unexpected, because the adjuvant effects of DEP in animals and humans had been detected only when DEP was coadministered with allergen and at relatively high doses (e.g., Diaz-Sanchez et al. 1994; Takano et al. 1997), and that was not the case in the current study.

The Panel also considered that the specific issues raised in the following sections were important in interpreting the results of the study.

Unexplained Variation in Control Values

The investigators noted large and unexplained variations in baseline (i.e., air-exposed control) plasma values of many of the measured lipids, proteins, and cytokines, which did not seem to be related to the age of the rodents per se. The investigators were unable to identify the source of this variability, but speculated that it might reflect responses to an external stressor such as noise or handling. The Panel thought this suggestion was not unreasonable, but considered that the variation in baseline levels made it difficult to draw conclusions about the effects of exposure to DE.

One example of such baseline variation was in cholesterol levels (see Figure 1 of the Investigators' Report). As the investigators point out, the level of cholesterol in female

rats exposed to air in the 3-month exposures was 14 mg/dL higher than in the female rats exposed to air at 1 month. This difference in background value was larger than the decrease observed when comparing the mid-level DE concentration to the control exposure; in other words, the cholesterol level in female rats at 3 months exposed to the mid-level DE concentration was significantly lower than the control value at the same time point, but very similar to the levels in female rats exposed to either air or DE at 1 month.

Interpretation of Cholesterol and Lipoprotein Level Changes

For several reasons, the Panel was uncertain as to the biologic significance of the changes in levels of cholesterol and HDL that were detected in some groups of rats exposed to DE. First, the variation in baseline levels described in the preceding section complicated the interpretation of comparisons among DE-exposed and control groups. Second, because the animals had been fed ad libitum before sacrifice, levels of cholesterol and HDL at the time of death — as the investigators point out and the Panel agreed — would most likely reflect catabolism of the food most recently eaten, rather than the effects of exposure. Third, although significant changes were detected in cholesterol and HDL levels between DE-exposed and control groups, they were not consistent in terms of the direction of the effect, the length of exposure, or the sex of the rats — that is, cholesterol and HDL levels increased by approximately 20% after 1-month exposure to mid- and high-level DE in male rats, but decreased by approximately 20% after 3-month exposure to mid-level DE in female rats.

Interestingly, McDonald and colleagues, in Part 1 of this report, also analyzed blood samples in parallel at 1 and 3 months and measured some of the same endpoints (including cholesterol), albeit in serum rather than plasma as was assessed in the current study. These findings provide a useful comparison: Similar to Conklin and Kong, McDonald and colleagues found a decrease of approximately 20% in cholesterol levels in female rats at 3 months after exposure to the mid-level DE concentration (see Table E.9 in Part 1 of this volume). However, they also reported an increase of approximately 20% in male rats at this time point at low DE exposure, which was not found by Conklin and Kong. Thus, whatever the pathophysiologic significance of the changes in cholesterol, the pattern of change — at least in female rats — was consistent at the only time point at which samples were analyzed for cholesterol in both the McDonald and Conklin studies.

As noted in the Scientific Background section, subchronic exposure to traditional DE has resulted in small decreases in serum cholesterol levels in rats but not mice (Reed et al. 2004, 2006). Interestingly, similar to the findings of the current study, Reed and colleagues (2004) also found small changes in a few serum markers (levels of clotting factor VII and gamma-glutamyl transferase) but concluded that "several other responses met screening criteria for significant exposure effects but were not consistent between genders or exposure times and were not corroborated by related parameters." Thus, taken overall, these results suggest that subchronic exposure to either traditional or 2007-compliant DE has few consistent effects on the cardiovascular endpoints that have been measured to date.

Mice and Rats as Models of Cardiovascular Effects

The Panel recognized that the investigators were interested in attempting to identify possible cardiovascular markers of exposure to DE from a 2007-compliant engine, with changes in thrombotic and inflammatory pathways as the key focus. However, the Panel noted that the use of "normal" rats and mice to evaluate cardiovascular changes of relevance to humans is challenging. This is because humans develop atherosclerosis as the result of an inflammatory condition in which increases in cholesterol levels and LDL are involved in the initiation of the lesion (Libby and Aikawa 2002). In contrast, strains of rats and mice such as the Wistar Han and the C57BL/6 used in this study do not develop hyperlipidemia or atherosclerosis. In rats and mice, 70% to 80% of circulating cholesterol is in the form of HDL, compared with only approximately 20% in humans (Li al. 2011). These differences among species in the distribution of plasma cholesterol between HDL and LDL likely result from differences in expression of cholesteryl ester transferase protein, which exchanges triglycerides from LDL for cholesteryl esters from HDL and vice versa. Humans express the protein but rats and mice lack the gene and so do not (Barter et al. 2003). In addition, rats efficiently convert cholesterol into bile (Li et al. 2011). Thus, because of these differences in cholesterol metabolism in different species, the relevance of changes in cardiovascular endpoints measured in normal rats and mice to the development of human cardiovascular disease is uncertain.

