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Advanced Collaborative Emissions Study (ACES): Lifetime Cancer and Non-Cancer Assessment in Rats Exposed to New-Technology Diesel Exhaust

Includes a Commentary by the Institute's ACES Review Panel

Advanced Collaborative Emissions Study (ACES): Lifetime Cancer and Non-Cancer Assessment in Rats Exposed to New-Technology Diesel Exhaust

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 330 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 more than 260 comprehensive reports published by HEI, as well as more than 1000 articles in the peer-reviewed literature.

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 184, *Advanced Collaborative Emissions Study (ACES): Lifetime Cancer and Non-Cancer Assessment in Rats Exposed to New-Technology Diesel Exhaust,* 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, 3, 12, and 24 months of exposure, and interpretations and conclusions of the studies. The report on the core bioassay by McDonald and colleagues also includes lifetime results (28 months for males; 30 months for females).

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. Each revised report was evaluated by the Panel, which prepared the Commentary based on the contents of all final reports.

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PREFACE

HEI's Advanced Collaborative Emissions Study: Phase 3B

INTRODUCTION

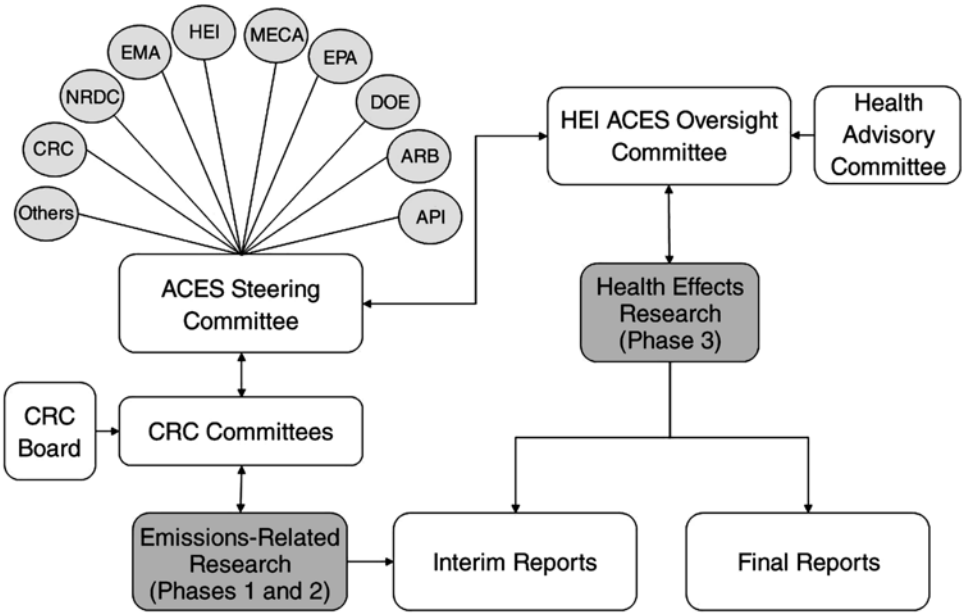
Because of health concerns related to exposure to diesel exhaust (DE*) emissions, the U.S. Environmental Protection Agency (EPA) and the California Air Resources Board (CARB) in 2001 adopted stringent new standards for diesel fuel and for heavy-duty diesel engine emissions. Starting with model-year 2007, engines were required to meet a new standard for particulate matter (PM) and, beginning 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 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. For clarity, exhaust from the modern advanced engines and emission control systems is sometimes referred to as "new-technology diesel exhaust" (NTDE) and exhaust from pre-2007 engine technology is referred to as "traditional-technology diesel exhaust" (TDE).

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 stringent 2010 standards (including more advanced NO_x controls). This phase was conducted at SwRI in 2012.
- **Phase 3**: Health effects assessment in rodents using the selected 2007-compliant heavy-duty diesel engine system among the four tested in Phase 1. 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 was 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 included a 90-day inhalation study in mice and a lifetime inhalation study in rats with health measurements at several time points, 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 were the responsibility of HEI and were overseen by the HEI ACES Oversight Committee

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



Preface Figure. ACES flow chart 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).

(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 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 Phases 1 and 2, the emissions characterization of four 2007-compliant engines and three 2010-compliant engines, respectively, have been published elsewhere (Khalek et al. 2009, 2011, 2013).

The investigators' reports in this Research Report describe the results from 12- and 24-month exposures in rats, including final pathology results in rats exposed for 28 months (males) or 30 months (females) in the core study. An earlier series of investigators' reports described interim 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 (ACES 2012). Both reports are accompanied by a detailed Commentary from a panel of expert reviewers appointed by HEI.

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 non-cancer 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 (see Table). After a review of the project

Preface Table. Summary of Studies Completed 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

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. The rationales for these choices are described below.

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 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. Three of the funded studies measured endpoints of genotoxicity and mutations, 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 Kong. Two of the ancillary studies 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 Additional Materials I (available on the Web at *http://pubs.healtheffects.org*) of the report by McDonald and colleagues in this volume.

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 bioassays of DE). During 2005 and 2006, the NTP was reconsidering its use of the F344 strain because of 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 within 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 aryl hydrocarbon 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 discussion of this issue is provided in the HEI ACES Review Panel Commentary which is a part of this report.

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 spontaneous 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 four 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 among rats was reduced from six to four.

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 three 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, any 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 were conducted 16 hours per day, 5 days a week for 30 months. 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 provided a longer time window to examine whether tumor formation occurs.

Because of lower survival rates of male rats compared with females, exposures of males were terminated after 28 months, as described in more detail in the report by McDonald and colleagues, Part 1 of this volume.

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 engines previously used at LRRI (which were light-duty), HEI funded LRRI to construct 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 *http://pubs.healtheffects.org*).

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 in Phase 3. 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. The 16-hour engine cycle was used to characterize the emissions of 2007- and 2010-compliant engines in Phases 1 and 2, as well as to expose animals in Phase 3.

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 *http://pubs.healtheffects.org*). A duplicate engine (referred to as *engine B′*) 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 features and emissions controls. At the time Phase 3B was about to start, engine B′ had a larger share of the market (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, medium, 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 only non-cancer health effects have been observed with exposure to NO_2 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 rats were exposed to NO_2 at 9.5 ppm for 7 hours per day, 5 days per week, for 24 months. This concentration would serve as the maximum tolerated dose (MTD). 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 (NOAEL).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

HEI initiated the multiparty ACES project to evaluate 2007- and 2010-compliant HHDD engine and control technologies. Phase 3 of ACES included 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 from one randomly selected 2007-compliant diesel engine for up to 30 months for all studies in rats and up to 3 months for all studies in mice (Phase 3B, described in this Research Report and in the interim report [ACES 2012]). This volume comprises detailed reports from all the investigators who completed health effects testing of DE from the 2007 engine, as well as a comprehensive Commentary by the HEI-appointed ACES Review Panel.

REFERENCES

Advanced Collaborative Emissions Study (ACES). 2012. Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Clark NN, Zhen F, Bedick C, Gautam M, Wayne W, Thompson G, et al. 2007. Creation of the 16-hour engine test schedule from the heavy heavy-duty diesel engine test schedule. CRC Report: ACES-1-a. Coordinating Research Council, Alpharetta, GA. Available at *www.crcao.org/publications/emissions/index.html* [accessed 10 January 2011].

Khalek IA, Blanks MG, Merritt PM. 2013. Phase 2 of the Advanced Collaborative Emissions Study. CRC Report: ACES Phase 2. Coordinating Research Council, Alpharetta, GA. Available at *www.crcao.org/publications/emissions/index.html* [accessed 30 October 2014].

Khalek IA, Bougher TL, Merritt PM. 2009. Phase 1 of the Advanced Collaborative Emissions Study. CRC Report: ACES Phase 1. Coordinating Research Council, Alpharetta, GA. Available at *www.crcao.org/publications/emissions/index.html* [accessed 10 January 2011].

Khalek IA, Bougher TL, Merritt PM, Zielinska B. 2011. Regulated and unregulated emissions from highway heavy-duty diesel engines complying with U.S. Environmental Protection Agency 2007 emissions standards. J Air Waste Manag Assoc 61:427–442.

King-Herbert AP, Sills RC, Bucher JR. 2010. Commentary: update on animal models for NTP studies. Toxicol Pathol 38:180–181.

King-Herbert A, Thayer K. 2006. NTP workshop: animal models for the NTP rodent cancer bioassay: stocks and strains — should we switch? Toxicol Pathol 34:802–805.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, et al. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust. Research Report 30. Cambridge, MA:Health Effects Institute.

Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study Phase 3A: Characterization of Engine-Exposure System Operation. Communication 17. Boston, MA:Health Effects Institute.

National Toxicology Program. 2011. Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP). National Toxicology Program, Research Triangle Park, NC.

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
EPA	U.S. Environmental Protection Agency
HHDD	heavy heavy-duty diesel
LRRI	Lovelace Respiratory Research Institute
MECA	Manufacturers of Emission Controls Association
MTD	maximum tolerated dose
NOAEL	no-observed-adverse-effect level
NO_x	nitrogen oxides
NRDC	Natural Resources Defense Council
NTDE	new-technology diesel exhaust
NTP	National Toxicology Program
PM	particulate matter
RFA	request for applications
RFP	request for proposals
SwRI	Southwest Research Institute
TDE	traditional-technology diesel exhaust

HEI STATEMENT

Synopsis of Research Report 184, Parts 1–4

Effects of Lifetime Exposure to Inhaled New-Technology Diesel Exhaust in Rats

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 chronic, lifetime exposures of rats (up to 30 months) and subchronic exposures (3 months) of mice to "new-technology diesel exhaust" (NTDE) — emissions from a heavy-duty diesel engine system compliant with 2007 U.S. Environmental Protection Agency (EPA) regulations. The studies were led by Drs. Jacob D. McDonald of the Lovelace Respiratory Research Institute (LRRI), 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. EPA and the California Air Resources Board adopted stringent regulations for heavy-duty highway diesel engines, which were required to meet a new standard for particulate matter (PM) 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 substantially reduce 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

What This Study Adds

- This is the first study to conduct a comprehensive evaluation of lifetime inhalation exposure to emissions from heavy-duty 2007-compliant engines (referred to as "new-technology diesel exhaust," or NTDE).
- The study evaluated the long-term effects of multiple concentrations of inhaled NTDE, which has greatly reduced particle emissions compared with "traditional-technology diesel exhaust" (TDE) in male and female rats on more than 100 different biologic endpoints, including tumor development, and compared the results with biologic effects seen in earlier studies in rats after exposure to TDE.
- Lifetime inhalation exposure of rats exposed to one of three levels of NTDE from a 2007-compliant engine, for 16 hours per day, 5 days a week, with use of a strenuous operating cycle that more accurately reflected the real-world operation of a modern engine than cycles used in previous studies, did not induce tumors or pre-cancerous changes in the lung and did not increase tumors that were considered to be related to NTDE in any other tissue. A few mild changes were seen in the lungs, consistent with long-term exposure to NO_2, a major component of NTDE, which is being further substantially reduced in 2010-compliant engines.

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 184 contains both the detailed Investigators' Reports and a Commentary on the study prepared by the Institute's ACES Review Panel.

exposure to these emissions, HEI, working in collaboration with the Coordinating Research Council, a nonprofit organization with expertise in emissions characterization, launched the multiphase ACES program. Phases 1 and 2 focused on emissions characterization, and Phase 3A established conditions for animal exposure. Phase 3B was designed to evaluate health outcomes in rats exposed to NTDE for up to 24 months, with the possibility of extension to 30 months, and in mice exposed for up to 3 months.

Through competitive processes, HEI funded several investigator teams in Phase 3B: a core study at LRRI, led by McDonald (who became principal investigator after the retirement of Dr. Joe L. Mauderly), and ancillary studies to evaluate endpoints not assessed in the core study. The overall hypothesis for ACES Phase 3B was that NTDE would *not* increase tumor formation or have 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. Reports from the investigator teams were reviewed by a specially convened ACES Review Panel, comprising members of HEI's Health Review Committee and outside experts. The current report focuses on findings in rats over the entire study; findings from subchronic exposures of mice and rats (up to 3 months of exposure) have already been published in HEI Research Report 166.

APPROACH

McDonald and colleagues generated exhaust from a 2007-compliant heavy heavy-duty diesel engine (defined as an engine installed in a vehicle with gross vehicle weight rating above 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.

The investigators exposed male and female 6-week-old Wistar Han rats (140 animals of each sex per exposure level) to one of three target 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 rather than PM, which had been used in previous studies of TDE, because the PM level in NTDE, identified in earlier phases of ACES, was so substantially reduced compared with TDE. Thus, calibrating exposures based on PM would have been problematic. 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) used in prior HEI-funded long-term inhalation studies in rats conducted by Mauderly and colleagues, in which minor biologic changes — but no cancer or pre-cancerous changes — were observed in the respiratory tract.

Exposures were conducted for 16 hours per day from approximately 1600 to 0800 hours for 5 days per week. The engine was run on a unique and strenuous operating cycle that represented more closely the behavior of modern engines than operating cycles used in older long-term studies of TDE. The emissions were characterized before they reached the animal exposure chambers as well as inside the chambers; in this way, the investigators could assess how the presence of the animals affected the composition of the exposure atmospheres.

Groups of male and female rats were euthanized at LRRI after 1, 3, 12, and 24 months of exposure, as well as at the terminal sacrifice — 28 months for males, 30 months for females. The LRRI investigators harvested blood and tissues for their analyses at these time points (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 evaluated animals histologically throughout the study for the presence of tumors and other types of lesions in the airways and in multiple tissues. In addition, they examined a vast array of biologic endpoints: 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 tissue injury), and pulmonary function.

For the assessments of genotoxicity, Bemis and colleagues measured the number of reticulocytes — immature red blood cells — containing micronuclei

in peripheral blood. Micronuclei can form as a result of a break in deoxyribonucleic acid (DNA) or from the disruption of chromosome segregation during cell 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, and whether chronic exposure had an effect on cardiac fibrosis or the remodeling of the aorta.

RESULTS AND CONCLUSIONS

In its independent review of the core ACES Phase 3B report by McDonald and colleagues, the HEI ACES Review Panel concluded that their study is the first to conduct a careful, comprehensive, and well-executed evaluation in rodents of lifetime inhalation exposure to NTDE from a 2007-compliant engine. Using appropriate statistical approaches to analyze the data from more than 100 endpoints in the broad areas of histology, serum chemistry, systemic and lung inflammation, and respiratory function, the investigators confirmed the a priori hypothesis, namely, that NTDE would *not* cause an increase in tumor formation or substantial toxic health effects in rats, although some biologic effects might occur.

Over the entire exposure period, the investigators attained NTDE exposure atmospheres within 20% of the target NO_2 levels. 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, NO, and NO_2. Concentrations of engine-generated PM were very low (<11 $\mu g/m^3$) at all exposure levels (in the ultrafine range of 20–40 nm in diameter), as were concentrations of sulfur dioxide and semivolatile and volatile organic species. These findings confirm that the concentrations of components of NTDE differ strikingly from those of older engines, in which the concentrations of PM, as well as volatile and PM-associated organic species, are much higher.

Most biologic endpoints evaluated showed no NTDE-associated changes after exposure of rats for up to 28 months in males and 30 months in females. In particular, chronic exposure to NTDE did not induce tumors or pre-cancerous changes in the lung and did not increase tumors that were considered to be related to NTDE in any other tissue. Some mild histologic changes were found in the lung; however, these were not pre-cancerous lesions, previously described in long-term exposure studies of rats to TDE. Rather, the histologic changes — periacinar epithelial hyperplasia, bronchiolization, accumulation of macrophages, and periacinar interstitial fibrosis — were confined to a small region, the centriacinus, which is involved in gas exchange.

HEI convened a separate panel of expert pathologists, the Pathology Working Group (PWG), to evaluate the histopathology data collected. The PWG findings confirmed the major histopathologic observations reported by the investigators. Also, the PWG, by evaluating the findings of this study side by side with findings from prior long-term exposure studies, provided a context with which to compare and contrast the current study findings with those of other relevant long-term studies of exposure to TDE and oxidant gases. The overall conclusion was that chronic exposure of rats to NTDE did not produce tumors in the lung, in marked contrast to the effects of chronic exposure to TDE observed in multiple previous rat studies, in which lung tumors, as well as inflammation and the deposition of soot in the lung, were observed. Rather, the effects of NTDE in the lung more closely resembled changes noted after long-term exposures to gaseous oxidant pollutants, in particular NO_2, and to TDE from which particles have been filtered out. It is possible that components of NTDE other than NO_2 may have contributed to the effects reported, but the low levels of other components suggest that they would not be primarily responsible.

The ACES Review Panel concluded that the multiple toxicity endpoints evaluated — including lung and serum chemistry and respiratory function — were appropriate for evaluating a wide range of possible biologic effects. There were small decreases in some respiratory endpoints, in particular those concerned with expiratory flow, predominantly at the highest exposure level and more in females than males. The diffusing capacity of carbon monoxide (DL_{CO}, a measure of alveolar–capillary gas exchange) showed a small effect of exposure to

NTDE. The Panel considered the small reductions in DL_{CO} to be consistent with the histopathologic findings of mild changes in the gas-exchange regions of the lung, indicating that the histologic changes might have had functional effects. In addition, some small changes in a few markers of oxidative stress and inflammation were detected in lung tissue, bronchoalveolar lavage fluid, and blood. The Panel identified a minor limitation to the study: some biochemical assays lacked positive controls (to determine that each was sensitive enough to detect any changes).

The Panel considered that the ancillary studies by Bemis et al., Hallberg et al., and Conklin and Kong were valuable extensions to the ACES core investigation. These generally well implemented studies took advantage of samples collected by McDonald and colleagues at several exposure time points up to 24 months to assess multiple endpoints that are not normally part of chronic inhalation bioassays. The genotoxicity studies assessed well-accepted endpoints — the frequency of micronucleated reticulocytes (immature red blood cells) in blood in the report by Bemis et al., and DNA damage and lipid peroxidation in the report by Hallberg et al. Conklin and Kong assessed a wide range of plasma markers associated with systemic inflammation and thrombosis, as well as markers of more chronic effects, to identify possible cardiovascular effects of NTDE. The Panel agreed with the conclusions of Bemis and colleagues and of Hallberg and colleagues that no genotoxic effects could be detected that were associated with exposure for up to 24 months to NTDE. However, the Panel noted that the assays measured relatively short-term effects (lasting 1 month or less), which somewhat reduced confidence in the utility of these negative findings. In Conklin and Kong's study, NTDE had no effect on cardiac fibrosis or aortic remodeling and few effects, predominantly in females and of uncertain pathophysiologic significance, on the inflammatory and thrombotic pathway endpoints measured in plasma over 24 months of exposure.

Overall, these results indicate that rats exposed to one of three levels of NTDE from a 2007-compliant engine for up to 30 months, for 16 hours per day, 5 days a week, with use of a strenuous operating cycle that more accurately reflected the real-world operation of a modern engine than cycles used in previous studies, showed few exposure-related biologic effects. In contrast to the findings in rats chronically exposed to TDE, there was no induction of tumors or pre-cancerous changes in the lung and no increase in tumors that were considered to be related to NTDE in any other tissue. The effects that were observed with NTDE were limited to the respiratory tract and were mild and generally seen only at the highest exposure level. The histologic changes in the lungs were consistent with previous findings in rats after long-term exposure to NO_2 — a major component of the exposure atmosphere, which is being substantially further reduced in 2010-compliant engines.

Part 1. Assessment of Carcinogenicity and Biologic Responses in Rats After Lifetime Inhalation of New-Technology Diesel Exhaust in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 1. Assessment of Carcinogenicity and Biologic Responses in Rats After Lifetime Inhalation of New-Technology Diesel Exhaust in the ACES Bioassay

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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 2007-compliant new-technology diesel exhaust (NTDE*). The a priori 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 was tested at the Lovelace Respiratory Research Institute (LRRI) by exposing rats by chronic inhalation as a carcinogenicity bioassay. Indicators of pulmonary toxicity in rats were measured after 1, 3, 12, 24, and 28–30 months of exposure. Similar indicators of pulmonary toxicity were measured in mice, as an interspecies comparison of the effects of subchronic exposure, after 1 and 3 months of exposure. A previous HEI report (Mauderly and McDonald 2012) described the operation of the engine and exposure systems and the characteristics of the exposure atmospheres during system commissioning. Another HEI report described the biologic responses in mice and rats after subchronic exposure to NTDE (McDonald et al. 2012). The primary motivation for the present chronic study was to evaluate the effects of NTDE in rats in the context of previous studies that had shown neoplastic lung lesions in rats exposed chronically to traditional technology diesel exhaust (TDE) (i.e., exhaust from diesel engines built before the 2007 U.S. requirements went into effect). The hypothesis was largely based on the marked reduction of diesel particulate matter (DPM) in NTDE compared with emissions from older diesel engine and fuel technologies, although other emissions were also reduced. The DPM component of TDE was considered the primary driver of lung tumorigenesis in rats exposed chronically to historical diesel emissions.

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 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

This Investigators' Report is one part of Health Effects Institute Research Report 184, 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; e-mail: *JMcDonal@lrri.org*.

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

components and periodic detailed physical–chemical characterizations. Exposures were conducted 16 hours/day (overnight, during the rats' most active period), 5 days/week. Responses to exposure were evaluated via hematology, serum chemistry, bronchoalveolar lavage (BAL), lung cell proliferation, histopathology, and pulmonary function.

The exposures were accomplished as planned, with average integrated exposure concentrations within 20% of the target dilutions. The major components from exhaust were the gaseous inorganic compounds, nitrogen monoxide (NO), NO_2, and carbon monoxide (CO). Minor components included low concentrations of DPM and volatile and semivolatile organic compounds (VOCs and SVOCs).

Among the more than 100 biologic response variables evaluated, the majority showed no significant difference from control as a result of exposure to NTDE. The major outcome of this study was the absence of pre-neoplastic lung lesions, primary lung neoplasia, or neoplasia of any type attributable to NTDE exposure. The lung lesions that did occur were minimal to mild, occurred only at the highest exposure level, and were characterized by an increased number and prominence of basophilic epithelial cells (considered reactive or regenerative) lining distal terminal bronchioles, alveolar ducts, and adjacent alveoli (termed in this report "Hyperplasia; Epithelial; Periacinar"), which often had a minimal increase in subjacent fibrous stroma (termed "Fibrosis; Interstitial; Periacinar"). Slight epithelial metaplastic change to a cuboidal morphology, often demonstrating cilia, was also noted in some animals (termed "Bronchiolization"). In addition to the epithelial proliferation, there was occasionally a subtle accumulation of pulmonary alveolar macrophages (termed "Accumulation; Macrophage") in affected areas. The findings in the lung progressed slightly from 3 to 12 months, without further progression between 12 months and the final sacrifice at 28 or 30 months. In addition to the histologic findings, there were biochemical changes in the lung tissue and lavage fluid that indicated mild inflammation and oxidative stress. Generally, these findings were observed only at the highest exposure level. There was also a mild progressive decrease in pulmonary function, which was more consistent in females than males. Limited nasal epithelial changes resulted from NTDE exposure, including increases in minor olfactory epithelial degeneration, hyperplasia, and/or metaplasia. Increases in these findings were present primarily at the highest exposure level, and their minor and variable nature renders their biologic significance uncertain.

Overall, the findings of this study demonstrated markedly less severe biologic responses to NTDE than observed previously in rats exposed similarly to TDE. Further, the effects of NTDE in the present study were generally consistent with those observed previously in rats exposed chronically to NO_2 alone. This suggests that NO_2 may have been the primary driver of the biologic responses to NTDE in the present study. There was little evidence of effects characteristic of rats exposed chronically to high concentrations of DPM in TDE, such as an extensive accumulation of DPM within alveolar macrophages and inflammation leading to neoplastic transformation of epithelia and lung tumors.

INTRODUCTION

The multidisciplinary ACES program was designed by HEI and the Coordinating Research Council (CRC) to better characterize the composition and potential toxicity of NTDE. The program addressed the advances in engine design, aftertreatment design, and reformulated fuels developed to meet 2007 and 2010 U.S. Environmental Protection Agency (EPA) emissions standards. The program consisted of three phases: characterization of 2007-compliant emissions (Phase 1; Khalek et al. 2011), characterization of 2010-compliant emissions (Phase 2; Khalek et al. 2013), and assessment of the toxicity of exhaust from 2007 technology (Phase 3; current report). McDonald and colleagues (2012) reported the findings in rats and mice exposed for 1 and 3 months (4 and 13 weeks), and the respiratory pathology and pulmonary function results in rats after 12 months of exposure.The current report adds the results of exposures of rats studied over the full length of the chronic bioassay (up to 30 months) and includes results from the interim sacrifices at 12 and 24 months. Appendix I of this report (available on the HEI Web site at *http://pubs.healtheffects.org*) provides details of the test atmosphere composition and the exposure system performance over the course of the study.

SPECIFIC AIM

The aim of this study was to determine biologic responses in rats exposed repeatedly from young adulthood to NTDE. The biologic response indicators included standardized toxicity endpoints such as tissue histopathology, clinical chemistry, clinical pathology, biochemical indicators of lung inflammation and oxidative stress, and pulmonary function. A major goal of the study was to evaluate the potential of NTDE to cause pre-neoplastic or neoplastic lesions in rats for comparison with the previously described effects of TDE under similar exposure conditions. To accomplish this, the overall study design was similar to that of an earlier HEI-funded study comparing lung carcinogenicity in rats after chronic exposure to TDE or carbon black (Mauderly et al. 1994; Nikula et al. 1995).

METHODS AND STUDY DESIGN

The animal groups used in this study are described in Table 1. Wistar Han rats (HsdRccHan:Wist, Harlan Laboratories) (10/sex/group for interim toxicity assays and 100/sex/group for long-term observation and histopathology) were exposed by inhalation 16 hours/day, 5 days/week to one of three dilutions of NTDE or to clean air as a control. Female rats were exposed for up to 30 months, but remaining males were euthanized at 28 months because their survival rate was lower than that of the females. Randomly selected rats were euthanized after 1, 3, 12, or 24 months of exposure for interim evaluations. Those evaluations included pulmonary function, necropsy, organ weights, bronchoalveolar lavage fluid (BALF) analysis, hematology, serum chemistry, lung cell proliferation, and histopathology of lesions and standard organ sections. Tissue and fluid samples were also collected from euthanized animals for the evaluation of additional responses in ancillary studies conducted by investigators outside LRRI (see the other Investigators' Reports in this volume).

ANIMALS AND MAINTENANCE

Rats were received at 6 weeks of age and quarantined and conditioned in inhalation exposure chambers for a period of no less than 14 days before being randomly assigned to exposure groups. They were housed individually in stainless-steel wire-mesh cages within stainless steel 1- or 2-m^3 Hazleton-type whole-body inhalation exposure chambers (H1000 and H2000, respectively; Lab Products, Seaford, DE) throughout the quarantine and exposure periods. The chambers were maintained daily by cleaning of excreta trays and visually inspecting the rats. Ad libitum water availability was verified daily by bleeding out the water lines. The 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 weekly, so that each cage unit was rotated through all positions within its chamber as the study progressed.

To verify the specific pathogen-free status of the rats, blood for serologic analysis was obtained from each group of animals before their entry into the exposure chambers (after release from quarantine). 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 and included H-1 parvovirus, Kilham rat virus, cilia-associated respiratory bacillus, pneumonia virus of rats, rat corona virus/Sialodacryoadenitis virus, and Sendai virus.

Exposure chamber flow rates were adjusted to maintain a minimum of 12 air changes per hour. The chambers were maintained at 2 to 5 cm of water pressure negative with respect to the room. Daily average temperatures were held within a target range of 18 to 26°C. The temperature fluctuated depending on the room temperature and would typically increase during each exposure period. In general, the highest exposure level had the highest temperature, which was typically 1.5° to 2° higher than the temperature at the lower exposure levels and in control chambers. Relative humidity was monitored, but not controlled or alarmed. A 12-hour light cycle (on from approximately 0600 to 1800 hours) was used. Tap water and 2016C Harlan Global Certified Rodent Chow were available ad libitum.

Table 1. Study Design to Examine Effects of Inhalation Exposure to NTDE in Wistar Han Rats[a]

Target NTDE Exposure	Sacrifice Time Point	Total No. of Rats per Sex per Exposure Level[b]	Endpoint Measurements
NO_2 (average integrated concentration) of 4.2 (high), 0.8 (mid), or 0.1 (low) ppm, or filtered air (control)	Terminal sacrifice (28 and 30 months)	100	Carcinogenesis and lifetime inhalation effects
	1 month	10	Pulmonary and systemic effects[c]
	3 months	10	Pulmonary and systemic effects[c]
	12 months	10	Pulmonary and systemic effects[c]
	24 months	10	Pulmonary and systemic effects[c]

[a] 6-Week-old male and female rats.

[b] The total number of rats used was a few more than the 1120 (140 × 4 exposure levels × 2 sexes) listed in the ACES Phase 3B Protocol (see Additional Materials 2 on the HEI Web site at *http://pubs.healtheffects.org*); additional animals were purchased in anticipation of losses during shipping from the breeding facility and were added to the chronic inhalation study.

[c] For a detailed list, see Table 2.

EXPOSURES AND MEASUREMENT OF EXPOSURE ATMOSPHERES

Detroit Diesel 2007 model Series 60 14.0L on-highway certified heavy-duty diesel engines and their stock aftertreatment systems were used to generate the test atmospheres for the ACES Phase 3 bioassay. This engine was selected from among the four candidate engines provided by different manufacturers and which were tested in Phase 1 of the ACES program at Southwest Research Institute (SwRI), San Antonio, Texas (Khalek et al., 2009). After the engine had been selected, the same engine manufacturer provided a backup engine, which was also characterized at SwRI. Characteristics of the emissions of these two engines, termed B and B′, and the process for engine selection are described in Khalek et al (2009 and 2011). Prestudy testing involved approximately 1200 hours of operation of each engine. Engine exchanges occurred during the exposure because of engine maintenance requirements. During the exposures, engine B′ was used from February 2010 to September 2011. That engine was replaced with engine B, which continued in operation until May 2012. Engine B′ was re-installed in June 2012 and operated until the end of the exposure (December 2012). At the end of the study, engine B′ had been operated for 10,090 hours, and engine B had been operated for 4031 hours. After each oil change and after each engine change-out, the engine's performance was "mapped" as required by 40 CFR Part 86. Speed, torque, and power measurements were compared with the speed, torque, and power cycle demand values using the "least squares" method. All engine maps met the criteria for performance.

The engine 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 (Dyne Systems, Jackson, WI) 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 variable-load duty cycle developed specifically for the ACES program (Clark et al. 2007). Diesel fuel meeting current on-road specifications ("ultra-low-sulfur") 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 CRC, which was provided by Lubrizol Corp., and was 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 I of this report (available on the HEI Web site, *http://pubs.healtheffects.org*).

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 and before entry into the dilution tunnel. The exhaust was diluted with filtered air under turbulent conditions at the injection point. 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 a portion of 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 (and in the exposure room), portions of the diluted exhaust were drawn through individual in-line extraction probes and routed through individual transit lines to each exposure chamber. The line to each chamber had its own secondary dilution system. Subsequent to extraction, the exhaust was diluted with filtered, compressed air provided through a rotary dilution and bypass system. Diluting flows were adjusted as needed to reach the final concentration targets. The residence time of diesel exhaust (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 nearly equal lengths for all exposure levels. Achieving the preselected target exposure concentrations of 4.2, 0.8, and 0.1 ppm NO_2 required total dilution ratios of approximately 25:1, 115:1, and 840:1. The dilution ratio in the primary dilution tunnel was approximately 5:1.

Exposures were conducted 16 hours/day, 5 days/week from approximately 1600 to 0800 hours Sunday through Thursday. Because the protocol required rats to be exposed on the day before necropsy, the Sunday–Thursday schedule allowed necropsies during the regular Monday–Friday workweek. 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.

Certain components of the exposure atmospheres were measured throughout each 16-hour exposure period.

Concentrations of NO and NO_2 were measured directly from each exposure level chamber throughout each exposure day. These samples were drawn from mid-chamber to represent the average of concentrations within the rats' breathing zone. Nitrogen oxides (NO_x) were also measured from the primary dilution tunnel to enable measurements of the secondary dilution ratio (the ratio of tunnel concentration to chamber concentration). CO, carbon dioxide (CO_2), non-methane hydrocarbons, particle mass (using a Dekati Mass Monitor), and black carbon (using a Photoacoustic Soot Spectrometer) were measured daily at the highest exposure level. These measurements were taken at the other exposure levels during periodic intensive characterizations and on those days the measurements were not made at the high level. Particle size was measured as part of the Dekati Mass Monitor analysis. A more detailed measurement of particle size was conducted once per week at each exposure level using a Fast Mobility Particle Sizer (TSI, St. Paul, MN). A periodic measurement of particle size in the chamber was conducted using an Aerodynamic Particle Sizer (TSI, St, Paul, MN). Particle mass concentration was measured once a week by gravimetric analysis of Teflon-membrane filters at the inlet of the chamber and at mid chamber at each exposure level. A detailed description of the composition of the test atmosphere is provided in Appendix I of this report (available on the HEI Web site, *http://pubs.healtheffects.org*).

EVALUATION OF HEALTH EFFECTS

The biologic response indicators are summarized in Table 2. Rats were observed twice daily for morbidity and mortality, and more detailed clinical evaluations using standardized criteria and computer entry of findings were performed during monthly weighing sessions. The rats were euthanized with an overdose of a pentobarbital–phenytoin euthanasia solution (Euthasol, VIRBAC, Fort Worth, TX). For interim assays, body and organ weights, blood, BALF, and frozen tissue specimens were collected in addition to fixed tissue specimens. The lungs were weighed, the left mainstem bronchus was clamped, and the right lung lobes were lavaged three times with phosphate-buffered saline (PBS) using 12 µL per gram of body weight (using average weights for females and males) at the time of euthanization. BAL was performed on the right lung only, preserving the left lung exclusively for histopathology. Right lung lobes were then frozen individually, and the left lung was instilled with neutral-buffered formalin (NBF) at 25 cm of hydrostatic pressure before immersion fixation. For rats designated for long-term observation, both right and left lung lobes were instilled with NBF and examined histologically. All major organs were examined and fixed in NBF.

For rats designated for clinical pathology analyses (i.e., hematology, serum chemistry, coagulation, as well as BALF cell count, cell differential, and chemistry), blood was collected at necropsy via open thorax cardiac puncture and immediately placed into potassium ethylenediaminetetraacetate anticoagulant tubes for hematology analysis, sodium citrate anticoagulant tubes for coagulation analyses, or a gel serum separator tube for serum chemistry analyses. All clinical pathology analyses were performed by American Society for Clinical Pathology–certified medical technologists. Hematology analysis was performed using an automated analyzer (Advia120 Hematology System, Siemens Medical Solutions Diagnostics, Tarrytown, NY). Serum and BAL supernatant 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 cells were counted manually using a Neubauer hemacytometer, and modified Wright-Giemsa stained cytospin preparations were used to conduct manual differential counts on BAL specimens.

Tissues for histologic examination (listed in the ACES protocol in Additional Materials 2 for this report at *http://pubs.healtheffects.org*) were trimmed per commonly accepted toxicologic pathology methodology, largely following published Registry of Industrial Toxicology animal-data guidance (Kittel et al. 2004; Morawietz et al. 2004; Ruehl-Fehlert et al. 2003). The left lung from interim sacrifices (1, 3, 12, and 24 months) and both right and left lung lobes from long-term observation (28–30 months) were sectioned per the published guidance to yield a single section of each lobe in a manner consistent with many historical toxicology studies in rats. Left lobes and right caudal lobes were sectioned in a plane to visualize primary and major secondary airways in the monopodial airway branching pattern of rats, right cranial and right middle lobes were sectioned parallel to the major intralobar airway, and right accessory lobes were sectioned transversely across the major intralobar airway. This method yielded five sections of lung with one section per lobe. Tissues were then processed routinely, embedded in paraffin, sectioned at approximately 4 µm, placed on glass slides, and stained with hematoxylin and eosin. Sections of selected target tissues (e.g., lung [five sections], nasal turbinates [four transverse sections], larynx [1 section at base of the epiglottis], trachea [cranial transverse section and frontal section at carina], kidney, liver, heart, brain, ovaries, and testes) were examined from all animals. Other tissues (e.g., pancreas, stomach, adrenal glands, and pituitary gland) were examined from all animals in the control and high-level exposure groups only. Non-target tissues

Table 2. Biologic Response Indicators Used to Indicate Effects of Inhaled NTDE

Hematology	Serum Chemistry	Pulmonary Function	Lung Lavage
Red blood cell count	Alanine aminotransferase (alanine transaminase)	Minute volume/body weight	Lactate dehydrogenase activity
Hemoglobin	Albumin	Total lung capacity	Protein
Hematocrit	Aspartate aminotransferase (aspartate transaminase)	Total lung capacity/kg	Albumin
Mean corpuscular volume		Dynamic lung compliance	Hemoglobin
Mean corpuscular hemoglobin concentration		Quasistatic lung compliance	Alkaline phosphatase
Mean corpuscular hemoglobin	Bilirubin (total)	CO diffusing capacity/alveolar volume	Total cell counts/differentials
Platelet count	Blood urea nitrogen	Forced expiratory flow	Total antioxidant capacity
Percent reticulocytes	Calcium	Mean mid-expiratory flow	Sodium (serum)
White blood cell count and absolute differential	Chloride	Other	Triglycerides
White blood cell count	Cholesterol (total)	**Other Clinical Observations**	**Lung Tissue Analysis**
Neutrophils	Creatinine		
Lymphocytes	Glucose	Mortality	Interleukin 1β
Monocytes	Gamma glutamyltransferase	Body weight	Tumor necrosis factor α
Eosinophils	Alkaline phosphatase	Organ weights	Macrophage inflammatory protein 2
Basophils	Phosphates	Tissue histopathology	Keratinocyte-derived chemokine
Large unstained cells	Potassium		Interleukin 6
Coagulation:	Protein (total)		Oxidized/reduced glutathione
Activated partial thromboplastin time	Sodium		Heme oxygenase 1
Prothrombin time	Triglycerides		Cell proliferation
	Calculated variables and ratios: Albumin/globulin Blood urea nitrogen/creatinine		
	Globulin		

were examined in animals from intermediate-level exposure groups only when the tissues exhibited gross abnormalities. If there was evidence of an exposure effect in a tissue from the high-level exposure group, that tissue was examined in all animals in intermediate groups (as occurred with thyroid glands in all but interim sacrifice rats); otherwise, the incidence in intermediate groups was based only on animals exhibiting gross lesions. Thus, incidences in non-target organs reflect a "gross lesion sampling bias" in the low- and mid-level exposure groups because the denominator of the computed incidence included only those animals with visually observed tissue abnormalities. In those instances, the observed incidence was reported, but no statistical testing was performed; age-adjusted rates (see Statistical Methods) were also reported, but they reflect the same sampling bias as the observed incidences and should be discounted. All lesions were graded subjectively by a single board-certified veterinary pathologist, and diagnoses received a severity grade on a 0–4 scale (where 0 indicated not present; 1, minimal; 2, mild; 3, moderate; and 4, marked). For example, in lung tissue, indicators of inflammation, cytotoxicity, and parenchymal changes (alveolar wall thickening, fibrosis, and pneumocyte hyperplasia) were graded for each animal.

In accordance with standard practice in toxicologic pathology as recommended by the Society of Toxicologic Pathology (Crissman et al. 2004), tissues were initially examined with knowledge of the exposure groups. Two experienced, board-certified pathologists performed the evaluations. The study pathologist (R.A.M., Experimental Pathology Laboratories [EPL]) coordinated the evaluations, examined all tissues, participated in a portion of the necropsies, and ensured consistency in nomenclature and grading. The on-site pathologist (A.P.G., LRRI) supervised and participated in the necropsies of both interim and long-term rats. Frequent communication between the study and on-site pathologists and image review of selected slides using the Internet ensured diagnostic consistency and lesion agreement. After the initial histopathology evaluation, the study pathologist and his in-house colleagues verified exposure-related changes observed in the lung by blinded evaluation. In addition, an independent pathologist consultant designated by HEI (E. McConnell, Tox Path) participated in pre-study discussions, observed a portion of the necropsies, and participated in example-based discussions of nomenclature and grading during the study. A second independent pathologist (Ronald Herbert, NIEHS, NTP), designated by HEI, participated with Dr. McConnell in reviewing pathology results at all interim exposure time points. An extensive peer review of the histopathologic findings was then performed. Finally, HEI commissioned a pathology working group (PWG; see the section of this volume titled "Pathology Working Group Report") to review the findings.

For biochemical analyses, lung tissue was homogenized in a buffer consisting of 1× Dulbecco phosphate-buffered saline (DPBS), 0.5% Triton X-100, and a proteinase cocktail including 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (serine proteinase inhibitor), 15 µM 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 (TeledyneTekmar, Mason, OH). 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., Rockford, IL) using bovine serum albumin as the standard. Hemoglobin in BALF was assayed using a hemoglobin reagent set (No. 6200-1, Eagle Diagnostics, Cedar Hill, TX) 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, which converts 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., St. Louis, MO) as a standard.

Albumin in BALF was measured using an enzyme-linked immunosorbant assay (ELISA) (Immunology Consultants Laboratory, Newberg, OR; kit E-25AL) diluted 1:1000 in PBS, according to the supplier's procedures. 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 were 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 was measured 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 described in the paragraph above. Cytokines (keratinocyte-derived chemokine [KC], interleukin-1β [IL-1β], interleukin-6 [IL-6], and tumor necrosis factor α [TNF-α]) were measured using a Luminex bead-based multiplex immunoassay (Milliplex, Millipore, Billerica, MA). Cytokine-induced neutrophil chemoattractant-3 (CINC-3) (also known as macrophage inflammatory protein-2 [MIP-2]) was assayed with an ELISA kit (cat. no. RCN300, R&D Systems, Goodhue, MN). Results were normalized to the protein content of the lysates. Lung tissue homogenates were analyzed for heme oxygenase-1 (HO-1) measured using a commercially available ELISA kit (Enzo Life Sciences, cat. no. ADI-EKS-810A). Lactate dehydrogenase (LDH) was measured using a microtiter plate method based on the oxidation of the reduced form of nicotinamide adenine dinucleotide in the presence of pyruvate. Alkaline phosphatase (ALP) was measured using a microtiter plate method based on hydrolysis of paranitrophenylphosphate (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 described in earlier studies (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 standards were combined with the Trolox solution, incubated for 5 minutes at room temperature, and then read in a spectrophotometric plate reader (VersaMax, Molecular Devices, Sunnyvale, CA) at 734 nm.

Cell proliferation was analyzed in lung tissue sections prepared from the paraffin-embedded, fixed left lung. Antigen was recovered by heating the tissue for 2 minutes under pressure in a commercial pressure cooker (Presto, 6 qt, National Presto Industries, Eau Claire, WI) 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 (rabbit polyclonal anti-Ki-67, Abcam, Cambridge, MA) 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-rabbit, Vectastain Elite kit, PK-610,Vector Laboratories, Burlingame, CA) 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 kit), and the slide was incubated for 5 minutes with diaminobenzidine (DAB) 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. Whole stained slides were scanned using a 20× objective (NanoZoomer Digital Pathology, Hamamatsu Photonics, Bridgewater, NJ) and then analyzed using the image quantification module in the Visiopharm Integrator System (Visiopharm, Copenhagen, Denmark). The software was used to randomly sample 50% of the tissue section. Within sampled areas, DAB-stained positive nuclei and hematoxylin-only stained negative nuclei were quantified, and a percentage labeling index ([positive nuclei/total nuclei] × 100) was determined across the sampled lung (on average over 100,000 total nuclei were counted for each lung slide analyzed).

Selected pulmonary function parameters were measured on 10 male and 10 female rats per exposure group after 3, 12, and 24 months of exposure using established methods (Harkema et al. 1982; Mauderly 1995). The measurements were performed immediately preceding necropsy. Rats were anesthetized with isofluorane, intubated by mouth with semi-rigid, thin-walled endotracheal catheters (modified 12- or 14-gauge intravenous catheters; Cathlon, Jelco, Raritan, NJ) (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 minimize dead volume as the rats increased in size. Flows were determined by measuring plethysmograph-to-room differences in pressure (MP-45, ± 2 cm H_2O, Validyne, Northridge, CA) 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, Buxco Electronics, Wilmington, NC). Flow, volume, and

P_{tp} signals were acquired via preamplifiers (Validyne) and a PC-based programmable system (Acknowledge V.3.7.2, Biopac Systems, Goleta, CA). Body temperature was measured using an electronic 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 re-induced, 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 then collected in a small side syringe. The CO and Ne concentrations in the collected sample were measured by gas chromatography (Model 111, Carle, Fullerton, CA). 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 kilogram of body weight (DL_{CO}/kg), and DL_{CO} normalized by the inflated alveolar volume estimated by Ne dilution (DL_{CO}/VA).

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

Another slow inhalation, but with forced exhalation, was performed as described above, but without intentional restriction of expiratory flow. Flow was driven by a vacuum reservoir maintained at −50 cm H_2O and connected to the airway via a solenoid valve having a 9.5 mm orifice (V52DA3012, Skinner, New Britain, CT) and an airway having a minimum internal diameter of 2.5 mm. The forced expiratory maneuver was performed at least twice with intervening recovery of spontaneous respiration, and was repeated 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 expiratory volume in 0.1 second ($FEV_{0.1}$, as mL and percentage of FVC), peak expiratory flow rate (PEFR), forced expiratory flow (FEF) 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 bedded plastic cages with food and water until necropsy the same day.

STATISTICAL METHODS AND DATA ANALYSIS

Survival Analysis

The probability of survival was determined by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs in Appendix A on the HEI Web site (*http://pubs.healtheffects.org*). Animals found dead of other than natural causes were censored; animals dying from natural causes were not censored. Statistical analyses for possible exposure-related effects on survival were performed using Cox's (1972) method for testing two groups for equality and Tarone's (1975) life-table test to identify exposure-related trends. All reported *P* values for the survival analyses are two-sided.

Histopathology Data

Neoplastic and non-neoplastic lesions that had an incidence greater than two in any exposure group were summarized and statistically analyzed. A modified Poly-3 method based on the Poly-k statistical test (Bailer and Portier 1988; Piegorsch and Bailer 1997; Portier and Bailer 1989) was used to assess the prevalence of non-neoplastic lesions and neoplasms. For analysis of a given tissue site, each rat was assigned a weighted risk factor. This value was 1 if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died before terminal sacrifice and did not have a lesion at that site, its weighted risk factor was the fraction of the entire study time that it survived, raised to the *kth* power., A value of $k = 3$ was used in the analysis of site-specific lesions in the present study. Bailer and Portier (1988) recommended this value after an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats (Portier et al. 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of *k* was anywhere in the range of 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variations introduced by the use of weighted risk factors, which reflect differential mortality, were

accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993). Tests of significance include pairwise comparisons of each exposed group with controls and a test for an overall exposure-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and one-sided *P* values were reported. For both neoplastic and non-neoplastic lesions, the modified Poly-3 method was used to estimate age-adjusted incidences; in the case of non-neoplastic lesions, average nonzero severity scores were computed.

In a previous report (McDonald et al. 2012), BALF, hematology, and serum chemistry measurements taken at 1 and 3 months of exposure were summarized and analyzed. This report provides data summaries for subsequent exposure time points (at 12 months, 24 months, and terminal sacrifice), as well as the results of comprehensive analyses across all time points (described below).

One-way analysis of variance (ANOVA) was used to evaluate exposure-related effects in organ and body weights at each exposure time point. For other endpoints, both one-way and two-way ANOVA tests were used to assess exposure effects at each exposure time point. Two-way ANOVA models contained terms for exposure group (control, low, mid, and high), sex, and sex × exposure group interaction effects. The comparability of sex-based differences in exposure-related responses 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. The Dunnett multiple comparison procedure (Dunnett 1955, 1980) was performed to compare NTDE-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 responses across 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). As there is no standard multiple comparison method for categorical data that is analogous to the Dunnett test, the *P* values for comparisons of individual NTDE exposure groups against the control group obtained from the categorical ANOVA were adjusted using the Bonferroni correction method.

Heteroscedasticity was significant 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 assumptions of homogeneity of variance 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 error of the mean. 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 $P = 0.01$.

Three-Way ANOVA

Three-way ANOVA models were employed to evaluate systematic response variation relative to sex, exposure times (at 1, 3, 12, and 24 months), exposure (control and low, mid, and high levels), and the interactions between these main effects (sex × time, exposure × time, sex × exposure, and sex × time × exposure). Although sex and exposure time effects (and the interactions between these sources of variation) are interesting from a biologic perspective, they are not indicators of exposure-related effects; instead, they indicate systematic differences in response (relative to sex and/or exposure time) across all exposure levels (including controls). However, their inclusion in the ANOVA model is important as it provides adjustment for systematic variation attributable to these factors (e.g., sex-related differences in baseline response levels, and differences in response levels at different points in time) that is needed to facilitate assessments of overall exposure effects and differences in exposure effects across time. Specifically, the *P* value for "exposure" assesses the equality of mean responses in exposure groups across time and sex. The "exposure × time" *P* value assesses the extent to which mean responses in exposure groups differ at different points in time (i.e., it tests the hypothesis that exposure effects are of the same magnitude across time, irrespective of sex). Similarly, the sex × exposure interaction tests the hypothesis that exposure effects were the same across sexes (irrespective of time), and the three-way interaction (sex × exposure × time) tests the extent to which exposure effects differed by sex at the measurement time points.

The three-way ANOVAs were used to identify endpoints with substantive evidence of exposure effects. Endpoints having statistically significant ($P < 0.05$) evidence of effects from exposure, exposure × time, sex × exposure, or exposure × sex × time were further examined with two-way and one-way ANOVAs at all measurement time points.

QUALITY ASSURANCE

This research was conducted in a manner consistent with most standards developed for the Good Laboratory Practices (GLP) requirements for regulatory studies, although full compliance with GLP was not a requirement of the protocol. Quality control (QC) consisted of conducting all work according to approved protocols and standard operating procedures, the inclusion of verified QC control standards for calibration and certification of system performance, and third-party verification of all data prior to 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, necropsy, tissue collection, histopathology, and clinical pathology were tracked through a validated software system (Provantis, Instem, Conshohocken, PA).

For each analytical instrument, calibration or "span" checks were conducted at each time of use. A wide array of biologic assays was used for this study, the sensitivity and range of which varied. They were, however, internally consistent with 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 biologic effect, but all assays had been shown to be responsive to treatment effects in previous studies.

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

RESULTS

GENERAL FINDINGS

Over 100 biologic response variables were assessed in exposed and control rats during either the interim or terminal evaluations. The target NO_2 concentrations were achieved, which resulted in a complex mixture characterized by low concentrations of particulate matter (PM), VOCs, and SVOCs, but relatively high concentrations of NO_x. The NO_2:NO ratio changed throughout the study as a result of a decrease in the performance of the diesel particle filter (DPF) catalyst. The PM remained below 15 $\mu g/m^3$ on average throughout the study. A summary of the biologic responses, including results of the statistical analyses (ANOVA and three-way ANOVA), is provided in Appendices A through H (available on the HEI Web site at *http://pubs.healtheffects.org*). Most indicators demonstrated no statistically significant differences from control as a result of exposure. Similar to findings from the subchronic sacrifices at 1 and 3 months (McDonald et al. 2012), most of the statistically significant responses at 12 and 24 months were accompanied by evidence of mild oxidative stress in the lung and increases in lung protein and cytokines that reflected a mild inflammatory response. There were minimal to mild exposure-related histopathologic changes in the lungs, but there was no evidence of neoplastic transformation. The non-neoplastic lesions were minimal to mild in severity and primarily characterized by an increase in the number and prominence of basophilic epithelial cells lining terminal bronchioles, alveolar ducts, and adjacent alveoli (those in close proximity to the terminal bronchioles and alveolar ducts). The severity and magnitude of the histopathologic findings progressed slightly from 3 to 12 months. However, further progression between 12 months and the terminal sacrifice was limited. The rats also had slight but statistically significant changes in pulmonary function at 24 months, a finding that was also observed at the earlier time points (McDonald et al. 2012). Although the impacts on pulmonary function were mild, they had progressed at the 24-month time point compared with the findings at 3 and 12 months.

EXPOSURE ATMOSPHERES

The exposure atmospheres are described in detail in Appendix I of this report (available on the HEI Web site at *http://pubs.healtheffects.org*). The mean concentrations of key components over the entire study are listed in Table 3. The concentrations of PM, SVOCs, and VOCs were generally very low. This confirmed the original expectations during the planning of ACES and predictions from Phase 1, which characterized the emissions from 2007-compliant engines (Khalek et al. 2011). The most abundant components were CO_2, CO, NO, and NO_2. The ratio of PM to NO_2 was at least 3 times lower than in earlier studies of inhaled TDE (e.g., Nikula et al., 1995). As discussed in more detail in Appendix I, the ratio of PM:NO_2 changed slightly throughout the study because of a decrease over time in the efficiency of the DPF in converting NO to NO_2. As a result, the dilution used to achieve the test atmospheres

Table 3. Average Atmosphere Composition (± SD[a]) during Exposure of Mice and Rats in ACES Phase 3B[b]

Exposure Atmosphere Composition	Units	Control	Low	Mid	High
Particle mass					
Dekati, inside chamber	µg/m³	0.3 ± 0.2	0.2 ± 0.2	0.9 ± 0.6	6.3 ± 4
Filter sample, chamber inlet	µg/m³	NA	2.5 ± 1.6	2.7 ± 1.2	7.8 ± 2.8
Filter sample, inside chamber	µg/m³	16.9 ± 6.7	23.3 ± 11.4	20 ± 5.4	27.3 ± 6.2
Particle mass (FMPS)[c]	µg/m³	0.2 ± 0.2	1.1 ± 0.9	2 ± 1.7	5.3 ± 2.9
Particle count (FMPS)[c]	particle/cm³	17,136 ± 23,420.5	215,225.5 ± 412,548.9	678,184 ± 1,247,908.6	828,814.3 ± 1,041,924.8
NMAD[a] (FMPS)[c]	nm	23 (1.8) ± 8.8 (0.5)	16.6 (1.5) ± 6.3 (0.2)	15.4 (1.5) ± 5.5 (0.2)	18.6 (1.6) ± 4.4 (0.1)
MMAD[a] (FMPS)[c]	nm	196.8 (1.7) ± 149 (0.2)	181.8 (2.7) ± 113.4 (0.6)	81.2 (2.9) ± 32 (0.8)	41.6 (2.2) ± 4.1 (0.5)
Nitrogen oxide (NO)	µg/m³ (ppm)	2 ± 3 (0.001 ± 0.002)	274.3 ± 186.3 (0.3 ± 0.2)	1,376.6 ± 390.4 (1.3 ± 0.4)	6,878.6 ± 2591.8 (6.6 ± 2.4)
Nitrogen dioxide (NO_2)	µg/m³ (ppm)	2.6 ± 5.2 (0.002 ± 0.004)	159.5 ± 16.6 (0.1 ± 0.01)	1,421.5 ± 83.7 (0.9 ± 0.05)	6,873.2 ± 489.4 (4.4 ± 0.3)
Carbon monoxide (CO)[c]	µg/m³ (ppm)	895.8 ± 883.2 (0.9 ± 0.9)	1,042.2 ± 510.1 (1.1 ± 0.5)	1,787.1 ± 444 (1.9 ± 0.5)	6,167.6 ± 2445.4 (6.4 ± 2.6)
Carbon dioxide (CO_2)[c]	mg/m³ (ppm)	2,110.6 ± 800.1 (1,402.2 ± 531.7)	2,695.7 ± 1,175.2 (1,790.9 ± 780.9)	3,599.1 ± 497.8 (2,391.2 ± 330.8)	7,215.6 ± 1456.4 (4,794.1 ± 967.5)
Sulfur dioxide (SO_2)	µg/m³ (ppb)	4.9 ± 1 (2.2 ± 0.4)	6.6 ± 1.5 (2.9 ± 0.7)	20.5 ± 6.2 (9.1 ± 2.7)	69.6 ± 28.6 (30.8 ± 12.6)
Elemental carbon[c]	µg/m³	0.4 ± 0.4	1.2 ± 1.2	0.4 ± 0.4	1.2 ± 1
Organic carbon[c]	µg/m³	4.5 ± 1.4	6.6 ± 1.4	4.8 ± 1.9	5.7 ± 2.4
Ammonium[c]	ng/m³	31.3 ± 31.3	57.2 ± 29	203.2 ± 150.6	1,312.2 ± 450.2
Sulfate[c]	ng/m³	101.6 ± 26	193.1 ± 153.1	644.8 ± 405.5	2,121.1 ± 968.2
Nitrate[c]	ng/m³	18.6 ± 16.7	181.2 ± 195.9	220.2 ± 73.9	2,087.3 ± 747
Elements (metals)[c]	ng/m³	461.3 ± 208.5	567.9 ± 224	661 ± 302.1	811.5 ± 336.3
Alkanes[c]	µg/m³	2.8 ± 0.5	4.7 ± 1.9	8.6 ± 2.8	26.6 ± 9.3
Carbonyl[c]	µg/m³	53.8 ± 12.1	71.2 ± 40.2	21.4 ± 5.1	1 ± 1.5
Polyaromatic hydrocarbons (PAHs)[c]	µg/m³	0.8 ± 0.5	1.1 ± 0.4	1.9 ± 0.7	4.7 ± 1.6
Nitro-PAHs[c]	ng/m³	0.4 ± 0.2	0.8 ± 0.4	1.6 ± 0.5	8 ± 2.5
Polars (acids)[c]	µg/m³	3.2 ± 1.2	4.1 ± 1.6	6.9 ± 2.9	16 ± 6
Hopane and steranes	ng/m³	2 ± 0.7	5.1 ± 3.5	2.5 ± 1.6	3.7 ± 3.9
Volatile organic compounds (VOC)	µg/m³	33.5 ± 21.3	50.3 ± 31.3	44.2 ± 15.3	148.9 ± 96.9

[a] Or geometric standard deviation where indicated.

[b] For composition during rat-only exposures, see Table 3B in Appendix I. NO_2 levels were 4.2, 0.8, and 0.1 ppm over the 30-month rat exposures.

[c] Derived from detailed characterizations (April 2010, September 2010, April 2011, April 2012). (See Appendix I.)

FMPS = fast mobility particle sizer; MMAD = mass median aerodynamic diameter; NA = not available (no data were collected during that period due to study design); NMAD = number median aerodynamic diameter.

was also changed by 35% to maintain the target NO_2 concentrations. However, this had only a minor impact on the test atmosphere composition in view of the variability of emissions and the low concentrations of most of the test atmosphere constituents. Analysis of the test atmospheres revealed that animals contributed to the overall composition of the chamber atmospheres by adding both PM (as dander) and VOCs, as well as products of reactions between animal "emissions" and components from the exhaust.

The PM mass concentrations were measured at the inlet to the chambers and at mid-chamber to allow the contribution of PM from the animals to be distinguished from the DPM from exhaust. As expected, the concentration in the chambers was much higher than at the chamber inlets, showing that the major portion of PM mass was due to contributions from animals. As a result of the "animal background," particle mass concentration within the chambers was not closely dilution-dependent. However, there was a dilution-dependent difference in the particle mass at the inlets, as well as a dilution-dependent difference in particle number count, with the highest particle number measured within the highest 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 size particles within the exposure chamber, but the mass concentrations were very low. The larger sized particles were likely attributed to the larger contribution from dander and background relative to the NTDE particles.

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. VOCs accounted for a small proportion of the mass in general, but because of the low concentrations of CO and NO_x, VOCs accounted for up to 10% of the mass at the low and control exposure levels. It is noteworthy that this result indicated that the animals contributed a major portion of the VOCs measured. The concentration of sulfur dioxide was low at all exposure levels.

The composition of the PM mass at mid-chamber resulted from a combination of DPM and the animal background PM. 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. 2004). As a result, the PM composition was slightly different at each exposure level. This was evidenced by differences in the proportion of ammonium, nitrates, and sulfates. The similar concentrations of particulate organic carbon across exposure groups suggested that the "animal background" PM was the dominant source. Because the sum of organic carbon and additional species exceeded the total measured mass, the organic carbon is 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 from dander and SVOC emissions from respiration. The high-level exposure atmosphere had the largest fraction of PM derived from the engine. 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.

Among the measured SVOCs, the alkanes and polar compounds contributed the most to the test atmospheres, followed by polyaromatic hydrocarbons (PAHs). The polar compounds were primarily benzoic acid derivatives, and the alkanes were compounds of higher molecular weight, perhaps derived mostly from unburned fuel because they are consistent with the fraction of petroleum distillate found in diesel fuel (Rogge et al. 1992). The PAHs were dominated by compounds of lower molecular weight, primarily in the gas phase. Concentrations of hopanes and steranes, as well as nitro-PAHs, were substantially less than those of lower molecular weight species, as expected.

BIOLOGIC RESPONSES IN THE WISTAR HAN RAT

Clinical Observations

A number of animals were found moribund throughout the study due to a range of causes that would be expected during the natural senescence of the rat. In addition, most animals in the study developed pododermatitis (ulcerative foot lesions) near the end of year 1. The pododermatitis was attributed to pressure on their feet from the wire-bottom construction that is typical of the Hazelton whole-body inhalation cages often used for such studies. The larger size of the Wistar Han rat may have elicited more of this response than observed in the smaller rat strains, such as Fischer 344, used in previous studies (e.g., Nikula et al. 1995). Supporting this explanation was the general trend toward higher prevalence and severity in the heavier animals (males). Severe pododermatitis became a criterion for euthanasia for a number of animals, especially in males. In all, 183 (of ~1000) animals were diagnosed with pododermatitis, resulting in euthanasia. At 13 months into the study, solid perches were added to the cages to reduce strain on the rats' feet. Animals that showed signs of pododermatitis were treated with a combination of cleaning and disinfecting with topical povidone-iodine (Biozide, St. Louis, MO).

Mortality, Body Weight, and Organ Weight

Mortality, body weight, and organ weight data are provided in Appendices A through C (available on the HEI Web site, *http://pubs.healtheffects.org*). Across the entire course of the study, there was a statistically significant increase in mortality among the male rats at the highest level of exposure (see Appendix A available on the HEI Web site at *http://pubs.healtheffects.org*). This was primarily the result of the substantial number of animals that were euthanized, many toward the end of year 1, due to pododermatitis-related moribund status. After day 485, when the preventative care and treatment for pododermatitis was in place, mortality rates among exposure groups did not differ significantly. Histopathology conducted on pododermatitis lesions from several animals from different exposure groups did not reveal exposure-related differences.

There was no overall relationship between body weight and exposure group. There were statistically significant differences in mean body weight among the groups during particular months (see Appendix B available on the HEI Web site at *http://pubs.healtheffects.org*). For example, body weights at the low levels of exposure were greater than that of controls during 5 out of 30 months.

There were only a few statistically significant exposure-related trends in raw organ weights or organ weights normalized to either body or brain weight, and none were concurrent in both sexes (see Appendix C available on the HEI Web site at *http://pubs.healtheffects.org*). There were no significant trends at 12 months. At 24 months, there were significant exposure-related downward trends in the ratios of heart and liver weight to body weight in males, but no trend in the raw organ weights. There were marginal downward trends among males in raw thymus weight and the ratios of thymus to body or brain weight ($P = 0.075$–0.091). There was a significant downward trend in raw liver weight among females at 24 months, but not in the normalized liver weights. At terminal sacrifice, there were significant upward trends in lung and thymus weights of females, but only marginal trends in the normalized weights ($P = 0.061$–0.081). There was also a marginal downward trend in the liver to body weight ratio of females ($P = 0.096$), but not in the raw or brain-normalized liver weights. There were neither significant nor marginal exposure-related trends in organ weights of males at terminal sacrifice. Overall, the magnitudes of the differences in organ weight among groups were small. None of the mean values of the high or mid exposure groups were significantly different from control mean values by pairwise comparison.

Hematology and Clinical Chemistry

Three-way ANOVA results (see Tables D.1, D.3, and D.5 in Appendix D on the HEI Web site at *http://pubs.healtheffects.org*) indicated that there was little, if any, evidence of consistent exposure-related effects in hematology, serum chemistry, or coagulation endpoints. For hematology endpoints, the only indication of a consistent exposure effect across time was for absolute eosinophils ($P = 0.029$), which were modestly elevated in low- and mid-level exposure groups at 12 and 24 months (Table D.1), but overall there was no substantial evidence of an exposure-related trend at any time point (Figure D.1). There was some evidence of exposure effects that differed across time for mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, shown in statistically significant increasing trends at 24 months across both sexes (Figures D.2 and D.3; Table D.2).

Of the clinical chemistry parameters, only ALP showed evidence of exposure effects consistent across time; these effects were manifested as trends toward decreasing response with increasing exposure level (Figure D.4), with statistically significant trends identified in both sexes at 12 months (Table D.4). Alanine aminotransferase (ALT) serum and chloride measurements showed statistically significant evidence of exposure effects that differed across time. In the case of ALT (Figure D.5), this was shown in a statistically significant decreasing trend in females at 12 months (Table D.4), which was not replicated at other time points or in males. For chloride, the significant exposure $\times$ time effect reflected a statistically significant decreasing trend in males at 24 months that appeared to be due to a high control mean (Figure D.6; Table D.4). Triglycerides (Figure D.7) had a statistically significant sex $\times$ exposure $\times$ time effect, which related to a nearly statistically significant increasing trend in males at terminal sacrifice and a nearly significant decreasing trend in females at 24 months (Table D.4) No other evidence of exposure-related effects was observed at other time points or in females.

Bronchoalveolar Lavage and Lung Tissue Analysis

Data summaries and time-specific statistical analysis results for biologic endpoints in BALF and lung tissue measured at the 12- and 24-month time points are in Tables E.3, E.4, and F.2 in Appendices E and F (on the HEI Web site at *http://pubs.healtheffects.org*). Several endpoints indicative of oxidative stress in the lung were upregulated. In general the responses were mild, and most showed only significant trends, not significant differences from controls.

Three-way ANOVA results for lavage fluid and biochemical endpoints (Table 4) provided evidence of consistent exposure effects across time and sex for alkaline

Table 4. Three-Way ANOVA Analysis (*P* Values) of Variance on Lung Lavage Cell and Biochemical Data in Rats at 1, 3, 12, and 24 Months of Exposure[a,b]

Endpoint	Sex	Time	Exposure	Sex × Exposure	Exposure × Time	Sex × Time	Sex × Exposure × Time
Alkaline phosphatase	0.433	0.009**	0.011*	0.700	0.359	0.567	0.367
µTP	< 0.001**	0.003**	< 0.001**	0.641	0.004**	0.021*	0.674
Eosinophils absolute count	0.133	0.525	0.307	0.409	0.844	0.233	0.870
Eosinophils differential cell count	0.140	0.898	0.178	0.355	0.794	0.213	0.799
LDH	0.011*	0.215	0.067	0.760	0.315	0.202	0.434
Lymphocytes absolute count	0.217	< 0.001**	0.717	0.219	0.835	0.483	0.197
Lymphocytes differential cell count	0.498	< 0.001**	0.572	0.316	0.469	0.126	0.121
Macrophages absolute count	< 0.001**	< 0.001**	0.001**	0.606	0.558	0.046*	0.196
Macrophages differential cell count	0.287	0.130	0.345	0.697	0.832	0.537	0.136
Number of epithelial cells per 100 cells absolute	0.819	< 0.01**	0.059	0.995	0.481	0.774	0.957
Number of epithelial cells per 100 cells differential	0.251	< 0.001**	0.075	0.954	0.504	0.601	0.967
Neutrophils absolute count	0.777	0.007**	0.635	0.601	0.965	0.420	0.217
Neutrophils differential cell count	0.644	0.002**	0.233	0.570	0.975	0.442	0.419
Total white blood cells	< 0.001**	< 0.001**	0.001**	0.696	0.547	0.053	0.173
Albumin	< 0.001**	< 0.001**	< 0.001**	0.978	0.208	0.842	0.200
GSH BALF	< 0.001**	< 0.001**	0.373	0.090	0.923	< 0.001**	0.255
GSSG BALF	0.021*	< 0.001**	0.016*	0.593	0.009**	0.001**	0.008**
Hemoglobin	0.016*	< 0.001**	0.001**	0.431	0.198	0.075	0.533
TEAC	0.384	< 0.001**	0.101	0.696	0.040*	0.111	0.563
Total GSH BALF	< 0.001**	< 0.001**	0.165	0.064	0.849	< 0.001**	0.375

[a] BALF = bronchoalveolar lavage fluid; GSH = glutathione; GSSG = oxidized glutathione; LDH = lactase dehydrogenase; µTP = micro–total protein; TEAC = Trolox equivalent antioxidant capacity.

[b] Degrees of freedom associated with sources of variation: sex (1); time (3); exposure (3); sex × exposure (3); sex × time (3); exposure × time (9); sex × exposure × time (9)

* $P < 0.05$.

** $P < 0.01$.

phosphatase, albumin, and hemoglobin. Significant (or marginally significant) exposure interaction effects for μTP, TEAC, GSSG, and some cellular counts (macrophages and total white blood cells) indicated exposure–response patterns that differed by time and/or sex. TEAC, which measures the ability of the BALF to prevent oxidation of a chromophore by a chemically generated free radical, was increased in females at 24 months, but showed substantial evidence of decreases in both sexes at 3 months and in females at 12 months (Figure E.1). Changes in hemoglobin and glutathione reflect a change in the oxidative balance in the lung. There was statistically significant evidence of exposure-related hemoglobin increases in BALF in both sexes at 24 months and in combined sexes at 12 months (Table E.4, Figure E.2), but only suggestive evidence of this pattern at earlier time points (McDonald et al. 2012). However, the response pattern of GSSG in BALF varied significantly relative to time point and sex (Figure E.3), with no substantial evidence of exposure differences at 1 or 3 months (McDonald et al. 2012), marginal ($P = 0.07$) evidence of an exposure-related increase for males at 12 months (Table E.4), and a statistically significant exposure-related increasing trend in females at 24 months.

In lung tissue, the three-way ANOVAs (Table 5) indicated exposure effects for HO-1 and the cytokines IL-6 and KC. There was evidence of exposure-related increases in HO-1 throughout the study (Figure 1). At 4 and 13 weeks, there were statistically significant exposure-related trends in both sexes (McDonald et al. 2012). At 12 months, there were significant increases in both sexes at the high exposure level followed by a significant increase in combined sexes at 24 months. In addition, there was evidence of exposure effects on the cytokines IL-6 (Figure 2) and KC (Figure F.1) in both sexes across time, with a statistically significant increasing trend identified in at least one sex (or combined sexes) at 12 and 24 months (Table F.2).

In addition to oxidative stress, some mild tissue injury was indicated by the lung tissue and BALF data at 12 and 24 months (Tables E.3, E.4, and F.1), primarily at the high-exposure level. Increases in μTP in BALF were observed in both sexes at 3, 12, and 24 months (Figure E.4), with statistical significance in high-level-exposure males at 3 months (McDonald et al. 2012), in females at 24 months (Table E.3), and in combined sexes at 3, 12, and 24 months. The increases in protein were paralleled by increases in

Table 5. Three-Way ANOVA (*P* Values) of Lung Tissue Data in Rats at 1, 3, 12, and 24 Months of Exposure[a,b]

Endpoint	Sex	Time	Exposure	Sex × Exposure	Exposure × Time	Sex × Time	Sex × Exposure × Time
Cytokine CINC-3 lung	< 0.001**	< 0.001**	0.870	0.516	0.340	0.477	0.614
Cytokine IL-6 lung	0.213	< 0.001**	0.002**	0.687	0.692	0.890	0.752
Cytokine IL-1β	0.098	< 0.001**	0.900	0.526	0.895	0.081	0.094
Cytokine KC lung	< 0.001**	< 0.001**	0.042*	0.547	0.290	0.432	0.589
Cytokine TNF-α[c]	0.226	< 0.001**	0.167	0.101	0.714	0.198	0.068
GSH lung	< 0.001**	< 0.001**	0.255	0.568	0.796	< 0.001**	0.762
GSSG lung	< 0.001**	< 0.001**	0.502	0.149	0.750	< 0.001**	0.880
HO-1	0.016*	< 0.001**	< 0.001**	0.812	0.179	0.366	0.919
Total GSH lung	< 0.001**	< 0.001**	0.146	0.265	0.569	< 0.001**	0.486

[a] CINC = cytokine-induced neutrophil chemoattractant; GSH= glutathione; GSSG = oxidized glutathione; HO-1 = heme oxygenase 1; IL = interleukin; KC = keratinocyte-derived chemokine; TNF-α = tumor necrosis factor α.

[b] Degrees of freedom associated with sources of variation: sex (1); time (3); exposure (3); sex × exposure (3); sex × time (3); exposure × time (9); sex × exposure × time (9).

[c] No 3-month data available.

* $P < 0.05$.

** $P < 0.01$.

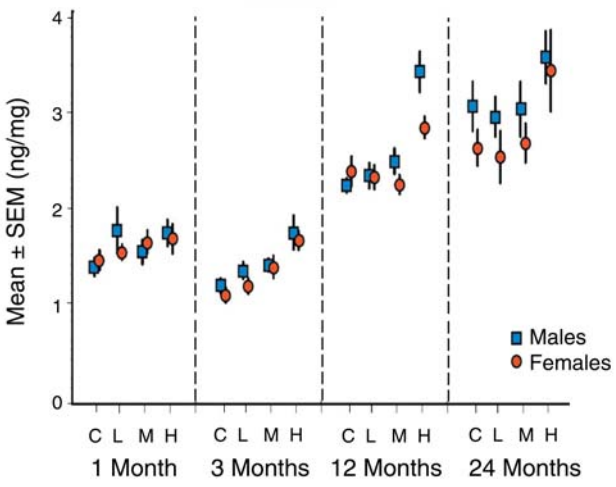

Figure 1. Pattern of response in HO-1 for males and females at 1, 3, 12, and 24 months (C = control, L = low-level exposure, M = mid-level exposure, H = high-level exposure). The 3-way ANOVA test and time-specific analyses indicated exposure-related increases across time (see Table F.1 in Appendix F).

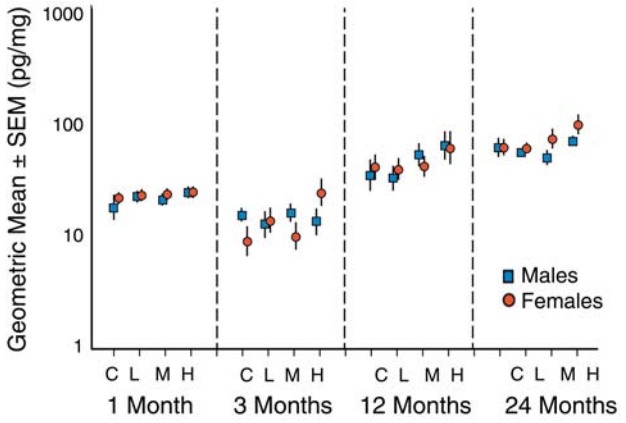

Figure 2. Pattern of response in IL-6 for males and females at 1, 3, 12, and 24 months (C = control, L = low-level exposure, M = mid-level exposure, H = high-level exposure). Note that data are plotted as geometric means and geometric standard errors. The 3-way ANOVA test revealed evidence of exposure effects across time points (see Table F.1 in Appendix F). Time-specific analysis identified statistically significant exposure-related trends at 12 and 24 months (Table F.2).

albumin (Figure E.5). There were also increased ALP levels in rats of both sexes exposed to the high level of NTDE at all points in time (Figure E.6), with the increase achieving statistical significance in combined sexes at 24 months (Table E.3). Figures E.7 and E.8 show evidence of exposure-related increases in total white blood cell and macrophage counts (which comprised most of the measured cells in BALF), but there was substantial variability in response, and statistically significant trends were observed only in males at 1 month (McDonald et al. 2012) and in females at 24 months (Table E.3). Even when exposure effects were noted in macrophage counts and total number of cells, the increases were modest (less than 2-fold), and the total number of cells was low.

Pulmonary Function

Three-way ANOVA of pulmonary function data (Table G.1 in Appendix G, available on the HEI Web site at *http://pubs.healtheffects.org*) indicated that exposure significantly effected forced expiratory flow rates and CO diffusing capacity, but had no significant affect on vital capacity (SVC or FVC) or Cqs.

Pairwise comparisons revealed that the reductions of forced flows were manifested more strongly in females than in males and, with only one exception, significant reductions occurred only at the highest exposure level in either sex (Table G.2). At 3 months, PEFR, FEF_{25}, and MMEF were significantly reduced in females exposed at the high level, but there were no significant differences in the volume-adjusted flows (PEFR/FVC, FEF_{25}/FVC, or MMEF/FVC). At 24 months, flows were significantly reduced in females exposed at the high level throughout the lung volume range (PEFR, FEF_{75}, FEF_{50}, FEF_{25}, FEF_{10}, and MMEF), and the volume-adjusted flows were also significantly reduced. There were no significant reductions of forced flows in females at 12 months or in males at 3 or 12 months. At 24 months, the FEF_{25}/FVC of males exposed at the mid level and FEF_{10} of males at the high level were significantly reduced. Both the unadjusted (raw) flow rates and flows adjusted by lung volume were affected more strongly at low lung volumes than at high lung volumes. Among females exposed at the high level, mean values for FEF_{75} and FEF_{75}/FVC were 18% and 14% lower than control mean values, respectively, while FEF_{10} and FEF_{10}/FVC were 50% and 46% lower than control. Among exposed males at 24 months, mean values differed significantly from control only at 25% and 10% of FVC at the high level. In general, flow performance at decreasing lung volumes is thought to reflect impacts on airways of decreasing size (Mauderly 1995). Accordingly, the results suggest that exposure affected the smallest airways more than larger airways.

Although ANOVA indicated an effect of exposure on DL_{CO} (Appendix Table G.1), pairwise comparisons between values for rats exposed at the high level and controls did not indicate significant reductions in either sex at any measurement time (Table G.2). At 24 months, the small nonsignificant differences in DL_{CO} among groups

resulted in significant downward trends in DL_{CO} across exposure groups of both males and females, and the mean value for the combined sexes exposed to the high level was significantly different from control. The magnitude of DL_{CO} is a combined function of the time over which the test gas is held in the lung, the efficiency of alveolar–capillary gas transfer, and lung size (affecting both the alveolar volume [VA] of test gas and the volume of blood in the alveolar capillary bed) (Mauderly 1995). Because the procedure is intended primarily as a test of the efficiency of gas transfer, the "breath-holding" time is held constant among subjects, and the result is divided by body weight or the estimated alveolar volume of test gas to adjust for lung size. At 24 months, adjustment by body weight (DL_{CO}/kg) eliminated the significance of the exposure-related trend in females, but not males or the combined sexes. Adjustment by alveolar volume (DL_{CO}/VA) eliminated the exposure-related trend in males, but the trend in females remained marginally significant ($P = 0.056$), and the trend for combined genders remained significant. These results suggest that the small effect of exposure on DL_{CO} at 24 months was in part due to a small reduction of lung size relative to control. Accordingly, the impact on the efficiency of gas transfer across the alveolar–capillary membrane, if any, must have been very slight.

Histopathology

Histologic analysis revealed a number of exposure-related findings, which were restricted to the respiratory tract. Chronic exposure of rat lungs to NTDE did not result in the induction of pre-neoplastic lung lesions or primary lung neoplasia. Neoplasms were not induced in the nasal cavity, and neoplasms in other organ systems were not associated with exposure and occurred within historical control values (Giknis and Clifford 2003; Poteracki and Walsh 1998; Tucker 1997; Walsh and Poteracki 1994). At the terminal sacrifice, the incidence of inflammatory changes in the lung related to exposure was not notable, and no DPM was detectable by light microscopy.

Results of statistical analyses of the histologic data are provided in Appendix H (available on the HEI Web site at *http://pubs.healtheffects.org*). Table H.2 lists the presence and type of lesions in each organ by sex and exposure group. The table provides the incidence rate (including adjustment for mortality), the time of first observation, and the mean lesion severity score. Table 6 summarizes the incidence and severity of selected findings in the lungs. Lesions interpreted to be related to exposure were observed at all time points beyond 1 month (3, 12, 24 months, and terminal sacrifice) in the lungs of both male and female rats at the high-level of exposure. These exposure-related lesions were not evident among the lower level groups or controls. Figure 3 provides illustrative photomicrographs of the lung findings that were typical at the high-exposure level. Tissues are shown from females in the control and high-level exposure groups at 30 months of exposure. These photographs are considered to be representative of lesions noted in both males and females from 12 months on. The lung lesions were primarily characterized by an increased number and prominence of basophilic epithelial cells, considered reactive or regenerative, lining pre-terminal and terminal bronchioles, alveolar ducts, and alveoli in close proximity to the terminal bronchiole and alveolar ducts. The distribution was rather uniform and focused at the central acinus, affecting many, but not all, centriacinar regions of any one lung section. This hyperplasia of the epithelium was minimal to mild, similar morphologically to these lesions seen at 12 months, but having mild-grade effects occurring at higher incidences at 24 months and at the terminal sacrifice. Often when the lesions were considered mild, epithelial cell proliferation within the pre-terminal bronchioles and terminal bronchioles formed unusual individual papillary projections into the lumina of those bronchioles. These projections were lined by epithelial cells and sometimes had a central collagenous stalk. The focal hyperplasia was observed in the absence of significant effects on cell proliferation as measured by Ki-67 staining (Table 7). Notably, the hyperplasia observed was confined to a quite limited area within the central acinus, while the labeling index was determined via image quantification using random fields involving 50% of the left lung. Thus the Ki-67 cell proliferation assay may not have been optimized to detect these subtle differences, which were observed in only a limited portion of the airways (i.e., any effect may have been diluted by the large sampling area). In addition to the epithelial proliferation, there was a slight amount of bronchiolization (a term used to describe metaplasia resulting in the presence of cuboidal, often ciliated, cells in the alveolar region where they are not typically present) in some animals.

The incidence of periacinar interstitial fibrosis was high at 12 months (80% in males and 100% in females), and remained high in both sexes at 24 months and terminal sacrifice (Table 6). Fibrosis occurred only around alveolar ducts and at the junctions of the alveolar ducts and alveolar walls in the centriacinar areas in the stroma underlying the epithelial change (Figure 4). The severity of the periacinar interstitial fibrosis remained mostly minimal even in animals at the terminal sacrifice. At 24 months and terminal sacrifice, the severity of fibrosis when present was only marginally increased relative to that seen at 3 months, and exposure-related fibrosis did not occur

Table 6. Summary of Incidence[a] Rate for Non-Neoplastic Lesions and Mean Severity Score[b] for Histopathologic Findings in the Lungs at Each of the Sacrifice Times by Sex and Exposure Group

	Males				Females			
Histopathologic Endpoint	Control (%)	Low (%)	Mid (%)	High (%)	Control (%)	Low (%)	Mid (%)	High (%)
Lung(s); Accumulation; Macrophage								
Incidence	0/139 (0)	0/141 (0)	0/136 (0)	8/131 (6)	0/139 (0)	0/142 (0)	0/139 (0)	6/134 (4)
Adjusted rate	0%	0%	0%	17%	0%	0%	0%	9%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
3-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	3/10 (30)	0/10 (0)	0/10 (0)	0/10 (0)	3/10 (30)
12-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	3/10 (30)	0/10 (0)	0/10 (0)	0/10 (0)	3/10 (30)
24-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	2/10 (20)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Terminal rate[c]	0/16 (0)	0/26 (0)	0/18 (0)	0/9 (0)	0/24 (0)	0/29 (0)	0/17 (0)	0/17 (0)
Natural death & moribund sacrifice rate	0/83 (0)	0/75 (0)	0/78 (0)	0/82 (0)	0/75 (0)	0/73 (0)	0/82 (0)	0/77 (0)
First incidence (days)	—	—	—	94	—	—	—	93
Poly-3 test	$P < 0.001$	—	—	$P = 0.001$	$P = 0.001$	—	—	$P = 0.013$
Mean lesion severity score	—	—	—	1.1	—	—	—	1.0
Lung(s); Bronchiolization								
Incidence	0/139 (0)	0/141 (0)	0/136 (0)	38/131 (29)	0/139 (0)	0/142 (0)	0/139 (0)	30/134 (22)
Adjusted Rate	0%	0%	0%	58%	0%	0%	0%	41%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
3-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
12-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)	0/10 (0)	0/10 (0)	2/10 (20)
24-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)
Terminal rate[c]	0/16 (0)	0/26 (0)	0/18 (0)	4/9 (44)	0/24 (0)	0/29 (0)	0/17 (0)	5/17 (29)
Natural death & moribund sacrifice rate	0/83 (0)	0/75 (0)	0/78 (0)	32/82 (39)	0/75 (0)	0/73 (0)	0/82 (0)	22/77 (29)
First incidence (days)	—	—	—	346	—	—	—	367
Poly-3 test	$P < 0.001$	—	—	$P < 0.001$	$P < 0.001$	—	—	$P < 0.001$
Mean lesion severity score	—	—	—	1.1	—	—	—	1.1
Lung(s); Fibrosis; Interstitial; Periacinar								
Incidence	0/139 (0)	0/141 (0)	0/136 (0)	93/131 (71)	0/139 (0)	0/142 (0)	0/139 (0)	101/134 (75)
Adjusted rate	0%	0%	0%	91%	0%	0%	0%	91%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
3-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	4/10 (40)	0/10 (0)	0/10 (0)	0/10 (0)	2/10 (20)
12-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	8/10 (80)	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)
24-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)
Terminal rate[c]	0/16 (0)	0/26 (0)	0/18 (0)	6/9 (67)	0/24 (0)	0/29 (0)	0/17 (0)	14/17 (82)
Natural death & moribund sacrifice rate	0/83 (0)	0/75 (0)	0/78 (0)	65/82 (79)	0/75 (0)	0/73 (0)	0/82 (0)	65/77 (84)
First incidence (days)	—	—	—	83	—	—	—	93
Poly-3 test	$P < 0.001$	—	—	$P < 0.001$	$P < 0.001$	—	—	$P < 0.001$
Mean lesion severity score	—	—	—	1.1	—	—	—	1.0

(Table continues next page)

[a] Number of lesion-bearing animals/number of animals examined. (In some cases, spare animals were included in groups based on total number of animals received and assigned.)

[b] Severity score: 0, not present; 1, minimal; 2, mild; 3, moderate; 4, marked.

[c] Observed incidence at terminal sacrifice.

Table 6 (*Continued*). Summary of Incidence[a] Rate for Non-Neoplastic Lesions and Mean Severity Score[b] for Histopathologic Findings in the Lungs at Each of the Sacrifice Times by Sex

	Males				Females			
Histopathologic Endpoint	Control (%)	Low (%)	Mid (%)	High (%)	Control (%)	Low (%)	Mid (%)	High (%)
Lung(s); Hyperplasia; Epithelium; Periacinar								
Incidence	0/139 (0)	0/141 (0)	0/136 (0)	117/131 (89)	0/139 (0)	0/142 (0)	0/139 (0)	116/134 (87)
Adjusted rate	0%	0%	0%	99%	0%	0%	0%	97%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
3-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	0/10 (0)	0/10 (0)	0/10 (0)	9/10 (90)
12-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)
24-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)
Terminal rate[c]	0/16 (0)	0/26 (0)	0/18 (0)	9/9 (100)	0/24 (0)	0/29 (0)	0/17 (0)	17/17 (100)
Natural death & moribund sacrifice rate	0/83 (0)	0/75 (0)	0/78 (0)	78/82 (95)	0/75 (0)	0/73 (0)	0/82 (0)	70/77 (91)
First incidence (days)	—	—	—	83	—	—	—	93
Poly-3 test	$P < 0.001$	—	—	$P < 0.001$	$P < 0.001$	—	—	$P < 0.001$
Mean lesion severity score	—	—	—	1.4	—	—	—	1.4
Nose/Turbinate 2 Level; Degeneration; Olfactory Epithelium								
Incidence	12/137 (9)	14/140 (10)	12/134 (9)	23/131 (18)	12/137 (9)	23/138 (17)	29/136 (21)	27/132 (20)
Adjusted rate	19%	20%	19%	40%	17%	30%	36%	36%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)	1/10 (10)	0/10 (0)	1/10 (10)
3-Month sacrifice rate	0/10 (0)	1/10 (10)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
12-Month sacrifice rate	0/10 (0)	1/10 (10)	2/10 (20)	5/10 (50)	0/10 (0)	1/10 (10)	4/10 (40)	2/10 (20)
24-Month sacrifice rate	3/10 (30)	1/10 (10)	1/10 (10)	1/10 (10)	1/10 (10)	3/10 (30)	5/10 (50)	1/10 (10)
Terminal rate[c]	2/16 (13)	4/26 (15)	2/18 (11)	0/9 (0)	1/24 (4)	7/29 (24)	3/17 (18)	1/17 (6)
Natural death & moribund sacrifice rate	7/81 (9)	7/74 (9)	7/76 (9)	16/82 (20)	10/73 (14)	11/69 (16)	17/79 (22)	22/75 (29)
First incidence (days)	177	94	366	31	650	31	326	31
Poly-3 test	$P = 0.009$	$P = 0.625$	$P = 0.588$	$P = 0.005$	$P = 0.008$	$P = 0.070$	$P = 0.007$	$P = 0.007$
Mean lesion severity score	1.3	1.5	1.7	1.6	1.8	1.5	1.5	1.5
Nose/Turbinate 3 Level; Degeneration; Olfactory Epithelium								
Incidence	2/139 (1)	7/138 (5)	4/134 (3)	11/132 (8)	4/137 (3)	14/137 (10)	13/134 (10)	15/134 (11)
Adjusted rate	3%	10%	7%	22%	6%	19%	18%	21%
1-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)
3-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)
12-Month sacrifice rate	0/10 (0)	3/10 (30)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	2/10 (20)	2/10 (20)
24-Month sacrifice rate	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	1/10 (10)	0/10 (0)
Terminal rate[c]	0/16 (0)	0/25 (0)	0/18 (0)	0/9 (0)	0/24 (0)	1/29 (3)	1/16 (6)	0/17 (0)
Natural death & moribund sacrifice rate	2/83 (2)	4/73 (5)	4/76 (5)	10/83 (12)	4/73 (5)	12/68 (18)	7/78 (9)	13/77 (17)
First incidence (days)	391	367	416	31	326	303	30	341
Poly-3 test	$P = 0.007$	$P = 0.208$	$P = 0.341$	$P = 0.002$	$P = 0.020$	$P = 0.032$	$P = 0.022$	$P = 0.007$
Mean lesion severity score	1.5	1.4	2.0	1.5	1.5	1.2	1.2	1.4

[a] Number of lesion-bearing animals/number of animals examined. (In some cases, spare animals were included in groups based on total number of animals received and assigned.)

[b] Severity score: 0, not present; 1, minimal; 2, mild; 3, moderate; 4, marked.

[c] Observed incidence at terminal sacrifice.

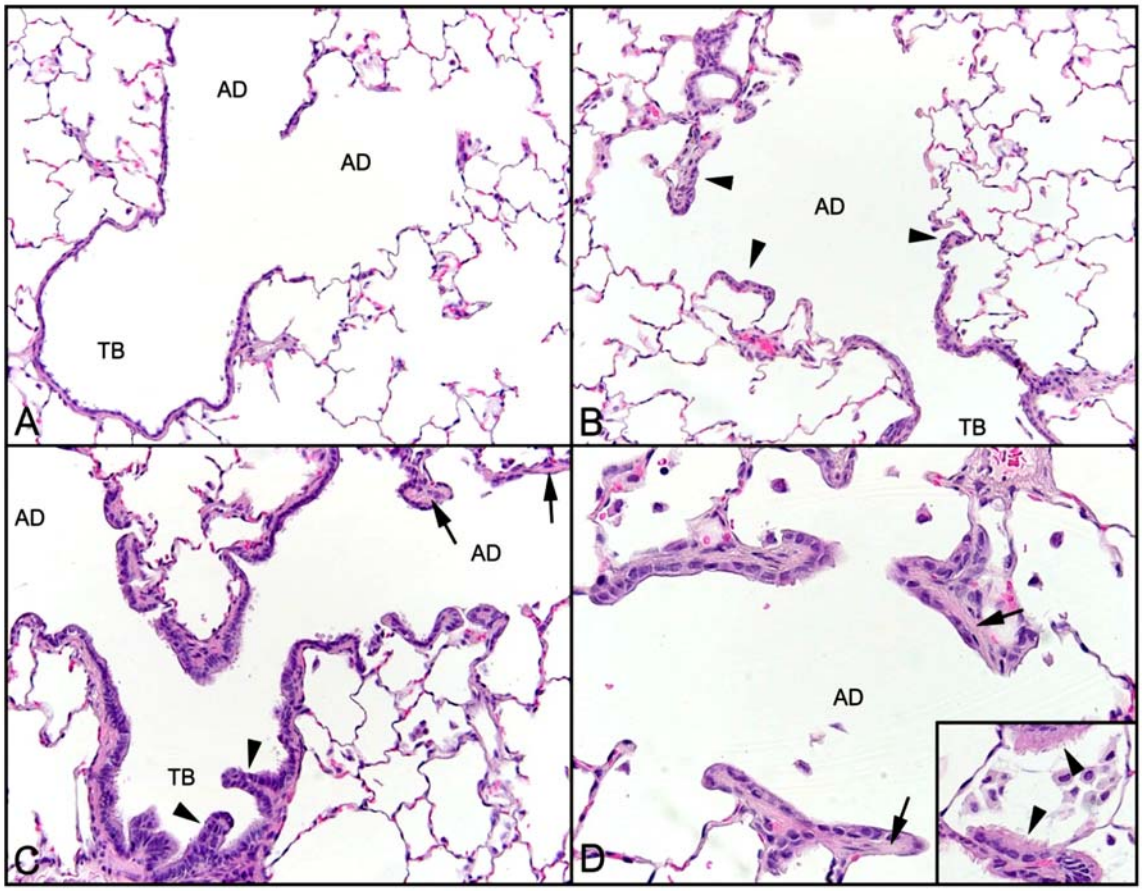

Figure 3. Illustrative photomicrographs of female rat lungs in the control and high-level groups after 30 months of exposure to NTDE, showing findings typical for this study: (A) control: normal appearance of a centriacinar area of the lung showing a terminal bronchiole (TB), alveolar ducts (AD), and alveoli (original magnification 200×); **(B) high-level group**: centriacinar area of the lung showing a minimally affected terminal bronchiole and an alveolar duct with increased numbers of epithelial cells lining the airways resulting in areas of increased thickening (arrowheads) of mainly the alveolar duct walls (termed "Hyperplasia; Epithelial; Periacinar"; original magnification 200×); **(C) high-level group:** more prominent increase in epithelial cells of the terminal bronchiole resulting in the projection of epithelial fronds into the lumen (arrowheads). Epithelial cells also increased in the lining of the alveolar ducts. The walls of some areas of the alveolar ducts are slightly thickened with an increase in fibrous connective tissue (arrows) ("Hyperplasia; Epithelial; Periacinar" and "Fibrosis; Interstitial; Periacinar"; original magnification 200×); **(D) high-level group:** the increased magnification of this image shows more cellular detail of the proliferating epithelial component alveolar duct and surrounding alveoli. Epithelial cell proliferation lines the alveolar duct and extends into some alveoli. The walls of the alveolar duct have increased fibrous connective tissue (arrows) (changes termed "Hyperplasia; Epithelial; Periacinar" and "Fibrosis; Interstitial; Periacinar"; original magnification 400×). ***Inset*** demonstrates that in some areas in some animals proliferating epithelial cells of alveolar ducts and alveoli are ciliated (arrowheads) and therefore allow confirmation of metaplasia of the alveolar epithelium to a bronchiolar cell type (i.e., "bronchiolization") (changes termed "Hyperplasia; Epithelial; Periacinar"; and "Bronchiolization"; original magnification 400×).

beyond the centriacinar areas. Since it was now clear that fibrosis had not affected the alveolar interstitium distal to the central acinus, we chose the term "fibrosis, interstitial, periacinar" to convey that the lesion distribution was not typical of the pattern generally associated with the term "interstitial fibrosis." The report of findings at earlier time points (McDonald et al. 2012) had used the "interstitial fibrosis" terminology.