Some special strains of mice (e.g., those with genes knocked out that are relevant to cholesterol metabolism or function, such as apolipoprotein E or the LDL receptor [Zaragoza et al. 2011]) and rats (e.g., the spontaneously hypertensive) do develop lipid-containing lesions or even frank atherosclerosis. Evaluating the effects of "new" diesel emissions in one or more of these rodent strains was discussed during the development of the original ACES protocol; such studies may be undertaken in the future to evaluate emissions from either 2007- or 2010-compliant systems.

Summary and Conclusions

In its independent review of the ancillary study by Conklin and Kong, the ACES Review Panel concluded that the investigators used appropriate statistical approaches to assess a wide-ranging panel of plasma markers, with the goal of finding one or more markers of cardiovascular effects that might result from subchronic exposure (1 or 3 months) to DE from a 2007-compliant engine.

The Panel agreed with the investigators' conclusions that most of the endpoints measured in thrombotic and inflammatory pathways (including the APR) were not affected by exposure for 1 or 3 months to the concentrations of DE (0.1, 0.8, or 4.2 ppm NO_2) tested in this study. Some scattered changes in plasma markers were detected in some groups of mice and rats exposed to DE, including changes in cholesterol and HDL levels in rats. The Panel noted, however, that several considerations make the pathophysiologic significance of these observations uncertain: The changes reported were seen at only one time point or in one sex, or were not consistent in terms of the direction of the effect at the two exposure time points. Similarly, inconsistent and scattered changes in levels of IgE were seen, but these changes did not support the interpretation that new-technology diesel emissions acted as an adjuvant — an enhancer of specific immune responses — for the allergic response, as has been suggested for the effects of DEP in some prior animal and human studies.

Unexplained variations in baseline (i.e., air-exposed control) plasma values of many of the markers measured at 1 and 3 months also complicated the interpretation of changes. It is further challenging to extrapolate to humans changes in cholesterol or HDL levels in rodents because of differences in cholesterol metabolism among species and because normal rats and mice do not develop atherosclerosis. Nonetheless, the paucity of changes reported in the study — in a large panel of endpoints assessed — suggests that exposure to the concentrations of DE employed in the study from a 2007-compliant engine triggers few, if any, cardiovascular responses that were sustained or detectable after 1 or 3 months.

OVERALL CONCLUSIONS FOR THE ACES PHASE 3B REPORTS

This report contains the results from a comprehensive and well-executed study by McDonald and colleagues to assess the effects of inhalation of a range of DE concentrations from a heavy heavy-duty engine compliant with 2007 emissions regulations. The McDonald report includes findings from rats exposed to DE for 1, 3, and 12 months and from mice exposed for 1 and 3 months. In addition, the report contains results from generally well-implemented ancillary studies by Bemis and Hallberg and their colleagues in mice and rats exposed for 1 and 3 months to assess genotoxicity endpoints that are not normally part of chronic inhalation bioassays. Conklin and Kong's study was also wide ranging in its attempt to measure multiple plasma markers (approximately 30) associated with inflammation and thrombosis to identify possible cardiovascular markers of DE exposure.

Based on its independent review, the HEI ACES Review Panel highlighted several strong points: The study by McDonald and colleagues is the first to conduct a careful and comprehensive evaluation of the subchronic effects in rodents of inhalation of a range of DE levels from a heavy heavy-duty 2007-compliant engine. Even while applying a unique and strenuous 16-hour engine operating cycle, McDonald and colleagues successfully maintained the continuous operation for more than 12 months of a facility in which engine exhaust was generated and transported to rodent exposure chambers. The investigators also attained DE exposure atmospheres within 20% of the designated target levels over the exposure period. Because PM levels in the engine exhaust were greatly reduced compared with emissions from older engines, the exposure concentrations were based on levels of NO_2 (4.2, 0.8, and 0.1 ppm) rather than PM. These NO_2 levels were chosen to provide a comparison with the same cumulative exposure to NO_2 (the product of concentration and exposure duration) used in prior HEI-funded rat inhalation studies by Mauderly and colleagues (1987, 1989).

In their extensive analysis of the physical and chemical composition of the emissions, McDonald and colleagues found that the most abundant pollutants were CO_2, CO, NO, and NO_2, whereas concentrations of particulate matter, SO_2, and semivolatile and volatile organic species were very low. These findings confirm that the components of emissions from the 2007-compliant engine differ strikingly from those of older engines, in which particulate matter concentrations are much higher.

The multiple standardized toxicity endpoints evaluated in this study — including histology, serum chemistry, and respiratory function — were appropriate for evaluating the hypothesis for the study, namely, that the emissions would not cause an increase in tumor formation or substantial toxic health effects in rats and mice, although some biologic effects might occur.