There was statistically significant evidence of exposure-related effects in the nasal cavity that were generally limited to the high-level exposure groups. The presence of some (usually) minimal- to mild-grade nasal lesions occurring across all groups throughout the study was noted. Rats of both sexes from all experimental groups had inflammation, squamous metaplasia, and epithelial hyperplasia in the nose/turbinate at one or more of the four nose/turbinate

Table 7. Cell Proliferation in Rat Lung Tissue at Different Exposure Time Points Measured by Percentage of Ki-67$^+$ Cells × 100[a]

% Positive	Group	Males Mean ± SEM (*n*)	Females Mean ± SEM (*n*)	Both Mean ± SEM (*n*)	Interaction[a]
1 Month	Control	3.5 ± 0.6 (10)	2.4 ± 0.3 (10)	3.0 ± 0.4 (20)	0.602
	High	3.6 ± 0.4 (10)	3.0 ± 0.3 (10)	3.3 ± 0.3 (20)	
3 Months	Control	4.2 ± 0.6 (10)	4.3 ± 0.6 (10)	4.2 ± 0.5 (20)	0.692
	High	3.9 ± 0.3 (10)	4.6 ± 0.8 (10)	4.3 ± 0.4 (20)	
12 Months	Control	1.5 ± 0.4 (10)	3.4 ± 1.1 (10)	2.5 ± 0.6 (20)	0.956
	High	1.9 ± 0.5 (10)	3.8 ± 1.0 (10)	2.8 ± 0.6 (20)	
24 Months	Control	1.9 ± 0.5 (9)	1.5 ± 0.2 (10)	1.7 ± 0.3 (19)	0.207
	High	3.2 ± 0.6 (8)	1.6 ± 0.5 (9)	2.4 ± 0.4 (17)	

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

sectioning levels. Minimal mucous cell metaplasia of the epithelium of the lateral wall often occurred in concert with those more prominent changes. The most consistent finding was minor olfactory epithelial degeneration within nose/turbinate levels 2 and 3, which exhibited statistically significant increases in both sexes (Table 6). In males, evidence of an increase was limited to the high exposure group, while females in all NTDE-exposed groups exhibited significant, or marginally significant, evidence of increased incidence relative to controls in both nasal levels. Other statistically significant increases in the rates of common spontaneously occurring lesions in nose/turbinate level 1 were found predominately in high-level-exposure animals relative to controls. These increases were small in magnitude: epithelial lateral wall hyperplasia (males 23% vs. 15%); suppurative inflammation (males 23% vs. 18%); lateral wall squamous metaplasia, (females 41% vs. 29%); lateral wall hyperplasia or squamous metaplasia (males 55% vs. 48%; females 64% vs. 55%). Statistically significant evidence in nose/turbinates levels 2 and 3 was limited to low incidences (5 or less) of two lesions that occurred in high-level-exposure males but were not found in controls.

A finding of note was that, in the rats under long-term observation, the incidence of thyroid follicular cell adenomas and combined incidence of follicular cell adenomas and carcinomas (Table H.1) were significantly increased in females exposed at the high level compared with control females (but not in males exposed at the high level compared with controls). The PWG members reviewed the thyroid follicular cell lesions and generally agreed with the diagnoses of the study pathologist. In the rare instances where the PWG consensus differed from the study pathologist's conclusion, the diagnoses were changed to reflect the PWG consensus opinion. Incidences in rats of both sexes exposed at the high level at the 1-, 3-, 12-, and 24-month interim sacrifices were unremarkable compared with controls (0% for all; no tumors of this type were seen in any rats exposed to the high level at interim sacrifice). In concert with the PWG, the decision was made to further investigate the increase in thyroid follicular cell tumors in females for a possible exposure–response effect by examining low- and mid-level exposure rats of both sexes. As there were no follicular cell tumors in any high-level interim-sacrifice rats, the PWG reasoned that it would be of little benefit to microscopically examine thyroids from low- and mid-level interim sacrifices. Thus low- and mid-level interim-sacrifice animals were excluded from histopathologic examination. However, all low- and mid-level animals from the terminal sacrifice, as well as any animals dying or euthanized moribund prior to the terminal sacrifice, were included. The combined incidences of follicular cell tumors in NTDE-exposed females were similar across exposure levels (low, 9%; mid, 9%; high, 6%) and consistent with historical control rates (see Discussion), but significantly higher than the control rate in the present study (1%). In males, there was no substantial statistical evidence that combined tumor incidence rates in exposed groups (low, 12%; mid, 5%; high, 2%) differed significantly from the concurrent control rate (6%).

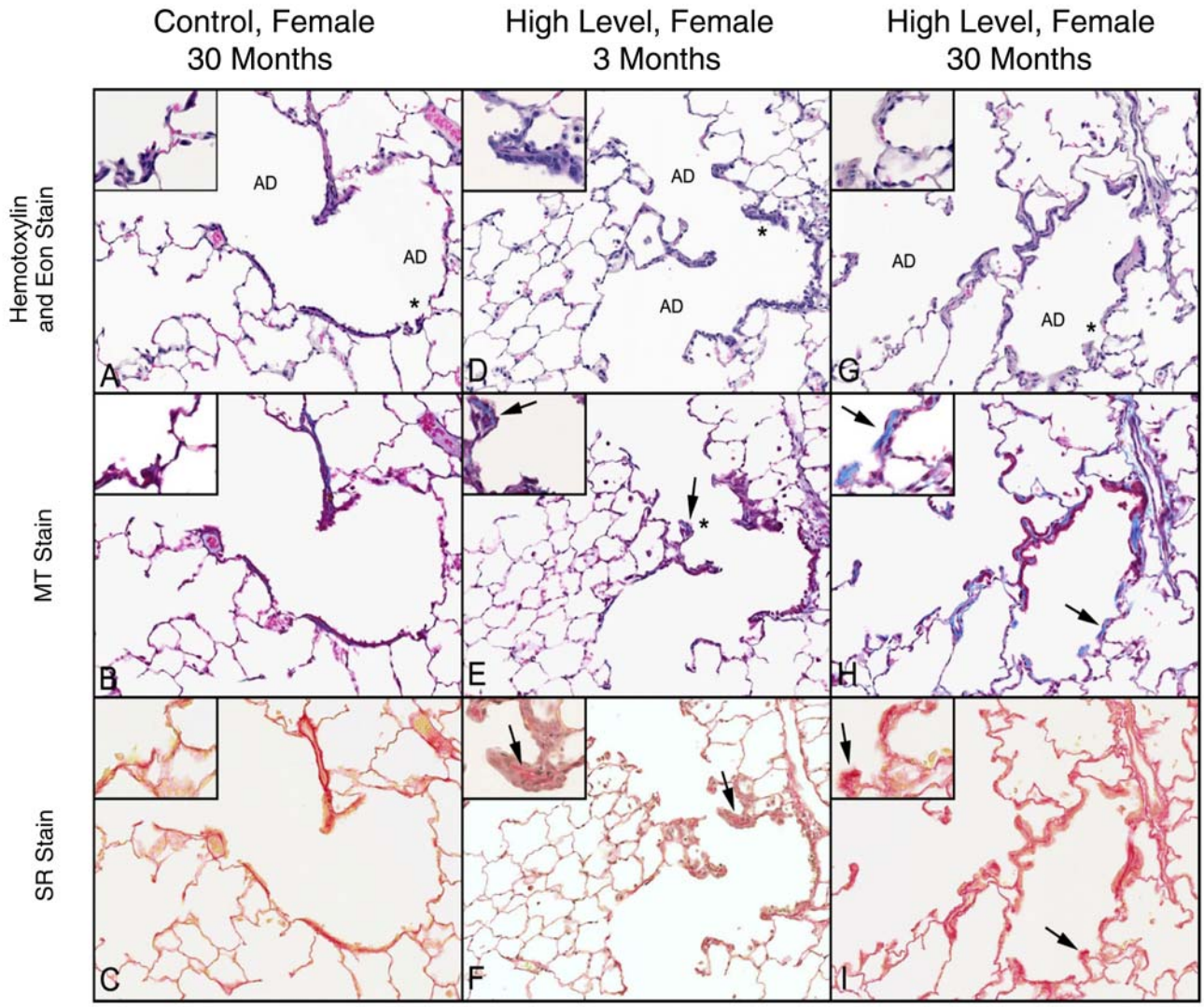

Figure 4. Illustrative photomicrographs of female rat lungs after 3 and 30 months of exposure to NTDE. Histochemical stains aid in demonstrating the minimal increase in mature collagenous connective tissue (termed "Fibrosis; Interstitial; Periacinar") sometimes occurring within areas of epithelial hyperplasia at 3 months and usually occurring after 30 months of exposure (all main panels original magnification 200×; all insets original magnification 400×; asterisk (*) designates region further magnified in insets; arrow indicates collagen): **(A–C) control female; 30 months: (A)** normal alveolar ducts (ADs) and adjacent alveoli as seen with hematoxylin and eosin stain, **(B)** Masson's Trichrome (MT) stain to highlight collagen in blue, and **(C)** Sirius Red (SR) stain to highlight collagen in red; **(D–F) high-group female; 3 months: (D)** centriacinar region demonstrates an increase in epithelial cells and connective tissue, **(E)** collagen within the stroma stains blue with the MT stain, and **(F)** red with the SR stain (see arrows in main panels and insets for examples). Note that area shown in inset for MT stain in panel E is slightly moved relative to D and F (as shown by asterisk in panel E) to better demonstrate a focus of collagen within the MT slide section; **(G–I) high-group female; 30 months:** centriacinar region demonstrating somewhat more prominent increase in collagenous tissue within alveolar duct region with hematoxylin and eosin stain (**G**), MT stain (**H**), and SR stain (**I**).

DISCUSSION AND CONCLUSIONS

This study was the first to evaluate potential biologic responses to repeatedly inhaled NTDE. It was designed with three exposure levels and a clean air control and with interim evaluations at 1, 3, 12, and 24 months, and terminal sacrifice (28 to 30 months). Rats were selected for this lifetime study both because they were expected, as observed in historical studies of TDE, to be more sensitive than mice to DE-induced lung neoplasia and because the previous hazard evaluations of TDE had focused primarily on responses in the rat (EPA 2002; Mauderly and Garshick 2009). The Wistar Han rat in particular was selected because of its relatively low incidence of background lung tumors and the concern that a higher background tumor incidence in F344 rats would make it potentially more difficult to distinguish subtle NTDE effects based on the number of animals in the study. A detailed description of the rationale for the decision is provided in Additional Materials 1 (available on the HEI Web site at *http:// pubs.healtheffects.org*). Although the Wistar Han rat is considered less sensitive to chemically induced neoplastic and non-neoplastic outcomes compared with the F344 rat (Weber et al. 2011), they have both been shown to be susceptible to the effects of high-level exposure to TDE (Heinrich et al. 1995; Mauderly et al. 1987a; Nikula et al. 1995). Previous responses in both rat strains are described later in the section of this volume titled "Pathology Working Group Comparison Statement." The primary motivation for this study was to investigate the potential for NTDE to cause pre-neoplastic lung lesions or primary lung neoplasia similar to what had been observed in previous studies of TDE. The outcome showed that NTDE exposures at the highest practical concentration resulted in a markedly less severe and different biologic responses compared with the effects of TDE and did not provide any indication of a cancer potential in the respiratory tract or other organs.

The study was intentionally designed to potentially cause statistically significant exposure-related effects at the most extreme (high) exposure level and to potentially demonstrate no statistically significant effects at one or more of the two lower exposure levels. It was anticipated that any biologic effects would most likely be related to the concentration of NO_2 in the chambers; NO_2 was thus used as the target component for setting and controlling the NTDE dilutions. The high 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 male Fischer-344 rats exposed to NO_2 alone (Mauderly et al. 1990). That study evaluated some biologic endpoints similar to those used in the present study. Although NO_2 concentration level was the primary indicator of dilution in the present study, it is important to acknowledge that the exposure atmosphere was a complex mixture containing many components. The present study design did not allow discrimination among the contributions of the different exposure components to the observed effects. However, as described below, considerable effort was expended in evaluating the potential roles of NO_2 and other reactive gases in causing the observed responses.

The major outcome of this study was the absence of lung lesions that may be considered potentially pre-neoplastic or primary lung neoplasia in rats exposed to NTDE. Spherical parenchymal hyperplastic lesions are classically considered potentially pre-neoplastic in the rat lung and are usually seen within the alveolar regions, where the alveolar walls are lined by densely proliferative epithelial cells, yet alveolar architecture remains intact. Such lesions are considered pre-neoplastic because they usually increase alongside lung tumors as a result of exposure to a carcinogen. The lung lesions that were found in this study occurred at the high level of exposure only and were characterized by an increased number and prominence of basophilic epithelial cells lining terminal bronchioles, alveolar ducts, and adjacent alveoli (those in close proximity to the terminal bronchioles and alveolar ducts). In addition to the epithelial proliferation, there was a slight bronchiolization in some rats. The histologic findings showed an increase in the extent of the tissue changes in the respiratory tract between 3 and 12 months (McDonald et al. 2012). Of interest is that the severity of the tissue injury that was considered related to NTDE exposure did not increase substantially after 12 months.

Changes attributed to exposure within the nose/turbinates were generally minimal to mild. These changes also demonstrated a high level of variability within a given nasal section (where extent and location varied), between sections within an animal, between rats within an exposure group, and through time as animals progressed through the study. The most consistent findings—those involving the olfactory epithelial changes—were mostly unilateral, generally single and very small, often scattered across interim sacrifices, and did not show a pattern of distribution within the nose typical of exposure to an inhaled agent. Frequently only a single small focus was observed anywhere within all four nasal sections. It is noted that the often inconsistent pattern of occurrence is difficult to reconcile with typical nasal responses to inhaled toxicants (i.e., ozone [NTP 1994]), which generally show a more consistent pattern within the nose and through time. Any clinical significance was not apparent, and the minor degree

and inconsistent nature of the change renders the biologic significance uncertain.

In the present study, the apparent increase in combined incidence of thyroid follicular cell tumors in exposed female rats (6%) was considered to be an artifact of the remarkably low incidence in controls (1%) and not to be related to exposure to NTDE for several reasons:

- The incidence of thyroid follicular cell neoplasms in control females (1%) was abnormally low compared with historical control values. Historical control data for female Wistar Han rats document that thyroid follicular cell adenomas and carcinomas often occur spontaneously at similar or higher incidences than those observed in the females exposed to a high level of NTDE. In one summary of historical control rates in female Wistar Han rats that was based on 10 two-year bioassays of animals from a single supplier (Giknis and Clifford 2003), the reported overall incidence rate of follicular cell adenomas across studies was 3.6% (with 4 of the 10 studies exceeding 5%, and a maximum rate of 9.1%); for thyroid follicular cell carcinomas, the overall rate was 1.62%, with a maximum of 3.64%. Similarly, in an examination of 50 two-year studies (RCC 2003), the overall rate of thyroid follicular adenomas was 1.9% (with 3 studies showing rates of 6% or higher, and a maximum rate of 8.5%); for thyroid follicular cell carcinomas, the overall rate was 1.0%, with a maximum rate of 5.8%.
- The 5% incidence of follicular cell adenomas in high-exposure NTDE females is remarkably similar to the rates reported by Giknis and Clifford (2003) and is well within the range reported by RCC (2003). The 2% incidence of follicular cell carcinomas is also consistent with these reports of historical data from 24-month studies. This is particularly noteworthy given that the present study was of 30 months' duration and would therefore be plausibly expected to have resulted in higher incidences than in those 24-month studies.
- There was no evidence of an increase in incidence with increasing exposure concentration (i.e., no exposure–response) in the combined incidence of follicular cell adenomas and carcinomas in females (low level, 9%; mid level, 9%; high level, 6%; mortality-adjusted rates 13%, 14%, 13%, respectively).
- The apparent evidence of exposure-related increase in incidence was limited to only one sex (female). The incidence in high level males (2%) was actually decreased relative to controls (6%).
- There was no corresponding evidence of an increase in thyroid follicular cell hyperplasia (3% in females exposed to the high level vs. 3% in controls). This is typically considered the relevant "pre-neoplastic" change, which would also be expected to increase if the tumor observation were truly exposure-related. In addition, no increase was observed in tumor multiplicity.

As described earlier in the Methods and Study Design, the findings from the study pathologist were confirmed in a number of ways. The chronic study findings were peer-reviewed internally at LRRI and EPL, followed by a second, independent, review by a PWG organized by HEI. The peer review was conducted on all tissues of five males and five females from the high-level group and the same number of animals from the control group. It also included all lung and nose sections from all rats and all thyroid glands from all female rats. Any differences were resolved, and the PWG then reviewed the study. The role of the PWG was to evaluate the conclusions of the study pathologist (having already incorporated the pathology peer-review findings and input) and to compare the findings to previous studies of TDE and oxidant gases. The PWG was composed of five pathologists who were voting members, one non-voting member, and five observers, including two of the study pathologists and the HEI study monitor. The PWG examined in a blind fashion all neoplastic and representative non-neoplastic proliferative lesions of the nasal cavity and lung, including neoplastic nasal lesions previously identified at the interim sacrifices. The group also evaluated all proliferative lesions of the female rat thyroid glands. No primary lung tumors were found by the study pathologist, the peer-review pathologist, or the PWG. The PWG examined slides and agreed that three proliferative pulmonary lesions (one in a control female, one in a low-level-exposure female, and one in a high-level-exposure female) — diagnosed as *alveolar epithelial hyperplasia* by the study pathologist — were indeed hyperplasia and not pulmonary tumors. The group agreed that there was no evidence of increased tumors in the upper or lower respiratory tract of the rats after exposure to NTDE in this study. The PWG's conclusion was that the centriacinar regions of the rats exposed to control and mid-level NTDE that were analyzed were within normal limits for this age of animal and that periacinar hyperplasia was not present. The PWG opinion was that NTDE-related pulmonary lesions were restricted to rats exposed to the high level of NTDE.

Because the historical studies of TDE were a primary driver for the present study, it is useful to consider the contrast in biologic responses to the emissions from the two generations of technologies (see below). Further, the presence of the high concentrations of NO_2 in NTDE led to lesions that merit comparisons with previous inhalation

studies with reactive gases (also discussed below). Interestingly, the PWG noted that the minimal to mild periacinar lesions in the lungs of rats exposed to high levels of NTDE were similar to those reported in Wistar Han rats continuously exposed to 4 ppm NO_2 for 27 months in an earlier study (Kubota et al. 1987). In addition, the PWG noted that the accumulation of soot particles in the lung and the accompanying substantial sustained lung inflammation typical of TDE exposure in rats were absent in rats exposed to NTDE. The comparisons are consistent with the premise that there should be fewer chronic effects associated with the accumulation of DPM in the lung in the present study than in historical studies of TDE. Both neoplastic and non-neoplastic 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 (Hesterberg et al. 2012; Mauderly and Garshick 2009; McClellan et al. 2012; Brightwell et al. 1989; Heinrich et al. 1986, 1995; Stinn et al. 2005). It is reasonable to speculate that the markedly lower, and possibly nearly negligible, levels of exposure to DPM in the present study may preclude effects attributable to DPM. The potential for a different type of progressive lung remodeling, with lesser severity, associated with exposure to NO_2 in NTDE is less certain, but certainly plausible — especially at the high level of exposure used in this study.

For ease of comparison, Figure 5 provides histologic findings from the current study and previous studies as evaluated by the PWG (see also "Pathology Working Group Comparison Statement" later in this volume). The six panels show examples from the current study (panels A and B), TDE studies conducted by Mauderly and colleagues (1994; panel C) and Heinrich and colleagues (1986; panel D), a chronic NO_2 study (Mauderly et al. 1989; panel E), and a chronic study of ozone conducted by the National Toxicology Program (NTP 1994; panel F). In general, the TDE examples reflect the chronic responses in rats in all chronic studies of solid particles (including carbon black) described below. As illustrated in Figure 5, the differences in appearance between the lungs exposed to TDE and those exposed to NTDE are dramatic. The PWG concluded that these differences related to DPM accumulation were the most notable of those observed among the comparisons. The respiratory tract response to NTDE was similar in the type, location, and severity of lesions to that observed in previous chronic inhalation studies of rats exposed to the reactive gases NO_2 and ozone (Mauderly et al. 1989; NTP 1994). The PWG noted that the severity of the lesions found in the NO_2 study (Mauderly et al. 1989) was less than what was found in the current study with NTDE, but that the NO_2 study examined was shorter in duration and involved a lower cumulative dose of NO_2 (9.5 ppm NO_2, 7 hours/day for up to 24 months in the study by Mauderly and colleagues [1989] vs. 4.2 ppm NO_2, 16 hours/day for up to 30 months in the current study).

Perhaps the study closest in design to the present study was the one funded by HEI and the Department of Energy in which rats were exposed for 24 months to compare the carcinogenicity of TDE and carbon black at similar PM concentrations (Mauderly et al. 1994; Nikula et al. 1995). In that study, F344 rats were exposed 16 hours/day, 5 days/week to exhaust from 1988 GM LH6 6.2-L engines burning then-contemporary D-2 certification fuel (300+ ppm sulfur) and operating on a variable-duty cycle without exhaust aftertreatment. As was typical of TDE, NO_2 constituted 10 to 20% of NO_x. Low- and high-level exposure atmospheres contained DPM (the concentration of which was estimated by subtracting the level of PM in control chambers from that in exposure 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. Those ratios were notably lower than the NO_2/DPM ratios in the present study. Lung tumors after long-term exposure were the focus of the study, but observations of tumors were limited in rats euthanized after 3, 6, 12, and 18 months of exposure. Exposure resulted in substantial accumulation of DPM in the lungs, accompanied by prominent lung inflammation and progressive epithelial and interstitial pulmonary pathology, including tumorigenesis. As illustrated in Figure 5, the most noticeable difference between the results of previous TDE studies (seen in panels C and D) and the present NTDE study (panels A and B) was the strikingly smaller accumulation of DPM in macrophages in the NTDE study. Figure 5 shows histologic findings from the study by Heinrich and colleagues (1986), which show a pattern of lung lesions in Wistar Han rats (the strain used in the present study) that is similar to that found in the F344 rats used in the study by Mauderly and colleagues (1989), results of which are shown in Figure 5C.

The characteristic lung pathology shown in Figure 5C and D that resulted from exposure to TDE was similar to those observed in several other TDE studies, including those in the Wistar Han rat (Brightwell et al. 1989; Heinrich et al. 1995; Stinn et al. 2005). Overall, the DPM exposure concentration–response relationship for the formation of tumors in chronically exposed rats was similar among those TDE studies (Mauderly and Garshick 2009). Lung carcinogenesis was strikingly less or absent in mice, hamsters, and guinea pigs exposed similarly. Exposure-related tumors in rats were observed only at high particle concentrations. Of interest is that similar concentrations of other inhaled solid

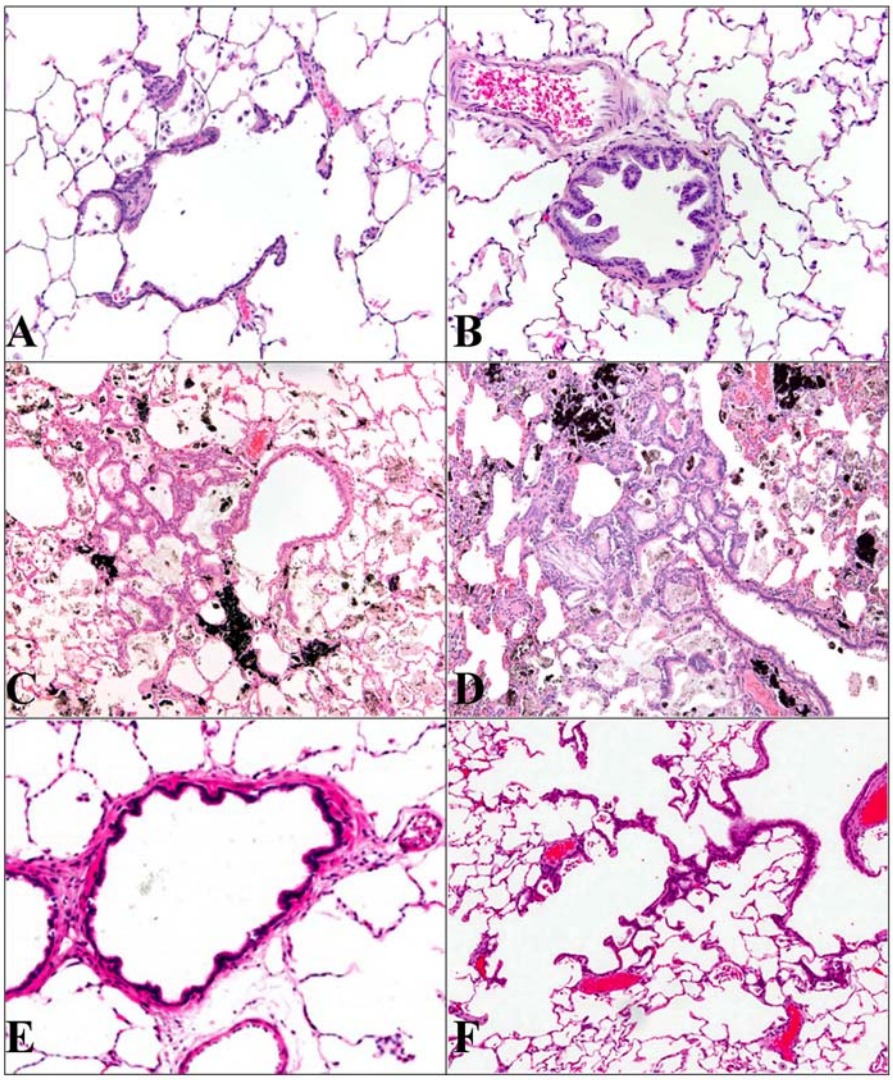

Figure 5. Comparison of illustrative photomicrographs of rat lungs from the current study and chronic studies with other exposure atmospheres of interest (for larger prints see Pathology Working Group Comparison Statement included in this volume): **(A) ACES investigation of NTDE (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×).** Minimal increase in thickening of alveolar walls with minimal increase in bronchiolar epithelium. While a few macrophages are present in this figure, inflammatory cells did not increase overall with exposure to the NTDE. These changes were found at only the highest exposures and not at the mid or low exposures (photograph by Rod Miller, EPL); **(B) ACES investigation of NTDE (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×).** Bronchiolar epithelial hyperplasia was an uncommon finding in rat lungs characterized by papillary projections into preterminal bronchiole in ACES (photograph by Rod Miller, EPL); **(C) LRRI TDE study (Mauderly et al. 1994) (6.5 mg/m^3 PM, male F344/N, 24 months, original magnification 100×).** Rat lung after exposure to diesel exhaust for up to 2 years. Prominent black pigment free or within macrophages. Hyperplasia of bronchiolar epithelium extending into centriacinar alveoli. Numerous alveolar macrophages throughout the lung often containing pigment (lung slide provided by Andrew Gigliotti, LRRI; photograph by Rod Miller, EPL). Contrast with Panel A resulting from NTDE where increased pigment was absent; **(D) Fraunhofer TDE study (Heinrich et al. 1986) (4.2 mg/m^3, female Wistar Han, 30 months, original magnification 100×).** Prominent black diesel soot particulates are present free in alveoli, in the numerous pulmonary alveolar macrophages, and in interstitial tissues. Centriacinar epithelial hyperplasia and bronchiolization were marked. Chronic inflammation, characterized by a mixed inflammatory cell infiltrate, fibrosis, and some sterol cleft formation, was marked. The entire lung was involved, and this picture is dramatically different from the findings in 2007 technology diesel (slide provided by Heinrich Ernst, Fraunhofer Institute; photograph by Rod Miller, EPL). Compare with Panel A; **(E) LRRI NO_2 study (Mauderly et al. 1989) (9.5 ppm NO_2, male F344, 24 months, original magnification 200×).** Preterminal bronchiole from the 24-month NO_2 rat study conducted at LRRI showing finding similar to ACES. These changes were uncommon in the NO_2 study and not as distinct as those shown in the Kubota et al. (1987) study in which animals were exposed to 4 ppm NO_2 for 27 months (lung slide provided by Andrew Gigliotti, LRRI; photograph by Rod Miller, EPL.); **(F) National Toxicology Program ozone study (NTP 1994) (1 ppm, male F344/N, 30 months, original magnification 100×).** Inhalation exposure up to 1 ppm for up to 30 months in F344/N male and female rats. The centriacinar area shows bronchiolar epithelial cells extending into centriacinar alveoli with minimal increase in thickening of alveolar walls and increased alveolar macrophages (photograph provided by Dr. Ron Herbert, U.S. National Toxicology Program, National Institute of Environmental Health Sciences).

particles used for comparison (e.g., carbon black and titanium dioxide) caused similar rates of tumor formation, suggesting that the formation of tumors was due to excess accumulation of particles and not to the composition of the particles per se (Heinrich et al. 1995; Hesterberg et al. 2012; Mauderly et al. 1994; Nikula et al. 1995).

Two previous HEI-funded studies of rats exposed repeatedly to NO_2 alone offer the closest comparison to the cumulative NO_2 exposures of the present study. In the first, Mauderly and colleagues (1987b) exposed 6-month-old male F344 rats 7 hours/day, 5 days/week 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 hours/week) and only somewhat less than the actual rate of 352 ppm•hour (4.4 ppm × 80 hours/week) for the average of the present study. In the 1987b study, after 6 months of exposure, lung weight was increased 7% compared with controls, but the increase was not statistically significant. The histologic appearance of the lungs was 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. Pulmonary function did not differ significantly from control values. Thus, the structural and functional effects of 6 months of exposure of male F344 rats to NO_2 at a rate similar to that in the current study appear similar to the effects of the highest concentration of NTDE on male Wistar Han rats at 12 months in the present study. Considering the differences in rat strain, study design, and endpoints, the comparison might be interpreted as suggesting that NO_2 could have largely caused the minimal pulmonary pathology in the present study.

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 hours/day, 5 days/week to 9.5 ppm NO_2 (332.5 ppm•hour/week), but the exposures continued for 24 months. Body weight and mortality were unaffected, and no clinical morbidity was observed throughout the exposure. At 24 months, the lung/body weight ratio was increased by 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. As illustrated in Figure 5E, there was mild hyperplasia of epithelium in terminal bronchioles and bronchiolization of proximal alveoli, accompanied by occasional foci of inflammatory cells in the same region. Overall, the inflammatory response was minimal. Responses in BALF endpoints reflected modest cytotoxicity and oxidative stress, and total lung collagen was increased. Clearance of inhaled radio-labeled tracer particles was not impaired. Two-way ANOVA identified 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 slightly increased, but differences were not significant by pairwise comparison. Three-way ANOVA indicated significant influences of NO_2 on lung volumes, but not compliance. Forced expiratory flows and DL_{CO} were not reduced by NO_2. Overall, the impacts on pulmonary function of exposure for 24 months at a rate of 332.5 ppm•hour/week in the 1989 study were less than those of exposure for 24 months at a rate of 352 ppm•hour/week in the present study. The most striking difference was the lack of impact on forced expiratory flows in the earlier study, although the magnitude of functional impairment was small in both studies. Whether the greater functional impact of the present exposure was due to the 6% higher exposure rate, the presence of other pollutants in the exposure, or the difference in rat strain is not known.

Other studies have demonstrated significant biochemical and histopathologic effects of repeated exposure of rats to NO_2, but useful comparisons to the present study are challenged by differences in study design and endpoints, and many involved extremely high concentrations that were not relevant to the present study. Results from the following somewhat comparable studies support the plausibility that NO_2 may have been the primary driver of the histopathologic and functional effects in the present study.

Beginning in the early 1960s, Freeman and colleagues at Stanford University conducted a series of studies of 1-year-old male Sprague-Dawley rats exposed "continuously" (assumed for the present comparison to have been approximately 23 hours/day to allow for chamber maintenance), 7 days/week to NO_2 at concentrations ranging from 0.8 to 50 ppm (reviewed in Freeman et al. 1968a). After first exploring mortality associated with high-level exposure, investigators in subsequent studies focused primarily on the development of pulmonary emphysema. Exposure for 2 years at 12 ppm produced microscopically visible emphysema (Haydon et al. 1965), but exposures for 2 years at 2 or 0.8 ppm did not (Freeman et al. 1968a), although morphometric measures demonstrated a slight increase in alveolar diameter (Freeman et al. 1968b). Exposure to 0.8 ppm (approximately 129 ppm•hour/week) for 2 years or longer caused "suggestive but equivocal" changes in terminal bronchiolar epithelium (Freeman et al. 1966), and higher concentrations caused more distinct "hypertrophy" of epithelium in the terminal bronchiolar–alveolar duct region.

Bronchiolar epithelial hypertrophy was evident after only 16 weeks of exposure at 4 ppm (approximately 644 ppm•hour/week) (Haydon et al. 1965). In a more recent morphometric study (Juhos et al. 1980), the same group compared the diameters of the distal portions of terminal bronchioles in controls and rats exposed to 15.7 ppm NO_2 (approximately 2528 ppm•hour/week) for times ranging from 1 week to 17 months and estimated airflow rates assuming a standard driving pressure. Diameters and estimated flows were reduced at all times, compared with controls, and the effect was especially evident at 3 months and later. Although the NO_2 studies conducted by Freeman and colleagues offer no direct comparisons to the present study, the epithelial changes related to level of exposure in terminal airways and reduced estimated flows parallel somewhat the histologic changes in the terminal bronchiole–alveolar duct region and the reduced forced expiratory flows at the high exposure level (352 ppm•hour/week) in the present study.

Gregory and colleagues (1983) exposed male and female F344 rats to NO_2 at 0.95 or 4.95 ppm for 7 hours/day, 5 days/week for up to 15 weeks and reported effects on histopathology and a few BALF and lung tissue variables in data from combined sexes. They observed significant changes in BALF ALP, LDH, and glutathione peroxidase, and lung tissue glutathione balance variables at earlier time points in the high-level group, but no significant differences from control at 15 weeks. At 15 weeks, they observed subpleural macrophage accumulations accompanied by local hyperinflation in lung sections at the high exposure level. The 13-week observations in the present study (reported in McDonald et al. 2012) were closest in time to the 15-week observations by Gregory and colleagues. At those times, the cumulative NO_2 exposures were 2599 ppm•hour in the study by Gregory and colleagues and 863 and 4222 ppm•hour at the mid and high levels, respectively, in the present study. Somewhat similar to the Gregory findings, the highest level exposure in the present study caused significant exposure-related differences in lung tissue (but not BALF), glutathione variables in females, and the combined data at 4 weeks (Table E.7 in McDonald et al. 2012), but no significant differences at 13 weeks. In contrast, however, neither ALP nor LDH in BALF were significantly affected at either 4 or 13 weeks in the present study, and no subpleural macrophage accumulations were noted. Instead, significant incidences of periacinar epithelial hyperplasia were present in both sexes at the high level at 13 weeks (Table 7 in McDonald et al. 2012). Despite differences in experimental design, rat strain, and cumulative exposure to NO_2, the study by Gregory and colleagues, which found that BALF and lung tissue effects of NO_2 can resolve with continued exposure, supports the notion that the time courses of certain exposure effects in the present study may have reflected the effects of NO_2 in the NTDE.

Kubota and colleagues (1987) exposed male JCL/Wistar rats "24 hours/day" (assumed for the present comparison to have been 23 hours/day to allow for chamber maintenance) for 9, 18, or 27 months to NO_2 at 0.04, 0.4, or 4.0 ppm (approximately 6, 64, or 644 ppm•hour/week). They then used light and electron microscopy to characterize histopathologic responses. Exposure at 0.04 ppm produced no lesions evident by either technique. Exposure at 0.4 ppm produced electron microscopic evidence of hyperplasia of epithelium at the "bronchopulmonary junction" at 18 months, and the lesion was evident by light microscopy at 27 months. Exposure at 4.0 ppm produced epithelial hyperplasia evident by light microscopy at 9 months that was accompanied by evidence of interstitial fibrosis. The hyperplasia and fibrosis were somewhat progressive at 18 and 27 months. The study also reported changes in alveolar epithelium at 9 months, but that the lesion resolved at a later time.

In a study by Juhos and colleagues (1980), the epithelial hyperplasia and interstitial fibrosis that was observed after exposure to 4.0 ppm NO_2 were generally consistent with the nature and location of lesions observed at the highest exposure level of the current study in the terminal bronchiolar–alveolar duct region. The description in the paper by Juhos and colleagues suggests more severe lesions than observed at similar times in the present study, which is consistent with the higher exposure rate (644 vs. 352 ppm•hour/week).

There were mild increases in inflammatory and oxidative stress indicators in the present study. Most of the statistically significant findings were significant only by trend analysis, without significant differences by pairwise comparisons. Moreover, they often lacked corroboration from complementary measures by different outcome variables, findings at different times, and effects in both sexes. The large number of responses measured in this study combined with a criterion for statistical significance of $P \leq 0.05$ could have resulted in a small number of statistically significant differences from control by chance. Statistically significant differences that might be suspected to have occurred by chance include those accompanied by lack of evidence of an exposure–response trend, lack of corroboration by occurrence in both sexes, lack of changes in biologically related parameters, or lack of responses for which the minimal differences were large in relation to mean values.

One of the more interesting findings in the current study was the mild yet progressive decline in pulmonary function. Overall, the effects of exposure on pulmonary function were small and were manifested more strongly in females than males. The difference between sexes has been hypothesized

to result from a greater "dose" of pollutant per unit of lung size in female than in male rats, due to the greater ventilation per unit of lung size in females (Mauderly 1986). Exposure to NTDE acted to reduce forced flow performance somewhat, with a greater effect at the lower lung volumes. The tests used in this study do not determine the cause of the flow limitation, but the overall evidence is consistent with morphologic changes in the smallest airways (terminal bronchiolar–alveolar duct region) being a likely cause. The efficiency of gas transfer across the alveolar–capillary membrane was not measurably affected, but the volume of gas transferred per unit of time was slightly reduced in parallel with a slight reduction of the volume of test gas in the lung.

Most of the NTDE exhaust mass 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 from this study and studies of newer TDE engines (e.g., McDonald et al. 2004) primarily consist of gaseous inorganic species with small amounts of PM and VOCs. The main differences between the exposure atmosphere in the present study and those of earlier TDE studies using pre-2007-compliant technologies were the concentration and composition of DPM relative to other pollutants. DPM mass comprised a significantly smaller portion of the total mass and had a markedly lower concentration in the present study than in earlier studies using TDE (e.g., Mauderly et al. 1987a; Nikula et al. 1995). However, there was a relatively high particle number concentration in the NTDE atmosphere. Indeed, the particle number concentration was similar to that of a previous inhalation study of TDE (McDonald et al. 2004). In the present study, the particle emissions occurred primarily during the trap regeneration. Because the nature of the observed biologic findings suggest that the responses may have been largely, if not entirely, attributable to a reactive gas (plausibly NO_2), it is doubtful that the particles played an important role in any of the observed effects.

The present study of NTDE also differs from earlier studies of TDE in that the concentration of NO_2 in NTDE was higher relative to concentrations of the other gases. The aftertreatment technology used in the present study oxidized NO to create NO_2, resulting in proportions of NO_2 that account for approximately 40 to 50% of the NO_x, compared with exhaust from previous technologies in which NO_2 comprised only 10% of the NO_x (e.g., Cheng et al. 1984; McDonald et al. 2004).

In the present study, changes in the output of NO_2 occurred largely due to changes in trap performance over time. Because NO_2 was the primary dilution indicator, the resulting dilution of the test atmosphere changed over time. This resulted in only minor changes in the concentrations of many of the minor constituents; however, the concentrations of NO and CO increased somewhat over time. It is not thought that changes in these gases had an important impact on the outcome of the study, because NO and CO are not perceived to have played an important role in the biologic responses observed. Further, despite the fact that the dilution ratio changed throughout the study because of the change in NO_2, the atmospheres did not have large changes because of the low and variable concentrations of the majority of the constituents. As a result, the changing dilution ratio likely had little impact on the outcome of the study in terms of biologic response.

The temperature of the test atmospheres differed due to the introduction of different concentrations of the warmer exhaust into the cooler diluting (and control) air. The high-level exposure atmosphere was an average of approximately 2°C warmer than the control atmosphere over the course of the study. It is not thought that this magnitude of temperature difference had any impact on the biologic responses observed in this study.

Both DPM and NO_2 have historically been primary targets of concern for potential TDE-related health hazards, although certainly other components could also exert toxicity. The DPM and NO_2 concentrations at the highest level over the entire course of the present study averaged approximately 8 μg/m^3 and 4.4 ppm (6873 μg/m^3) (Table 3) for a NO_2/PM mass ratio of 859:1. The health studies of humans and animals exposed to TDE have mostly employed exposure atmospheres having very different NO_2/PM mass ratios. Mauderly (2010) reviewed the relevance of exposure atmospheres employed in published TDE studies to contemporary (TDE) emissions. At that time (mid-2010), the most contemporary engine system used in a published study was 19 years old and did not include aftertreatment to reduce PM emissions (Barath et al. 2010). The same group (Lucking et al. 2011) subsequently used the same engine (1991 Volvo TD40 GJE 4.0L) operated on a variable-duty cycle burning contemporary European fuel (5–7 ppm sulfur, 2–6% PAHs) and equipped with or without a continuously regenerating trap (CRT) for PM (DPF-CRT, Johnson Matthey, Royston, UK) to evaluate the effects of filtration on vascular responses in human subjects (Lucking et al. 2011). They reported that exposure to DE without the CRT (PM = 320 μg/m^3, NO_2 = 0.7 ppm, NO_x = 6.4 ppm) significantly reduced the response of forearm blood flow to vasodilators and significantly increased ex vivo thrombus formation. Exposure to DE having passed through the CRT (PM = 7 μg/m^3, NO_2 = 3.4 ppm, NO_x = 5.5 ppm) did not produce

significant responses. That study was the first to employ a NO_2/PM mass ratio (approximately 760:1) approaching that of the present study (859:1). Although the present study did not include the responses measured by Lucking and colleagues, the Lucking study clearly demonstrated that contemporary aftertreatment can reduce or eliminate certain biologic effects.

Overall, the findings of the present study of NTDE contrast markedly with those of similarly designed rat studies of TDE. The most notable differences were the absence of pre-neoplastic or neoplastic lung lesions, the lack of large accumulations of intra-alveolar macrophages filled with DPM, and the lack of lung inflammation, such as were observed in rats exposed to high concentrations of TDE. Although the highest exposure level was selected based on the NO_2 concentration, the dilution proved to be at or near the minimum at which chamber temperature could be maintained within the limits of current animal care guidelines.

The parallels between the present findings and results from chronic exposures of rats to NO_2, suggest that the biologic responses in the present study may have been caused largely, if indeed not entirely, by NO_2. In that regard, it is noteworthy that starting in 2010 in the United States, additional changes in technology in response to the more stringent NO_2 emissions standard have resulted in dramatic further reductions in NO_x, including NO_2. It is therefore reasonable to speculate that a similar chronic inhalation study involving 2010 NTDE technology may demonstrate no significant pathologic responses at the minimum exhaust dilution (maximum concentration) consistent with permissible animal housing conditions.

SUMMARY

This report provides the results of biologic responses in Wistar Han rats exposed from young adulthood up to the end of their life span to inhalation exposures to combined tailpipe exhaust and crankcase emissions from a heavy-duty diesel engine system and fuel meeting U.S.-2007 on-road emission standards (NTDE). The highest exposure concentration (minimum exhaust dilution) was based on a target concentration of NO_2 that alone might be anticipated to elicit biologic responses. In addition to NO_2, 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 and NO.

The major outcome of this study was the absence of pre-neoplastic lung lesions, primary lung neoplasia, and neoplasia of any type related to NTDE exposure. The minimal lung lesions that did result occurred only at the highest level of exposure and were characterized by an increased number (proliferation) and prominence of basophilic epithelial cells (considered reactive or regenerative) lining terminal bronchioles, alveolar ducts, and adjacent alveoli, with slight metaplastic change ("bronchiolization") noted in some animals. In addition to the epithelial proliferation, minimal to mild fibrosis occurred only around the centriacinar areas within the stroma underlying the epithelial change, and sometimes there was a subtle accumulation of pulmonary macrophages in affected areas. The histologic findings showed an increase in the extent of the tissue changes in the respiratory tract between 3 and 12 months (McDonald et al. 2012), but the lesions progressed little after 12 months. The type and extent of the lesions were similar to those observed in previous studies of NO_2 alone (e.g., Freeman et al. 1968a; Mauderly et al. 1989), which was a major constituent of the NTDE test atmosphere in the present study.

The rats exhibited statistically significant, exposure-level–related responses in indices that correlate with oxidative stress in the lung and had increased lung protein and cytokines that correlate with a mild inflammatory response. Similar to the lung histology findings, such responses have been observed in previous studies of NO_2. Pulmonary function tests revealed a limitation of forced expiratory flows, most pronounced at low lung volumes. The functional impairment was consistent with the mild histologic changes in the terminal airway and proximal alveolar region. There was an absence of several effects characteristic of responses to TDE, such as the extensive accumulation of DPM-containing alveolar macrophages and persistent inflammation that led to neoplastic lesions in rats.

IMPLICATIONS OF FINDINGS

This was the first study to evaluate the biologic responses of rats to repeated, chronic inhalation of emissions from a fuel, engine, and aftertreatment system compliant with U.S. 2007 on-road standards. The primary motivation for this study was to investigate the potential for NTDE to cause pre-neoplastic lung lesions or primary lung neoplasia similar to those observed in previous studies of TDE. The literature suggests that the rat is the most sensitive laboratory animal species for evaluation of TDE, because of its sensitivity to high concentrations of particles (present in TDE), compared with other species (including humans) (Hesterberg et al. 2012; Mauderly 1995; McClellan et al. 2012). The outcome showed that the NTDE exposures resulted in markedly fewer and different

biologic responses than caused by similar exposures to TDE. Perhaps most important, the pre-neoplastic and neoplastic lung lesions characteristic of rats exposed chronically to TDE were absent in the present study of NTDE. These studies with NTDE were conducted in a rat strain (Wistar) that was shown in previous studies to develop neoplastic lesions in response to TDE exposures. Of additional importance is that the responses to NTDE might have been caused primarily or solely by NO_2. NO_2 emissions are now reduced considerably further under the current U.S. 2010 on-road emissions standards for DE.

In 2012 the International Agency for Research on Cancer (IARC) classified "diesel exhaust" as a class 1 carcinogen (IARC 2012). That conclusion was based on the epidemiologic and animal study literature on TDE, because no such literature existed for NTDE. IARC recognized the possibility that because NTDE has a composition different from that of TDE, there may be a corresponding change in the level of hazard. The IARC committee stopped short of making the distinction between TDE and NTDE, however, because of a lack of empirical evidence on the hazards of NTDE. Both McClellan and colleagues (2012) and Hesterberg and colleagues (2012) reviewed the rationale for distinguishing between the potential health hazards and risks from NTDE and TDE. They noted that in addition to the marked decrease in the concentrations of most individual components of emissions in NTDE compared with TDE, the composition of the exhaust is also quite different and that those differences may confer a difference in the hazard of the material. This report and the ancillary ACES reports included in this volume provide new empirical evidence supporting the importance of distinguishing between the potential health impacts of TDE and NTDE. The present findings strongly support the premise that advances in engine, fuel, and combustion technologies have substantially reduced the potential health impacts of DE and that estimates of hazard and risk based on laboratory or epidemiologic studies of the health impacts of TDE exposures most likely do not reflect either the hazards or the risks from NTDE.

ACKNOWLEDGMENTS

We would like to acknowledge the many technical staff members at LRRI and EPL who contributed to the successful completion of this study. This study was also aided by the support of Craig Kazmierczak and Donald Keski-Hynnila of Daimler, the engine manufacturer representatives.

REFERENCES

Anderson ME. 1985. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 113:548–555.

Bailer AJ, Portier CJ. 1988. Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. Biometrics 44(2):417–31.

Barath S, Mills NL, Lundbäck M, Törnqvist H, Lucking AJ, Langrish JP, et al. 2010. Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions. Part Fibre Toxicol 7:19. Available: *www.particleandfibretoxicology.com/content/7/1/19*.

Bieler GS, Williams RL.1993. Ratio estimates, the delta method, and quantal response tests for increased carcinogenicity. Biometrics 49(3):793–801.

Brightwell J, Fouillet X, Cassano-Zoppi AL, Bernstein D, Crawley F, Duchosal F, et al. 1989. Tumours of the respiratory tract in rats and hamsters following chronic inhalation of engine exhaust emissions. J Appl Toxicol 9:23–31.

Cheng YS, Yeh HC, Mauderly JL, Mokler BV. 1984. Characterization of diesel exhaust in a chronic inhalation study. Am Ind Hyg Assoc J 45(8):547–555.

Clark NN, Zhen F, Bedick C, Gautam M, Wayne W, Thompson G, et al. 2007. Creation of the 16-hour engine test schedule from the heavy heavy-duty diesel engine test schedule. CRC Report: ACES-1-a. Alpharetta, GA:Coordinating Research Council. Available: *www.crcao.org/publications/emissions/index.html* [accessed 10 January 2011].

Cox DR. 1972. Regression models and life-tables. J R Stat Soc. Ser B Methodol 34(2):187–220.

Crissman JW, Goodman DG, Hildebrandt PK, Maronpot RR, Prater DA, Riley JH, et al. 2004. Best practices guideline: toxicologic histopathology. Toxicol Pathol 32:126–131.

Dunnett CW. 1955. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 50:1096–1121.

Dunnett CW. 1980. Pairwise multiple comparisons in the unequal variance case. J Am Stat 75:796–800.

Freeman G, Crane SC, Stephens RJ, Furiosi NJ. 1968a. Pathogenesis of the nitrogen dioxide-induced lesion in the rat lung: a review and presentation of new observations. Am Rev Respir Dis 98:429–443.

Freeman G, Furiosi NJ, Haydon GB. 1966. Effects of continuous exposure of 0.8 ppm NO_2 on respiration of rats. Arch Environ Health 13:454–456.

Freeman G, Stephens RJ, Crane SC, Furiosi MS. 1968b. Lesion of the lung in rats continuously exposed to two parts per million of nitrogen dioxide. Arch Environ Health 17:181–192.

Giknis M, Clifford C. 2003. Spontaneous Neoplasms and Survival in Wistar Han Rats: Compilation of Control Group Data. Wilmington, MA:Charles River Laboratories.

Gregory RE, Pickrell JA, Hahn FF, Hobbs CH. 1983. Pulmonary effects of intermittent subacute exposure to low-level nitrogen dioxide. J Toxicol Environ Health 11:404–414.

Grizzle JE, Starmer CF, Koch GG. 1969. Analysis of categorical data by linear models. Biometrics 25(3):489–504.

Harkema JR, Mauderly JL, Hahn FF. 1982. The effects of emphysema on oxygen toxicity in rats. Am Rev Respir Dis 126:1058–1065.

Haydon GB, Freeman G, Furiosi NJ. 1965. Covert pathogenesis of NO_2 induced emphysema in the rat. Arch Environ Health 11:776–783.

Heinrich U, Fuhst R, Rittinghausen S, Creutzenberg O, Bellmann B, Koch W, et al. 1995. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inhal Toxicol 7:533–556.

Heinrich U, Muhle H, Takenaka S, Ernst H, Fuhst R, Mohr U, et al. 1986. Chronic effect on the respiratory tract of hamsters, mice, and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol 6:383–395.

Hesterberg TW, Long CM, Bunn WB, Lapin CA, McClellan RO, Valberg PA. 2012. Health effects research and regulation of diesel exhaust: an historical overview focused on lung cancer risk. Inhal Toxicol 24. Suppl 1:1–45.

International Agency for Research on Cancer (World Health Organization). GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. Available: *http://globocan.iarc.fr/Default.aspx*.

Juhos LT, Green DP, Furiosi NJ, Freeman G. 1980. A quantitative study of stenosis in the respiratory bronchiole of the rat in NO_2-induced emphysema. Am Rev Resp Dis 121:541–549.

Kaplan EL, Meier P. 1958. Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481.

Khalek IA, Blanks MG, Merritt P. 2013. Phase 2 of the Advanced Collaborative Emissions Study. Coordinating Research Council Report. Available: *www.crcao.org/publications/emissions/index.html* [accessed 5 August 2014].

Khalek IA, Bougher T, Merritt P. 2009. Phase 1 of the Advanced Collaborative Emissions Study. Coordinating Research Council Report. Available: *www.crcao.org/publications/emissions/index.html* [accessed 5 August 2014].

Khalek IA, Bougher TL, Merritt PM, Zielinska B. 2011. Regulated and unregulated emissions from highway heavy-duty diesel engines complying with U.S. Environmental Protection Agency 2007 emissions standards. J Air Waste Manag Assoc 61(4):427–442.

Kittel B, Ruehl-Fehlert C, Morawietz G, Klapwijk J, Elwell MR, Lenz B, et al. 2004. Revised guides for organ sampling and trimming in rats and mice — part 2. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol 55(6):413-431.

Kubota K, Murakami M, Takenaka S, Kawai K, Kyono H. 1987. Effects of long-term nitrogen dioxide exposure on rat lung: Morphological observations. Environ Health Perspect 73:157–169.

Lucking AJ, Lundbäck M, Barath SL, Mills NL, Sidhu MK, Langrish JP, et al. 2011. Particle traps prevent adverse vascular and prothrombotic effects of diesel engine exhaust inhalation in men. Circulation 123:1721–1728.

March TH, Barr EB, Finch GL, Nikula KJ, Seagrave JC. 2002. Effects of concurrent ozone exposure on the pathogenesis of cigarette smoke-induced emphysema in B6C3F1 mice. Inhal Toxicol 14:1187–1213.

Mauderly JL, 1995. Assessment of pulmonary function and the effects of inhaled toxicants. In: Concepts in Inhalation Toxicology (McClellan RO, Henderson RF, eds). 2nd ed. Washington, DC:Taylor & Francis, 355–412.

Mauderly JL, 1977. Bronchopulmonary lavage of small laboratory animals. Lab Anim Sci 27(2):255–261.

Mauderly JL, 2010. Current status of the toxicology of diesel engine exhaust — and the ACES project. Zbl Arbeitsmed 60(12):412–417.

Mauderly JL, 1986. Respiration of F344 rats in nose-only inhalation exposure tubes. J Appl Toxicol 6:25–30.

Mauderly JL, Bice DE, Carpenter RL, Gillett NA, Henderson RF, Pickrell JA, et al. 1987a. Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung. Research Report 8, Cambridge, MA:Health Effects Institute.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, et al. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust.Research Report 30. Cambridge, MA:Health Effects Institute.

Mauderly JL, Cheng YS, Gillett NA, Griffith WC, Henderson RF, Pickrell JA, et al. 1990. Influence of pre-existing pulmonary emphysema on susceptibility of rats to chronic inhalation exposure to nitrogen dioxide. Inhal Toxicol 2:129–150.

Mauderly JL, Garshick E. 2009. Diesel exhaust. In: Environmental Toxicants (Lippmann M, ed). NewYork:Wiley, 551–631.

Mauderly JL, Jones RK, Griffith WC, Henderson RF, McClellan RO. 1987b. Diesel exhaust is a pulmonary carcinogen in rats exposed chronically by inhalation. Fundam Appl Toxicol 9:208–221.

Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study (ACES) Phase 3A: Characterization of U.S. 2007-Compliant Diesel Engine and Exposure System Operation. Communication 17. Boston, MA:Health Effects Institute.

Mauderly JL, Snipes MB, Barr EB, Belinsky SA, Bond JA, Brooks AL, et al. 1994. Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats. Research Report 68. Cambridge, MA:Health Effects Institute.

McClellan RO, Hesterberg TW, Wall JC. 2012. Evaluation of carcinogenic hazard of diesel engine exhaust needs to consider revolutionary changes in diesel technology. Regul Toxicol Pharacol 63(2): 225–258.

McDonald JD, Barr EB, White RK, Chow JC, Schauer JJ, Zielinska B, et al. 2004. Generation and characterization of four dilutions of diesel engine exhaust for a subchronic inhalation study. Environ Sci Technol 38(9):2513–2522.

McDonald JD, Doyle-Eisele M, Gigliotti A, Miller RA, Seilkop S, Mauderly, JL, et al. 2012. 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. Research Report 166. Boston, MA:Health Effects Institute.

Morawietz G, Ruehl-Fehlert C, Kittel B, Bube A, Keane K, Halm S, et al. 2004. Revised guides for organ sampling and trimming in rats and mice — part 3. A joint publication of the RITA and NACAD groups. Exp Toxicol Pathol 55(6):433–449.

National Toxicology Program. 1994. Toxicology and Carcinogenesis Studies of Ozone (CAS No. 10028-15-6) and Ozone/NNK (CAS No. 10028-15-6/ 64091-91-4) in F344/N Rats and B6C3F1 Mice (Inhalation Studies). National Toxicology Program Technical Report Series 440. Research Triangle Park:Department of Health and Human Services.

Nikula KJ, Snipes MB, Barr EB, Griffith WC, Henderson RF, Mauderly JL. 1995. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. Fundam Appl Toxicol 25:80–94.

Ogilvie CM, Forster RE, Blackmore WS, Morton SW. 1957. A standardized breath holding technique for the clinical measurement of the diffusing capacity for carbon monoxide. J Clin Invest 36:1–17.

Piegorsch WW, Bailer AJ. 1997. Statistics for Environmental Biology and Toxicology. Boca Raton, FL:Chapman & Hall/CRC Press.

Portier CJ, Bailer AJ. 1989. Two-stage models of tumor incidence for historical control animals in the National Toxicology Program's carcinogenicity experiments. J Toxicol Environ Health 27(1):21–45.

Portier CJ, Hedges JC, Hoel DG. 1986. Age-specific models of mortality and tumor onset for historical control animals in the National Toxicology Program's carcinogenicity experiments. Cancer Res 46:4372–4378.

Poteracki J, Walsh KM. 1998. Spontaneous neoplasms in control Wistar rats: a comparison of reviews. Toxicol Sci 45(1): 1–8.

RCC Ltd. 2003. Historical control data on neoplastic findings in Wistar rats (planned sacrifice after 103 weeks): Compiled from 2-year bioassays. Itingen, Switzerland:RCC, Ltd.

Rocke DM, Durbin B. 2003. Approximate variance-stabilizing transformations for gene-expression microarray data. Bioinformatics 19(8):966–972.

Rogge WF, Hildemann LM, Mazurek MA, Cass GR. 1992. Sources of fine organic aerosol: 1. charbroilers and meat cooking operations. Environ Sci Technol 25:1112–1125.

Ruehl-Fehlert C, Kittel B, Morawietz G, Deslex P, Keenan C, Mahrt CR, et al. 2003. Revised guides for organ sampling and trimming in rats and mice — part 1. Exp Toxicol Pathol 55(2–3):91–106.

Stinn W, Teredesai A, Anskeit E, Rustemeier K, Schepers G, Schnell P, et al. 2005. Chronic nose-only inhalation study in rats, comparing room- aged sidestream cigarette smoke and diesel engine exhaust. Inhal Toxicol 17:549–576.

Tarone RE. 1975. Tests for trend in life table analysis. Biometrika 62(3):679–690.

Tietze F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 27:502–522.

Troost FJ, Saris WH, Haenen GR, Bast A, Brummer RJ. 2003. New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. Am J Physiol Gastrointest Liver Physiol 285:G354–G359.

Tucker MJ. 1997. Diseases of the Wistar Rat. London:Taylor & Francis.

U.S. Environmental Protection Agency. 2002. Health Assessment Document for Diesel Engine Exhaust. EPA 600/8-90/057F. Washington, DC:National Center for Environmental Assessment, Office of Research and Development.

Walsh KM, Poteracki J. 1994. Spontaneous neoplasms in control Wistar rats. Fundam Appl Toxicol 22(1): 65–72.

Weber K, Razinger T, Hardisty JF, Mann P, Martel KC, Frische EA, et al. 2011. Differences in rat models used in routine toxicity studies. Int J Toxicol 30:162–73.

MATERIALS AVAILABLE ON THE WEB

Appendices A through I and Additional Materials 1 and 2 contain supplemental material not included in the printed report. They are available on the HEI Web site at *http://pubs.healtheffects.org*.

Appendix A. Survival Rates for Rats in the Chronic Exposure Group

Appendix B. Body Weight

Appendix C. Organ Weights

Appendix D. Hematology/ Serum Chemistry and Coagulation Factors

Appendix E. Bronchoalveolar Lavage

Appendix F. Lung Tissue

Appendix G. Respiratory Function

Appendix H. Histopathology

Appendix I. Characterization of Exposure Atmospheres in the ACES Bioassay

Additional Materials 1: Memorandum on Rat Strain Decision

Additional Materials 2: ACES Phase 3B Protocol

ABOUT THE AUTHORS

Jacob D. McDonald received a Ph.D. in environmental chemistry with an emphasis in atmospheric chemistry from the University of Nevada, Reno. He is the vice president for applied sciences as well as the director of the Environmental Respiratory Health Program at LRRI. He has oversight of all work conducted at the LRRI Applied Science facility (formerly the Inhalation Toxicology Research Institute facility). He served 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 GLP and non-GLP toxicologic studies that evaluate acute toxicity, deposition, pharmacokinetics, and efficacy using a variety of respiratory disease models at LRRI. She was the study director for Phase 3 of the ACES program.

JeanClare Seagrave holds a Ph.D. in medical sciences/biochemistry. She was the cell biologist at LRRI responsible for the bronchoalveolar lavage and cell proliferation assays conducted for Phase 3 of ACES.

Andrew P. Gigliotti, D.V.M., D.A.C.V.P., also holds a Ph.D. in experimental pathobiology and is an associate contract scientist and veterinary pathologist in the LRRI Experimental Toxicology Program. He served as the necropsy/clinical pathology manager and 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.

Judith Chow, Sc.D., environmental science and physiology, is research professor and director of the Environmental Analysis Facility of the Desert Research Institute. She conducted analyses of metals and organics in emissions and test atmosphere samples for Phase 3 of ACES.

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Joe L. Mauderly, D.V.M., is an LRRI senior scientist emeritus. He was the original principal investigator and then served as a consultant to the HEI-funded ACES program. Mauderly was the principal investigator for all past subchronic and chronic studies of diesel emissions conducted at LRRI and has written extensively in the field. He supervised the assessments of pulmonary function.

Steven K. Seilkop, has an M.S. in statistics and holds an adjunct scientist appointment at LRRI. He also has served as a statistical consultant in the fields of health studies in animals, air pollution, and human health risks for more than 25 years. He served as principal investigator of a statistical support contract with NIEHS/NTP for the development

and refinement of statistical methods for analyzing data from carcinogenicity and toxicity bioassays. He was the study statistician for Phase 3 of ACES.

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

OTHER PUBLICATION RESULTING FROM THIS RESEARCH

McDonald JD, Doyle-Eisele M, Gigliotti A, Miller RA, Seilkop S, Mauderly JL, et al. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

ABBREVIATIONS AND OTHER TERMS

ACES	Advanced Collaborative Emissions Study
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CINC	cytokine-induced neutrophil chemoattractant
CO	carbon monoxide
CO_2	carbon dioxide
Cqs	quasistatic lung compliance
CRC	Coordinating Research Council
CRT	continuously regenerating trap
DAB	diaminobenzidine
DE	diesel exhaust
DL_{CO}	lung diffusing capacity for carbon monoxide
DPBS	Dulbecco phosphate-buffered saline
DPF	diesel particle filter
DPM	diesel particulate matter
ELISA	enzyme-linked immunosorbant assay
EPA	U.S. Environmental Protection Agency
EPL	Experimental Pathology Laboratories
FEF	forced expiratory flow (at 10%, 25%, 50%, and 75% of total vital capacity)
$FEV_{0.1}$	forced expiratory volume in 0.1 second
FVC	forced vital capacity
GLP	Good Laboratory Practices
GSH	glutathione
GSSG	oxidized glutathione
HO-1	heme oxygenase 1
IARC	International Agency for Research on Cancer
IL	interleukin
KC	keratinocyte-derived chemokine
LDH	lactate dehydrogenase
LRRI	Lovelace Respiratory Research Institute
µTP	micro–total protein
MIP-2	macrophage inflammatory protein 2
MMEF	mean mid-expiratory flow
NBF	neutral-buffered formalin
Ne	neon
NO	nitrogen monoxide
NO_x	nitrogen oxides
NO_2	nitrogen dioxide
NTDE	new-technology diesel exhaust
NTP	National Toxicology Program
PAHs	polyaromatic hydrocarbons
PBS	phosphate-buffered saline
PEFR	peak expiratory flow rate
PM	particulate matter
PMN	polymorphonuclear
P_{tp}	transpulmonary pressure
PWG	Pathology Working Group
QC	quality control
SwRI	Southwest Research Institute
SSA	sulfosalicylic acid
SVC	slow vital capacity
SVOC	semivolatile organic compound
T_{90}	time to reach 90% of the target atmosphere
TDE	traditional-technology diesel exhaust
TEAC	Trolox equivalent antioxidant capacity
TNF-α	tumor necrosis factor α
VA	alveolar volume
VOC	volatile organic compound

Pathology Working Group
Report and Comparison Statement

CONTENTS

PATHOLOGY WORKING GROUP REPORT AND COMPARISON STATEMENT

PATHOLOGY WORKING GROUP REPORT

Study Title

Pathology Working Group (PWG) Report for Advanced Collaborative Emissions Study (ACES)

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DISCUSSION

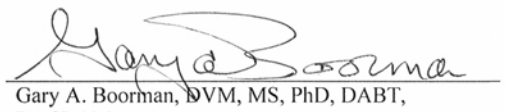
A Pathology Working Group (PWG) met on 29 May 2013 in Chantilly, Virginia, to review pathology findings of the Advanced Collaborative Emissions Study (ACES), a 30-month inhalation study in rats. The testing facility was Lovelace Respiratory Research Institute (LRRI) Inhalation Toxicology Laboratory in Albuquerque, New Mexico. The study sponsor was the Health Effects Institute (HEI) of Boston, Massachusetts. The PWG review was held at the Covance-Chantilly facility in Chantilly, Virginia. The study pathologist responsible for the microscopic evaluation was Rodney Miller of Experimental Pathology Laboratories, Inc. Gary Boorman of Covance was the peer-review pathologist who selected slides for the PWG review. The list of participants is shown the table later in this section.

Annemoon van Erp presented an overview of the goals and objectives of ACES. Rodney Miller presented an overview of the lesions and the issues to the PWG members.

The purpose of ACES was to determine biologic responses of rats and mice to repeated inhalation exposure to exhaust from diesel engines compliant with U.S. 2007 emissions standards. Since mice were not exposed for more than 3 months and were not part of this review, further details are not provided.

SIGNATURE PAGE

I, the undersigned, hereby declare the work was performed under my supervision and the findings provide a true and accurate record of the results obtained.



21 Feb 2014

Gary A. Boorman, DVM, MS, PhD, DABT,
DACLAM
Diplomate, ACVP
Anatomic Pathologist and PWG Chair
Covance Laboratories Inc.

Date

Male and female HsdRccHan:Wist rats were assigned to groups (140 rats/sex/exposure group) and exposed by inhalation (16 hours/day, 5 days/week) to exhaust at one of three dilutions from a diesel engine designed to meet 2007 on-road emissions standards of the United States. Exposures were based on nitrogen dioxide (NO_2) levels with target concentrations of 4.2, 0.8, and 0.1 ppm NO_2. The study included interim sacrifices of 10 animals/sex/exposure group at 1, 3, 12, and 24 months. The interim sacrifice pathology results have been subjected to peer review, and these slides were not included for PWG review, with the exception of several proliferative lesions of the lung and nasal cavity identified by the study pathologist at the 12- and 24-month interim sacrifices (see below). Phase 3B of ACES involved exposures up to 30 months in rats to determine potential carcinogenicity with exposure to this new-technology diesel exhaust. Approximately 100 animals/sex/exposure group were available for examination in the Phase 3B 30-month toxicology studies (actual numbers in each group varied between 93 and 102 rats/group). Because of decreasing survival, all remaining males were terminated at 28 months, while females reached the scheduled sacrifice at 30 months.

PATHOLOGY WORKING GROUP PARTICIPANT SIGNATURES

PWG PARTICIPANTS' SIGNATURE PAGE
09-111 RAT ACES – Advanced Collaborative Emissions Study (ACES)

Chair

Gary Boorman, DVM, Diplomate ACVP, Ph.D. — 5/29/2013
Covance, Inc — Date

PWG Members

Ann F. Hubbs, DVM, Ph.D., Diplomate ACVP — 5/29/2013
National Institute for Occupation Safety and Health CVC — Date

Heinrich Ernst, Dr. Med. Vet. — 5/29/2013
Fraunhofer ITEM — Date

Roger Renne, DVM, Diplomate ACVP — 5/29/2013
Roger Renne ToxPath Consulting, Inc — Date

Jack Harkema, DVM, Diplomate ACVP, Ph.D. — 5/29/2013
Michigan State University — Date

The study pathologist performed a histopathologic examination on all tissues for the control and high-exposure animals, including five sections of lung and nasal cavity at four levels. For Groups 2 (low-exposure) and 3 (mid-exposure), five sections of lung and nasal cavity at four levels were examined as well as macroscopic lesions. The peer-review pathologist examined all lung and nasal cavity sections from all exposure groups. The peer-review pathologist examined thyroid from high-exposure (Group 4) and control (Group 1) animals and macroscopic lesions in the intermediate-exposure groups.

The PWG examined in a blind fashion all proliferative lesions of the nasal cavity and lung, including lesions previously identified at the interim sacrifices. Primary lung tumors were not found by the study pathologist, peer-review pathologist, or PWG members. The PWG examined the slides and agreed that three proliferative pulmonary lesions (one control female, one low-exposure female, and one high-exposure female) diagnosed as alveolar epithelial hyperplasia by the study pathologist were hyperplasia and not pulmonary tumors. Papilloma/adenomas of the nasal respiratory epithelium were found in one 12-month

PWG PARTICIPANTS' SIGNATURE PAGE
09-111 RAT ACES – Advanced Collaborative Emissions Study (ACES)

Observers

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Gene McConnell, DVM, MS(Path), ACVP, ABT — Date
Toxpath, Inc

Charles Plopper — 29 May 13
Charles Plopper, Ph.D. — Date
ucdavis

Andrew Gigliotti — 29 May 2013
Andrew Gigliotti, DVM, Ph.D., Diplomate ACVP — Date
LRRI

Ronald A. Herbert — 5/29/13
Ron Herbert, DVM, Ph.D. — Date
NIEHS

Annemoon van Erp — 5/29/13
Annemoon van Erp, Ph.D. — Date
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Study Pathologist

Rodney A. Miller — 5-29-13
Rodney A. Miller, DVM, Diplomate ACVP, Ph.D. — Date
EPL

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Observer Participants	
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Gene McConnell, DVM, MS, Diplomate, ACVP (ACES Oversight Committee)	Tox Path Raleigh, North Carolina
Charlie Plopper, Ph.D. (ACES Oversight Committee)	University of California–Davis Davis, California
Annemoon van Erp, Ph.D. (ACES Project Manager)	Health Effects Institute Boston, Massachusetts

[a] Participated in 30 May 2013 meeting only.

interim-sacrifice control male, one 24-month interim-sacrifice mid-exposure male, and one high-exposure male examined after 28 months of exposure. All potential nasal tumors were reviewed from the 12- and 24-month interim-sacrifice animals and from animals examined at the end of the 30-month study. The PWG agreed that no evidence of increased tumors was noted in the upper or lower respiratory tract of rats following exposure to 2007-technology diesel exhaust in this study.

The incidence of thyroid follicular cell adenomas and follicular cell carcinomas was higher in high-exposure females (8) compared with concurrent control females (1). An increased incidence of thyroid follicular cell tumors was not found in males in this study. The PWG members reviewed the thyroid follicular cell lesions (hyperplasia, adenoma, and carcinoma) from females, including the mid-exposure groups where only macroscopic lesions were examined. The PWG members generally agreed with the diagnoses of the study pathologist; thus, more thyroid follicular cell tumors were found in the high-exposure female group. The thyroids were examined in a coded fashion to mask group identity, and where the PWG consensus differed from the study pathologist, the diagnoses were changed to reflect the PWG opinion. The PWG recommendation was that the thyroid be examined from the low- and mid-exposure groups (Groups 2 and 3).

The PWG members evaluated slides of lungs from high-exposure males and females and assessed the severity of

the lesions found. The slides were selected to illustrate periacinar interstitial fibrosis, periacinar epithelial hyperplasia, and bronchiolization; these findings were considered by the study pathologist to be related to inhalation of 2007-technology diesel exhaust. These slides also included examples of focal alveolar histiocytosis, considered a common but incidental lesion. Most of the lesions were of minimal to slight severity, and the PWG agreed the histiocytosis was an incidental lesion not related to exposure. Periacinar interstitial fibrosis was usually a very minimal to mild lesion involving the wall at the terminal bronchiole and proximal alveolar ducts. Periacinar epithelial hyperplasia was characterized by increased number of airway epithelial cells and generally affected only a portion of the centriacinar regions on the slide (PWG Report Figure 1). A sometimes prominent feature was the small papillary-like projections into the lumen of preterminal and terminal bronchioles (PWG Report Figure 2). These projections were lined by epithelium, sometimes with a central stalk. The PWG members did not consider the periacinar epithelial hyperplasia a pre-neoplastic lesion but rather a response to the inhalation of the diesel exhaust. Bronchiolization was a less common finding, characterized by bronchiolar cells extending into alveolar ducts and centriacinar alveoli. Bronchiolization generally includes the presence of ciliated cells, Clara cells, and/or hypertrophied Type II alveolar cells in alveolar ducts and adjacent alveoli. For this study, the study pathologist used the presence of ciliated cells as a hallmark of bronchiolization. In some cases, increased numbers of macrophages accompanied bronchiolization, but a remarkable finding was the general lack of inflammatory cells associated with the inhalation of the 2007-technology diesel exhaust.

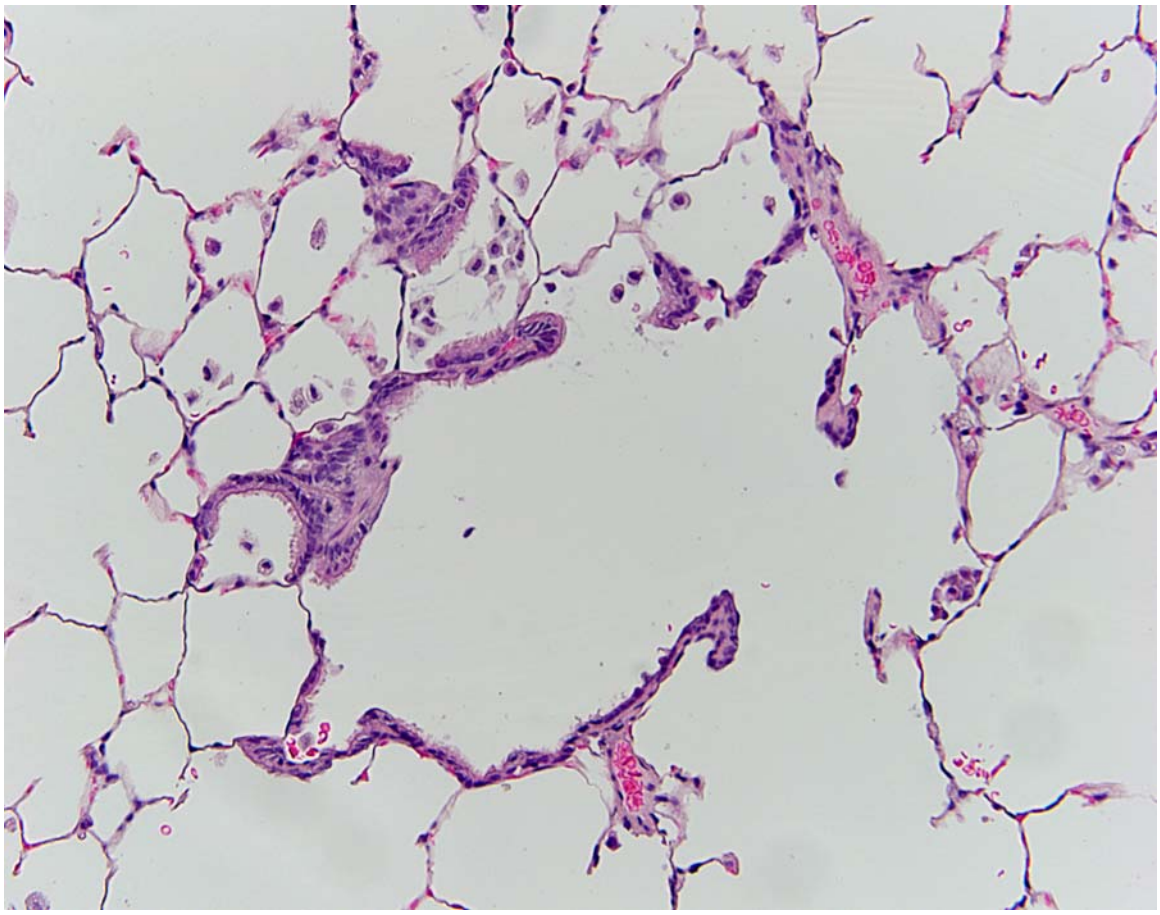
In addition, PWG members noted the presence of mucus-containing cells in the larger axial airways, which sometimes extended into small-diameter preterminal bronchioles, suggesting this lesion might be an uncommon metaplastic change.

Four high-exposure females had pinpoint focal dark spots on the lungs at necropsy when the lungs were examined under a dissecting microscope. Two lesions were examined by the PWG. These dark spots were subpleural foci of pigment-containing macrophages and considered incidental findings but were distinct from the alveolar histiocytosis found in most animals.

The PWG members evaluated slides of lungs from control and mid-exposure groups of male and female rats. Samples were selected from rats that had reached scheduled sacrifice and had 30 months (females) or 28 months (males) of exposure for 16 hours/day, 5 days/week. All lung sections from two mid-exposure animals diagnosed with peribronchiolar hyperplasia were also examined in a coded fashion. The PWG conclusion was that the centriacinar regions were within normal limits for this age of animal, and periacinar hyperplasia was not present. The PWG opinion was that diesel-exhaust–related pulmonary lesions were restricted to high-exposure animals.

The PWG examined slides of the nasal cavity for the presence of suppurative inflammatory lesions and the presence of degeneration of olfactory epithelium. The PWG agreed with the diagnoses of suppurative inflammation and olfactory epithelial degeneration. It was noted that olfactory lesions were very small, focal, usually unilateral, and showed no pattern related to diesel exposure. Often, only one small focus was present in the four sections of the nasal cavity. Olfactory epithelial degeneration was considered an incidental finding. It was also noted that this might have been an aging phenomenon and that these rats were up to 30 months old. Suppurative inflammation generally occurred only at the Level I nasal section, and the incidence was not exposure-related. The PWG members noted an increase of mucous cells on the nasal septum and lateral wall. The PWG members recommended evaluation of the nasal cavity from a subset of untreated control animals and high-exposure animals for the presence of mucous cells to determine whether mucous cell hyperplasia/metaplasia was incidental or exposure-related.

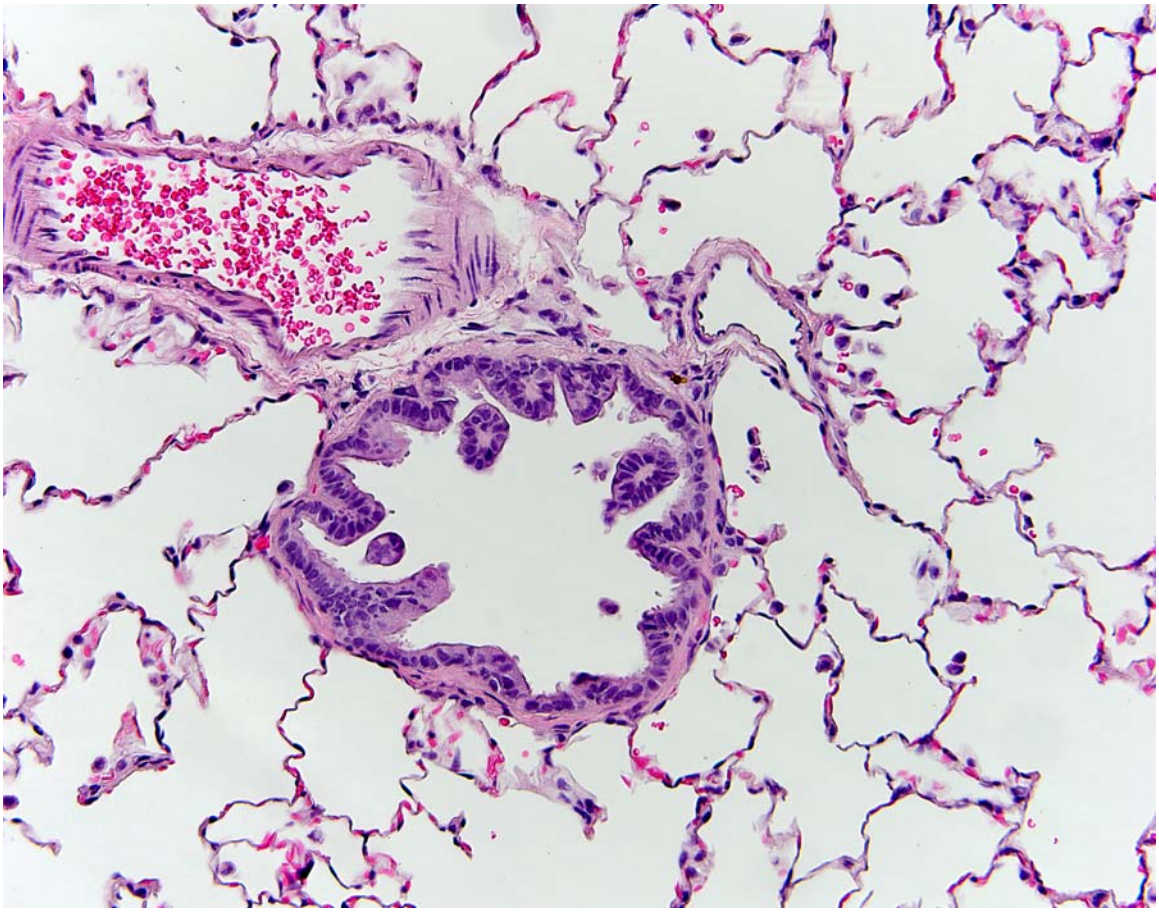
Following the PWG review, the chair and study pathologist reviewed a subset of nasal cavity slides containing a Level I section from control and high-exposure animals from the end of the 30-month study. Slides containing a Level I section of the nasal cavity were selected randomly from 10 animals/sex/exposure group for control and high-exposure rats and coded to mask group identity. The presence of mucous cells in the epithelium of the nasal section and the lateral wall at Level I was graded independently and in a blind fashion by the study and peer-review pathologists. There was good agreement on the independent review by the two pathologists for incidence and severity of lesions. The review did not reveal a clear difference between controls and high-exposure animals for incidence and/or severity of lesions. Mucous cells in the epithelial lining of the nasal cavity were usually part of a complex of lesions that included suppurative inflammation, squamous metaplasia, and respiratory epithelial hyperplasia. Squamous metaplasia and respiratory epithelial hyperplasia were recorded separately for the nasal septum and for the lateral wall in the study. The review suggested mucous cells, mostly in the nasal cavity but also in the pulmonary airways, were not uncommon in the study and may have been related to the strain of rat and/or length of the study.



PWG Report Figure 1. ACES 2007-technology diesel exhaust study (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×). Minimal increase in thickening of alveolar walls with minimal increase in bronchiolar epithelium. While a few macrophages are present in this figure, no overall increase in inflammatory cells was noted with exposure to the 2007-technology diesel exhaust. These changes were found at only the highest exposures and not at the mid or low exposures. (Photograph by Rod Miller, EPL.)

CONCLUSION

Overall, the study was considered well conducted and was a good test for potential carcinogenicity with approximately 100 animals/exposure group and exposures of up to 30 months. The PWG concluded that no evidence of carcinogenicity in the respiratory tract was noted with exposure to exhaust from U.S. 2007-compliant diesel technology in this study. The thyroid follicular cell tumors in female rats were considered of uncertain significance. Examination of mid-exposure males and females may provide some evidence for the presence or lack of an exposure–response relationship. It was noted that the incidence of these tumors in high-exposure females was not unlike historical control rates for this strain in 24-month studies. Little historical control data are available for this strain in comparable 30-month studies. Pulmonary lesions were restricted to high-exposure animals, were often slight to minimal severity, and affected a subpopulation of the acinar regions. The PWG noted the lungs were dramatically less affected than those in the reported studies of exposure to



PWG Report Figure 2. ACES 2007-technology diesel exhaust study (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×). Bronchiolar hyperplasia was an uncommon finding in rat lungs in ACES, characterized by papillary projections into preterminal bronchioles. (Photograph by Rod Miller, EPL.)

old-technology diesel exhaust in the literature, where particulate overload was often a problem. Interestingly, it was noted by the panel that the minimal to mild periacinar lesions in the lungs of high-exposure rats were similar to those previously reported in Wistar rats continuously exposed to 4 ppm NO_2 for 27 months (Kubota et al. 1987).

ARCHIVE STATEMENT

The original copy of this report will be stored in the Covance archives for at least 5 years.

REFERENCE

Kubota K, Murakami M, Takenaka S, Kyono H. 1987. Effects of long-term nitrogen dioxide exposure on the rat lung: morphological observations. Environ Health Perspect 73:157–169.

PATHOLOGY WORKING GROUP COMPARISON STATEMENT

Study Title

Pathology Working Group (PWG) Statement Comparing Pulmonary Morphology from the Advanced Collaborative Emissions Study (ACES) with the National Toxicology Program (NTP) 30-Month Ozone Study, Lovelace Respiratory Research Institute (LRRI) Diesel Exhaust Study, LRRI Nitrogen Dioxide (NO_2) Study, and the Fraunhofer Institute Long-term Inhalation of Filtered and Unfiltered Diesel Engine Emissions Study

SIGNATURE PAGE

I, the undersigned, hereby declare the work was performed under my supervision and the findings provide a true and accurate record of the results obtained.

Gary A. Boorman, DVM, MS, PhD DABT, DACLAM
Diplomate, ACVP
Anatomic Pathologist and PWG Chair
Covance Laboratories Inc.

21 Feb 2014
Date

The PWG members and observers participated in drafting of the report.

PWG PARTICIPANTS' SIGNATURE PAGE
Comparison of ACES Study with IITRI NO_2, IITRI Old Diesel and NTP Ozone

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Gary Boorman, DVM, Diplomate ACVP, Ph.D.
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Date

PWG Members

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Report Issue Date

21 February 2014

DISCUSSION

A Pathology Working Group (PWG) met on 30 May 2013 in Chantilly, Virginia, to compare the pulmonary pathology findings of the Advanced Collaborative Emissions Study (ACES), a 30-month inhalation study of 2007-technology diesel exhaust in rats, with previous inhalation studies examining other air toxicants. The day prior, the PWG members reviewed the lungs of rats in the ACES bioassay as well as other tissues of interest. The goal of the second day was to compare the lung lesions in rats from ACES with lung lesions in rats exposed to other inhalation toxicants. This review was held at the Covance-Chantilly facility in Chantilly, Virginia. The PWG members and participants (see list in PWG Report table earlier in this volume) reviewed scanned lung slides from the National Toxicology Program (NTP) 30-month ozone lifetime study in rats (NTP 1994). In addition, they reviewed histologic slides of the lung from rats that had been exposed for 24 months to old-technology diesel exhaust (Mauderly et al. 1994) and to nitrogen dioxide (NO_2; Mauderly et al. 1989), both conducted at the Lovelace Respiratory Research Institute (LRRI).

Subsequent to the 30 May 2013 slide review, Drs. Rod Miller and Gary Boorman reviewed additional slides from the NO_2 study at lower levels of exposure, additional low-exposure slides from the LRRI study of old-technology diesel exhaust (Mauderly et al. 1994), and slides from a 1986 Fraunhofer Institute lifetime inhalation study in which rats had been exposed to clean air, filtered diesel exhaust without particulate matter (PM), or unfiltered diesel exhaust (including PM; Heinrich et al. 1986). Dr. Miller took photographs of representative lesions from ACES, the NO_2 study, the Fraunhofer Institute diesel exhaust study, and from scanned slides from the NTP 30-month ozone inhalation study. These photomicrographs are used in this report to illustrate differences and similarities between ACES and other air toxicant studies.

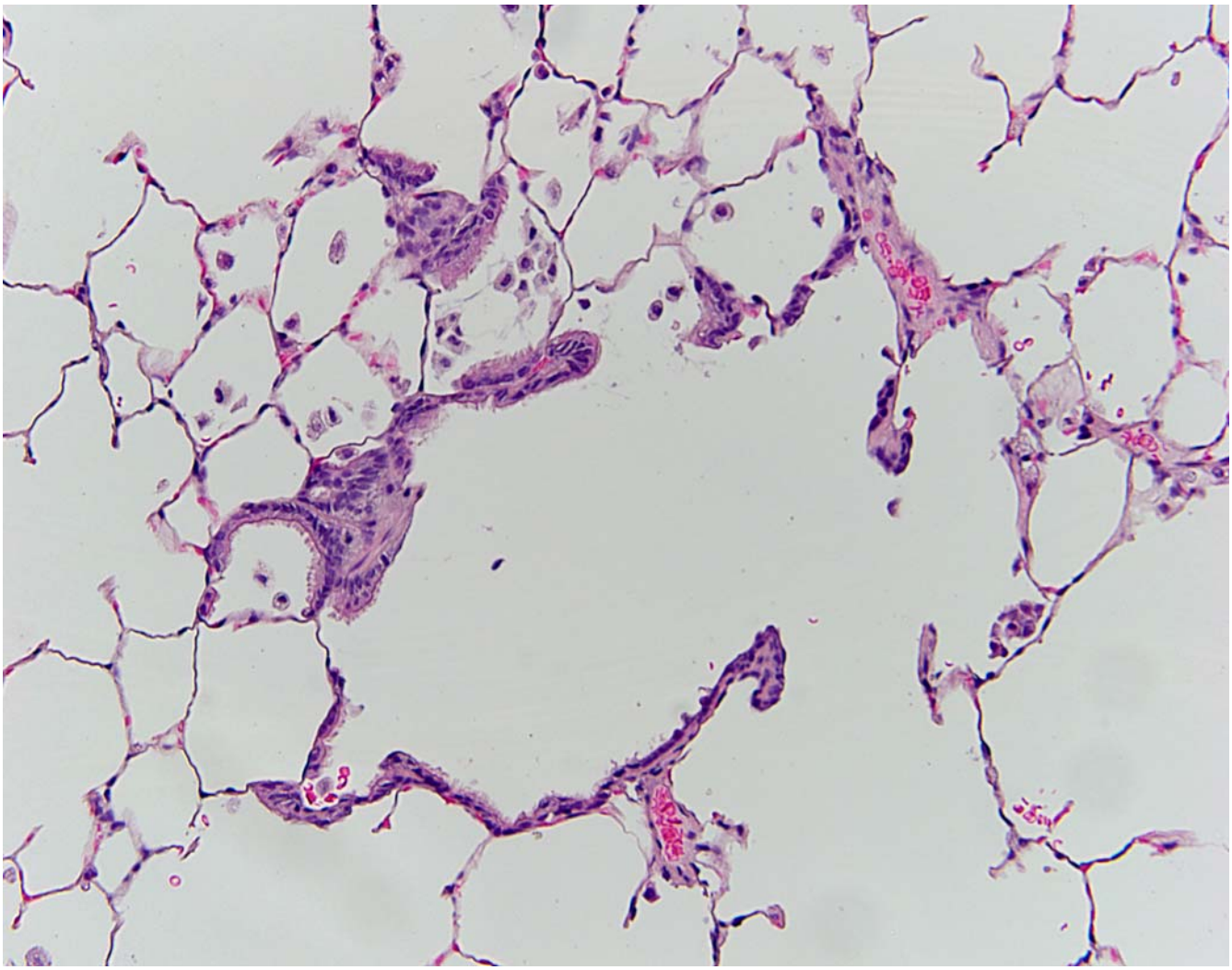
ACES Investigation of 2007-Technology Diesel Exhaust

The PWG reviewed slides from ACES (McDonald et al. 2014) as described in the accompanying PWG report. In brief, high-exposure (4.2 ppm NO_2) male and female Wistar Han rats had periacinar interstitial fibrosis, periacinar epithelial hyperplasia, and bronchiolization considered related to inhalation of 2007-technology diesel exhaust. Most of the lesions were of minimal to mild severity. Periacinar interstitial fibrosis was usually a very minimal to mild lesion involving the wall at the terminal bronchiole and proximal alveolar ducts. Periacinar epithelial hyperplasia was characterized by increased number of airway epithelial cells and generally affected only a portion of the centriacinar regions on the slide (PWG Comparison Statement Figure 1). An occasional finding was the small papillary-like projections into the lumen of preterminal and terminal bronchioles (PWG Comparison Statement Figure 2). These projections were lined by epithelium, sometimes with a central stalk. These bronchiolar lesions were uncommon and not present in most slides. Bronchiolization was a less common finding characterized by bronchiolar cells extending into alveolar ducts and centriacinar alveoli. Bronchiolization generally included the presence of ciliated cells, Clara cells, and/or hypertrophied Type II alveolar cells in alveolar ducts and adjacent alveoli. A remarkable finding was the general lack of inflammatory cells associated with inhalation of the 2007-technology diesel exhaust and lack of pigment in the lung.

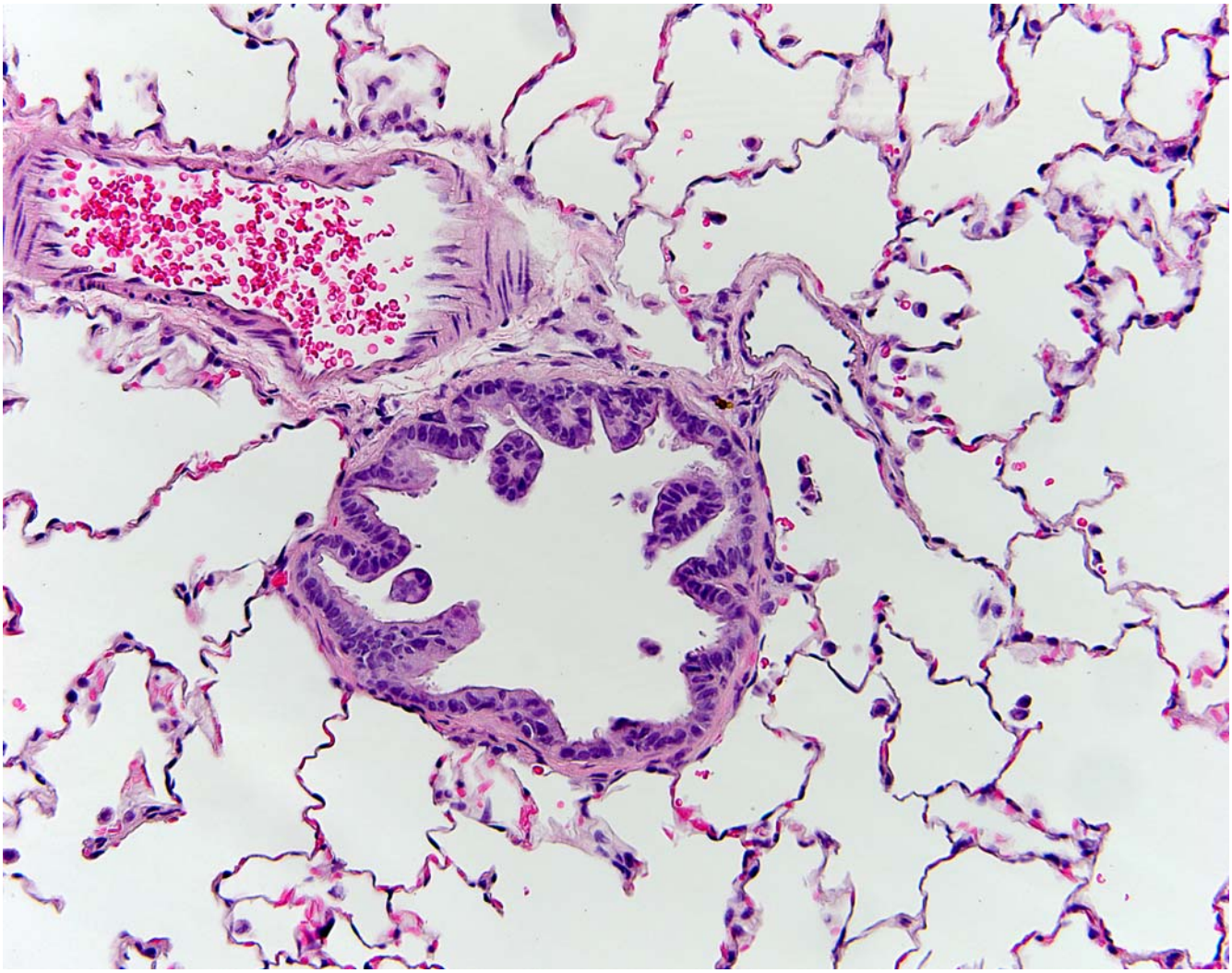
The PWG members concluded that the lungs of low-exposure (0.1 ppm NO_2) and mid-exposure (0.8 ppm NO_2) rats were within normal limits for the age of the animals, and periacinar hyperplasia was not present. The PWG opinion was that diesel-exhaust–related pulmonary lesions were restricted to high-exposure animals.