The panel agreed with McDonald and colleagues that there were no changes in health endpoints for the majority of biologic tests conducted in rats and mice. Comparing results at 1 and 3 months across the species, the few

changes observed were reported more often in rats than mice and occurred almost exclusively with exposure to high-level DE.

The investigators reported mild histologic changes in the respiratory tract of rats: In the lung, changes were detected after 3 months of exposure to high-level DE, and the changes progressed at the 12-month exposure time point, in that they were more widespread within the lung and were found in more animals. Nonetheless, the histologic changes were still mild as defined by the investigators' scoring system (i.e., a score of 1 on a 4-point scale). Mild thickening of the central acinus, the junction of the conducting airways and the gas exchange region of the lung, was noted. In addition, the nose and turbinate of a very small number of rats showed scattered changes after 3 months of exposure, and these generally mild changes were detected in a few more male and female rats at all DE exposure levels at 12 months.

The panel agreed with the investigators' suggestion that the changes in lung histology detected in the current study after exposure to 2007-compliant DE are consistent with effects observed in Mauderly and colleagues' earlier studies of long-term exposure to NO_2. However, the effects of other gaseous components of DE cannot be ruled out.

Some small changes in respiratory function of rats were noted at 3 months, but of these, only a decrement in DL_{CO} may have persisted at 12 months. A decrease in DL_{CO} suggests the possibility of effects on pulmonary gas transfer or the pulmonary circulation, which would be consistent with the observed histologic changes in the gas exchange region of the lung. Some small changes in biochemical endpoints, particularly related to oxidant stress pathways, were also noted in rats in BALF and lung tissue at 1 and 3 months. (The results of 12-month biochemical assessments will be included in the final report from the investigators.) Overall though, these changes were small, and there was a lack of coherence among the endpoints; that is, endpoints that might have been expected to change in concert — because they share a common pathway — did not do so. These discrepancies among endpoints in the same pathway may reflect the different sensitivities of the individual assays used to measure changes, or they may be just anomalous observations.

The panel noted that the study design did not include a side-by-side comparison with an older pre-2007 model-year engine. While recognizing that such a "positive control" could not be included as it would have substantially increased the complexity and cost of the study and would have posed enormous logistical challenges, the panel thought that such a side-by-side comparison could have enhanced the study.

The panel also identified some other limitations to the study — some biochemical assays lacked positive controls (to determine that each was sensitive enough to detect changes). And, rather than using a standard three-way analysis of variance on the entire data set, in some statistical approaches the investigators combined data from both sexes and used a trend analysis. The panel also thought that more precise quantitative histopathologic information (such as morphometric readings in the lung) would have enhanced the study. The panel concluded that the ancillary studies assessed generally well-accepted markers of both genotoxicity — MN formation in RETs (in the report by Bemis et al.) and DNA damage and lipid peroxidation (in the report by Hallberg et al.) — and systemic inflammation and thrombosis (in the report by Conklin and Kong), and that these studies were valuable extensions to the ACES core study. The panel agreed with the investigators' conclusions that no genotoxic effects could be detected that were associated with exposure for up to 3 months to any level of DE (0.1, 0.8, or 4.2 ppm NO_2) from the 2007-compliant engine. The small group size (only 5 animals of each sex per exposure group) and the assessment of genotoxic endpoints that, although well validated, are relatively short term (lasting 1 month or less) slightly reduced confidence in the utility of these negative findings.

Most of the thrombotic and inflammatory endpoints measured in plasma in Conklin and Kong's study were not affected by exposure to DE, but some scattered changes — including changes in rat levels of cholesterol and high density lipoprotein — were detected. The pathophysiologic significance of these scattered changes was uncertain, however, because they were seen at only one of the two exposure time points in each sex, and the direction of the change (positive or negative) differed at the different exposure time points. Similarly, inconsistent and scattered changes in levels of IgE were seen, but these changes did not support the interpretation that new-technology diesel emissions acted as an adjuvant — an enhancer of specific immune responses — for the allergic response, as has been suggested for the effects of DEP in some prior animal and human studies.

Overall, these results indicate that rats exposed to one of three levels of DE from a 2007-compliant engine for up to 12 months, for 16 hours per day, 5 days a week, with use of a strenuous operating cycle that was more realistic than cycles used in previous studies, showed few DE exposure-related biologic effects. Even fewer exposure-related biologic effects were found in mice exposed for 3 months to DE. In rats, the effects that were observed were limited to the respiratory tract and were mild, and the changes in lungs were consistent with previous findings after long-term exposure to NO_2 — a major component of

the exposure atmosphere. No exposure-related genotoxic effects were found in rats or mice after 3 months of exposure. Rats will continue to be exposed for up to 30 months. In 2013, McDonald, Bemis, Hallberg, and their colleagues, and Conklin and Kong will submit reports from later and final exposure time points that will provide further comprehensive reviews of the effects of long-term exposure to DE emitted by a 2007-compliant engine.

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