NTP 30-Month Rat Ozone Study

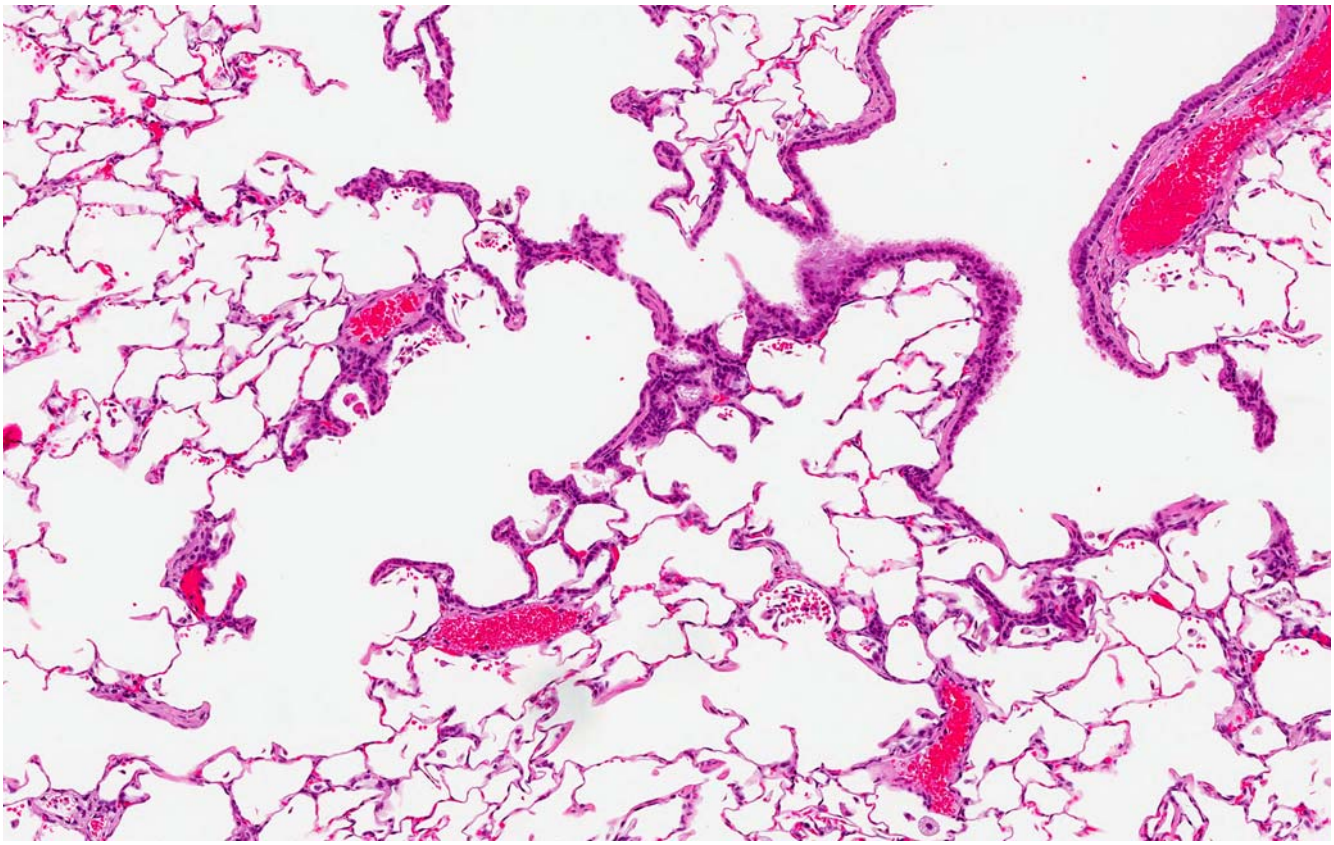
The PWG reviewed scanned slides of the NTP ozone study, in which male and female F344/N rats were exposed to 0.5 or 1 ppm ozone for 6 hours/day, 5 days/week for up to 30 months, with remote access to slides on file at NTP (courtesy of Dr. Ron Herbert). The lungs of rats exposed to 1 ppm ozone showed minimal centriacinar changes. The changes were characterized by few centriacinar alveolar macrophages and slight thickening of the centriacinar wall. Preterminal lesions, as seen in ACES, were not seen in scanned slides from ozone-exposed animals. However, centriacinar lesions (PWG Comparison Statement Figure 3) were seen in these animals, not unlike those in



PWG Comparison Statement Figure 1. ACES 2007-technology diesel exhaust study (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×). Minimal increase in thickening of alveolar walls with minimal increase in bronchiolar epithelium. While a few macrophages are present in this figure, no overall increase in inflammatory cells was noted with exposure to the 2007-technology diesel exhaust. These changes were found at only the highest exposures and not at the mid or low exposures. (Photograph by Rod Miller, EPL.)



PWG Comparison Statement Figure 2. ACES 2007-technology diesel exhaust study (4.2 ppm NO_2, female Wistar Han, 30 months, original magnification 200×). Bronchiolar hyperplasia was an uncommon finding in rat lungs in ACES, characterized by papillary projections into preterminal bronchiole. (Photograph by Rod Miller, EPL.)

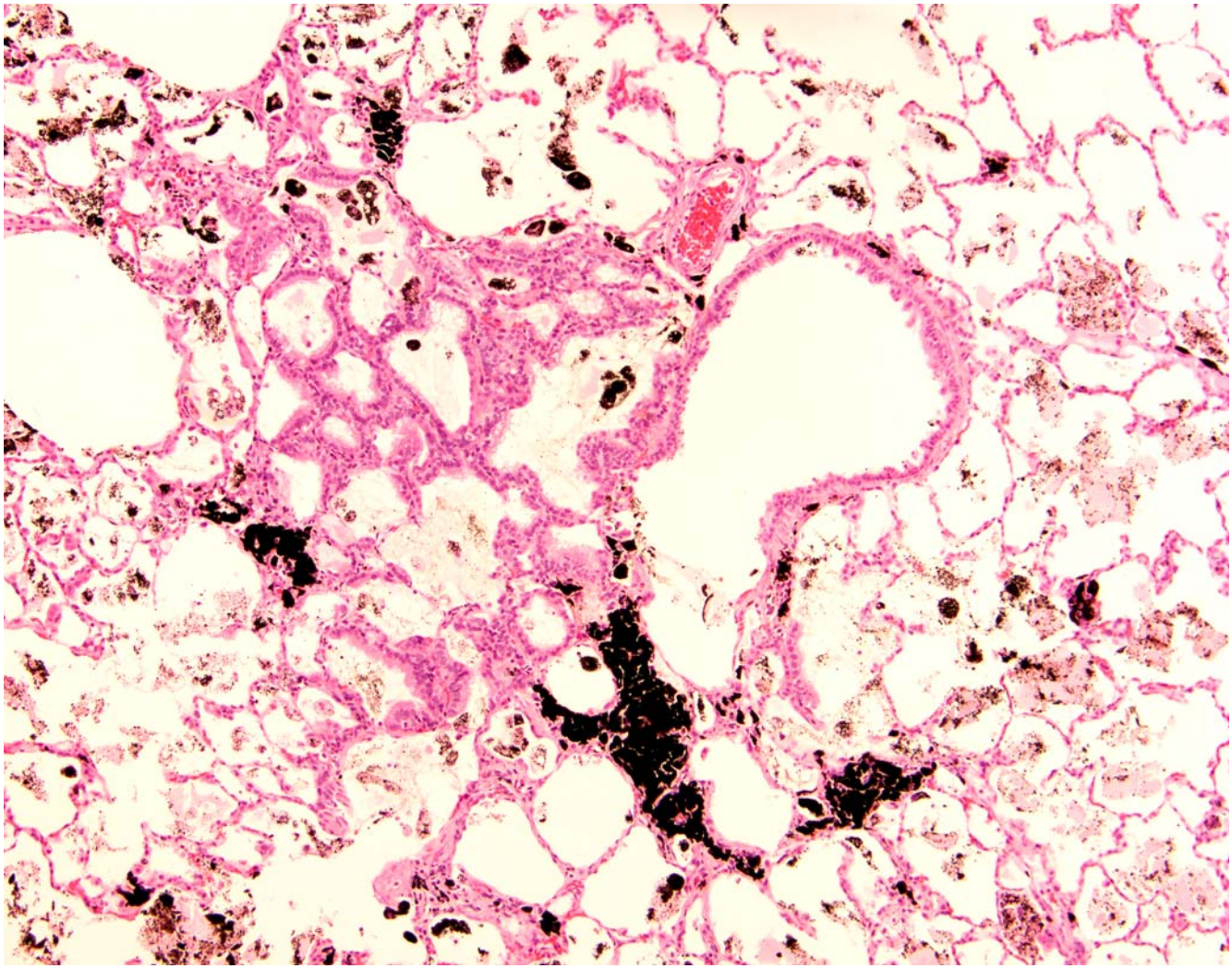


PWG Comparison Statement Figure 3. NTP ozone study (NTP 1994) (1 ppm, male F344/N, 30 months, original magnification 100×). Inhalation exposure up to 1 ppm for up to 30 months in F344/N male and female rats. The centriacinar area shows bronchiolar epithelial cells extending into centriacinar alveoli with minimal increase in thickening of alveolar walls and increased alveolar macrophages. (Photograph provided by Dr. Ron Herbert of the U.S. National Toxicology Program, National Institute of Environmental Health Sciences.)

ACES. The NTP report (1994) described the ozone lesions as multifocal and centriacinar. The ozone lesions in rats exposed up to 30 months were characterized by the presence of cuboidal epithelium (ciliated and nonciliated) along the alveolar ducts where Type I epithelium is normally present. Histiocytic infiltration and interstitial fibrosis were also noted.

LRRI Old-Technology Diesel Exhaust Study

Seven slides were available from the old-technology diesel exhaust study conducted at LRRI (Mauderly et al. 1994) in which male and female F344/N rats were exposed to diesel exhaust at PM concentrations of 2.5 or 6.5 mg/m^3 for 16 hours/day, 5 days/week, for 24 months. The PWG members agreed the lungs of high-exposure animals were strikingly different from those in ACES. Alveoli containing numerous pigment-laden macrophages, squamous metaplasia, and squamous cell carcinomas were present in some lungs. Inflammation and respiratory epithelial hyperplasia are shown in PWG Comparison Statement Figure 4. The pulmonary lesions were very similar to those observed in the Fraunhofer Institute study in which rats were exposed to whole diesel exhaust including PM (see later section on the

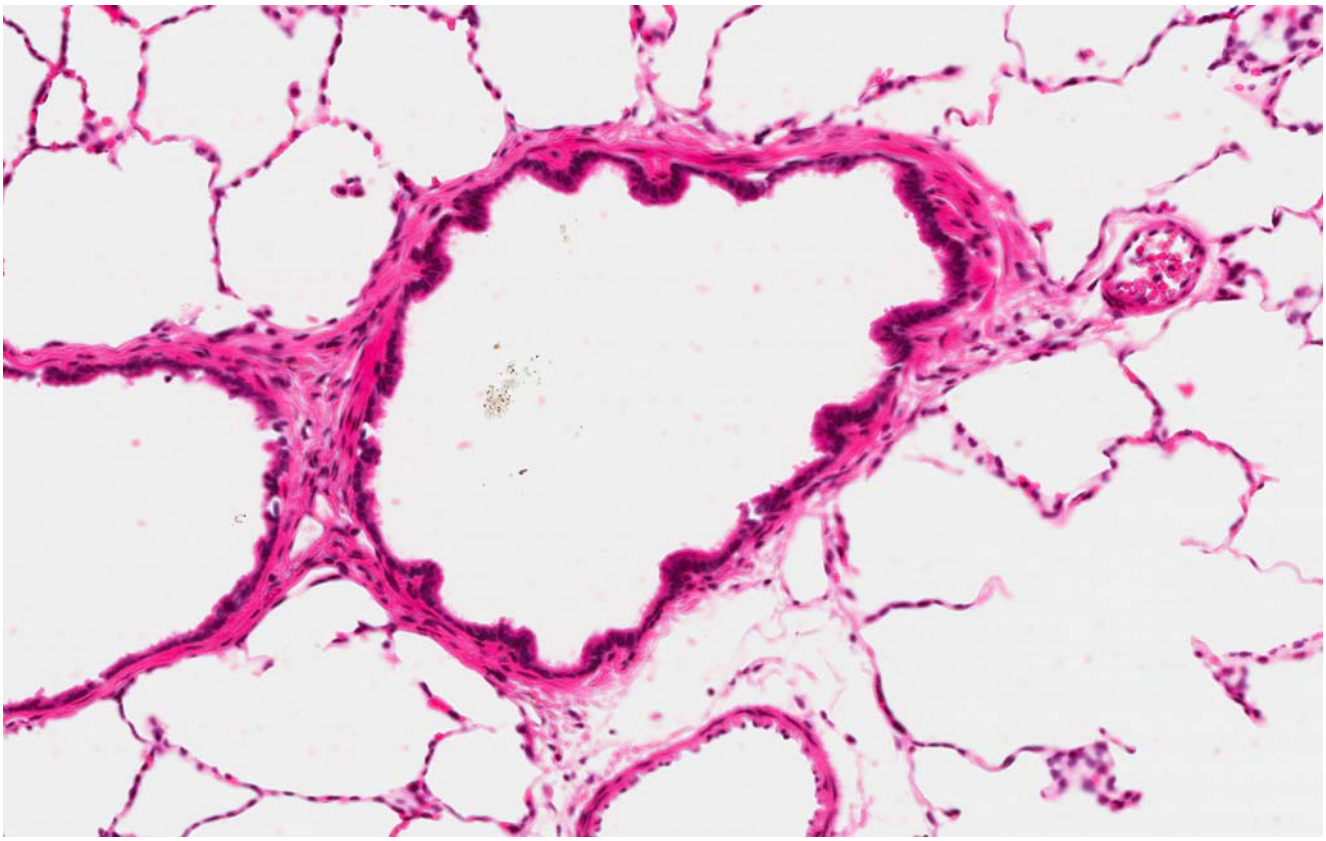


PWG Comparison Statement Figure 4. LRRI old-technology diesel exhaust study (Mauderly et al. 1994) (6.5 mg/m^3 PM, male F344/N, 24 months, original magnification 100×). Rat lung after exposure to diesel exhaust for up to 2 years, showing prominent black pigment free or within macrophages, hyperplasia of bronchial epithelium extending into centriacinar alveoli, and numerous alveolar macrophages throughout the lung often containing pigment. Contrast with Figure 1 from the 2007-technology diesel exhaust study where increased pigment was not present. (Lung slide provided by Andrew Gigliotti, LRRI; photograph by Rod Miller, EPL.)

Fraunhofer Institute study). However, the lungs of rats in the LRRI old-technology diesel exhaust study were dramatically different from those in ACES, in which only slight to minimal lesions were seen with no pigment and no evidence of cancer or precancerous lesions. Even the low-exposure animals reviewed by Drs. Rod Miller and Gary Boorman at a later date had marked deposition of pigment, numerous macrophages, and hyperplasia that was less than that in the high-exposure animals, but again the lungs were dramatically more affected than the lungs from high-exposure animals in ACES.

LRRI Nitrogen Dioxide Study

Five slides were available from the NO_2 rat study conducted at LRRI (Mauderly et al. 1989) in which male F344/N rats were exposed to up to 9.5 ppm NO_2 for 7 hours/day, 5 days/week, for up to 2 years. Animals reviewed by the PWG were from the 24-month study. The PWG members

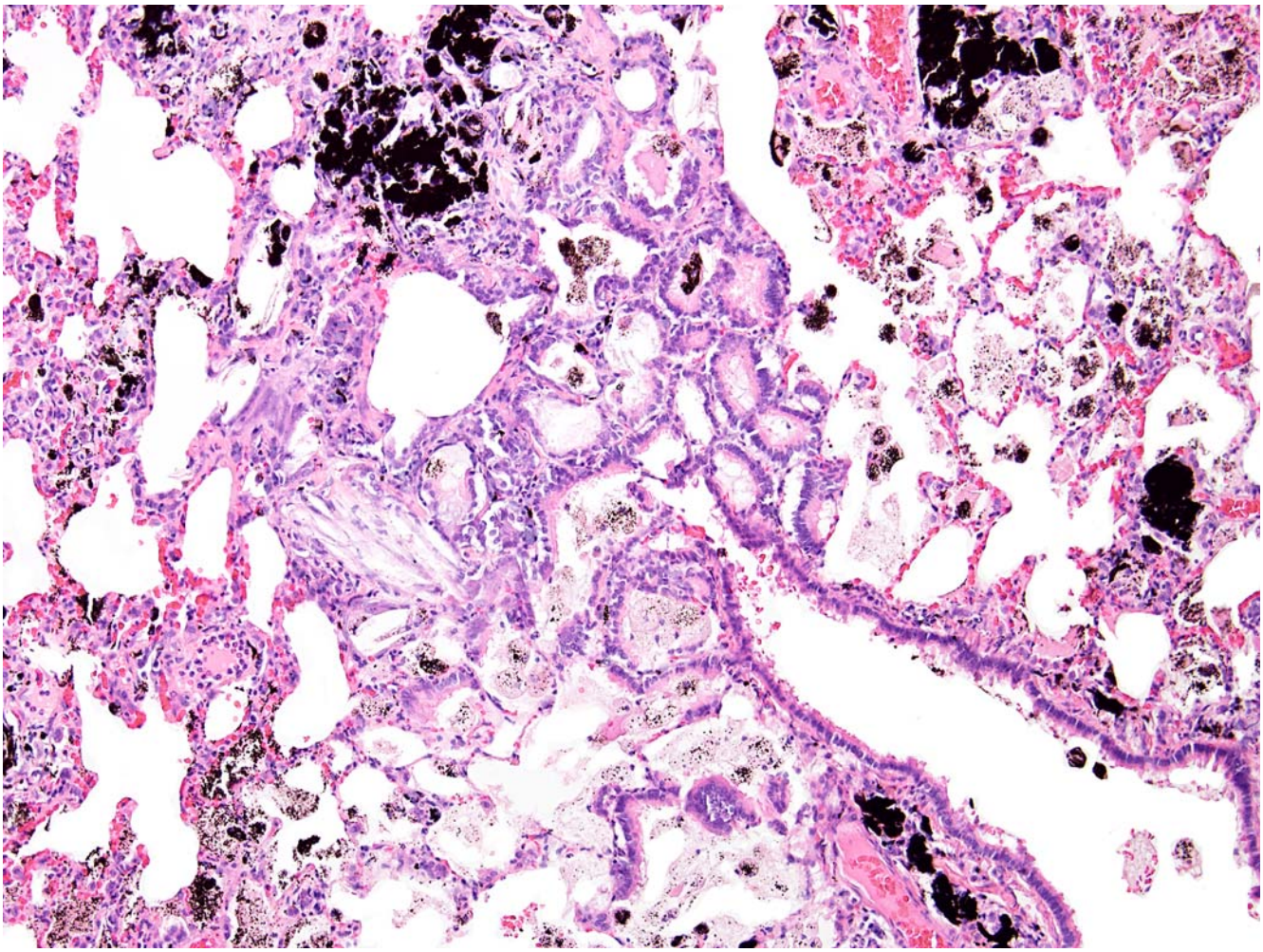


PWG Comparison Statement Figure 5. LRRI NO_2 study (Mauderly et al. 1989) (9.5 ppm NO_2, male F344, 24 months, original magnification 200×). Preterminal bronchiole from the 24-month NO_2 rat study conducted at LRRI showing findings similar to ACES. These changes were uncommon in the NO_2 study and not as distinct as those shown in the Kubota et al. study (1987) in which animals were exposed to 4 ppm NO_2 for 27 months. (Lung slide provided by Andrew Gigliotti, LRRI; photograph by Rod Miller, EPL.)

found very few minimal centriacinar lesions that had some resemblance to those in ACES with slight thickening of the centriacinar walls. A review of the literature (Kubota et al. 1987) showed that Wistar rats continuously exposed to 4 ppm NO_2 for 27 months had papillary-like projections into the lumen of preterminal and terminal bronchioles similar to what was seen in ACES (compare PWG Comparison Statement Figure 2 with Figure 2 in Kubota et al. 1987). Further review of additional lung slides from the NO_2 study also showed very minimal papillary-like projections into the lumen of preterminal and terminal bronchioles (PWG Comparison Statement Figure 5). Since the Kubota et al. study was 27 months of continuous exposure (24 hours/day) and ACES was 28 to 30 months with 16 hours of exposure, the length of exposure may have been important for the development of the papillary-like projections into the lumen of preterminal and terminal bronchioles. The LRRI NO_2 inhalation slides examined by the PWG members were from rats exposed only 7 hours/day for 2 years, and the papillary projections were quite minimal.

Fraunhofer Institute Old-Technology Diesel Exhaust Study

Drs. Rod Miller and Gary Boorman reviewed 77 slides from a Fraunhofer Institute diesel exhaust study in which rats were exposed to clean air, filtered diesel exhaust, or the complete unfiltered diesel exhaust mixture (Heinrich et al. 1986). Female Wistar Han rats were exposed to whole diesel exhaust (4.2 mg/m^3 PM) or filtered exhaust (1.2 ppm NO_2) for 17 hours/day, 5 days/ week, for 30 months. Animals exposed to filtered diesel exhaust (without PM) had minimal increase in centriacinar macrophages and very minimal thickening of the alveolar walls in the centriacinar regions (not shown). These changes were not unlike what was found in ACES with no pigment and very minimal centriacinar



PWG Comparison Statement Figure 6. Fraunhofer Institute old-technology diesel exhaust study (Heinrich et al. 1986) (4.2 mg/m^3, female Wistar Han, 30 months, original magnification 100×). Prominent black diesel soot particulates are present free in alveoli, in the numerous pulmonary alveolar macrophages, and in interstitial tissues. Marked centriacinar epithelial hyperplasia and bronchiolization occurred. Marked chronic inflammation also occurred characterized by a mixed inflammatory cell infiltrate, fibrosis, and some sterol cleft formation. The entire lung was involved, and this picture is dramatically different from what was found in the 2007-technology diesel exhaust study. Compare with PWG Comparison Statement Figure 1. (Lung slide provided by Heinrich Ernst, Fraunhofer Institute; photograph by Rod Miller, EPL.)

changes. In contrast, rats exposed to the complete diesel exhaust mixture had lungs with extensive infiltrates of inflammatory cells, proliferation and extension of bronchiolar cells into the centriacinar alveoli, and extensive deposition of pigment (PWG Comparison Statement Figure 6). Lung lesions observed in the Fraunhofer Institute and LRRI old-technology diesel exhaust studies in rats were very similar. One representative figure of the Fraunhofer Institute study is shown here. The difference between the lungs from the ACES rat bioassay and the LRRI or Fraunhofer Institute studies was striking.

CONCLUSION

The pulmonary lesions in ACES were dramatically different from those in the old-technology diesel exhaust studies. Many of the acini appear normal in the lungs of high-exposure ACES rats with minimal thickening of

alveoli walls in some centriacinar regions. The ACES lungs lacked the prominent inflammation, pigment accumulation, hyperplasia, and neoplasia seen in the old-technology diesel exhaust studies. Changes in the lungs in ACES were more similar to those in the NO_2 study in which centriacinar thickening and occasional preterminal bronchiole hyperplasia were found. The NO_2 bronchiolar hyperplasia in the 24-month exposure studies that Drs. Rod Miller and Gary Boorman examined was less than seen in ACES. In ACES, animals were exposed to 4.2 ppm NO_2 (as part of the diesel exhaust mixture) 16 hours/day for up to 28 months (males) or 30 months (females). In the NO_2 study, exposure concentrations were higher (9.5 ppm), but the exposure duration was for 7 hours/day for up to 24 months. It was interesting that the bronchiolar lesion most like that in ACES was reported in the 24-hours/day, 27-month NO_2 study (Kubota et al. 1987).

The PWG members noted that the most dramatic differences were between the new 2007-technology diesel exhaust study, in which few, and mostly minimal, lesions were found, and the old-technology diesel exhaust studies in which the lungs were loaded with pigment, inflammatory changes, hyperplasia, and cancer.

ARCHIVE STATEMENT

The original copy of this report will be stored in the Covance archives for 5 years.

REFERENCES

Heinrich U, Muhle H, Takenaka S, Ernst H, Fuhst R, Mohr U, et al. 1986. Chronic effects on the respiratory tract of hamsters, mice and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol 6(6):383–395.

Kubota K, Murakami M, Takenaka S, Kyono H. 1987. Effects of long-term nitrogen dioxide exposure on the rat lung: morphological observations. Environ Health Perspect 73:157–169.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, et al. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust. Research Report 30. Cambridge, MA:Health Effects Institute.

Mauderly JL, Snipes MB, Barr EB, Belinsky SA, Bond JA, Brooks AL, et al. 1994. Part I. Neoplastic and nonneoplastic lung lesions. In: Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats. Research Report 68. Cambridge, MA:Health Effects Institute.

McDonald JD, Doyle-Eisele M, Seagrave J, Gigliotti AP, Mauderly JL, Seilkop SK, et al. 2014. Part 1. Assessment of carcinogenicity and biologic responses in rats after lifetime inhalation of new-technology diesel exhaust in the ACES bioassay. In: Technology Diesel Exhaust in the ACES Bioassay. Research Report 184. Boston,MA:Health Effects Institute.

National Toxicology Program. 1994. Toxicology and Carcinogenesis Studies of Ozone (CAS No. 10028-15-6) and Ozone/NNK (CAS No. 10028-15-6/ 64091-91-4) in F344/N Rats and B6C3F1 Mice (inhalation studies). National Toxicology Program Technical Report Series. 440:1–314. Research Triangle Park:Department of Health and Human Services.

Part 2. Assessment of Micronucleus Formation in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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Part 2. Assessment of Micronucleus Formation in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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ABSTRACT

The formation of micronuclei (MN*) 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 increases in MN formation frequency. This endpoint is therefore commonly used in preclinical studies designed to assess the potential risks to humans of exposure to a myriad of chemical and physical agents, including inhaled diesel exhaust (DE). As part of the Advanced Collaborative Emissions Study (ACES) Phase 3B, which examined numerous additional toxicity endpoints associated with lifetime exposure to DE in a rodent model, this ancillary 24-month investigation examined the potential of inhaled DE to induce chromosome damage in chronically exposed rodents.

The ACES design included exposure of both mice and rats to DE derived from heavy-duty engines that met U.S. Environmental Protection Agency (EPA) 2007 standards for diesel-exhaust emissions (new-technology diesel exhaust). The exposure conditions consisted of air (the control) and three dilutions of DE, resulting in four levels of exposure. At specific times, blood samples were collected, fixed, and shipped by the bioassay staff at Lovelace Respiratory Research Institute (LRRI) to Litron Laboratories (Rochester, NY) for further processing and analysis. In recent years, significant improvements have been made to MN scoring by using objective, automated methods such as flow cytometry, which allows the detection of micronucleated reticulocytes (MN-RET), micronucleated normochromatic erythrocytes (MN-NCE), and reticulocytes (RET) in peripheral blood samples from mice and rats. By using a simple staining procedure coupled with rapid and efficient analysis, many more cells can be examined in less time than was possible using traditional, microscopy-based MN assays. Thus, for each sample in the current study, 20,000 RET were scored for the presence of MN. In the chronic-exposure (12 and 24 months) bioassay, blood samples were obtained from separate groups of exposed animals at specific time points throughout the course of the study. The automated method using flow cytometry has found widespread use in safety assessment and is supported by regulatory guidelines, including International Conference on Harmonisation (ICH) S2(R1) (2011). Statistical analyses included the use of analysis of variance (ANOVA) to compare the effects of sex, exposure condition, and duration, as well as the interactions between them.

Analyses of blood samples from rats combined data from our earlier 1- and 3-month exposure studies (Bemis et al. 2012) with data from our current 12- and 24-month exposure studies. Consistent with findings from the preliminary studies, no sex-based differences in MN frequency were observed in the rats. An initial examination of mean frequencies across the treatment groups and durations of exposure showed no evidence of treatment-related increases in MN at any of the time points studied. Further statistical analyses did not reveal any significant exposure-related effects.

This Investigators' Report is one part of Health Effects Institute Research Report 184, 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, 3500 Winton Place, Rochester, NY 14623; e-mail: *jbemis@ litronlabs.com.*

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

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

An examination of the potential genotoxic effects of DE is clearly valuable as part of a large-scale chronic exposure bioassay. The results described in this report provide a comprehensive examination of chronic exposure to DE in a rodent model. Our investigation of chromosomal damage also plays an important role in the context of ACES, which was designed to assess the safety of emissions from 2007-compliant diesel engines.

INTRODUCTION

BACKGROUND ON GENOTOXICITY ASSESSMENT

The exposure of humans to by-products of combustion engines is an unavoidable fact of modern life. The potential effects of such exposure, both in the environment and in 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 the use of such devices. However, regulatory agencies require extensive testing of new engines in order to determine if their designs meet or exceed established standards. As a means of obtaining a sufficient assessment of the potential risks to human populations associated with exposure to DE, a large-scale, multi-endpoint study such as ACES must be conducted. One important component of this study is the examination of the genotoxicity associated with exposure to DE.

Regulatory guidelines require the implementation of several genotoxicity 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 (ICH 2011). MN formation can indicate the effect of a treatment or exposure on the structure and function of the chromosomes in the erythrocyte. Any effect on the strictly regulated chromosomal processes can have both immediate and long-lasting consequences for the cell and for the organism as a whole. Outcomes such as genomic instability and cancer are often associated with the damage indicated by MN formation. MN formation can thus be considered a short-term indicator of damage that can lead to long-term effects, and the MN test has become a well-established part of the safety and risk assessments used for 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 chromosomes) (Cimini and Degrassi 2005). The rodent MN test is designed to examine peripheral blood samples from rodents exposed to the agent(s) of interest by way of various routes. Although some MN tests use bone-marrow–derived erythrocytes for analysis, peripheral blood samples have the advantages of ease of integration into chronic animal studies and ease of collection. Historically, microscopy-based analyses were done on bone-marrow samples collected for MN enumeration and involved manual scoring. This process was labor intensive, required specialized training, and suffered from the inherent subjectivity of the human operator. Litron Laboratories developed an automated, flow cytometry–based method for analyzing MN-RET that provides greater speed, efficiency, and accuracy when compared with the microscopy-based method. Known commercially as In Vivo MicroFlow, it has been in use since 1996. It has been used to assess 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 Organisation for Economic Co-operation and Development (OECD) Guideline 474 as a suitable alternative to the microscopy-based method. For rodent blood analyses, this method 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 ICH S2(R1) guidelines (ICH 2011).

Two of the advantages of the automated flow cytometry–based method, as compared with the traditional method, are the greater objectivity it affords 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 flow cytometry–based method routinely takes approximately 2 minutes to count 20,000 cells.

In addition to measuring MN-RET frequency, the flow cytometry–based method can count MN in NCE. This more mature population of red blood cells can be used to examine long-term or cumulative damage in mice. However, because of differences in the splenic morphology of rats that result in the removal of MN-NCE from circulation, MN-NCE are not a reliable indicator of chromosome damage in rats (and data on MN-NCE for samples derived from rats are not shown in the current study).

RATIONALE FOR MICRONUCLEUS ASSESSMENT AFTER DIESEL EXHAUST EXPOSURE

A large body of literature describes the genotoxic or carcinogenic potential of DE in various in vivo and in vitro models (see examples below). MN tests (especially rodent in vivo MN tests) were often used in these studies, thus it is useful to consider the existing body of work in the context of the current report.

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 in vitro mammalian cell genotoxicity assays (Dybdahl et al. 2004), in vivo carcinogenicity studies show both positive and negative results depending on the species evaluated (Mauderly et al. 1996). 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 ACES. Older engine designs can be expected to have considerably different emission profiles than present-day or future engines. Indeed, the characterizations of DE constituents associated with the current study demonstrated that 2007-compliant diesel engines yielded significant reductions in, for example, particulate matter (PM) (Mauderly and McDonald 2012). Also, many of the previous studies exposed rodents to much higher concentrations of DE than would be found in normal ambient or even extreme occupational conditions (U.S. EPA 2002). Finally, epidemiologic data on the carcinogenicity of DE in humans has provided only weak evidence of effects in occupationally exposed workers (Jarvholm and Silverman 2003).

The study whose design perhaps most closely resembled that of ACES was conducted by Reed and colleagues (2004) at LRRI (Albuquerque, NM). The investigators exposed mice and rats by whole-body inhalation to DE for 7 days per week, 6 hours per day, for 6 months. Genotoxic effects were studied in the mice only, and the endpoints examined were MN-RET counts and the proliferation of adenomas. Compared with controls, no significant changes in results for either endpoint were found. The study was part of a larger effort organized by the National Environmental Respiratory Center and sought to compare the effects of numerous inhalation conditions to which humans are regularly exposed (Reed et al. 2006).

The specificity of the MN endpoint in characterizing chromosome damage makes the MN test a powerful tool for predicting the potential of a chemical or exposure condition to induce damage that might lead to cancer. However, no single test conducted in isolation is an acceptable indicator of carcinogenic potential, especially when the results must be extrapolated from a rodent model to humans. Perhaps the greatest advantage of using the In Vivo MicroFlow method for MN testing in a study such as ours was that we were routinely able to evaluate 10 times the number of cells examined using the standard microscopy method, allowing the reliable detection even of weak responses and providing increased confidence in negative results.

It should be noted that the peripheral-blood MN test as described in the current report was a direct indicator of the impact of exposures on hematopoiesis in bone marrow. Any impact of exposures that do not directly target bone marrow should be considered a response to systemic effects of the exposure conditions being investigated. In the case of inhalation exposures to what could be considered primarily a lung-targeting agent, there are examples of increased peripheral-blood MN frequencies associated with exposure to cigarette smoke (De Flora et al. 2007; Marchetti et al. 2011). There have also been numerous studies of the systemic effects of DE or DE constituents. Burchiel and colleagues (2004), for example, found systemic immunotoxicity in mice after whole-body exposure to DE, and Song and Ye (1995) found increased frequencies of peripheral-blood MN-RET in mice after exposure to DE extracts. Arlt and colleagues (2008) examined genotoxicity in a transgenic mouse model after exposure to 3-nitrobenzanthrone, a carcinogen found in DE, and its metabolite 3-aminobenzanthrone. 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 very commonly used across numerous disciplines and industries; it continues to be an important part of regulatory guidelines and risk assessment for many types of exposure conditions.

VALIDATION OF FLOW-CYTOMETRY MICRONUCLEUS TESTING

Litron Laboratories has an extensive history of conducting in vivo rodent MN testing and analysis. During the development of the In Vivo MicroFlow assay, multiple interlaboratory validation trials were conducted that demonstrated the portability and reproducibility of the assay across laboratories (Torous et al. 2001, 2005). Table 1 shows an aggregate correlation coefficient of 0.943 for a nine-laboratory trial that examined the MN-RET response of mice exposed to various chemical genotoxicants.

In addition to these validation trials, considerable testing was done to compare MN-RET scoring by the microscopy and flow-cytometry methods (Table 1, Figure 1). Both inter- and intra-laboratory studies showed excellent agreement between results for the two methods (Dertinger et al. 2006).

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).

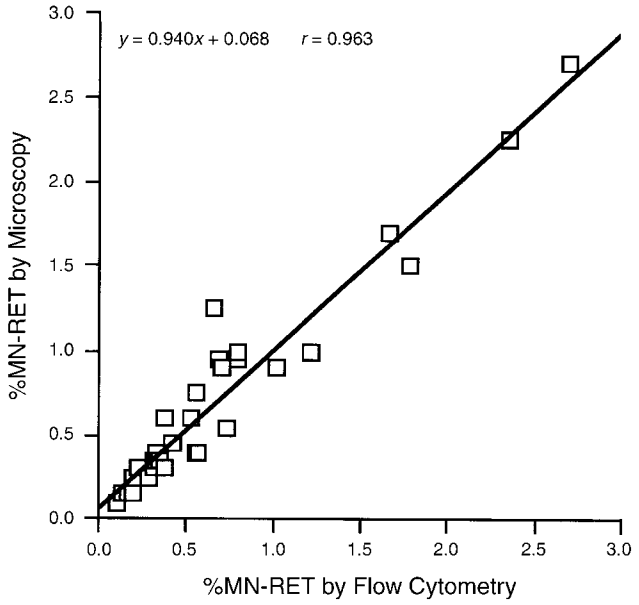

Figure 1. Comparison between microscopy and flow-cytometry analyses of frequency of MN-RET in mouse blood after consecutive 3-day exposures to various doses of cyclophosphamide (r = bivariate Spearman correlation coefficient).

The previous studies were conducted using mice; however, In Vivo MicroFlow technology can also be used to analyze rat blood samples (Torous et al. 2003). Both MacGregor and colleagues (2006) (Figure 2) and Hayashi and colleagues (2007) reported the ability to count genotoxicant-induced MN-RET reliably and accurately in the peripheral blood of rats.

Figure 2 also shows the transient nature of MN-RET frequencies (%MN-RET) after a bolus administration of a chromosome-damaging agent; the frequencies peaked at about 48 hours. This means that when measured in the context of a chronic study, the MN-RET frequency reflects the most recent (i.e., past several days) exposure.

As the In Vivo MicroFlow method gained greater acceptance, more investigators began using it in their studies. It was used by De Boeck and colleagues (2005), for example, who conducted a multiple-sampling time-course study in mice exposed to various mutagens, and by Cammerer and colleagues (2007), who compared the responses of mice and rats to aneugenic compounds. In addition, as part of its efforts to evaluate the genotoxicity of substances of public health concern, the National Toxicology Program compared the microscopy and flow-cytometry methods to validate the use of the latter as a replacement for the former (Witt et al. 2008). The In Vivo MicroFlow method was also used in the chronic inhalation studies of Reed and colleagues (2004, 2006) to investigate the incidence of MN-RET in the peripheral blood of mice exposed to DE or wood smoke.

In addition to the efforts made to validate the In Vivo MicroFlow method, an HEI-funded pilot study was conducted that demonstrated the ability of the method to detect genotoxicant-induced changes in MN-RET frequencies in older rats (see Additional Materials 2, available on the HEI Web site at *http://pubs.healtheffects.org*). Blood samples from male and female rats ranging in age from 10 to 21 months showed cyclophosphamide-induced increases in MN-RET frequencies. Thus, for rats at ages approximately equivalent to those we studied in the 12- and 24-month ACES exposures, the HEI pilot study showed that the In Vivo MicroFlow method was able to identify a genotoxic response in peripheral blood cells.

Kissling and colleagues (2007) assessed the statistical power and sensitivity of the in vivo MN test across several

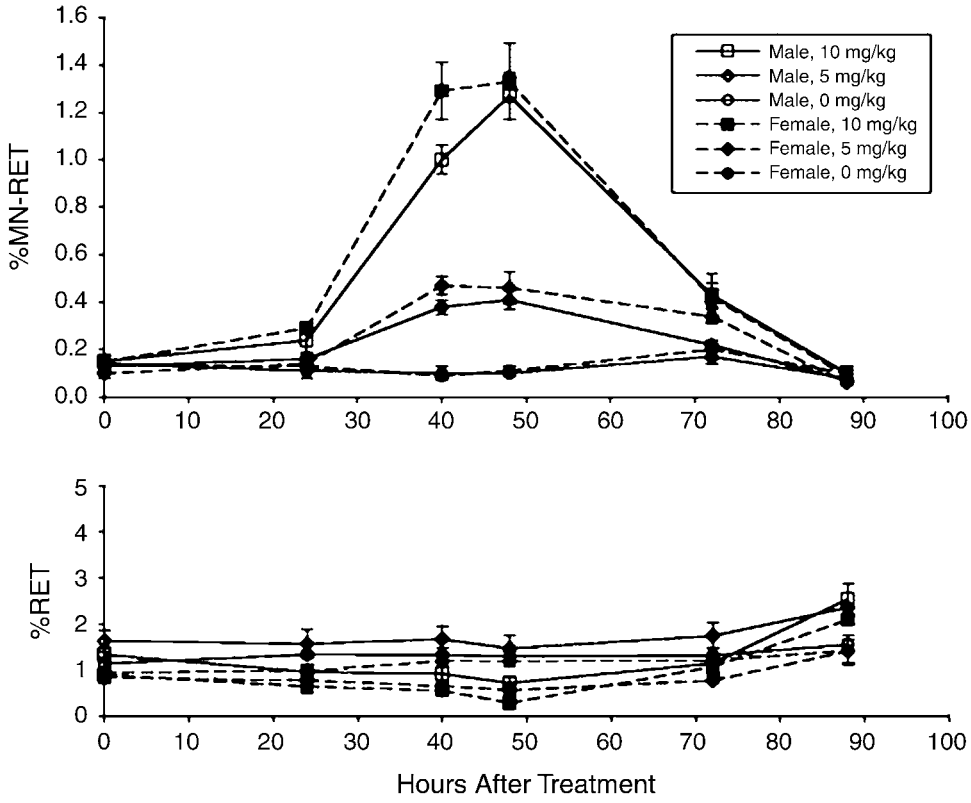

Figure 2. Time course of the frequency of MN-RET in rat blood after a single oral dose of 5 or 10 mg/kg cyclophosphamide. (Reprinted from MacGregor et al. 2006 with permission of Kluwer Academic Publishers [Dordrecht].)

species and experimental parameters. The investigators used a standard experimental design of five animals per group and examined factors such as background MN-RET frequency, inter- and intra-animal variation, number of cells scored, and associated counting errors in order to determine the statistical power of the test. As shown in Table 2, for the rat model with a spontaneous MN-RET frequency of 0.10 (standard deviation [SD] = 0.045), scoring 20,000 RET 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. The reported baseline values from Kissling and colleagues (2007) were similar to those observed in the current study, in which the spontaneous frequency of MN-RET in the control rats (pooled across sex and duration, $n = 40$) was 0.12% (SD = 0.033), and the MN-RET frequency for all the rats in the study ($n = 158$) was 0.12% (SD = 0.039) (see also Additional Materials 1 on the HEI Web site at *http://pubs.healtheffects.org*).

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 MN frequency. Adler and colleagues (1998) argued that a suitable minimum standard for in vivo mutagenicity tests (which include the in vivo MN test) is "the ability to detect a doubling of the observed endpoint at an α-level of 0.05 and a power of 80%." They offered the following as a rationale for this approach: "the increment in mutational events over the spontaneous level to be recognized is determined by biologic criteria rather than by purely statistically achievable precision." As reported by Kissling and colleagues (2007), the currently employed analytic approach (i.e., the standard In Vivo MicroFlow approach based on an analysis of 20,000 RET) can resolve a two- to threefold increase from baseline with 90% power at a significance level of 0.05.

OECD Guideline 474 (1997), which defined operating parameters for the in vivo MN test, stated that five animals per dose group were sufficient. For the current study, the majority of experiments met this OECD minimum requirement. In fact, because the rat data could be pooled across

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

Spontaneous Frequency (%MN-RET)	RET 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 RET, 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.

sex, most experimental conditions exceeded the requirement, examining up to 10 animals per exposure group. The experimental groups in the current report that did not meet the recommended five animals per group are clearly identified.

The studies described in this section demonstrated that the In Vivo MicroFlow method can be used to investigate chromosomal damage in mice and rats in a rapid and efficient manner. The method is easily incorporated into a standard chronic bioassay, as is readily apparent from the examples described above.

SPECIFIC AIMS

The broader goal of the ACES bioassay was to provide information on the safety of 2007-compliant heavy-duty diesel engines. As one of the ancillary ACES bioassay studies associated with ACES, our study sought to evaluate MN formation, a specific biomarker of chromosome damage, as a means to assess the genotoxic potential of DE emitted 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. The study used a rapid and efficient automated flow-cytometry–based

method to accurately enumerate MN-RET in the blood of rodents exposed to DE by inhalation.

In this report, the assessment of MN was performed in rats exposed to DE for 1, 3, 12, or 24 months. In order to assess the potential impact of DE over the complete time course of ACES, the data previously reported for the 1- and 3-month exposure groups (Bemis et al. 2012) were combined with new data from the 12- and 24-month exposure groups.

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 LRRI's Institutional Animal Care and Use Committee. McDonald and colleagues (Mauderly and McDonald 2012; McDonald et al. 2012) provided detailed descriptions of the exposure facility, engine specifications, DE characterization, and animal exposure conditions. For the MN data reported here, blood samples were obtained from male and female Wistar Han rats under four DE exposure conditions (i.e., air control and low, mid, and high exposures). These exposure levels were based on dilutions of the whole engine exhaust to achieve 0.1, 0.8, and 4.2 ppm NO_2 in the low, mid, and high exposures, respectively. All inhalation exposures were whole-body and occurred under the conditions described by McDonald and colleagues (2012). For the majority of experiments in the current study, each exposure group consisted of five males and five females. However, there was an error in the 12-month exposures, with the result that the numbers of animals in the control and low-, mid-, and high-exposure groups were 5, 4, 5, and 5 for the males and 5, 6, 5, and 5 for the females, respectively. There was also an error in the 24-month exposures, with the result that the numbers of animals in the control and low-, mid-, and high-exposure groups were 5, 5, 5, and 4 for the males and 5, 5, 4, and 6 for the females, respectively. In addition, one female rat in the mid-exposure group died before the 24-month endpoint.

ANALYSIS OF MICRONUCLEATED ERYTHROCYTES BY FLOW CYTOMETRY

All of the solutions and materials discussed below are commercially available as part of In Vivo MicroFlow kits (Litron Laboratories). The procedures for using them are the same for samples derived from mice and rats unless otherwise noted.

Sample Collection and Fixation

Whole-blood samples from rats were collected at LRRI into anticoagulant solution and well mixed. A portion of these diluted blood samples was fixed by rapid introduction into ultracold (−80°C) methanol fixative and stored at −80°C until further processing. After the samples were in the fixative a minimum of 48 hours, the methanol was washed out of the samples using the kit's wash buffer and transferred to the kit's long-term storage solution. The samples were maintained at −80°C until they were shipped to Litron Laboratories under controlled-cold conditions (−20°C ice packs) for further processing and analysis.

Sample Processing and Staining

At Litron Laboratories, the samples were stored at −80 to −90°C until scheduled for analysis. Samples were first washed to remove the long-term storage solution and then labeled with anti-CD71-FITC and anti-CD61-PE antibodies (see below) in the presence of RNase. DNA-staining solution was added to the antibody-labeled samples just before analysis by flow cytometry.

Flow-Cytometry Instrument Set-up and Calibration

Before analyzing the samples, the flow cytometer was calibrated with a CD71− blood sample and a malaria biostandard that were provided with the kit. This calibration was done daily and ensured that instrument parameters, such as photomultiplier voltages and compensation settings, were appropriately and consistently applied to all subsequent analyses. The CD71− blood samples and malaria biostandard were processed in parallel with the experimental samples.

ANALYSIS OF EXPERIMENTAL SAMPLES

A more detailed description of sample processing and analysis can be found in Dertinger and colleagues (2004). The analytic scheme was based on the selection and enumeration of specific cells of interest (i.e., RET and MN-RET) using flow-cytometry software and a specialized template. The fluorescent labeling of cells depended on three main cell markers (CD61, propidium iodide [PI], and CD71). CD61 is expressed on platelets and, in conjunction with light scatter, is a marker used to exclude platelets from analysis. The DNA stain noted above contains PI, which labels both DNA and RNA, but the inclusion of RNase in the sample processing made the staining DNA-specific. This allowed the PI fluorescence to be used as a marker for all nucleated cells as well as red blood cells that contained MN. With this staining, nucleated cells exhibit very bright fluorescence in comparison with the absent or

dim staining associated with normal red blood cells and those that contain MN. The bright fluorescence of the stained nucleated cells allows them to be excluded from analysis. The third marker, CD71, is expressed on the surface of RET and is gradually lost as these cells mature; the anti-CD71 fluorescent label thus permits the differentiation of RET from mature NCE. This labeling and selection scheme was executed by way of an assembly of gates and inclusion–exclusion logic in the software template. Figure 3 shows two examples of final plots in the template used to quantify the number of events in the respective quadrants that correspond to RET ($CD71^+$, PI^-), MN-RET ($CD71^+$, PI^+), NCE ($CD71^-$, PI^-), and MN-NCE ($CD71^-$, PI^+). The plot on the left was obtained from a control-exposed rat; the plot on the right was from a rat exposed to a known genotoxic agent. In the plot on the right, the obvious increase in the frequency of MN-RET in the upper right quadrant is a consequence of MN formation in the bone marrow and indicates that chromosomal damage has occurred.

Once the flow cytometer was properly calibrated using the malaria biostandard, the processed experimental samples were analyzed. Instrument settings and gating were kept consistent across all the samples in a given analytic run. The typical stop point for each analysis was the collection of 20,000 RET per sample.

STATISTICAL METHODS AND DATA ANALYSIS

After the experimental samples were analyzed, the data were transferred to a spreadsheet (Excel 2008 for Mac; Microsoft, Redmond, WA). These data were used to calculate the percentages of RET (%RET) and MN-RET (%MN-RET), based on the number of events found in each of the defined quadrants (see Figure 3) — upper left (UL), upper right (UR), lower left (LL), and lower right (LR). %RET was calculated as %RET = [(UL + UR) / (UL + UR + LL + LR)] × 100, and %MN-RET was calculated as %MN-RET = [(UR) / (UR + UL)] × 100.

The means and standard errors of the means were then calculated. Once these initial calculations were made, the %MN-RET values were converted to a proportion, and an arcsinSqrt transformation was made in Excel using the following equation: new_value = ASIN(SQRT(old_value)). This transformation was used to stabilize group variance and normalize the data and is based on a consensus opinion of experts in genetic toxicology statistics associated with the Pharmaceutical Research and Manufacturers of America consortium. Because the arcsinSqrt transformation did not achieve a suitably normal distribution for the %RET data, an alternative strategy was used. The values for %RET were transformed by taking the natural log, applying a consistent correction factor to shift the data into positivity, and then taking the natural log again. This double-logarithmic transformation yielded the best

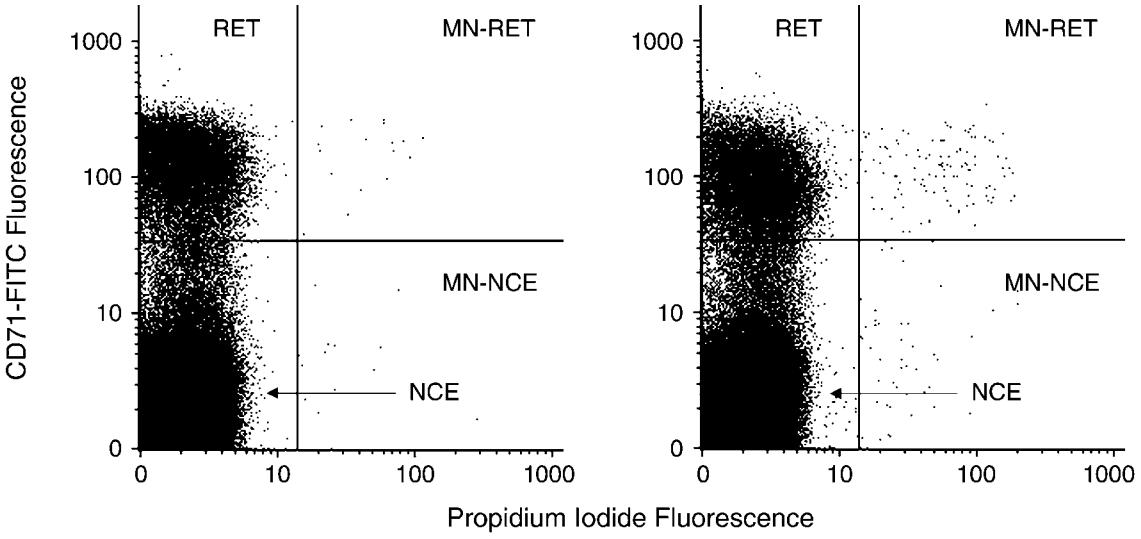

Figure 3. Representative flow-cytometry plots of blood samples obtained from a rat treated with vehicle control (left) or a rat treated with a known genotoxic agent (right). In each plot, the upper left quadrant corresponds to RET, the upper right to MN-RET, the lower left to NCE, and the lower right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

approximation of a normal distribution for rat data sets. After transformation, the data were imported into a statistics program (JMP, v.8 for Mac; SAS, Cary, NC) for further statistical analysis.

For the statistical analyses associated with ACES, the ANOVA model was chosen as the preferred method for comparison. The data in the Results section, below, were analyzed using a three-way ANOVA that included the factors of sex, exposure condition, and exposure duration. Significance was set at $P \leq 0.05$. As described above, the data for male and female rats were pooled in order to maximize the number of animals per exposure group, such that there were 10 animals per exposure condition, unless otherwise noted.

RESULTS

Summary tables of the raw and calculated data for rats are shown in Additional Materials 1 (available on the HEI Web site). The raw data were used for the figures in this report; the calculated data were used for the statistical analyses.

Table 3 and Table 4 show the results of the three-way ANOVA analyses performed on the %MN-RET and %RET values, respectively, comparing sex, exposure condition, and duration of exposure.

For the %MN-RET data, there was a statistically significant increase in %MN-RET associated with an increase in

Table 3. Three-Way ANOVA for Rat %MN-RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Sex	1	1	0.00003438	1.1429	0.2871
Exposure condition	3	3	0.00001085	0.1202	0.9481
Duration of exposure	3	3	0.00052674	5.8370	0.0009[b]
Sex × Exposure condition	3	3	0.00003610	0.4000	0.7532
Sex × Duration of exposure	3	3	0.00006108	0.6768	0.5678
Exposure condition × Duration of exposure	9	9	0.00029573	1.0923	0.3731
Sex × Exposure condition × Duration of exposure	9	9	0.00015661	0.5785	0.8127

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

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

Table 4. Three-Way ANOVA for Rat %RET Data[a]

Source	Nparm	Degrees of Freedom	Sum of Squares	*F* Ratio	Prob > *F*
Sex	1	1	0.00625716	0.1593	0.6905
Exposure condition	3	3	0.17289149	1.4673	0.2267
Duration of exposure	3	3	0.22519773	1.9112	0.1311
Sex × Exposure condition	3	3	0.07739805	0.6568	0.5801
Sex × Duration of exposure	3	3	0.18165203	1.5416	0.2070
Exposure condition × Duration of exposure	9	9	0.31298205	0.8854	0.5403
Sex × Exposure condition × Duration of exposure	9	9	0.18009236	0.5095	0.8655

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

the duration of exposure (Table 3 and Figure 4). Elevated MN-RET frequency is commonly observed with increased age (Kaspler et al. 2011; Sato et al. 1995). However, when duration of exposure was combined with sex, exposure condition, or sex and exposure condition together, the effect was no longer significant.

For the %RET data, only two individual rats in the low- and mid-level exposure groups of the 12-month studies had high RET frequencies. The %RET data obtained as part of our MN analysis were corroborated by data from similar analyses performed at LRRI (see Part 1 of this volume). Analysis of the data for the effect of DE on %RET as a marker of general bone-marrow toxicity did not reveal any statistically significant effects after any of the exposure conditions examined (Table 4 and Figure 5).

DISCUSSION AND CONCLUSIONS

This study examined the potential for DE to cause chromosome damage in Wistar Han rats after 1-, 3-, 12-, or 24-month exposures. Peripheral-blood MN-RET, a well-established indicator of chromosome damage, was analyzed by flow-cytometry methods. The study was conducted as one of the ancillary studies associated with the core ACES Phase 3B chronic-inhalation study (see Part 1 of this volume).

Our overall conclusion was that there was no apparent effect of the study's DE exposures on the chromosome-damage endpoint tested. Thus, although some comparisons in the ANOVA analyses resulted in statistically significant effects for various factors, additional comparisons did not support interactions that were related to the exposure

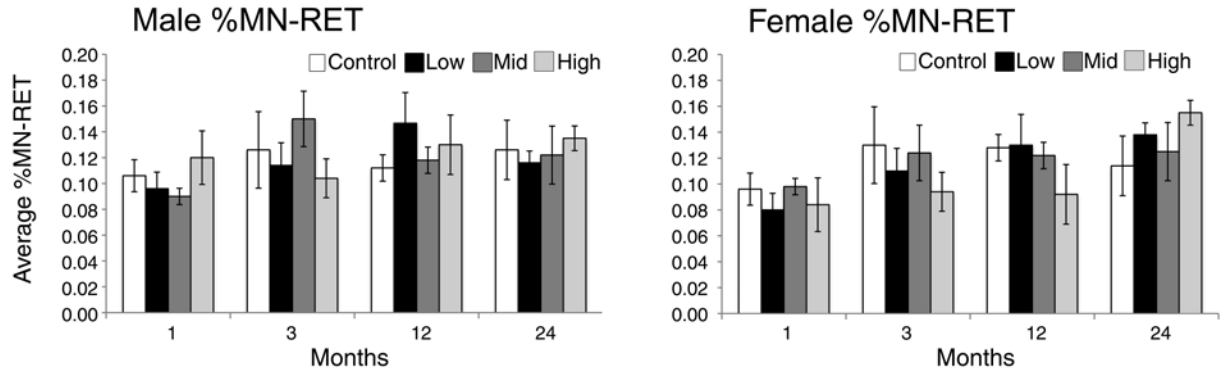

Figure 4. Mean frequency of MN-RET in rats exposed to DE for 1 to 24 months. Control, low, mid, and high correspond to the air control, low, mid, and high levels of DE as described in the text. Error bars indicate standard error of the mean.

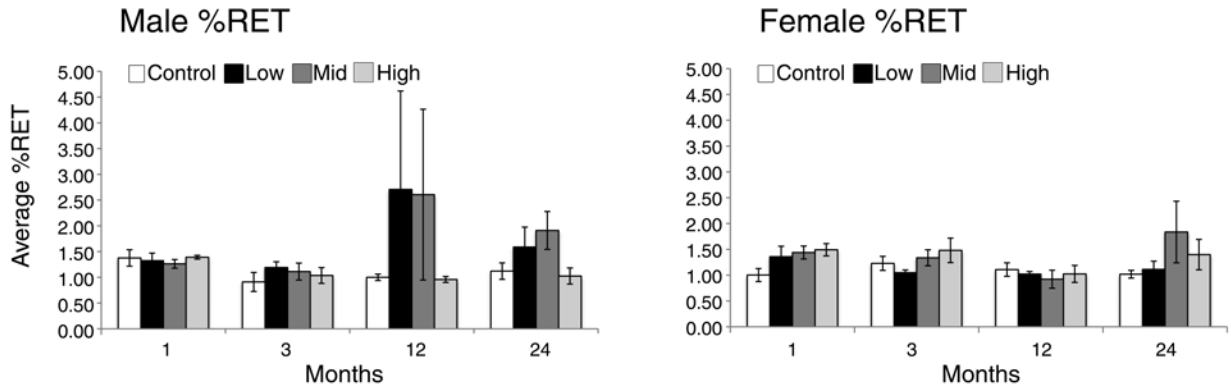

Figure 5. Mean frequency of RET in rats exposed to DE for 1 to 24 months. Control, low, mid, and high correspond to the air control, low, mid, and high levels of DE as described in the text. Error bars indicate standard error of the mean.

conditions or that demonstrated a consistent exposure–response relationship. As mentioned earlier, biologically relevant responses of these genotoxicity markers are typically described as being on the order of a twofold increase from baseline. The data collected for the current study did not show any effects that approached this magnitude (Figure 4). The lack of any obvious increases in the chromosome-damage endpoint associated with increasing exposure levels, even after 24 months of exposure, strongly suggests that under the experimental conditions studied here whole-body DE exposures had no discernible genotoxic impact on hematopoietic cells.

IMPLICATIONS OF FINDINGS

Our data have demonstrated an apparent lack of effect of DE inhalation on MN-RET levels in the peripheral blood of rats after 1, 3, 12, or 24 months of exposure. This lack of genotoxicity extends observations made in the subchronic study (Bemis et al. 2012) and shows that chronic exposure to DE from 2007-compliant engines at the levels tested does not elicit a systemic chromosome-damage response, as indicated by the in vivo MN test. These observations should be interpreted in the context of ACES Phase 3B as a whole, which examined numerous additional toxicity endpoints associated with lifetime exposure to DE in a rodent model.

REFERENCES

Adler ID, Bootman J, Favor J, Hook G, Schriever-Schwemmer G, Welzl G, et al. 1998. Recommendations for statistical designs of in vivo mutagenicity tests with regard to subsequent statistical analysis. Mutat Res 417:19–30.

Arlt VM, Gingerich J, Schmeiser HH, Phillips DH, Douglas GR, White PA. 2008. Genotoxicity of 3-nitrobenzanthrone and 3-aminobenzanthrone in MutaMouse and lung epithelial cells derived from MutaMouse. Mutagenesis 23:483–490.

Bemis JC, Torous DK, Dertinger SD. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Brown BG, Lee CK, Bombick BR, Ayres PH, Mosberg AT, Doolittle DJ. 1997. Comparative study of DNA adduct formation in mice following inhalation of smoke from cigarettes that burn or primarily heat tobacco. Environ Mol Mutagen 29:303–311.

Burchiel SW, Lauer FT, McDonald JD, Reed MD. 2004. Systemic immunotoxicity in A/J mice following 6-month whole body inhalation exposure to diesel exhaust. Toxicol Appl Pharmacol 196:337–345.

Cammerer Z, Elhajouji A, Kirsch-Volders M, Suter W. 2007. Comparison of the peripheral blood micronucleus test using flow cytometry in rat and mouse to aneugens after single-dose applications. Mutagenesis 22:129–134.

Cimini D, Degrassi F. 2005. Aneuploidy: a matter of bad connections. Trends Cell Biol 15:442–451.

De Boeck, M, van der Leede BJ, Van Goethem F, De Smedt A, Steemans M, Lampo A, et al. 2005. Flow cytometric analysis of micronucleated reticulocytes: time-and dose-dependent response of known mutagens in mice, using multiple blood sampling. Environ Mol Mutagen 46:30–42.

De Flora S, D'Agostini F, Izzotti A, Zanesi N, Croce CM, Balansky R. 2007. Molecular and cytogenetical alterations induced by environmental cigarette smoke in mice heterozygous for Fhit. Cancer Res 67:1001–1006.

Dertinger SD, Bishop ME, McNamee JP, Hayashi M, Suzuki T, Asano N, et al. 2006. Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: I. intra- and inter-laboratory comparison with microscopic scoring. Toxicol Sci 94:83–91.

Dertinger SD, Camphausen K, MacGregor JT, Bishop ME, Torous DK, Avlasevich S, et al. 2004. Three-color labeling method for flow cytometric measurement of cytogenetic damage in rodent and human blood. Environ Mol Mutagen 44:427–435.

Dybdahl M, Rison L, Bornholdt J, Autrup H, Loft S, Wallin H. 2004. Inflammatory and genotoxic effects of diesel particles in vitro and in vivo. Mutat Res 562:199–131.

Hayashi M, MacGregor JT, Gatehouse DG, Blakley DH, Dertinger SD, Abramson-Zetterberg L, et al. 2007. In vivo erythrocyte micronucleus assay III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of the non-hematopoietic target cells and a single dose-level limit test. Mutat Res 627:10–30.

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. 2011. Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use S2(R1). Geneva, Switzerland:International Conference on Harmonisation.

Ishinishi N, Kuwabara N, Nagase S, Susuki T, Ishiwata S, Kohno T. 1986. Long-term inhalation studies on effects of exhaust from heavy and light duty diesel engines in F344 rats. Dev Toxicol Environ Sci 13:329–348.

Jarvholm B, Silverman D. 2003. Lung cancer in heavy equipment operators and truck drivers with diesel exhaust exposure in the construction industry. Occup Environ Med 60:516–520.

Kaspler P, Chen R, Hyrien O, Jelveh S, Bristow RG, Hill RP. 2011. Biodosimetry using radiation-induced micronuclei in skin fibroblasts. Int J Radiat Biol. 87:824–838.

Kirkland DJ, Henderson L, Marzin D, Muller L, Parry JM, Speit G, et al. 2005. Testing strategies in mutagenicity and genetic toxicology: an appraisal of the guidelines of the European Scientific Committee for Cosmetics and Non-Food Products for the evaluation of hair dyes. Mutat Res 588:88–105.

Kissling GE, Dertinger SD, Hayashi H, MacGregor JT. 2007. Sensitivity of the erythrocyte micronucleus assay: dependence on number of cells scored and inter-animal variability. Mutat Res 634:235–240.

MacGregor JT, Bishop ME, McNamee JP, Hayashi M, Asano N, Wakata A, et al. 2006. Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: II. An efficient method of monitoring chromosomal damage in the rat. Toxicol Sci 94:92–107.

Marchetti F, Rowan-Carroll A, Williams A, Polyzos A, Berndt-Weis ML, Yauk CL. 2011. Sidestream tobacco smoke is a male germ cell mutagen. Proc Natl Acad Sci USA 108:12811–12814.

Mauderly JL, Banas DA, Griffith WC, Hahn FF, Henderson RF, McClellan RO. 1996. Diesel exhaust is not a pulmonary carcinogen in CD-1 mice exposed under conditions carcinogenic to F344 rats. Fundam Appl Toxicol 30:233–242.

Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study (ACES) Phase 3A: Characterization of U.S. 2007-Compliant Diesel Engine and Exposure System Operation. Communication 17. Boston, MA:Health Effects Institute.

Mauderly JL, Snipes MB, Barr EB, Belinsky SA, Bond JA, Brooks AL, et al. 1994. Part I. Neoplastic and nonneoplastic lung lesions. In: Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats. Research Report 68. Cambridge, MA:Health Effects Institute.

McDonald JD, Doyle-Eisele M, Gigliotti A, Miller RA, Seilkop S, Mauderly JL, et al. 2012. 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. In: Advanced Collaborative Emissions Study (ACES). Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Oh SM, Chung KH. 2006. Identification of mammalian cell genotoxins in respirable diesel exhaust particles by bioassay-directed chemical analysis. Toxicol Lett 161:226.

Organisation for Economic Co-operation and Development (OECD). 1997. Guideline for the Testing of Chemicals. Guideline 474. Mammalian erythrocyte micronucleus test. Paris:Organization for Economic Co-operation and Development.

Reed MD, Campen MJ, Gigliotti AP, Harrod KS, McDonald JD, Seagrave JC, et al. 2006. Health effects of subchronic exposure to environmental levels of hardwood smoke. Inhal Toxicol 18:523–539.

Reed MD, Campen MJ, Gigliotti AP, McDonald JD, Seagrave JC, Sielkop SK, et al. 2004. Health effects of subchronic exposure to environmental levels of diesel smoke. Inhal Toxicol 6:177–193.

Sato S, Taketomi M, Nakajima M, Kitazawa M, Shimada H, Itoh S, et al. 1995. Effect of aging on spontaneous micronucleus frequencies in peripheral blood of nine mouse strains: the results of the 7th collaborative study organized by CSGMT/JEMS.MMS. Collaborative Study Group for the Micronucleus Test. Environmental Mutagen Society of Japan. Mammalian Mutagenesis Study Group. Mutat Res 338:51–57. Erratum in: Mutat Res 316:287–288.

Song J, Ye SH. 1995. Study on the mutagenicity of diesel exhaust particles. Biomed Environ Sci 8:240–245.

Soni MG, Burdock GA, Christian MS, Bitler CM, Crea R. 2006. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. Food Chem Toxicol 44:903–915.

Torous DK, Hall NE, Dertinger SD, Diehl MS, Illi-Love AH, Cederbrant K, et al. 2001. Flow cytometric enumeration of micronucleated reticulocytes: high transferability among 14 laboratories. Environ Mol Mutagen 38:59–68.

Torous DK, Hall NE, Illi-Love AH, Diehl MS, Cederbrant K, Sandelin K, et al. 2005. Interlaboratory validation of a CD71-based flow cytometric method (Microflow) for the scoring of micronucleated reticulocytes in mouse peripheral blood. Environ Mol Mutagen 45:44–55.

Torous DK, Hall NE, Murante FG, Gleason SE, Tometsko CR, Dertinger SD. 2003. Comparative scoring of micronucleated reticulocytes in rat peripheral blood by flow cytometry and microscopy. Toxicol Sci 74:309–314.

U.S. Environmental Protection Agency. 2002. Health Assessment Document for Diesel Engine Exhaust. EPA/600/8-90/057F. Washington, DC:U.S. Environmental Protection Agency, Office of Research and Development.

Witt KL, Livanos E, Kissling GE, Torous DK, Caspary W, Tice RR, et al. 2008. Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals. Mutat Res 649:101–113.

Zeiger E. 1998. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: premises, promises and performance. Regul Toxicol Pharmacol 28:85–95.

ADDITIONAL MATERIALS AVAILABLE ON THE WEB

Additional Materials 1 and 2 are supplemental materials not included in the printed report. They are available on the HEI Web site at *http://pubs.healtheffects.org*.

Additional Materials 1: Summary Tables of Raw and Calculated Data

Additional Materials 2: ACES Pilot Study to Determine Efficacy of Flow Cytometric Assessment of Chromosome Damage in Aged Rats

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Jeffrey C. Bemis received his Ph.D. in environmental health and toxicology from the School of Public Health, University at Albany–State University of New York, in Albany, New York. His thesis focused on the effects of combined exposures to environmental contaminants such as polychlorinated biphenyls and methyl mercury on central neurotransmitter systems. He 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, where he studied the effects of polychlorinated biphenyls and dioxin on the nervous and immune systems and identified target tissues in the developing fetus. He joined Litron Laboratories in 2006 as a research scientist and is currently the director of clinical studies.

Dorothea K. Torous received her BS in biology from the University at Albany–State University of New York, in Albany, New York. She began her 27-year tenure at Litron Laboratories as a research technician and became proficient with the standard battery of genotoxicity tests. She played a leading role in developing the flow-cytometry method for scoring in vivo MN (commercially known as the In Vivo MicroFlow test). She organized and led an international, multi-laboratory study, funded by the National Institutes of Health, that validated the method, leading to its widespread utilization. She is also responsible for training numerous investigators to perform the MicroFlow procedures, and she provides technical support for MicroFlow products. Since 1998, she has served as the director of Microflow and as principal investigator. In this capacity, she is responsible for phases of multisite studies that include the flow-cytometry MN scoring of client-supplied specimens.

Stephen D. Dertinger received his Ph.D. at the Department of Environmental Medicine at the University of Rochester, in Rochester, New York, where he studied the role of the aryl hydrocarbon receptor in mediating the toxicity of cigarette smoke. Since then, he has served as the director of research at Litron Laboratories. He has overseen the development of high-throughput in vitro and in vivo cytogenetic-damage assays, most notably automated procedures for scoring MN in mammalian cell cultures and blood reticulocytes. These methods, commercially available as the In Vitro MicroFlow and In Vivo MicroFlow kits, are used by many pharmaceutical and chemical companies throughout the world to assess compounds for genotoxic activity.

OTHER PUBLICATION RESULTING FROM THIS RESEARCH

Bemis JC, Torous DK, Dertinger SD. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

ABBREVIATIONS AND OTHER TERMS

%MN-RET	percentage of micronucleated reticulocytes
%RET	percentage of reticulocytes
ACES	Advanced Collaborative Emissions Study
ANOVA	analysis of variance
ASIN	arcsine
DE	diesel exhaust
FDA	Food and Drug Administration
ICH	International Conference on Harmonisation
LL	lower left
LR	lower right
LRRI	Lovelace Respiratory Research Institute
MN	micronuclei
MN-RET	micronucleated reticulocytes
MN-NCE	micronucleated normochromatic erythrocytes
NCE	normochromatic erythrocytes
OECD	Organisation for Economic Co-operation and Development
PI	propidium iodide
PM	particulate matter
RET	reticulocytes
SD	standard deviation
UL	upper left
UR	upper right
U.S. EPA	United States Environmental Protection Agency

Part 3. Assessment of Genotoxicity and Oxidative Damage in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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Bill T. Ameredes, and Jeffrey K. Wickliffe

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INVESTIGATORS' REPORT

Part 3. Assessment of Genotoxicity and Oxidative Damage in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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ABSTRACT

In 2001, the U.S. Environmental Protection Agency (EPA*) and the California Air Resources Board (CARB) adopted new standards for diesel fuel and emissions from heavy-duty diesel engines. By 2007, diesel engines were required to meet these new standards for particulate matter (PM), with other standards to follow. Through a combination of advanced compression-ignition engine technology, development of exhaust aftertreatment systems, and reformulated fuels, stringent standards were introduced.

Before the 2007 standards were put in place by the EPA, human health effects linked to diesel exhaust (DE) exposure had been associated with diesel-fuel solvent and combustion components. In earlier research, diesel engine exhaust components were, in turn, linked to increased mutagenicity in cultures of *Salmonella typhimurium* and mammalian cells (Tokiwa and Ohnishi 1986). In addition, DE was shown to increase both the incidence of tumors and the induction of 8-hydroxy-deoxyguanosine (8-OHdG) adducts in rodents (Ichinose et al. 1997) and total DNA adducts in rats (Bond et al. 1990). Furthermore, DE is composed of a complex mixture of polycyclic aromatic hydrocarbons (PAHs) and particulates. One such PAH, 3-nitrobenzanthrone (3-NBA), is also found in urban air. 3-NBA has been observed to induce micronucleus formation in the DNA of human hepatoma cells (Lamy et al. 2004).

The current study is part of the Advanced Collaborative Emissions Study (ACES), a multidisciplinary program carried out by the Health Effects Institute and the Coordinating Research Council. Its purpose was to determine whether recent improvements in the engineering of heavy-duty diesel engines reduce the toxicity associated with exposure to DE components. To this end, we evaluated potential genotoxicity and induction of oxidative stress in bioassays of serum and tissues from Wistar Han rats chronically exposed — for up to 24 months — to DE from a 2007-compliant diesel engine (new-technology diesel exhaust, or NTDE). Genotoxicity was measured as DNA strand breaks in lung tissue, using an alkaline-modified comet assay. As a correlate of possible DNA damage evaluated in the comet assay, concentrations of the free DNA adduct 8-OHdG were evaluated in serum by a competitive enzyme-linked immunosorbent assay (ELISA). The 8-OHdG fragment found in the serum is a specific biomarker for the repair of oxidative DNA damage. In addition, an assay for thiobarbituric acid reactive substances (TBARS) was used to assess oxidative stress and damage in the form of lipid peroxidation in the hippocampus region of the brains of the DE-exposed animals. These endpoints were evaluated at 1, 3, 12, and 24 months of exposure to DE or to a control atmosphere (filtered air).

At the concentrations of DE evaluated, there were no significant effects of exposure in male or female rats after 1, 3, 12, or 24 months in any measure of DNA damage in the comet assay (%DNA in tail, tail length, tail moment, or olive moment).

This Investigators' Report is one part of Health Effects Institute Research Report 184, 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, Texas 77555-1110; e-mail: *lmhallbe@utmb.edu*.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83467701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

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

The comparison of exposure groups versus control and the comparison of groups by sex for 1 and 3 months of exposure showed no significant differences in serum 8-OHdG concentrations ($P > 0.05$). The concentrations of 8-OHdG in all exposure groups at 3 months were higher than those in exposure groups at any other time point ($P < 0.05$). Looking at the levels of 8-OHdG in serum in the 12-month and 24-month groups, we saw a significant difference from control in the 12-month group at the mid and high levels ($P < 0.05$), as well as some other scattered changes. Sex differences were noted in the 12-month high-level group ($P < 0.05$). However, these differences did not follow an exposure-dependent pattern. All other comparisons were not significant ($P > 0.05$).

Hippocampal concentrations of TBARs, measured as malondialdehyde (MDA), showed some small and scattered changes in groups exposed to different levels of DE and at different time points, but we did not consider these to be exposure-related. We concluded that exposure to DE in these rats did not produce any significant increase in oxidative damage to lipids or damage to DNA in the form of strand breaks.

INTRODUCTION

In 2001, the EPA and CARB adopted new standards for diesel fuel and emissions from heavy-duty diesel engines. By 2007, diesel engines were required to meet this new standard for PM and, in 2010, to conform to an additional standard for nitrogen oxides (NO_x). A combination of advanced compression-ignition engines, exhaust aftertreatment systems, and reformulated fuels was used to meet these stringent standards, which are expected to result in substantially reduced emissions of PM, NO_x, and other exhaust constituents. These changes were anticipated to reduce emissions by 90% compared with those of pre-2007 heavy-duty diesel engines, providing substantial public health benefits.

The study known as ACES was designed in an effort to assess the potential health effects of these engineering advances in diesel engines. ACES was carried out in three phases: Phase 1, conducted at Southwest Research Institute in San Antonio, Texas, was an extensive characterization of the emissions of production-ready heavy-duty diesel engines designed to meet the 2007 standards. One engine was chosen (see the ACES Final Plan for Engine Selection in Appendix A of HEI Communication 17 [Mauderly and McDonald 2012], available on the Web at *http://pubs.healtheffect.org*) to be used in the health effects assessments during Phase 3, conducted at Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico. (In Phase 2, investigators at the Southwest Research Institute characterized emissions from a group of diesel engine and control systems designed to meet the more stringent 2010 standards.) Phase 3 was separated into two parts. Phase 3A carefully characterized the emission profile in the exposure chambers of the selected engine, which was then used in Phase 3B as the emission source for animal exposure studies.

The current study conducting research on the genotoxicity of DE emissions from 2007-compliant diesel engines was a part of Phase 3B. In this report we describe the results from our testing of 1-, 3-, 12-, and 24-month exposures.

Human health effects linked to DE exposure have been associated with both diesel-fuel solvent and combustion components. In the past, DE 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-OHdG adducts in rodents (Ichinose et al. 1997). DE is a complex mixture of PAHs and particulates. One such PAH, 3-NBA, has been identified in DE and is found in urban air. 3-NBA has been observed to induce micronucleus formation in the DNA of human hepatoma cells (HepG2) (Lamy et al. 2004). Thus, the purpose of the current research was to determine if improvements in the engineering of heavy-duty diesel engines have reduced the oxidative stress and genotoxic risk associated with DE components. To this end, the genotoxicity of DE from an improved diesel engine was evaluated in bioassays of tissues from Wistar Han rats exposed to DE. The endpoints measured included those of the comet assay used to examine DNA damage in the lung, the primary target tissue, as indicated by strand breaks. To correlate possible DNA damage found by the comet assay, DNA adduct formation was measured by a competitive ELISA assay to determine the levels of free 8-OHdG found in serum. 8-OHdG is a specific modified base that indicates an oxidative type of DNA damage to DNA nucleotides. In addition, a TBARS assay was used to assess oxidative stress and damage in the form of lipid peroxidation in the hippocampus of the brains of DE-exposed animals.

Historically, exposure to DE (which includes volatile organic compounds [VOCs], PAHs, divalent metals, and PM components) has been associated with human health concerns. In particular, it is suspected of contributing to lung cancer and cardiopulmonary diseases (Pope et al. 2002). To strengthen this suspicion, DE would have to be linked to increased mutagenicity, because the induction of mutagenic or tumorigenic events is likely involved in carcinogenesis. Such mutagenicity has been observed in both bacterial and mammalian cell systems (Tokiwa and

Ohnishi 1986). In studies of human–hamster hybrid (A/L) cells it has been demonstrated that National Institute of Standards and Technology Standard Reference Material 2975 DE particulates (DEP) can be transported inside cells through phagocytic action and that this transport resulted in a greater than twofold increase in mutation frequency (Bao et al. 2007). DE includes a complex mixture of PAHs, including 3-NBA, which has been identified in urban air and has been found to induce micronucleus formation in HepG2 cells (Lamy et al. 2004). Additionally, studies have shown increases in both the incidence of tumor formation and the formation of 8-OHdG adducts after exposure to DE and DEP in rodents (Ichinose et al. 1997). Other studies in rodents have demonstrated increased 8-OHdG adduct formation in lung tissue after a single exposure to DEP. In these experiments, 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) 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 DE's genotoxic effects. This oxidative potential can oxidize DNA nucleotides such as guanine. Exposure to PM with an aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$) and smaller ultrafine particles (UFPs; PM with an aerodynamic diameter $\leq$ 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 human biomonitoring for exposure to DE.

In proposing its standards, the EPA anticipated that improvements in the engineering of 2007-compliant diesel engines and in the reformulation of diesel fuel would result in reduced emissions. Reduction in these emissions might then result in reduced genotoxicity compared with that of older diesel engines. If advances in diesel engine design effectively reduce total emissions, as anticipated, then the hypothesized reductions in the levels of oxidized nucleotides and adduct formation would be observed. This in turn would result in reductions in the numbers of mutagenic lesions, abasic (apurinic/apyrimidinic) sites, and resultant strand breaks. However, although the new engineering standards might reduce particulate and total DE emissions, it is possible that even these lower emissions levels could 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 suggests that the 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, through production of ROS, or indirectly, through induction of the inflammatory response, as a major underlying mechanism (Donaldson et al. 2002; Riedl and Diaz-Sanchez 2005; Terzano et al. 2010). This being the case, the use of endpoints that indicate oxidative-type DNA damage, as was done in the past, 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 can be shown to have a reduced ability to damage DNA, then 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. It has been shown that $PM_{2.5}$ induces damage directly or indirectly (Dybdahl et al. 2004; Kenyon and Liu 2011; Risom et al. 2005). For example, one direct mechanism is the induction of ROS by the particle surface, which contains redox-reactive metal. UFPs are phagocytized by alveolar macrophages and can elicit macrophage-induced inflammatory responses — resulting in ROS production, oxidative stress, and resultant DNA damage (Beck-Speier et al. 2005; Lundborg et al. 2007). However, if this reduction is limited to larger particulates, then UFPs could potentially continue to have a health impact. For example, it has been observed that UFPs are inhaled deeper into the lungs, from where they can pass into the circulatory system (Mühlfeld et al. 2008). These particles can also pass through the blood–brain barrier, inducing a neurologic inflammatory response (Chen et al. 2007; Peters et al. 2006). Because the majority of health effects caused by UFPs arise from apparent increased oxidative stress, the endpoints we selected for the current study should be suitable for investigating this effect.

SPECIFIC AIMS

Numerous studies have shown associations between exposure to air pollutants and environmental health effects. Among the pollutants of concern are the criteria pollutants sulfur dioxide (SO_2), NO_x, and PM. These pollutants are 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 PM 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-exposure testing of newly engineered diesel engines. Induction of oxidative stress in the human body can result from exposure to various components of DE, including organic and inorganic gases, metals, and PM. A result of this induction could be the production of oxidative damage in DNA, proteins, and lipids. This project was therefore designed to answer the following questions:

1. Does exposure of a primary target tissue, the lung, to DE from a 2007-compliant diesel engine lead to oxidative damage of 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, resulting in lipid peroxidation and the formation of TBARs?

Specific Aim 1: Determine whether exposure to DE results in the induction of DNA damage. We used the comet assay to determine whether oxidative damage occurred in the DNA of exposed animals. We used the alkaline-modified comet assay to determine the presence of DNA strand breaks in lung tissue. To support the findings of the comet assay, we performed an 8-OHdG assay to measure 8-OHdG residues, a result of the repair of DNA adducts, in serum.

Specific Aim 2: Determine whether exposure to DE results in the induction of lipid damage. We used 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.

These endpoints were measured in samples of serum, lung, and brain tissue at multiple exposure time points (1, 3, 12, and 24 months).

METHODS AND STUDY DESIGN

EXHAUST DILUTIONS

Four chamber-exposure levels were used for the rat bioassays: low, medium, and high DE concentrations 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 concentration 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. 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 them were as follows:

1. The highest concentration of NO_2 was 4.2 ppm. This concentration was derived from an earlier study of chronic NO_2 exposures (Mauderly 1989) in which animals were exposed to 9.5 ppm NO_2 for 7 hours per day, 5 days per week, for 24 months. 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 exposures 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. It was thought that it was possible at this concentration to maintain the exposure chamber temperature within the specified range.
2. The targeted medium concentration of NO_2 was 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 this, to provide a concentration that would likely have no observed adverse effect. This concentration was near the 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.

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 of the ACES Oversight Committee was to use Wistar Han rats. (See Additional Materials 1 in Part 1 of this volume.)

NUMBER OF ANIMALS

We determined the number of animals needed to perform our statistical analysis based on a power analysis of

data from our previous studies (see Hallberg et al. 2012). We found that four animals would be sufficient to detect differences between exposure levels (Table A.1 in Appendix A on the HEI Web site, at *http://pubs.healtheffects.org*), but we increased the number to five animals of each sex to look for sex differences. Further discussion of the rationale for this power calculation is provided in Appendix A. All assays were performed blind. If our findings showed that our assays were unable to detect any differences by sex associated with DE exposure, we pooled the data for the males and females, giving us an $n = 10$, and repeated the analysis.

MODIFIED COMET ASSAY

The comet assay is a sensitive technique that is able to detect DNA damage at the cellular level. It is now a standard laboratory tool for the evaluation of DNA damage and repair and genotoxicity testing and biomonitoring. Samples for the comet assay were processed at LRRI. Data from LRRI showed that animal tissues were processed within 1.5 to 4 hours after exposure. The standard neutral comet assay, which is a single-cell gel electrophoresis assay used to asses DNA damage, was slightly modified to incorporate an alkaline nucleus treatment before electrophoresis. (The neutral comet assay does not incorporate this modification and is primarily capable of detecting only genomic double-strand breaks.) The alkaline modification, used in our analysis of lung tissues from rats exposed to defined dilutions of DE, theoretically increases the sensitivity of the assay, allowing for increased resolution of both double- and single-strand DNA breaks. In addition, some fraction of alkali-sensitive sites can also be resolved using this modified assay. However, use of the modified assay does not allow for distinctions between single- and double-strand breaks or alkali-sensitive sites.

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. To determine the optimal amount of starting material necessary for generating an adequate cell suspension, various sizes of blocks of frozen lung tissue from a laboratory rodent (not one from the experimental groups) were minced, and cells were counted on a hemocytometer. Intact cells were embedded in agarose on microscope slides and lysed in situ. The manufacturer's suggested procedure for the neutral comet assay was followed, modified by way of an additional step in which the agarose-immobilized nuclei on microscope slides were immersed in an alkaline solution (pH > 13) for 60 minutes in the dark at room temperature. The slides were then rinsed twice in distilled water and placed in a neutral electrophoresis buffer for 5 minutes at room temperature. The remaining steps of the standard neutral comet assay were then followed.

The nuclei were imaged and captured using a microscope (Eclipse 90i, Nikon Instruments, Melville, NY) at 10× magnification fitted with a camera (SensiCam QE, PCO, Kelheim, Germany) using image-capturing software (version 3.7, IP Labs, Bonn, Germany). All images were autoexposed before capture to objectively minimize variable background staining among the images. Pyknotic (shrunken and condensed) nuclei, which are characteristic of degenerate or dying cells, can result from biologic phenomena such as apoptotic processes or from technical deficiencies in sample processing and handling. In the comet assay, pyknotic nuclei were distinguished from normal nuclei by the smaller size of their head diameter.

Comet profiles were fitted to captured images, and associated metrics (%DNA in tail, defined as the percentage of DNA in the comet tail and computed as the total comet tail intensity divided by the total comet intensity, multiplied by 100; tail moment, computed as the %DNA in the comet tail multiplied by the tail length; tail length, computed as the comet head diameter subtracted from the overall comet length; and olive moment, computed as the summation of each tail intensity integral value, multiplied by its relative distance from the center of the head [the point at which the head integral was mirrored], and divided by the total comet intensity) were estimated using specialized software (CASP version 1.2.2, Comet Assay Software Project, *http://casplab.com/*). To verify that this freely available software was suitable, five randomly selected samples of rat lung were analyzed using it and commercially available software (CometScore, TriTek Corp., Sumerduck, VA). Correlation coefficients for the two software programs were > 95% for the comet assay metrics tail moment, tail length, and olive moment. Preparation of samples, imaging, capture, and analysis were conducted using a double-blind approach.

Controls for the modified comet assay are discussed in Appendix C (on the HEI Web site, at *http://pubs.healtheffects.org*).

BRADFORD PROTEIN ASSAY

To normalize data for the 8-OHdG ELISA assay, DNA quantification was considered. However, in a small pilot study, we were not able to isolate sufficient amounts of DNA to allow for duplicate samples when assaying for 8-OHdG. The decision was therefore made to use whole serum and to use protein content to correct the results. In addition, because 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. The Bradford protein microassay (Cell BioLabs, San Diego, CA) was therefore used, per the manufacturer's instructions.

Preparation of Standards

Bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used to produce a standard curve for the analysis of protein content, as follows. The BSA was weighed and then diluted with molecular-grade water to prepare a concentrated (> 10 mg/mL) solution. The actual protein concentration of the stock solution 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 spectrophotometric reading. The standard was aliquoted and kept frozen until use. On the day of the assay the 10 mg/mL stock solution was diluted with molecular-grade water to 1.6 mg/mL, which was then further diluted to prepare additional standard solutions of 800, 400, 200, and 100 µg/mL. The remaining standards were prepared by serially diluting the 1.6 mg/mL solution 1:1 with water. Twenty-five µL of these standards were aliquoted into tubes, in which they were mixed with 6.25 µL Bradford dye reagent concentrate (Bio-Rad, Hercules, CA) and incubated for 5 minutes at room temperature before being read in a spectrophotometer (Nanodrop, 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 animal was transferred to a 1-mL centrifuge tube (Eppendorf North America, New York, NY) and diluted 1:10 with molecular-grade water, free of DNase and RNase, to a final volume of 50 µL. The samples were assayed in small batches to prevent long-term incubation. Bradford dye reagent concentrate (6.25 µL) was added to each batch as in the standard preparation protocol and mixed, and the mixture was allowed to incubate for 5 minutes at room temperature. Three-µL aliquots were placed on the platform of the spectrophotometer and were measured 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, as a result of exposure to carcinogenic compounds. The formation of 8-OHdG, a byproduct of the removal of oxidative DNA damage by exonucleases, serves as a useful biologic marker of DNA oxidative damage. After excision, 8-OHdG is excreted without being metabolized further. For the detection and quantitation of 8-OHdG in serum samples, we used a competitive enzyme immunoassay (OxiSelect Oxidative DNA Damage ELISA Kit; Cell BioLabs, San Diego, CA) according to the manufacturer's recommendations. In this immunoassay, an 8-OHdG standard curve, measured at 450 nm, was used to quantify 8-OHdG in the samples. The sensitivity range of the immunoassay was 100 to 2000 pg/mL, and concentrations of 8-OHdG in samples were expressed as pg/mL/mg protein.

Controls for the 8-OHdG assay are discussed in Appendix C.

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 MDA. The measurement of lipid peroxide byproducts is one of the most widely accepted assays for oxidative damage. We used a commercial assay (OxiSelect TBARS Assay, Cell BioLabs) to assess 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. The TBARS concentrations in samples were expressed as µM MDA/mg protein in tissue.

Preparation of Samples for Assay

Whole-brain tissues from rats were rapidly extracted and snap frozen at –80°C. Tissues were received from LRRI and were stored at –80°C. For processing, whole or half brains were thawed and then dissected in chilled PBS supplemented with 1× butylated hydroxytoluene (BHT) (to inhibit further oxidation of the tissue during excision and assay) to isolate hippocampi. The 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 was transferred to a new tube for protein and TBARS analysis.

Controls for the TBARS assay are discussed in Appendix C.

STATISTICAL METHODS AND DATA ANALYSIS

Comet Assay

Differences among treatment groups at the specific time points were tested using general linear models (analysis of variance, or ANOVA). Post-hoc comparisons were made only when the overall ANOVA was significant using a Bonferroni correction. In those cases, interactions among other factors (e.g., sex) could be examined as well. These analyses were conducted using statistical software (SPSS 16.1, IBM, Armonk, NY). A P value of less than 0.05 was used as our cutoff for statistical significance.

We also analyzed a subset of the data with and without pyknotic nuclei to explore possible differences in statistical test outcomes. Whether or not samples with such nuclei were included did not affect the results or statistical test outcomes. Pyknotic samples were not restricted to any one treatment group, exposure duration, or sex.

8-OHdG Statistical Analysis

We first examined the distribution of the 8-OHdG data. Descriptive statistics were used to examine the mean and standard error (SE) of the outcomes by sex, exposure groups (low, mid, and high exposures versus control), and duration. ANOVA was used when the data were normally distributed. If the data did not meet normality or variance criteria, the Student–Newman–Keuls nonparametric method was used to examine the exposure effects, controlling for sex and stratifying by duration. Because the analysis of the total data set showed the main effect to be from duration, individual duration time-point data were subjected to two-way ANOVA comparing sex and exposure effects. If the data did not meet normality or variability criteria, the Student–Newman–Keuls method was again used for comparisons. The concentrations of 8-OHdG (expressed as pg/mL/mg protein) were analyzed using general linear models in data analysis software (SigmaPlot, version 11, SYSTAT, San Jose, CA).

TBARS Statistical Analysis

The distribution of the TBARS data was examined. Descriptive statistics were used to examine the mean and standard deviation (SD) of the outcomes (TBARS corrected for protein, expressed as μM MDA/mg tissue) by sex, exposure groups (low, mid, and high exposures versus control), and duration. ANOVA was used when data were normally distributed. If the data did not meet normality or variance criteria, the Student–Newman–Keuls nonparametric method was used to examine the exposure effects, controlling for sex and stratifying by duration. Because the analysis of the total data set showed the main effect to be from duration, individual duration time-point data were subjected to two-way ANOVA comparing sex and exposure effects. If the data did not meet normality or variability criteria, the Student–Newman–Keuls method was again used for comparisons. Analysis was performed using SigmaPlot software.

RESULTS

DNA DAMAGE: MODIFIED COMET ASSAY IN LUNG TISSUE

The modified comet assay was performed on the lung tissue from a total of 155 rats. Samples were obtained from five animals of each sex in each (exposure or control) group at every exposure time point, except at 1 month, when there were only 4 females in each group and 4 males in the highest DE group. Analysis of the data from the assay was conducted on tail length (i.e., the comet head diameter subtracted from the overall comet length) and %DNA in tail. Analyses of exposure groups, exposure duration, and sex effects were also performed.

1- and 3-Month Exposures

After 1 month of exposure in rats, no significant differences were noted in any comparisons of groups exposed to various levels of DE and the control animals, nor were there any statistically significant effects by sex when tail moment data were used (Table B.1 in Appendix B, available on the HEI Web site; Figure 1A). Similar results were observed for tail length data (Table B.2; Figure 2A). All samples were visually screened for the presence of pyknotic cells (Figure 3). Pyknotic cells were scored, along with non-pyknotic cells, because of the blind manner in which the data were collected. Analyses of comet measures were conducted both with and without the pyknotic cells. No significant differences with respect to sex or exposure levels versus the controls were noted for the %DNA in the tail in the 1-month group (Table B.3; Figure 4A).

After 3 months of exposure in rats, no significant differences were noted using either tail moment (Table B.4; Figure 1B) or tail length (Table B.5). No significant differences with respect to sex or exposure levels versus the controls were noted for the %DNA in the tail in the 3-month group (Table B.6; Figure 4B).

Our results showed no significant differences between the 1- and 3-month samples and no significant interactions among the factors. A few samples had cells with pyknotic nuclei (i.e., four rats in the 1-month group, including one control and three in the high-exposure group, and five rats in the 3-month group, including one in the low-exposure

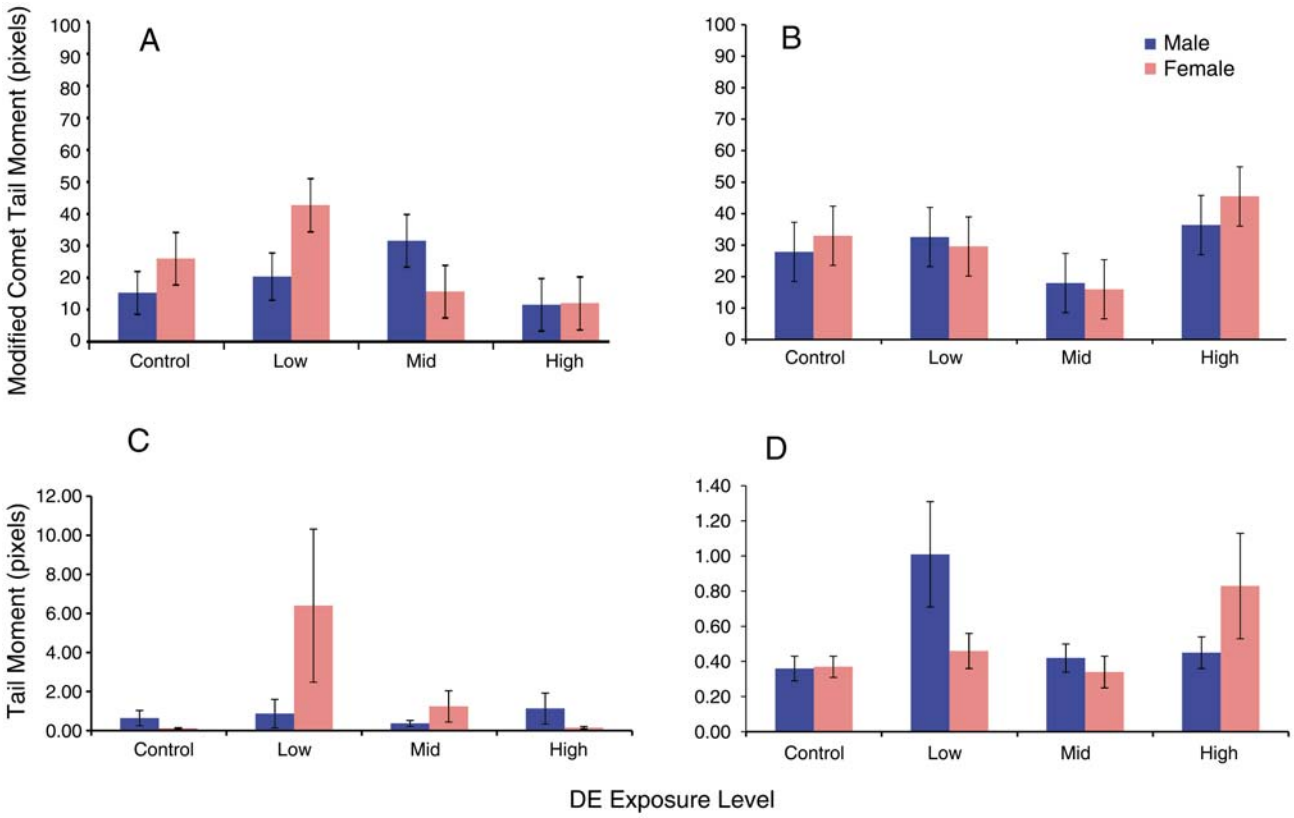

Figure 1. Tail moment data from modified comet assay in rat lung tissue (for details on analyses see Methods section). No significant differences were found for **(A)** the 1-month-exposure group, **(B)** the 3-month-exposure group, **(C)** the 12-month-exposure group, and **(D)** the 24-month-exposure group. (Please note differences in *y*-axis scales.) No values significant; all *P* values > 0.05.

group, three in the mid-exposure group, and one in the high-exposure group), but these were not restricted to any exposure group, sex, exposure duration, or sample processing batch. Analyses of the %DNA in the tail were done with and without these samples. The results were unchanged (Figure 4).

12- and 24-Month Exposures

Forty rat samples (20 female and 20 male) in each of the 12- and 24-month-exposure experiments were prepared and analyzed. Two rats in each experiment had pyknotic nuclei and were excluded from further analyses. One rat in each experiment failed to yield an adequate sample after multiple attempts and was also excluded from further analyses. In the 12-month-exposure experiment, an average of 68.8 nuclei (ranging from 57 to 94 nuclei) per rat sample were analyzed. In the 24-month-exposure experiment, an average of 60.0 nuclei (ranging from 46 to 86 nuclei) per rat sample were analyzed, with the exception of two rat samples in which a total of 25 and 26 nuclei, respectively, were analyzed.

In the 12-month experiment, there were no significant differences in comet assay measures between females and males (tail moment [Table B.7; Figure 1C], tail length

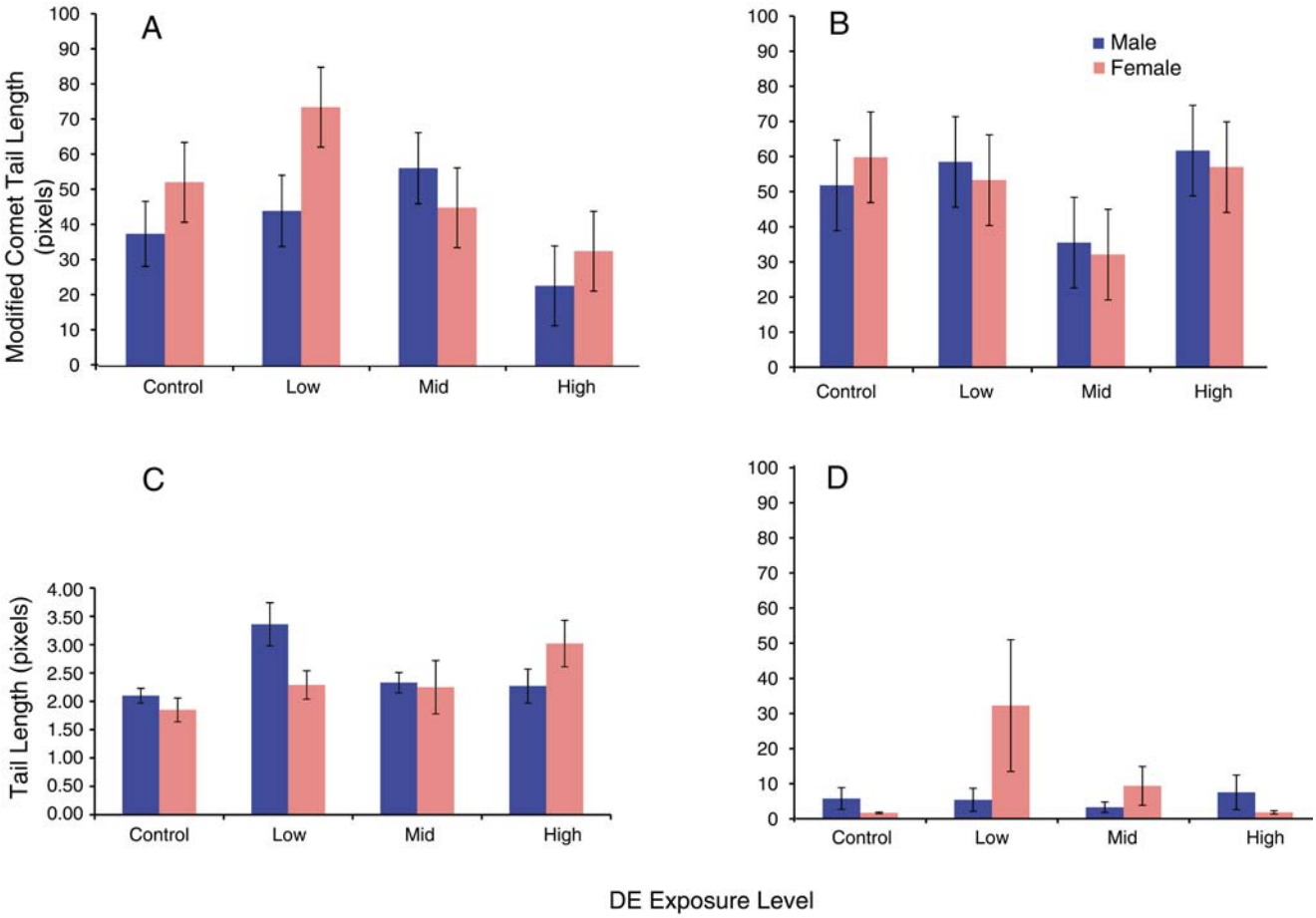

Figure 2. Tail length data from modified comet assay in rat lung tissue. For the 1-month exposure group **(A)**, a significant difference was found between the low- and high-exposure levels ($P = 0.04$). For the 3-month **(B)**, 12-month **(C)**, and 24-month **(D)** exposure groups, no values were significant; all P values > 0.05. (Please note differences in y-axis scales.)

[Table B.8; Figure 2C], %DNA in tail [Table B.9; Figure 5A], and olive moment [Table B.9; Figure 6A). Likewise, in the 24-month experiment, there were no significant differences in comet measures between females and males (tail moment [Table B.10; Figure 1D], tail length [Table B.11; Figure 2D], %DNA in tail [Table B.12; Figure 5B], and olive moment [Table B.12; Figure 6B]). For all subsequent statistical testing, the sexes were combined. No significant differences were observed in the 12-month experiment among any of the exposure groups (for tail length, %DNA in tail, tail moment, and olive moment). Similarly, no significant differences were observed in the 24-month experiment among any of the exposure groups (for tail length, for %DNA in tail, for tail moment, and for olive moment).

DNA DAMAGE: 8-OHdG ELISA ASSAY IN SERUM

Samples of serum from 151 rats were analyzed using the 8-OHdG assay. The samples were obtained from five animals of each sex from each exposure group (including the control) and each exposure duration, except the 12-month exposure group (which had four males and four females in the control and low-exposure groups, four males and three females in the mid-exposure group, and three males in the

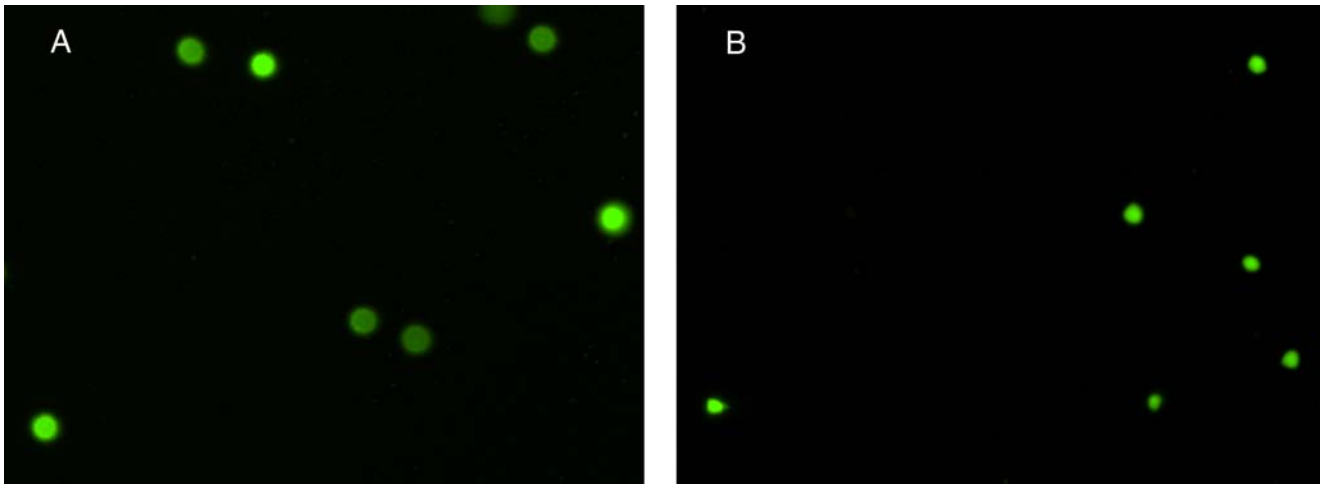

Figure 3. Representative photographs of comet assay results showing non-pyknotic and pyknotic nuclei. (A) Typical nuclei that were not considered to be comparatively pyknotic (average head diameter = 105 pixels). **(B)** A rare sample considered to be comparatively pyknotic (average head diameter = 44 pixels).

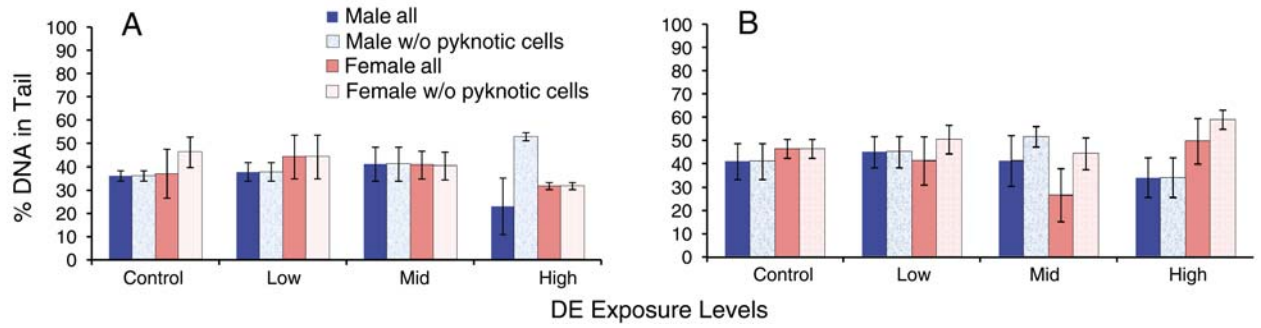

Figure 4. %DNA tail data from modified comet assay in rat lung tissue. (A) For 1-month-exposure group. **(B)** For 3-month-exposure group. Data were analyzed with or without pyknotic cells. No significant differences were noted between exposure levels and control. Data shown are the mean %DNA ± SEM.

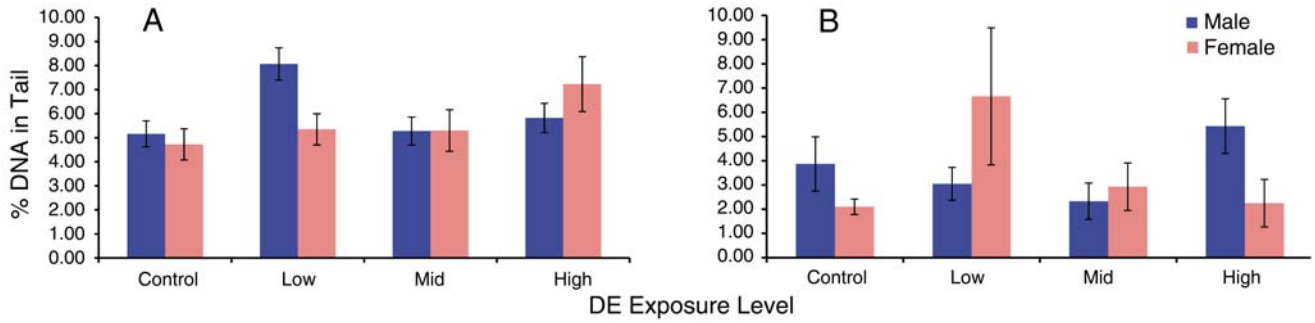

Figure 5. %DNA tail data from modified comet assay in rat lung tissue. (A) For 12-month-exposure group. **(B)** For 24-month-exposure group. No values significant; all *P* values > 0.05.

high-exposure group). Table B.13A shows the results for the 1- and 3-month exposure durations. There were no significant differences between controls and exposure groups or differences by sex (Table B.13B; Figure 7A and B; $P > 0.05$). The concentrations of 8-OHdG in all exposure groups at 3 months were higher than those of exposure groups at any other time point ($P < 0.05$). Looking at the levels of 8-OHdG in serum in the 12- and 24-month groups, we saw a significant difference from control in the 12-month group at the mid level in males and at the high level in

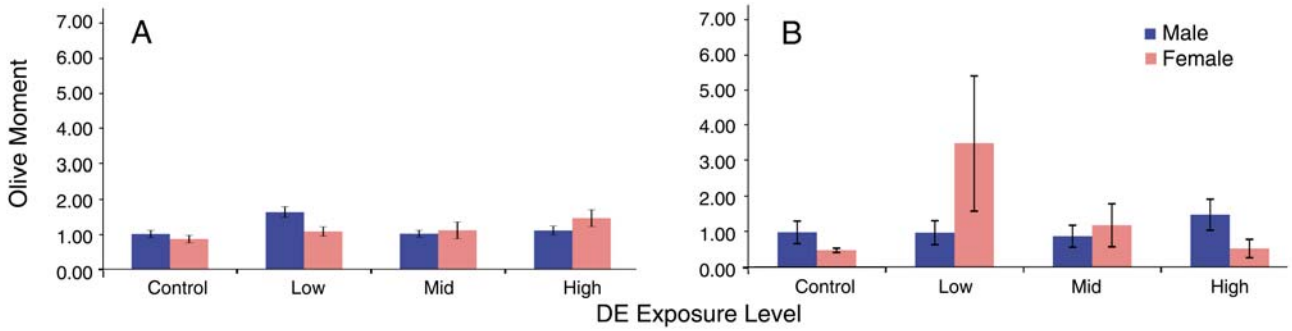

Figure 6. Olive moment data from modified comet assay in rat lung tissue. (A) For 12-month-exposure group. **(B)** For 24-month-exposure group. No values significant; all P values > 0.05.

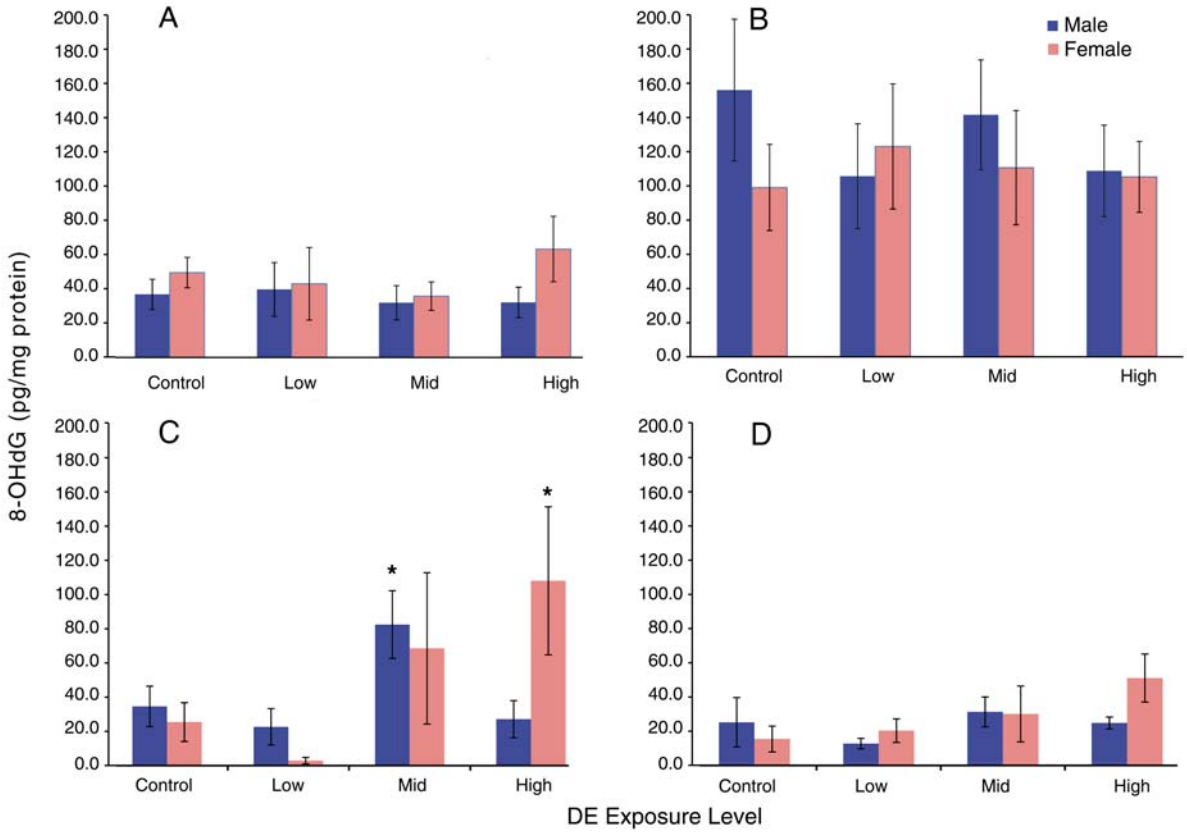

Figure 7. 8-OHdG assay data in rat serum. (A) For 1-month-exposure group, **(B)** for 3-month-exposure group, **(C)** for 12-month-exposure group, and **(D)** for 24-month-exposure group. * $P < 0.05$.

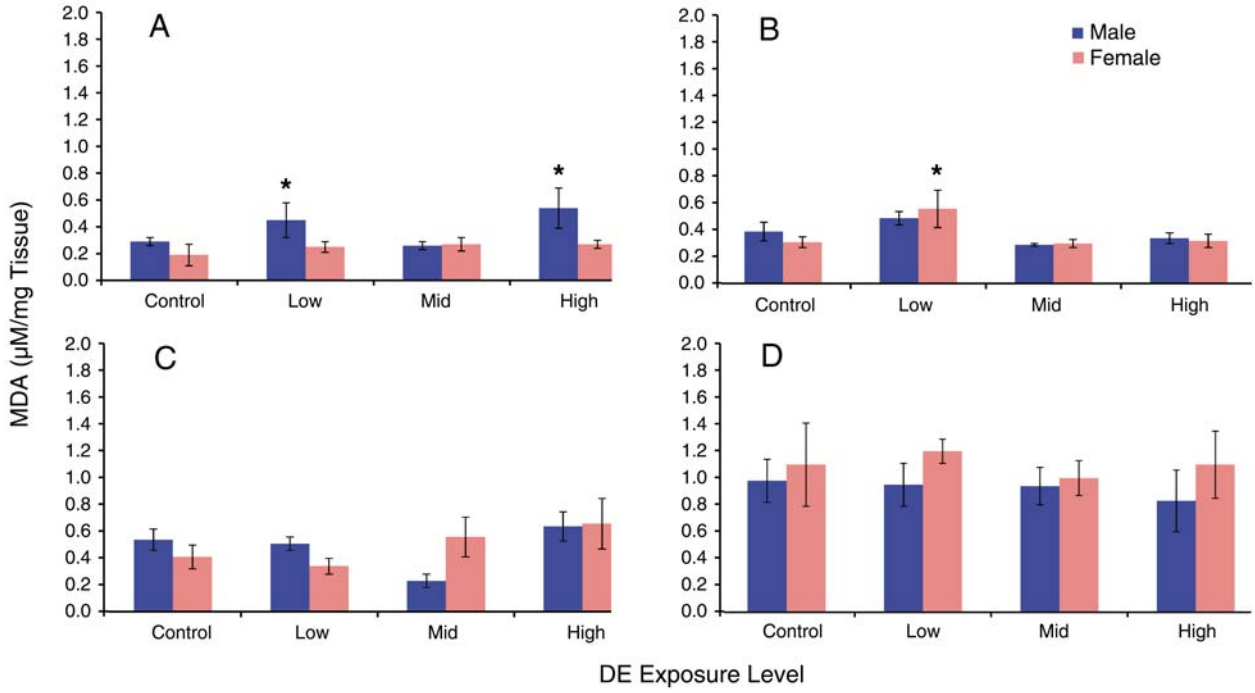

Figure 8. TBARs assay data in rat hippocampal tissue. (A) For 1-month-exposure group (* $P < 0.05$ for the low and high groups, males), **(B)** for 3-month-exposure group (*$P < 0.05$ for the low group, females), **(C)** for 12-month-exposure group, and **(D)** for 24-month-exposure group.

females ($P < 0.05$), as well as some other scattered changes. Sex differences were noted in the 12-month high-level group ($P < 0.05$). However, these differences did not follow an exposure-dependent pattern. All other comparisons were not significant (Tables B.14A and B.14B; Figure 7C and D; $P > 0.05$).

LIPID PEROXIDATION: TBARS ASSAY IN HIPPOCAMPUS

Samples of cells from the hippocampi of 158 rats were analyzed using the TBARS assay. The samples were obtained from five rats of each sex in each exposure group (including the control) and each exposure duration, except the 3-month exposure group, which had only four males in the control and mid-exposure groups. Figure 8 and Tables B.15A (1 and 3 months) and B.16A (12 and 24 months) show the results for all time points. Analysis (see Methods section) of the TBARS data (Tables B.15B and B.16B) showed some small and scattered changes in groups exposed to different levels of DE and at different time points, but we did not consider these to be exposure-related. There was a duration effect — specifically, the baseline MDA levels in all groups at 24 months were higher than at 1, 3, and 12 months.

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 of DE have also been attributed to its ability to induce genotoxicity, caused to a large extent by DE's particulate fraction, especially UFPs (Peters et al. 2006; Risom et al. 2005). This attribution is based on the supposition that particulates can exert their effects either directly on tissue, by producing oxidative damage to lipids, proteins, or DNA, or indirectly, by the induction of an inflammatory response and the recruitment of inflammatory cells that in turn induce oxidative damage. UFPs tend to be deposited deeper in the lungs than larger particulates and to have easier access to the bloodstream and tissue. DEPs, in particular, have hundreds of chemicals adsorbed onto them (Lai et al. 2005), including PAHs, which are thought to be a substantial contributor to the health effects of PM from motor vehicles (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 motor vehicles. NO_x can be transformed into NO_2 in the presence of ozone (WHO 2000). In vitro and in vivo studies in humans and animals all demonstrate that NO_2 can activate oxidant pathways (Bayram et al. 2001).

Earlier 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 DE. In the current study, we sought to determine whether DNA damage and lipid peroxidation would continue to be problematic with emissions from 2007-compliant engines.

Our approach to answering this question was to determine the extent of DNA damage as a function of adduct formation and strand breaks in male and female rats exposed to DE. Additionally, we wanted to confirm our findings by looking for the stable DNA-adduct excision products resulting from DNA repair found in serum in the form of 8-OHdG, a ubiquitous marker of oxidative stress. 8-OHdG is physiologically formed and can be increased 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.

To further confirm the presence of systemic oxidative stress, we investigated whether DE-induced oxidative stress could occur in the brain. Previous studies (Calderón-Garcidueñas et al. 2003, 2010) have indicated that toxicants might be able to find their way into the brain, by either bypassing the blood–brain barrier or passing along the olfactory bulb and migrating into the hippocampus. We therefore investigated whether lipid peroxidation, a well-defined mechanism of cellular damage in animals, was taking place in the hippocampal region. Lipid peroxides, formed by peroxidation, are unstable indicators of oxidative stress that decompose to form more complex and reactive compounds, such as MDA and 4-hydroxynonenal, natural byproducts of lipid peroxidation.

When our study was first proposed, we hypothesized that the induction of oxidative damage would be the result of exposure to DE gases and especially DEP. However, 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 DEP-induced oxidative stress would likely decrease, because it was DEP and in particular its PAH components that were most often associated with the observed health effects. However, because NO_x was still present in DE and could induce oxidative stress, it remained a concern for adverse health effects.

The assays we used in this study were selected based on the experience and expertise in our group. There was some concern that these assays would record only short-term damage from the exposures, and to a certain extent this is true. However, if the body suffers an exposure to a genotoxicant, thus damaging DNA, but is able to repair the damage efficiently by way of a mechanism with a low error rate, then the exposure might be of reduced biologic relevance.

GENOTOXICITY OF DE IN RATS AND HUMANS

The comet assay is an appropriate test for DNA damage, because it examines a broad range of oxidized purines and pyrimidines in DNA (Møller 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 the DNA of lymphocytes to be higher among the workers than among the controls (Bagryantseva et al. 2010). Similarly, in a Danish study using the comet assay, increased DNA damage was detected in people who bicycled in traffic (Vinzents et al. 2005). The results were attributed to UFPs in the air. UFP concentrations were associated with CO concentrations, temperature, and NO_2 concentrations, as measured at fixed stations. In a study by Müller 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 did not show any significant exposure-related increases in DNA damage in rats as measured by the comet assay, compared with controls. Further, no differences in DNA damage were found between sexes or in comparisons between the exposure groups. It was noted that the background (i.e., control) levels for the comet assay for the 1- and 3-month samples on the one hand and the 12- and 24-month samples on the other hand were different. It is possible there is some age-related biologic explanation for these differences. However, we are not aware of any study specifically examining age-related changes in genomic damage as measured by the comet assay in rodent lung tissues to either support or refute this possibility. No changes were made to the laboratory methods or source materials used for the comet assay.

Another possible explanation for the difference is that we had to change microscope platforms during the experiments. The long-term nature of the study and normal problems with core equipment dictated that we make this switch.

For the 1- and 3-month samples, which were all processed at the same time, data were captured using the core (standard fluorescent) microscope in the J. Bennett Johnson facility at Tulane University in New Orleans, Louisiana. For the 12- and 24-month samples, processed together but not at the same time as the 1- and 3-month exposure samples, data were captured using the new core (inverted fluorescent) microscope in the Louisiana Cancer Research Consortium facility in New Orleans. Our interpretation is that the differences between background (control) concentrations in the 1- and 3-month samples and the 12- and 24-month samples likely resulted from technical differences in sample preparation and data capture. Consequently, comparing results from the 1- and 3-month samples with those from the 12- and 24-month samples is not advised or should be undertaken with caution. However, comparing exposure–response data within individual duration groups, across the 1- and 3-month groups, and across the 12- and 24-month groups, would be appropriate.

We also analyzed the data for samples with and without the pyknotic nuclei to explore possible differences in statistical test outcomes. This approach did not affect the results or statistical test outcomes.

The comet assay as conducted under these conditions can detect a limited number of types of known DNA damage. If there are repair processes for these types of damage, then we would argue that the repaired damage poses little or no future health or disease risk. This argument assumes that the rats used in our study adequately modeled the complexity of human DNA repair processes — an assumption that is likely not justified for all possibilities when considering the variations in human responses to toxicologic and genotoxicologic insults. We would argue that every exposure has relevance, though whether every exposure results in an increased health risk to the exposed individuals is a separate issue. It is possible that other types of DNA damage occurred as a result of the exposures in the study that our assays were incapable of detecting. If that is the case, these undetected or undetectable instances of DNA damage might well pose a future health or disease risk in exposed individuals. Variation in 8-OHdG concentrations has been noted in epidemiologic studies in populations exposed to DE. In a recent study (Lee et al. 2012), it was observed that an increase in urinary 8-OHdG in DE emissions inspectors was correlated with increases in PAH concentrations, but in a study by Harri and colleagues (2005), no differences in urinary 8-OHdG were observed between garage workers, waste collectors, and controls. Our findings using the 8-OHdG assay supported the lack of DNA damage (with no sex effects) seen in the comet assay in all four exposure-duration groups at all three exposure levels.

LIPID PEROXIDATION IN BRAIN TISSUE

Acute, subchronic, and chronic exposures to PM and pollutant gases can affect people (Anderson et al. 2012). 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. The researchers found that nasal, respiratory, and olfactory epithelia were early targets of air pollution. Both the olfactory bulbs and the hippocampi of the exposed dogs were found to have significantly higher numbers of abasic sites than those of the control animals. Additionally, increased inflammation-distress proteins were found in the brains of the exposed dogs, providing evidence that respiratory tract inflammation and deterioration of the olfactory barrier might play an important role in this neuropathy. Calderón-Garcidueñas and colleagues (2004) found that autopsied human brain tissue from cognitively and neurologically intact individuals who had been exposed to high concentrations of air pollution had significantly higher COX-2 expression in the frontal cortex of the hippocampus compared with the brain tissue of individuals who had been residents of cities with no air pollution (controls). These findings suggest that exposure to severe air pollution is associated with inflammation responses in the brain (Calderón-Garcidueñas et al. 2004). Additionally, Mexico City residents exposed to severe air pollution exhibited olfactory-bulb inflammation and endothelial hyperplasia. UFPs were observed in olfactory endothelial cytoplasm and basement membranes (Calderón-Garcidueñas et al. 2010). Furthermore, Gerlofs-Nijland and colleagues (2010) investigated the effects of controlled exposure to DE in rats. The rats 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 brains were dissected into cerebellum, frontal cortex, hippocampus, olfactory bulb and tubercles, and striatum. Baseline levels of pro-inflammatory 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 rats revealed no significant differences between exposed groups. A nearly significant difference between sexes in the rats was observed.

STUDY LIMITATIONS

A critical limitation of the study we conducted was the lack of a DE-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 those samples with the current samples might have given us a better benchmark for the effects of the engineering changes associated with the newer type of engine. Nonetheless, the use of cumulative NO_2 exposures in our study that were very similar to those of earlier studies by Mauderly and colleagues (1987, 1989) was a key feature that facilitated comparisons between the studies.

In the newer engines, particles in the range of 2 to 10 μm would be decreased in level, but would still emit UFPs, possibly even at a higher level than in the older engines, leaving open the possibility of adverse health effects as a result of particle-associated oxidative stress. Because the DE in our study was not aged, as might normally be encountered in ambient air, we speculated that any particulates released by the study engine would be more reactive and therefore more potent than those in aged DE. Thus, the lack of oxidative-type damage, as assessed by our study, provides evidence that the newer engines appear to be running cleaner, with less induction of stress-associated effects.

Another concern involved the amount of time between the end of the exposures and the harvesting of tissues. It was pointed out that this was a possible confounder, that is, a possible explanation for the lower levels of oxidative damage noted in the study, in that from the time the exposures ended until the tissue samples were processed cellular DNA was being repaired. We therefore measured the time between the end of exposures and the end of sample processing. In the rats exposed for 1 month, the processing of samples from females averaged 2 hours and 31 minutes and of samples from males, 3 hours and 41 minutes. In the rats exposed for 3 months, the processing of samples from females averaged 4 hours and 11 minutes (which was longer than the averages for the other groups because one female was processed earlier in the day than the rest) and of samples for males, 3 hours and 21 minutes. In summary, processing time ranged from 1 hour and 23 minutes to 4 hours and 11 minutes for the females and from 1 hour and 37 minutes to 3 hours and 42 minutes for the males. It would be expected that DNA repair mechanisms would continue to mitigate damage caused by the exposure to DE during these processing times. However, two points need to be made: (1) if DNA was being repaired during these times, one would expect that 8-OHdG concentrations would have increased in the serum as a result of the repairs and should have been detected; and (2) if any damage induced by the exposure could be repaired in an efficient, error-free manner in such a short amount of time, it is our contention that it could be considered biologically irrelevant. The more important concern would involve DNA damage that was persistent. This type of damage would have several biologic fates: (1) it could induce cell-cycle delay, insofar as the 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. Mutation 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. Additionally, as stated in our discussion above, this assumes that the rats used in the study adequately modeled the complexity of humans in terms of DNA repair processes — an assumption that is likely not justified for all possibilities when considering the variations in human responses to toxicologic and genotoxicologic insults. We would argue that every exposure has relevance, though whether every exposure results in an increased health risk to the exposed individual is a separate issue. It is possible that other types of DNA damage occurred as a result of the exposures in the study that our assays were incapable of detecting. If that were the case, these undetected or undetectable instances of DNA damage might well pose a future health or disease risk in exposed individuals.

IMPLICATIONS OF FINDINGS

In our experimental design, we anticipated that the presence of particulates, SO_2, and NO_x in the DE exposures evaluated, which have been implicated in the induction of oxidative stress, might result in DNA damage. No statistically significant differences between results for controls and exposure levels were observed, and there were no statistically significant differences in any of the DE exposure groups we tested, as compared with controls. Thus, although a few of our analyses showed statistically significant differences between some groups — and are intriguing as observations — they are not likely to be biologically important from the perspective of DE effects.

The exposures in this study were chronic and continuous in nature and not short-term or acute. There was not a lengthy recovery period following the exposures, based on our understanding of the exposure design. The assays we used might thus record a chronic or continuous increase in oxidative stress or DNA damage that might result from such exposures. The exposures ended just before killing the rats and harvesting their tissue. It is possible that oxidative stress or DNA damage was induced either early in

the exposures or throughout. If the oxidative stress or DNA damage occurred early and the animals adapted in some way to mitigate either or both biomarkers, then our assays would not have been able to measure these processes, and we would not have found increases in either biomarker. If the increased oxidative stress or DNA damage occurred throughout the exposures but antioxidant capacity or DNA repair were upregulated, it is possible that the brief time between the end of the exposures and death might have allowed for mitigation of such chronic effects. In this case, too, our assays would not have found increases in their respective endpoints. It is also possible that such adaptations simply restored the biomarker responses as we measured them to levels that resembled those of the controls. Therefore, based on the endpoints we evaluated and the assays used, we conclude that the 2007-compliant DE did not produce significant or persistent oxidatively induced DNA damage to rats at the exposure levels we tested.

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REFERENCES

Anderson JO, Thundiyil JG, Stolbach A. 2012. Clearing the air: a review of the effects of particulate matter air pollution on human health. J Med Toxicol 8:166–175.

Ayres JG, Borm P, Cassee FR, Castranova V, Donaldson K, Ghio A, et al. 2008. Evaluating the toxicity of airborne particulate matter and nanoparticles by measuring oxidative stress potential — a workshop report and consensus statement. Inhal Toxicol 20:75–99.

Bagryantseva Y, Novotna B, Rossner P Jr, Chvatalova I, Milcova A, Svecova V, et al. 2010. Oxidative damage to biological macromolecules in Prague bus drivers and garagemen: impact of air pollution and genetic polymorphisms. Toxicol Lett 199:60–68.

Bao L, Chen S, Wu L, Hei TK, Wu Y, Yu Z, et al. 2007. Mutagenicity of diesel exhaust particles mediated by cell-particle interaction in mammalian cells. Toxicology 229:91–100.

Bayram H, Sapsford RJ, Abdelaziz MM, Khair OA. 2001. Effect of ozone and nitrogen dioxide on the release of pro-inflammatory mediators from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients in vitro. J Allergy Clin Immunol 107:287–294.

Beck-Speier I, Dayal N, Karg E, Maier KL, Schumann G, Schulz H, et al. 2005. Oxidative stress and lipid mediators induced in alveolar macrophages by ultrafine particles. Free Radic Biol Med 38:1080–1092.

Bond JA, Johnson NF, Snipes MB, Mauderly JL. 1990. DNA adduct formation in rat alveolar type II cells: cells potentially at risk for inhaled diesel exhaust. Environ Mol Mutagen. 16:64–69.

Calderón-Garcidueñas L, Franco-Lira M, Henríquez-Roldán C, Osnaya N, González-Maciel A, Reynoso-Robles R, et al. 2010. Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. Exp Toxicol Pathol 62:91–102.

Calderón-Garcidueñas L, Maronpot RR, Torres-Jardon R, Henríquez-Roldán C, Schoonhoven R, Acuña-Ayala H, et al. 2003. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. Toxicol Pathol 31:524–538.

Calderón-Garcidueñas L, Reed W, Maronpot RR, Henríquez-Roldán C, Delgado-Chavez R, Calderón-Garcidueñas A, et al. 2004. Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. Toxicol Pathol 32:650–658.

Chen L, Lee HM, Greeley GH Jr, Englander EW. 2007. Accumulation of oxidatively generated DNA damage in the brain: a mechanism of neurotoxicity. Free Radic Biol Med 42:385–393.

Donaldson K, Brown D, Clouter A, Duffin R, MacNee W, Renwick L, et al. 2002. The pulmonary toxicology of ultrafine particles. J Aerosol Med 15:213–220.

Dybdahl M, Risom L, Bornholdt J, Autrup H, Loft S, Wallin H. 2004. Inflammatory and genotoxic effects of diesel particles in vitro and in vivo. Mutat Res 562:119–131.

Gerlofs-Nijland ME, van Berlo D, Cassee FR, Schins RP, Wang K, Campbell A. 2010. Effect of prolonged exposure to diesel engine exhaust on proinflammatory markers in different regions of the rat brain. Part Fibre Toxicol 7:12.

Hallberg LM, Ward JB, Hernandez C, Ameredes BT, Wickliffe JK. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Harri M, Svoboda P, Mori T, Mutanen P, Kasai H, Savela K. 2005. Analysis of 8-hydroxydeoxyguanosine among workers exposed to diesel particulate exhaust: comparison with urinary metabolites and PAH air monitoring. Free Radic Res 39:963–972.

Ichinose T, Yajima Y, Nagashima M, Takenoshita S, Nagamachi Y, Sagai M. 1997. Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. Carcinogenesis 18:185–192.

Kenyon N, Liu F-T. 2011. Pulmonary effects of diesel exhaust: neutrophilic inflammation, oxidative injury, and asthma. Am J Pathol 179:2678–2682.

Lai CH, Liou SH, Lin HC, Shih TS, Tsai PJ, Chen JS, et al. 2005. Exposure to traffic exhausts and oxidative DNA damage. Occup Environ Med 62:216–222.

Lamy E, Kassie F, Gminski R, Schmeiser HH, Mersch-Sundermann V. 2004. 3-Nitrobenzanthrone (3-NBA) induced micronucleus formation and DNA damage in human hepatoma (HepG2) cells. Toxicol Lett 146:103–109.

Lee MW, Chen ML, Lung SC, Tsai CJ, Lai CF, Yang SC, et al. 2012. Increase of urinary concentrations of 8-hydroxy-2′-deoxyguanosine in diesel exhaust emission inspector exposed to polycyclic aromatic hydrocarbons. Int Arch Occup Environ Health 85:273–282.

Lewtas J. 2007. Air pollution combustion emissions: characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. Mutat Res 636:95–133.

Lundborg M, Bouhafs R, Gerde P, Ewing P, Camner P, Dahlén SE, et al. 2007. Aggregates of ultrafine particles modulate lipid peroxidation and bacterial killing by alveolar macrophages. Environ Res 104:250–257.

Mauderly JL. 2000. Animal models for the effect of age on susceptibility to inhaled particulate matter. Inhal Toxicol 12:863–900.

Mauderly JL, Bice DE, Carpenter RL, Gillett NA, Henderson RF, Pickrell JA, et al. 1987. Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung. Research Report 8. Health Effects Institute, Cambridge, MA.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, et al. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust. Research Report 30. Health Effects Institute, Cambridge, MA.

Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study (ACES). Phase 3A: Characterization of U.S. 2007-Compliant Diesel Engine and Exposure System Operation. Communication 17. Boston, MA:Health Effects Institute.

Møller P. 2006. The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. Basic Clin Pharmacol Toxicol 98:336–345.

Mühlfeld C, Rothen-Rutishauser B, Blank F, Vanhecke D, Ochs M, Gehr P. 2008. Interactions of nanoparticles with pulmonary structures and cellular responses. Am J Physiol Lung Cell Mol Physiol 294:L817–L829.

Müller AK, Farombi EO, Møller P, Autrup HN, Vogel U, Wallin H, et al. 2004. DNA damage in lung after oral exposure to diesel exhaust particles in Big Blue rats. Mutat Res 550:123–132.

Peters A, Veronesi B, Calderón-Garcidueñas L, Gehr P, Chen LC, Geiser M, et al. 2006. Translocation and potential neurological effects of fine and ultrafine particles: a critical update. Part Fibre Toxicol 3:13.

Pope CA III, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, et al. 2002. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. JAMA 287:1132–1141.

Riedl M, Diaz-Sanchez D. 2005. Biology of diesel exhaust effects on respiratory function. J Allergy Clin Immunol 115:221–228.

Risom L, Dybdahl M, Bornholdt J, Vogel U, Wallin H, Møller P, et al. 2003. Oxidative DNA damage and defence gene expression in the mouse lung after short-term exposure to diesel exhaust particles by inhalation. Carcinogenesis 24:1847–1852.

Risom L, Møller P, Loft S. 2005. Oxidative stress-induced DNA damage by particulate air pollution. Mutat Res 592:119–137.

Terzano C, Di Stefano F, Conti V, Graziani E, Petroianni A. 2010. Air pollution ultrafine particles: toxicity beyond the lung. Eur Rev Med Pharmacol Sci 14:809–821.

Tokiwa H, Ohnishi Y. 1986. Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. Crit Rev Toxicol 17:23–60.

Tokiwa H, Sera N, Nakanishi Y. 2005. Involvement of alveolar macrophages in the formation of 8-oxodeoxyguanosine associated with exogenous particles in human lungs. Inhal Toxicol 17:577–585.

Tokiwa H, Sera N, Nakanishi Y, Sagai M. 1999. 8-Hydroxyguanosine formed in human lung tissues and the association with diesel exhaust particles. Free Radic Biol Med 27: 1251–1258.

Vinzents PS, Møller P, Sørensen M, Knudsen LE, Hertel O, Jensen FP, et al. 2005. Personal exposure to ultrafine particles and oxidative DNA damage. Environ Health Perspect 113:1485–1490.

World Health Organization. 2000. Air Quality Guidelines for Europe, 2nd edit. Regional Publications, European Series No. 91. WHO, Copenhagen, Denmark.

MATERIALS AVAILABLE ON THE WEB

Appendices A, B, and C contain supplemental material not included in the printed report. They are available on the HEI Web site *http://pubs.healtheffects.org*.

Appendix A. Power Calculation

Appendix B. Data Tables for All Results

Appendix C. Positive Technical Controls

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OTHER PUBLICATION RESULTING FROM THIS RESEARCH

Hallberg LM, Ward JB, Hernandez C, Ameredes BT, Wickliffe JK. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

ABBREVIATIONS AND OTHER TERMS

3-NBA	3-nitrobenzanthrone
8-OHdG	8-hydroxy-deoxyguanosine
ACES	Advanced Collaborative Emissions Study
A/L	human–hamster hybrid
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CARB	California Air Resources Board
CO	carbon monoxide
DE	diesel exhaust
DEP	diesel exhaust particulate
ELISA	enzyme-linked immunosorbent assay
EPA	(U.S.) Environmental Protection Agency
HepG2	a human hepatoma cell line
LRRI	Lovelace Respiratory Research Institute
MDA	malondialdehyde
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
NTDE	new-technology diesel exhaust
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate-buffered saline
PM	particulate matter
$PM_{2.5}$	particulate matter $\leq$ 2.5 μm in aerodynamic diameter
ROS	reactive oxygen species
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SO_2	sulfur dioxide
TBARS	thiobarbituric acid reactive substances
UFP	ultrafine particle ($\leq$ 0.1 μm in aerodynamic diameter)
UTMB	University of Texas Medical Branch
VOCs	volatile organic compounds

Part 4. Assessment of Plasma Markers and Cardiovascular Responses in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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INVESTIGATORS' REPORT

Part 4. Assessment of Plasma Markers and Cardiovascular Responses in Rats After Chronic Exposure to New-Technology Diesel Exhaust in the ACES Bioassay

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ABSTRACT

Although epidemiologic and experimental studies suggest that chronic 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. Because advances in new technologies have resulted in cleaner fuels and decreased engine emissions, uncertainty about the relationship between DE exposure and human cardiovascular health effects has increased. To address this ever-changing baseline of DE emissions, as part of the larger Advanced Collaborative Emissions Study (ACES) bioassay studying the health effects of 2007-compliant diesel engine emissions (new-technology diesel exhaust), we examined whether plasma markers of vascular inflammation, thrombosis, cardiovascular aging, cardiac fibrosis, and aorta morphometry were changed over 24 months in an exposure-level-, sex-, or exposure-duration-dependent manner. Many plasma markers — several recognized as human CVD risk factors — were measured in the plasma of rats exposed for up to 24 months to filtered air (the control) or DE. Few changes in plasma markers resulted from 12 months of DE exposure, but significant exposure-level-dependent increases in soluble intercellular adhesion molecule 1 (sICAM-1) and interleukin-6 (IL-6) levels, as well as decreases in total and non-high-density-lipoprotein cholesterol (non-HDL) levels in plasma, were observed in female rats after 24 months of DE exposure. These effects were not observed in male rats, and no changes in cardiac fibrosis or aorta morphometry resulting from DE exposure were observed in either sex. Collectively, the significant changes may reflect an enhanced sensitivity of the female cardiovascular system to chronic DE exposure; however, this conclusion should be interpreted within both the context and limitations of the current study.

INTRODUCTION

Air pollution exposure is associated with increased cardiovascular morbidity and mortality, and is especially associated with ischemic heart disease in elderly populations (Brook et al. 2010; Pope and Dockery 2006; Pope et al. 2004). 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 and Russell 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 reassessed repeatedly. Previous studies have shown that acute and chronic DE exposures alter plasma cholesterol levels in rodents (Reed et al. 2004, 2006b), perhaps reflecting the activation of an acute phase response (APR) as observed in sepsis (Harris et al. 2000). Moreover, DE-induced changes in cholesterol level can

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

include lipoprotein function and oxidation state (Araujo et al. 2008). For example, cholesterol levels may be increased or decreased, but more subtle changes may be restricted to one lipoprotein class (e.g., very low-density lipoprotein, low-density lipoprotein [LDL, oxidized LDL], or HDL that may remain unidentified. Additionally, in normolipidemic rodents, the majority of circulating cholesterol is carried by HDL not by LDL, as it is in humans. Thus, decreased total cholesterol (CHOL) level in rodents could very well be the result of a loss of HDL (or "good cholesterol"), as proposed in studies of acrolein exposure in mice (Shao et al. 2005a, 2005b), or of no change in CHOL but a loss of HDL antioxidant capacity (Araujo et al. 2008). Because a decreased cholesterol level in rodents has been reported in previous studies of DE exposure (Reed et al. 2004, 2006a), lipid levels should be monitored and changes carefully interpreted.

The mechanism or mechanisms by which air pollution increases human CVD risk are unknown, although several hypotheses have been proposed (Brook et al. 2010). Time-series analyses of epidemiologic data have shown that CVD risk increases chronically and also acutely within discrete time lags following exposure to peak air pollution. Changes in plasma markers may precipitate or reflect cardiovascular injury or causally induce long-term structural changes; thus, evaluating changes in plasma markers can contribute to the understanding of the mechanisms by which air pollution leads to subsequent pathogenic events. Moreover, chronic changes in plasma markers indicate that three general and overlapping mechanisms are likely to 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) induction of APR (systemic inflammation) (Brook 2008; Brook et al. 2010). The overlap between these pathways is incontrovertible, and yet studying the initiation and sequence of the events that govern the long-term relationship between these pathways is likely critical to unraveling the complex actions of air pollution (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 groups of humans, especially aging populations (Peters et al. 2001; Pope et al. 2004). Certain markers reflect a coordinated biologic response — as in an APR (e.g., serum amyloid A [SAA]) (Cabana et al. 1999; Kindy et al. 2000), vascular inflammation (e.g., sICAM-1) (Kitagawa et al. 2002), or thrombosis (e.g., platelet factor 4) (Sithu et al. 2010; Srivastava et al. 2011) — whereas some markers participate in more than one pathway. For example, fibrinogen, a soluble plasma protein, is converted to fibrin when the coagulation cascade is activated and represents a general marker of thrombosis (Reinhart 2003). Fibrinogen is also an APR reactant, as its level of expression is increased during the APR via transcriptional upregulation in the liver that is dependent on IL-6 (Gervois et al. 2004; Zambon et al. 2006).

A variety of cytokines and chemokines, including tumor necrosis factor-α (TNF-α), IL-6, monocyte chemotactic protein 1 (MCP-1), and keratinocyte-derived chemoattractant (KC) (also called CXCL1), have been associated with pulmonary injury following exposure to pollutants such as DE emissions (Arimoto et al. 2007; Fujimaki et al. 2006; Hiramatsu et al. 2003; Inoue et al. 2006; Li et al. 2007; Saber et al. 2006; Saito et al. 2002; Seagrave et al. 2005). The association of these markers with cardiovascular injury and systemic inflammation, however, requires more investigation (Erdely et al. 2011). For example, vascular injury and endothelial activation are typically reflected in increased plasma levels of endothelial cell markers, such as vascular endothelial growth factor (VEGF), and soluble adhesion molecules, such as sICAM-1, that are secreted or shed into the blood. VEGF is a chemokine and a growth factor that reflects acute endothelium injury and is also a marker of hypoxia and sepsis; secreted VEGF increases vascular permeability, mobilization of endothelial progenitor cells, and angiogenesis (Bateman et al. 2007; Grad et al. 1998; Nolan et al. 2004; Pickkers et al. 2005; Yano et al. 2006). However, even though pollutant exposure causes endothelium injury, it may not necessarily increase VEGF and sICAM-1. Our recent studies show that acrolein, a gaseous pollutant that results from frying food and is found in tobacco smoke, decreases plasma levels of sICAM-1 in vivo (Conklin et al. 2011b) and that this may result from the inhibition of intercellular adhesion molecule-1 (ICAM-1) shedding from endothelial cells (Tsakadze et al. 2004), which would increase the level of ICAM-1 expressed on the endothelium. Moreover, cardiovascular and systemic "VEGF resistance" has been reported in mice exposed short term to concentrated ambient particles (CAPs) or to acrolein (Haberzettl et al. 2012; Wheat et al. 2011). In these studies, neither CAPs nor acrolein exposure had an effect on blood levels of VEGF. This indicates that plasma marker levels may not change even under conditions that promote endothelium injury, which implies that the overall utility of plasma markers for detection of endothelium injury perhaps is limited.

On the other hand, some markers also influence other systemic functions; for example, leptin is considered a positive APR reactant but also is an important regulator of feeding behavior and overall body metabolism (Fantuzzi and Faggioni 2000). Increased systemic or pulmonary oxidative stress has been reported in a number of pollutant-exposure models and has been linked to injury of extrapulmonary targets and cardiovascular disease progression (Campen et al. 2010; Hirano et al. 2003; Ikeda et al. 1995a, 1995b; Reed et al. 2004; Seagrave et al. 2005). Because of the importance of

structural remodeling of the aorta and heart in chronic CVD such as atherosclerosis, hypertension, cardiomyopathy, and heart failure — and especially given the identified effects of pollutant exposures on these endpoints in rodents (Chen et al. 2013; Kodavanti et al. 2011; Wold et al. 2012; Ying et al. 2009) — we also assessed the aging thoracic aorta and heart. Thus, the purpose of this study was to analyze the effects of chronic DE exposure on the APR, vascular inflammation, and thrombosis pathways as well as on structural changes in the heart and aorta that may provide evidence of direct or indirect cardiovascular toxicity. Our study will provide the basis for subsequent studies assessing how real-world DE exposure contributes to increased human CVD risk (Channell et al. 2012; Ghio et al. 2012).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Core Study

The experimental time course and levels of exposure to DE emissions from 2007-compliant diesel engines followed the procedure carried out at the Lovelace Respiratory Research Institute (LRRI) for the DE-exposure chronic bioassay study, as described elsewhere (see Part 1 of this volume). In brief, young, healthy male and female Wistar Han rats (12 weeks old) were used for all exposures. Up to 14 rats were assigned to one of four exposure groups: those exposed to filtered air (the control), or low (0.1 ppm nitrogen dioxide [NO_2], mid (0.8 ppm NO_2), or high (4.2 ppm NO_2) levels of DE. Rats were exposed 16 hr/dy, 5 dy/wk from approximately 1600 to 0800 hours on Sunday through Thursday, and tissues and blood sample were taken at 1, 3, 12, and 24 months (see Part 1). Plasma samples were shipped overnight on dry ice from LRRI to the University of Louisville.

Positive Controls

Plasma samples (from rats) stored frozen at −80°C from previously conducted studies at the University of Louisville were used for internal validation of plasma markers. That is, plasma samples were initially collected 24 hours after 12- to 16-week-old male Sprague Dawley rats were gavage fed either water ($n = 3$) or acrolein (0.5 mg/kg; $n = 3$) (Conklin et al. 2011a). Samples were thawed and assayed in parallel with plasma samples from the current study. Control values and those of acrolein-treated rats were compared using the Student *t*-test (unequal variance), and a P value < 0.05 was considered statistically significant. Measures of acrolein-dependent changes in some blood markers (see Additional Materials 3) were similar to those reported previously (Conklin and Kong 2012).

PLASMA MARKERS

Groups of 10 rats were used for all measurements of lipids, albumin (ALB), total protein (TP), cytokines and chemokines, and systemic markers of toxicity except where indicated in the tables in the Results section.

Plasma Lipids and Proteins

Lipids and proteins in 150 µL of previously frozen plasma were measured using a Cobas Mira Plus clinical chemistry autoanalyzer (Roche, Indianapolis, IN) with calibrated standards and clinical quality reagents (Wako Chemicals; Randox; ThermoElectron) (Conklin et al. 2010). CHOL, HDL cholesterol, LDL cholesterol, and triglycerides (TRIG) were measured in mg/dL. Based on Wako's insert, HDL cholesterol was measured using an apolipoprotein-specific precipitating antibody, 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).

Cytokines, Chemokines, and Other Plasma Proteins

For measuring chemokines, cytokines, and growth factors (Saber et al. 2005, 2006), plasma thawed for lipid and protein analyses, as described above, was refrozen in 100-µL aliquots and shipped overnight on dry ice to AssayGate (Ijamsville, MD). AssayGate used a bead-based antibody technology and a custom-designed array to measure protein levels of the following rat 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, and CRP.

Thrombosis Markers

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 enzyme-linked immunosorbent assay (ELISA) system (Sithu et al. 2010). Notably, significant concordance was found between the values measured by commercial array and those values measured by in-house ELISA (Conklin and Kong 2012).

Systemic Organ Toxicity Markers

To discern whether DE exposure stimulated general or specific 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. The following

were measured: plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST), both 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) (Conklin and Kong 2012).

CARDIOVASCULAR ORGAN HISTOPATHOLOGIC ANALYSES

Cardiac Fibrosis

The hearts of male and female Wistar Han rats (n = 7/group) exposed to air or DE emissions for 12 or 24 months were isolated, cut longitudinally into dorsal and ventral halves, and formalin-fixed and paraffin-embedded for sectioning. Longitudinal sections (5 µm) were stained with Sirius Red (RA Lamb LLC; Apex, NC) and Fast Green FCF (Sigma-Aldrich) to detect collagen fibers (red staining). Slides were viewed on an Olympus IOM microscope and imaged with a SPOT camera and SPOT advanced image-capture software (20× magnification; SPOT Imaging Solutions, Sterling Heights, MI). Three images were taken (base, midventricle, apex), and each digital image was embedded with a SPOT software-generated calibration line (1000 µm) stamp for subsequent image analysis (see Additional Materials 2, Figure B.1.A, available on the HEI Web site).

Aortic Remodeling

The proximal thoracic aorta of male and female Wistar Han rats (n = 7/group) exposed to air or DE emissions for 1, 3, 12, or 24 months were isolated, cut into sections (up to three), and formalin-fixed and paraffin-embedded for sectioning. Cross sections (5 µm) were stained with Sirius Red and Fast Green FCF to demarcate the outer elastic lamina (OEL; border) between the tunica media and tunica adventitia. Up to three cross sections were viewed on an Olympus IOM microscope and imaged with a SPOT camera and SPOT advanced image-capture software (40× magnification). Each image was embedded with a SPOT software-generated calibration line (1000 µm) stamp for subsequent image analysis (see Figure 1; Additional Materials 2, Figure B.2.B, inset).

Image Analysis

Cardiac fibrosis and aortic structural analyses were performed using the National Institutes of Health free software ImageJ, version 1.45s (Schneider et al. 2012). Each digital photomicrograph (with up to three different aorta sections per slide) was analyzed as follows:

Heart The extent of fibrosis was the red-stained area measured as the percentage of the total area assessed. Following a standard protocol (using ImageJ; see examples given by the National Institutes of Health at *http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html*), images were split into red, green, and blue channels, and the green channel (red staining) was thresholded for appropriate contrast. The numerical threshold level was recorded for each image. Because red staining (i.e., Sirius Red-stained collagen fibers) was measured as a function of a threshold setting (i.e., numerical contrast) in ImageJ, two validation tests were performed. For test 1, 10 different images were measured for the percentage of fibrosis at variable thresholds chosen by the observer, and the results were compared with those at a single preset threshold level. For test 2, 10 different images were thresholded for the percentage of fibrosis at three different prechosen threshold levels (e.g., 170, 175, 180), which encompassed the typical threshold range used when the observer was allowed to choose a threshold by eye. The results of test 1 indicated that allowing the observer to choose the threshold resulted in less variation around the mean compared with using a single preset threshold level (data not shown). The results of test 2 showed that the percentage of fibrosis was a linear function of the threshold setting (data not shown). Based on these tests, we chose to have the observer set the threshold level by eye. Each image was analyzed for total, vascular, and avascular fibrosis as a percentage of the area covered. Vascular fibrosis comprised all easily identifiable blood vessels (> 50 µm diameter) and their surrounding connective tissue. Avascular fibrosis was assessed in cardiac tissue absent of obvious relatively large diameter vasculature (vessels ≥ 100 µm).

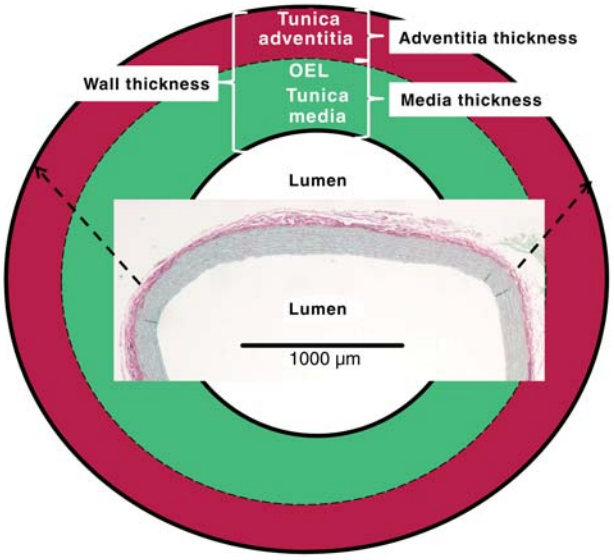

Figure 1. Schematic of aortic cross section used for analysis of aorta wall morphometry. Cross sections (5 µm) were stained with Sirius Red and Fast Green to demarcate the OEL (border) between the tunica media (green) and tunica adventitia (red). *Inset*: Portion of rat thoracic aorta wall stained with Sirius Red (adventitia) and Fast Green (media). The image is stamped with a calibration line (1000 µm).

Aorta Area (in μm^2) was measured for the following: lumen, wall, media, and adventitia. Thickness (in μm) was measured for the following: wall, media, and adventitia. The measurements of some parameters were used to calculate other parameters, as described next (see Figure 1).

Luminal Area The luminal area (LA) was measured by tracing the inner border (intima) of the aorta.

Wall Area The aortic wall area (WA) was calculated by first measuring the area of the aortic cross section (i.e., the outer circumference of the aorta was traced) and then subtracting the LA.

WA/LA Ratio This was the WA divided by the LA.

Media Area The media area was measured by first tracing the OEL to get the OEL area and then subtracting the LA from the OEL area.

Adventitia Area The adventitia area was calculated by first measuring the area of the aortic cross section and then subtracting the OEL area.

Wall Thickness The aortic wall thickness (WT) was calculated as the mean of four measurements per cross section; each measurement, made at 90° intervals, was the distance from outer adventitial border to the luminal border.

Media Thickness The media thickness (MT) was measured and calculated as the WT was, but the distance measured was from the OEL to the luminal border.

Adventitia Thickness The adventitia thickness was the difference between the WT and the MT.

STATISTICAL ANALYSES

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 methodologic validity. Among these flagged values, we removed only those values that were both improbable (based on our experience) and that exceeded 4 × SD. The resulting data set excluding outlier values was used for subsequent statistical analyses. The mean and standard error (SE) for each group based on these data sets are reported in the Results section.

Note that all measurements (both bioanalytic and morphologic) were made and recorded in a blinded manner (i.e., the observer was unaware of the exposure treatment of the sample), which should have prevented observer-dependent bias in the cardiovascular histopathology analyses. Similarly, statistical analyses were performed only after all data were collected for 12- and 24-month exposures. Potential general limitations include the sample size (n = 10/group for bioanalytic and n = 7/group for morphologic endpoints), that the samples were not formalin-fixed under physiologic pressure conditions, and that the rats were unfasted at euthanization.

Statistical Analysis Methods

For each marker, we first carried out three-way analysis of variance (ANOVA), with exposure level, sex, and exposure duration as factors, and included all two-way and three-way interactions. The normality of the residuals was assessed using the Shapiro-Wilk test. If the normality assumption was violated, the Box-Cox transformation was applied to meet the normality assumption. Otherwise, a rank-based transformation was applied (Venables and Ripley 2002). The three-way ANOVA was applied to the transformed data, and the mean squares for each factor and their interactions and the P values for these significance tests are reported in the Results section.

Additionally, for each marker with significant exposure-level effect or significant two-way or three-way interaction with the exposure level, we applied the Dunnett multiple comparisons test to determine whether the measurements 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 the Results section. A P value less than 0.05 was considered statistically significant, and all analyses were carried out using the statistics software R (*www.r-project.org*).

RESULTS

EFFECTS OF DE EXPOSURE LEVEL

Plasma Markers

Summary data for plasma markers in rats exposed for 12 and 24 months are shown in Tables 1 and 2, and summary data for short-term exposures (1 and 3 months) are compiled in Additional Materials 1, available on the HEI Web site (in

Table 1. Plasma Levels (Mean ± SE) of Markers Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 12 or 24 Months[a]

	CHOL (N = 157) (mg/dL)	HDL (N = 158) (mg/dL)	LDL (N = 158) (mg/dL)	HDL/LDL (N = 157)	TRIG (N = 156) (mg/dL)	ALB (N = 158) (g/dL)	TP (N = 158) (g/dL)	NAP[b] (N = 157) (g/dL)
12-Month Exposure: Male Rats								
Air	115.69 ± 10.86	64.03 ± 6.23	20.15 ± 1.88	3.30 ± 0.35	107.46 ± 27.83	4.05 ± 0.16 (9)	6.45 ± 0.38	2.14 ± 0.17 (9)
Low	95.56 ± 6.55	51.64 ± 2.99	18.30 ± 2.33	3.14 ± 0.31	96.68 ± 14.43	4.07 ± 0.15	6.31 ± 0.34	2.24 ± 0.19
Mid	95.60 ± 4.16 (9)	52.76 ± 2.41 (9)	18.18 ± 2.11 (9)	3.20 ± 0.38 (9)	102.95 ± 12.74 (9)	4.05 ± 0.10 (9)	6.21 ± 0.19 (9)	2.16 ± 0.11 (9)
High	97.14 ± 5.53	52.29 ± 2.62	20.99 ± 2.17	2.70 ± 0.26	63.39 ± 5.29	4.00 ± 0.12	5.90 ± 0.19	1.90 ± 0.09
12-Month Exposure: Female Rats								
Air	88.51 ± 9.35	55.45 ± 5.27	13.94 ± 1.96	4.25 ± 0.33	53.63 ± 14.36	4.36 ± 0.22	6.33 ± 0.35	1.98 ± 0.14
Low	93.97 ± 7.31	57.63 ± 4.43	15.71 ± 1.55	3.81 ± 0.26	79.32 ± 18.87	4.16 ± 0.14	6.15 ± 0.28	1.99 ± 0.16
Mid	93.83 ± 7.06	57.62 ± 4.02	16.59 ± 2.41	3.92 ± 0.43	43.15 ± 7.61 (9)	4.16 ± 0.12	5.94 ± 0.24	1.79 ± 0.12
High	96.50 ± 10.47	56.98 ± 5.71	16.53 ± 2.45	3.75 ± 0.30	90.00 ± 24.37	4.20 ± 0.13	6.04 ± 0.23	1.84 ± 0.13
24-Month Exposure: Male Rats								
Air	139.81 ± 15.16	73.85 ± 8.15	31.61 ± 2.98	2.40 ± 0.27	90.33 ± 21.72	3.82 ± 0.17	6.42 ± 0.29	2.60 ± 0.15
Low	145.98 ± 18.43	77.44 ± 8.55	32.79 ± 4.76 (9)	2.66 ± 0.42 (9)	67.55 ± 16.11 (9)	3.97 ± 0.07	6.61 ± 0.10 (9)	2.67 ± 0.13 (9)
Mid	147.61 ± 11.11	78.69 ± 6.57	31.26 ± 2.82	2.56 ± 0.14	117.34 ± 20.89	4.02 ± 0.06	6.76 ± 0.17	2.75 ± 0.14
High	152.62 ± 16.84	80.19 ± 7.48	31.32 ± 3.92	2.71 ± 0.26	91.82 ± 16.91	4.07 ± 0.09	6.67 ± 0.19	2.60 ± 0.13
24-Month Exposure: Female Rats								
Air	138.42 ± 11.43 (9)	80.50 ± 5.77 (9)	24.18 ± 3.28	3.94 ± 0.44 (9)	143.84 ± 33.29	4.25 ± 0.08	6.92 ± 0.24	2.67 ± 0.20
Low	135.88 ± 12.31	76.84 ± 7.31	24.61 ± 3.21	3.37 ± 0.37	113.64 ± 18.49	4.32 ± 0.09	6.97 ± 0.17	2.65 ± 0.11
Mid	146.88 ± 9.77	85.52 ± 5.75	27.72 ± 3.04	3.29 ± 0.26	65.64 ± 12.94 (9)	4.36 ± 0.10	7.11 ± 0.23	2.75 ± 0.18
High	99.29 ± 3.62 (9)	64.80 ± 7.39	20.11 ± 3.31	3.53 ± 0.33	92.45 ± 21.80	4.23 ± 0.12	6.94 ± 0.18	2.71 ± 0.12

(Table continues next page)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value. N = total number of samples.

[b] Non-albumin protein.

Table 1 (*Continued*). Plasma Levels (Mean ± SE) of Markers Measured in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 12 or 24 Months[a]

	ALT (N = 158) (U/L)	AST (N = 157) (U/L)	CK (N = 156) (U/L)	CREA (N = 158) (mg/dL)	LDH (N = 156) (U/L)	Non-HDL (N = 157) (mg/dL)	HDL + LDL (N = 157) (mg/dL)
12-Month Exposure: Male Rats							
Air	46.56 ± 3.91	76.84 ± 6.13	255.40 ± 47.16	0.59 ± 0.03	271.14 ± 28.20	51.66 ± 5.12	84.18 ± 7.27
Low	46.43 ± 2.08	70.78 ± 6.18	193.00 ± 33.91	0.60 ± 0.04	279.77 ± 70.96	43.91 ± 4.00	69.94 ± 4.69
Mid	42.07 ± 2.55 (9)	74.31 ± 7.41 (9)	180.11 ± 22.01 (9)	0.61 ± 0.03 (9)	195.59 ± 10.73 (9)	42.84 ± 2.72 (9)	70.94 ± 3.46 (9)
High	54.49 ± 6.64	71.51 ± 7.91	181.28 ± 28.87	0.58 ± 0.03	207.92 ± 17.29	44.85 ± 3.47	73.28 ± 4.12
12-Month Exposure: Female Rats							
Air	79.64 ± 8.06 (9)	132.98 ± 17.89	217.52 ± 32.21	0.57 ± 0.04 (9)	276.51 ± 25.30	33.07 ± 4.28	69.38 ± 6.94
Low	83.12 ± 10.14	122.26 ± 18.89	187.15 ± 30.39	0.55 ± 0.03	282.86 ± 30.34	36.34 ± 3.09	73.34 ± 5.54
Mid	54.52 ± 5.73	104.68 ± 15.02	143.92 ± 18.59	0.54 ± 0.01	256.70 ± 17.78	36.22 ± 3.43	74.21 ± 5.88
High	67.43 ± 6.83	97.68 ± 12.62	134.61 ± 10.98	0.50 ± 0.02	247.09 ± 24.56	39.52 ± 5.17	73.51 ± 7.57
24-Month Exposure: Male Rats							
Air	47.57 ± 4.54	86.40 ± 17.03 (9)	282.28 ± 47.25	0.56 ± 0.02	337.03 ± 61.98	65.95 ± 7.28	105.47 ± 9.92
Low	49.42 ± 2.95	78.22 ± 9.87	177.92 ± 50.99 (9)	0.58 ± 0.02	240.53 ± 79.10 (9)	68.54 ± 10.34	110.77 ± 13.75 (9)
Mid	67.30 ± 6.97	118.29 ± 15.87	346.92 ± 80.16	0.57 ± 0.03	480.48 ± 103.34	68.92 ± 4.87	109.95 ± 8.78
High	58.50 ± 8.16	104.71 ± 12.71	385.42 ± 78.47	0.59 ± 0.03	477.69 ± 151.36	72.43 ± 9.97	111.51 ± 10.44
24-Month Exposure: Female Rats							
Air	67.04 ± 7.26	110.57 ± 14.02 (9)	225.37 ± 64.33 (9)	0.52 ± 0.02	313.40 ± 91.83 (9)	57.91 ± 6.34 (9)	102.68 ± 7.84 (9)
Low	69.51 ± 7.20	122.52 ± 25.00 (9)	201.81 ± 44.94 (9)	0.57 ± 0.04	336.63 ± 83.49 (9)	59.03 ± 5.23	101.45 ± 9.71
Mid	81.67 ± 10.50	139.96 ± 19.75	250.04 ± 90.47	0.48 ± 0.03	389.17 ± 145.07	61.36 ± 4.63	113.24 ± 8.09
High	66.75 ± 9.18	139.39 ± 19.72	362.68 ± 90.76	0.52 ± 0.02	533.02 ± 169.30	41.71 ± 2.20 (9)	84.91 ± 10.43

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value. N = total number of samples.

Table 2. 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 12 or 24 Months[a]

	GM-CSF (N = 156) (pg/mL)	MCP-1 (N = 154) (pg/mL)	IL-1β (N = 150) (pg/mL)	IL-6 (N = 152) (pg/mL)	IL-10 (N = 153) (pg/mL)	IFN-γ (N = 153) (pg/mL)	KC (N = 155) (pg/mL)
12-Month Exposure: Male Rats							
Air	61.03 ± 6.08	259.14 ± 108.31	267.85 ± 129.77	466.29 ± 128.44	168.27 ± 85.06	21.15 ± 10.63	4.31 ± 0.78
Low	80.08 ± 6.26	148.30 ± 15.12	186.47 ± 63.73	374.50 ± 69.52	120.59 ± 43.94	24.86 ± 13.54	3.92 ± 0.54
Mid	82.06 ± 6.84	122.60 ± 27.19	224.18 ± 97.85	857.64 ± 339.89	126.12 ± 43.59	10.16 ± 1.19	3.54 ± 0.39
High	72.92 ± 4.62	163.69 ± 52.85	272.27 ± 151.92	1118.78 ± 270.44	259.67 ± 153.92	45.70 ± 14.60	9.78 ± 4.48
12-Month Exposure: Female Rats							
Air	61.27 ± 7.17	98.93 ± 20.35	76.41 ± 29.40	772.36 ± 182.63	31.73 ± 9.68	37.04 ± 15.13	6.66 ± 2.49
Low	60.32 ± 3.48	101.68 ± 14.48	112.42 ± 28.62	620.12 ± 135.57	192.41 ± 108.61	73.14 ± 41.78	6.08 ± 2.13
Mid	50.09 ± 6.33	93.57 ± 12.78	121.84 ± 40.61	434.18 ± 79.18	52.38 ± 10.26	20.25 ± 10.47	5.80 ± 2.04
High	59.56 ± 4.65	82.85 ± 10.91	176.74 ± 95.61 (9)	390.86 ± 62.77	32.54 ± 7.79 (9)	22.89 ± 13.51	8.88 ± 2.65
24-Month Exposure: Male Rats							
Air	162.50 ± 60.67	298.42 ± 90.38	490.41 ± 210.18 (9)	355.18 ± 80.46 (9)	223.57 ± 105.96	53.94 ± 23.94 (9)	8.64 ± 3.12
Low	223.83 ± 79.20 (9)	167.43 ± 33.26 (9)	217.97 ± 119.05 (9)	1148.03 ± 565.18 (9)	223.02 ± 115.97	60.74 ± 41.85 (9)	5.55 ± 1.92
Mid	266.75 ± 80.27	242.70 ± 48.48	389.50 ± 285.13 (9)	583.69 ± 172.42	161.09 ± 80.63 (9)	59.98 ± 24.19	5.06 ± 1.87 (9)
High	184.07 ± 79.04 (9)	185.94 ± 49.56 (9)	244.81 ± 129.50 (9)	287.97 ± 155.16 (8)	53.16 ± 23.15 (9)	88.96 ± 65.92 (8)	9.41 ± 3.46 (9)
24-Month Exposure: Female Rats							
Air	162.60 ± 75.41	192.22 ± 49.37 (9)	71.64 ± 42.34	77.97 ± 23.36	100.77 ± 89.72	18.11 ± 2.98	7.63 ± 2.39
Low	112.20 ± 39.98 (8)	242.94 ± 68.71 (8)	77.29 ± 39.56 (6)	552.26 ± 247.07 (8)	200.55 ± 162.32 (7)	175.50 ± 100.27 (8)	8.02 ± 1.57 (8)
Mid	109.72 ± 73.87	207.52 ± 38.94	173.21 ± 85.51 (9)	470.01 ± 121.09	88.95 ± 59.27 (9)	33.20 ± 9.59	8.84 ± 2.13 (9)
High	108.91 ± 26.38	117.27 ± 31.76 (9)	73.83 ± 17.48	974.26 ± 389.26 (8)	35.73 ± 13.03	248.82 ± 133.93 (9)	10.65 ± 1.60

(Table continues next page)

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value. N = total number of samples.

Table 2. (*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 12 or 24 Months[a]

	TNF-α (N = 156) (pg/mL)	VEGF (N = 153) (pg/mL)	sICAM-1 (N = 155) (pg/mL)	Fibrinogen (N = 157) (pg/mL)	Leptin (N = 157) (pg/mL)	CRP (N = 157) (pg/mL)
12-Month Exposure: Male Rats						
Air	6.09 ± 1.92	15.55 ± 1.45	4745.23 ± 2042.97	1654.34 ± 99.83	119.02 ± 12.23	418.64 ± 24.52
Low	4.06 ± 0.10	22.67 ± 2.72	4227.32 ± 854.60	1680.01 ± 224.61	124.06 ± 8.96	408.21 ± 37.16
Mid	4.00 ± 0.12	50.40 ± 22.69	5451.53 ± 2203.12	1862.99 ± 198.19	121.98 ± 7.14	395.57 ± 25.70
High	3.84 ± 0.07	51.06 ± 17.20	5063.65 ± 937.57	1622.40 ± 149.39	104.04 ± 9.96	376.43 ± 22.34
12-Month Exposure: Female Rats						
Air	4.06 ± 0.22	59.27 ± 33.37	3427.54 ± 664.73	1985.65 ± 120.27	50.66 ± 10.69	347.73 ± 30.39
Low	4.51 ± 0.55	25.23 ± 3.52	2596.09 ± 606.66	2120.87 ± 222.41	63.09 ± 13.03	376.28 ± 34.45
Mid	4.90 ± 0.88	37.75 ± 10.94 (9)	1067.43 ± 489.23	1804.56 ± 184.25	58.02 ± 5.90	291.78 ± 11.69
High	3.81 ± 0.08	22.89 ± 2.93 (9)	1534.37 ± 383.95	1734.07 ± 124.72	64.99 ± 8.47	295.17 ± 26.92
24-Month Exposure: Male Rats						
Air	6.31 ± 1.32	43.36 ± 16.36	11,885.15 ± 2503.63	2667.06 ± 378.77	75.67 ± 8.31	388.93 ± 31.36
Low	13.80 ± 7.59	51.88 ± 23.13	9868.69 ± 2918.25	2946.71 ± 318.21	100.78 ± 8.04	447.62 ± 25.20
Mid	7.79 ± 1.53	23.04 ± 3.89	15,833.35 ± 3666.47	2561.22 ± 263.54	107.78 ± 11.12	446.93 ± 25.17
High	6.79 ± 2.18 (9)	31.40 ± 5.49 (9)	12,460.72 ± 3772.46 (7)	2582.76 ± 274.27 (9)	94.21 ± 17.26 (9)	428.30 ± 25.09 (9)
24-Month Exposure: Female Rats						
Air	5.41 ± 0.88 (9)	64.80 ± 30.89	3505.02 ± 973.69	2333.92 ± 282.72	61.21 ± 10.53	446.83 ± 40.77
Low	8.80 ± 2.89 (8)	66.16 ± 15.24 (8)	8719.85 ± 1624.18 (8)	2419.96 ± 237.81 (8)	100.16 ± 15.02 (8)	500.49 ± 49.75 (8)
Mid	7.25 ± 1.59	27.81 ± 5.02 (9)	6247.14 ± 1642.65	2892.09 ± 287.12	73.91 ± 11.75	428.49 ± 32.16
High	5.95 ± 0.89	48.29 ± 20.04 (9)	11,245.42 ± 2447.96	3141.97 ± 294.84	65.20 ± 9.55	426.17 ± 41.43

[a] The sample size was 10 per group unless otherwise indicated by the number in the parentheses (n) after the value. N = total number of samples.

all cases, $P \leq 0.05$ was considered statistically significant). We used three-way ANOVA testing on data for all groups (1, 3, 12, and 24 months), and the results of statistical testing are summarized in Tables 3 and 4. Significant interactions involving DE exposure level were observed for CHOL, non-HDL, TRIG, ALT, CK, IL-6, leptin, and sICAM-1 (Tables 3 and 4). These significant effects of DE exposure on plasma markers were then further analyzed with Dunnett tests to compare individual DE levels and the air control (Table 5). The Dunnett tests revealed the following: (1) mean plasma CHOL was decreased in female rats exposed to both mid-level DE for 3 months and high-level DE for 24 months compared with matched air controls (Figure 2A); (2) no significant differences were detected in HDL between female rats exposed to DE at all levels and the controls, as shown in Figure 2C; (3) mean plasma non-HDL cholesterol (i.e., total cholesterol minus HDL cholesterol) was significantly lower in female rats exposed to high-level DE for 24 months than in the air controls (Figure 2E); (4) mean plasma TRIG was significantly decreased in female rats exposed to mid-level DE for 24 months compared with the air controls (Figure 3A); (5) mean plasma ALT in female rats exposed to mid-level DE for 12 months was significantly lower than in the air control group (see Table 1);

Table 3. 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, 3, 12, and 24 Months[a,b]

	CHOL Box–Cox Transformation		HDL Box–Cox Transformation		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	0.003	0.640	0.002	0.867	0.016	0.755	0.014	0.922
Sex	0.042	**0.003**	0.001	0.726	0.759	**< 0.001**	2.837	**< 0.001**
Duration	0.363	**< 0.001**	0.278	**< 0.001**	2.306	**< 0.001**	1.571	**< 0.001**
Exposure level:Sex	0.005	0.330	0.008	0.280	0.043	0.354	0.03	0.793
Exposure level:Duration	0.007	0.173	0.011	0.105	0.024	0.784	0.073	0.587
Sex:Duration	0.002	0.698	0.002	0.782	0.087	0.089	0.354	**0.008**
Exposure level:Sex:Duration	0.011	**0.018**	0.012	0.069	0.041	0.414	0.032	0.952
Residuals	0.005	NA	0.006	NA	0.04	NA	0.088	NA

	TRIG Box–Cox Transformation		ALB Rank Transformation		TP Rank Transformation		NAP[c] Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	0.665	0.878	1338.9	0.852	3542.511	0.640	3610.438	0.583
Sex	92.141	**< 0.001**	792,130.3	**< 0.001**	278,560.4	**< 0.001**	2078.091	0.541
Duration	6.557	0.084	65,863.27	**< 0.001**	108,767.2	**< 0.001**	315,865.9	**< 0.001**
Exposure level:Sex	1.340	0.712	5648.845	0.346	9429.392	0.215	9579.557	0.162
Exposure level:Duration	1.584	0.845	2513.594	0.879	5007.276	0.621	1973.17	0.955
Sex:Duration	17.721	**0.001**	54,534.5	**< 0.001**	54,760.28	**< 0.001**	13,572.49	0.064
Exposure level:Sex:Duration	8.011	**0.004**	2742.741	0.846	2848.359	0.905	2642.416	0.890
Residuals	2.931	NA	5097.1	NA	6296.559	NA	5554.037	NA

(Table continues next page)

[a] NA indicates not available.

[b] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

[c] Non-albumin protein.

(6) compared with matched air controls, mean plasma CK levels were decreased in female rats exposed to high-level DE for 12 months (see Table 1); (7) compared with matched air controls, mean plasma IL-6 levels were significantly higher in female rats exposed 24 months to both mid- and high-level DE and in male rats exposed for 12 months to high-level DE (see Table 2); (8) mean plasma sICAM-1 levels were significantly lower in female rats exposed to mid-level DE exposure for 12 months than in the air control group, and yet, compared with air controls, sICAM-1 levels were elevated in female rats exposed for 24 months to low- and high-level DE (Table 2, Figure 4A); and (9) the mean plasma leptin levels were significantly decreased in males exposed for 3 months to mid-level DE compared with the matched air control group (Figure 5B, Table 5, Additional Materials 1).

Cardiovascular Histopathology

Cardiac Fibrosis To estimate cardiac fibrosis, longitudinal sections of hearts from rats exposed for 12 and 24 months to air or DE were stained with Sirius Red and Fast Green FCF to label collagen fibers. Photomicrographs of the midleft ventricular wall were examined by a blinded

Table 3 (*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, 3, 12, and 24 Months[a,b]

	ALT Box–Cox Transformation		AST Box–Cox 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	0.014	0.387	0.005	0.786	11,483.4	0.192	2210.572	0.675
Sex	0.468	**< 0.001**	0.43	**< 0.001**	41,332.25	**0.017**	88,447.14	**< 0.001**
Duration	0.513	**< 0.001**	0.216	**< 0.001**	97,168.35	**< 0.001**	406,139.2	**< 0.001**
Exposure level:Sex	0.030	0.094	0.021	0.206	8507.144	0.318	928.523	0.886
Exposure level:Duration	0.032	**0.015**	0.02	0.166	14,767.96	**0.034**	3632.713	0.581
Sex:Duration	0.067	**0.003**	0.038	**0.043**	1577.413	0.883	16,502.48	**0.011**
Exposure level:Sex:Duration	0.006	0.933	0.003	0.992	5535.5	0.647	2039.106	0.894
Residuals	0.014	NA	0.014	NA	7215.636	NA	4331.631	NA

	LDH Rank Transformation		Non-HDL Box–Cox Transformation		HDL+LDL Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	1151.987	0.935	0.006	0.602	0.002	0.783
Sex	54,402.97	**0.010**	0.436	**< 0.001**	0.011	0.166
Duration	23,025.84	**0.038**	1.142	**< 0.001**	0.329	**< 0.001**
Exposure level:Sex	5614.88	0.557	0.004	0.750	0.008	0.245
Exposure level:Duration	11,310.02	0.189	0.009	0.518	0.007	0.241
Sex:Duration	4563.07	0.640	0.010	0.412	0.003	0.610
Exposure level:Sex:Duration	5964.959	0.675	0.020	**0.043**	0.010	0.061
Residuals	8097.812	NA	0.010	NA	0.006	NA

[a] NA indicates not available.

[b] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

observer for the percentage of red-stained collagen, as described in Materials and Methods. As rats age, their levels of cardiac fibrosis increase (Annoni et al. 1998; Cornwell et al. 1991), and so, as expected, the percentage of red-stained collagen was significantly increased with age in both male and female rats (Table 6; Figure B.1.B–D in Additional Materials 2). However, DE exposure had no significant effect on the progression of cardiac fibrosis (Figure B.1.B–D in Additional Materials 2). As basal collagen deposition is intrinsically greater in perivascular than in avascular regions of heart, vascular fibrosis and avascular fibrosis were quantified separately. Yet, no significant differences in vascular and avascular fibrosis staining were observed between the groups exposed to air and those exposed to DE at either time point (Table 6; Figure B.1 in Additional Materials 2). Thus, DE exposure of up to 24 months had no effect on progressive age-related cardiac fibrosis in rats.

Aorta Morphometry We measured a number of thoracic aorta parameters to assess whether any changes in wall architecture indicative of vascular wall remodeling were induced by DE exposure independent of aging yet resulting from potentially deleterious forces such as hypertension. The vascular fibrosis area was usually less than 10% of the

Table 4. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or DE for 1, 3, 12 and 24 Months[a,b]

	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	12,397.67	0.184	8816.597	0.191	0.382	0.844	10,901.68	0.234
Sex	120,567.4	**< 0.001**	255,232.6	**< 0.001**	17.045	**0.001**	16,419.73	0.143
Duration	34,466.77	**0.004**	200,525.7	**< 0.001**	44.349	**< 0.001**	40,699.71	**0.001**
Exposure level:Sex	5504.867	0.540	4493.028	0.488	0.258	0.906	2398.443	0.814
Exposure level:Duration	4869.557	0.764	5304.186	0.475	0.265	0.995	9032.481	0.303
Sex:Duration	3723.967	0.691	12,679.25	0.078	2.651	0.130	1569.042	0.892
Exposure level:Sex:Duration	14,439.79	0.053	1798.484	0.966	0.868	0.778	14,938.22	**0.044**
Residuals	7629.052	NA	5534.389	NA	1.394	NA	7611.519	NA

	IL-10 Box–Cox Transformation		IFN-γ Rank Transformation		KC Rank Transformation		TNF-α Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	10.179	0.104	5279.846	0.428	2349.881	0.323	2208.239	0.771
Sex	62.239	**< 0.001**	11,977.53	0.148	17,977.05	**0.003**	3324.654	0.453
Duration	29.246	**0.001**	268,500.5	**< 0.001**	649,097.8	**< 0.001**	272,968.0	**< 0.001**
Exposure level:Sex	2.497	0.676	3732.765	0.580	179.365	0.966	2261.232	0.764
Exposure level:Duration	3.665	0.666	2976.704	0.857	683.852	0.961	4943.059	0.579
Sex:Duration	2.582	0.665	3108.007	0.651	2138.189	0.366	977.785	0.919
Exposure level:Sex:Duration	3.566	0.684	7921.849	0.191	437.758	0.992	3831.618	0.752
Residuals	4.907	NA	5690.673	NA	2013.775	NA	5879.505	NA

(Table continues next page)

[a] NA indicates not available.

[b] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

total ventricle area, but its fibrosis values (as percentages) were greater than 10 times the fibrosis values of avascular areas (e.g., > 30% vascular and < 3% avascular; see Table 6). As with cardiac fibrosis, we expected (and observed) obvious changes in aortic wall dimensions as rats grew and aged over the 24 months of air or DE exposure (Table 6 and Table 7; Figure B.2 in Additional Materials 2). However, we observed no statistically significant differences in measured aortic wall parameters between the air and DE exposure groups at any time point (Table 6). This suggests that DE exposure at these levels and under these specific conditions had little influence on aortic wall changes related to growth and aging in male or female rats. Because of the obvious difference in absolute size between female and male rats, numerous sex-dependent differences in aortic parameters were observed (Tables 6 and 7).

EFFECTS OF SEX AND DURATION ON PLASMA MARKERS AND CARDIOVASCULAR AGING IN RATS

In addition to DE-level effects, we identified numerous statistically significant changes in plasma levels of markers, cardiac fibrosis, and aorta dimensions that were dependent on sex or exposure duration or on an interaction between sex and duration (see Tables 3, 4, and 7, rows labeled "Sex," "Duration," and "Sex:Duration").

Table 4 (*Continued*). Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Plasma Values (Transformed) of Cytokines and Chemokines and APR Reactants Measured in Male and Female Rats Exposed to Air or DE for 1, 3, 12 and 24 Months[a,b]

	VEGF Rank Transformation		sICAM-1 Rank Transformation		Fibrinogen Rank Transformation		Leptin Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	2392.633	0.614	8396.223	0.119	3065.25	0.176	9133.602	**0.001**
Sex	2734.728	0.407	539,007.1	**< 0.001**	860.61	0.495	277,886.7	**< 0.001**
Duration	416,364.2	**< 0.001**	200,530.8	**< 0.001**	693,545.7	**< 0.001**	603,828.8	**< 0.001**
Exposure level:Sex	382.445	0.962	3126.409	0.533	1505.76	0.486	256.6	0.922
Exposure level:Duration	5907.561	0.152	7286.745	0.087	939.25	0.868	2041.189	0.245
Sex:Duration	2092.115	0.664	9671.058	0.081	1127.68	0.608	9583.414	**0.001**
Exposure level:Sex:Duration	6363.497	0.114	11,891.69	**0.004**	2397.953	0.237	1481.424	0.498
Residuals	3969.838	NA	4265.668	NA	1845.361	NA	1589.838	NA

	CRP Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value
Exposure level	0.003	0.403
Sex	0.002	0.393
Duration	0.331	**< 0.001**
Exposure level:Sex	0.002	0.427
Exposure level:Duration	0.003	0.241
Sex:Duration	0.033	**< 0.001**
Exposure level:Sex:Duration	0.001	0.883
Residuals	0.003	NA

[a] NA indicates not available.

[b] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

Table 5. Comparisons of *P* Values for DE Exposure Groups and Controls for the Plasma Markers, Cytokine and Chemokine Markers, and Cardiac Fibrosis and Aorta Morphometry Parameters Whose Final ANOVA Models Included Statistically Significant Exposure Effect or Statistically Significant Interaction of Exposure with Sex or Duration[a]

	CHOL	TRIG	ALT	CK	Non-HDL	IL-6	sICAM-1	Leptin	Adventitia Area	Mean Media Thickness
1-Month Exposure: Male Rat										
Low vs. Control	0.734	0.160	0.962	0.982	0.684	0.973	0.731	0.087	0.419	0.993
Mid vs. Control	0.069	0.530	0.260	0.992	0.161	0.571	0.674	0.964	0.166	0.638
High vs. Control	0.076	0.842	0.994	0.982	0.384	0.94	0.994	0.594	0.112	0.999
1-Month Exposure: Female Rat										
Low vs. Control	0.352	0.997	0.132	0.921	0.874	0.416	0.927	0.220	0.788	0.897
Mid vs. Control	0.435	0.724	0.292	0.061	0.872	0.652	0.494	0.905	0.824	0.964
High vs. Control	0.691	0.994	0.339	0.792	0.497	0.108	0.310	0.608	0.895	0.440
3-Month Exposure: Male Rat										
Low vs. Control	0.356	0.996	0.805	0.990	0.763	0.691	0.908	0.856	0.992	0.588
Mid vs. Control	0.998	0.076	1	0.744	1	0.998	0.641	**0.007**	1	0.853
High vs. Control	1	0.706	0.995	0.746	0.818	0.996	0.965	0.856	0.661	0.443
3-Month Exposure: Female Rat										
Low vs. Control	0.252	0.999	0.991	0.810	0.548	0.937	0.991	0.999	0.595	0.145
Mid vs. Control	**0.015**	0.745	0.369	0.988	0.089	0.994	0.329	0.903	0.390	0.459
High vs. Control	0.908	0.981	0.387	0.999	0.962	0.998	0.722	0.589	0.177	0.433
12-Month Exposure: Male Rat										
Low vs. Control	0.240	0.996	0.989	0.440	0.423	0.933	0.724	0.865	0.899	0.993
Mid vs. Control	0.345	0.972	0.849	0.440	0.464	0.806	0.916	0.955	0.999	0.999
High vs. Control	0.358	0.236	0.560	0.414	0.617	**0.035**	0.409	0.660	0.312	0.792
12-Month Exposure: Female Rat										
Low vs. Control	0.819	0.470	1	0.510	0.628	0.974	0.761	0.661	1	0.618
Mid vs. Control	0.794	0.999	**0.031**	0.076	0.649	0.257	**0.032**	0.961	0.181	0.446
High vs. Control	0.793	0.234	0.574	**0.027**	0.433	0.150	0.069	0.602	0.279	0.560
24-Month Exposure: Male Rat										
Low vs. Control	0.989	0.638	0.940	0.115	1	0.400	0.994	0.241	0.091	0.141
Mid vs. Control	0.844	0.445	0.085	1	0.890	0.821	0.834	0.126	0.984	0.684
High vs. Control	0.845	0.955	0.712	0.781	0.915	0.667	0.988	0.588	1	0.141
24-Month Exposure: Female Rat										
Low vs. Control	0.982	0.847	0.990	0.999	0.980	0.151	**0.021**	0.078	0.459	0.447
Mid vs. Control	0.862	**0.032**	0.678	0.998	0.828	**0.037**	0.346	0.694	0.996	0.149
High vs. Control	**0.009**	0.239	0.993	0.624	**0.034**	**0.027**	**0.005**	0.991	0.530	0.996

[a] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

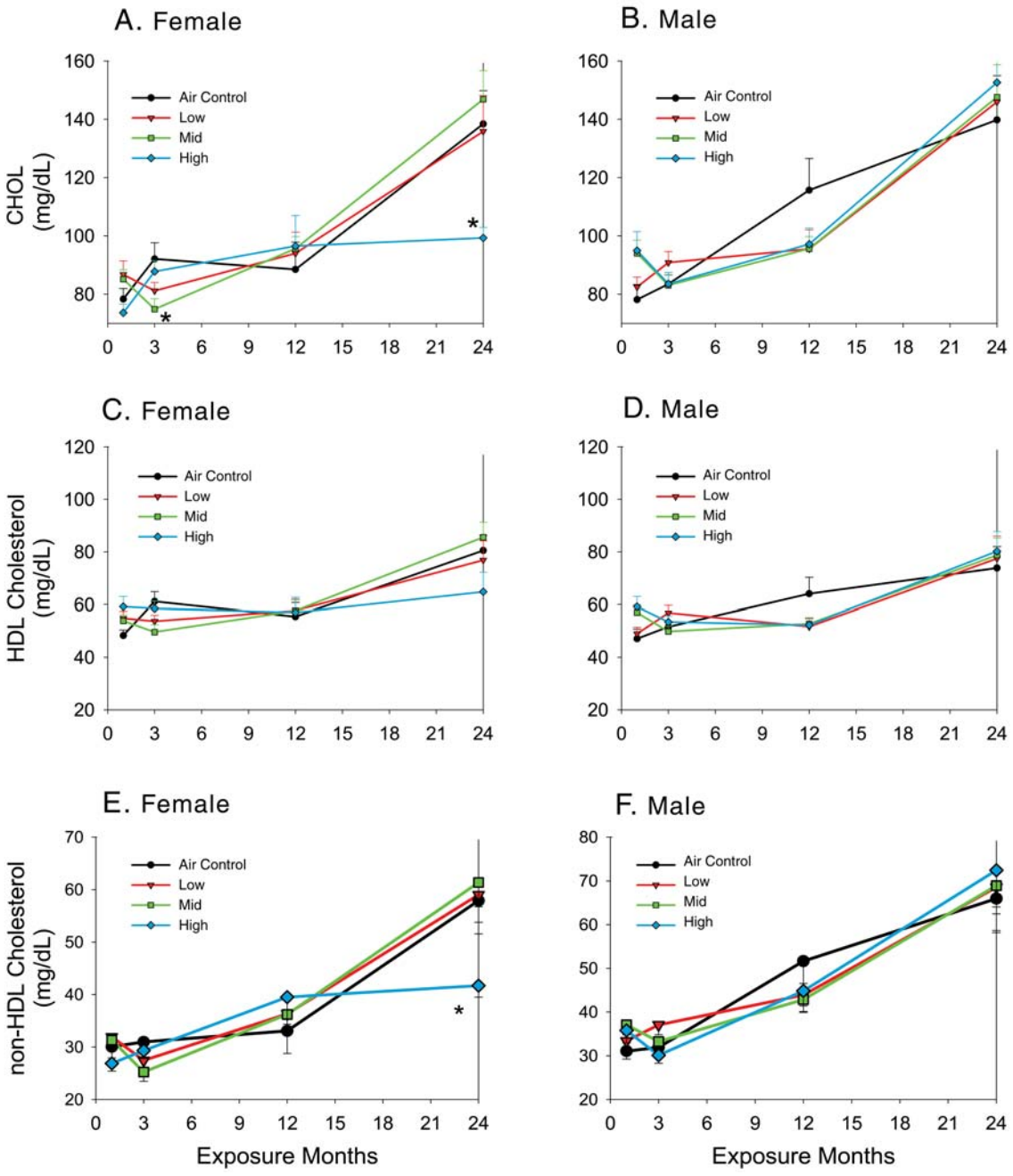

Figure 2. Plasma CHOL, HDL cholesterol, and non-HDL cholesterol levels in female (A, C, E) and male (B, D, F) rats over the 24 months of exposure to filtered air or DE at the low, mid, or high level. Values are the mean ± SE. *N* = 9 to 10 rats/group (see Table 1 and Additional Materials 1). *indicates a significant difference between the air control and DE group.

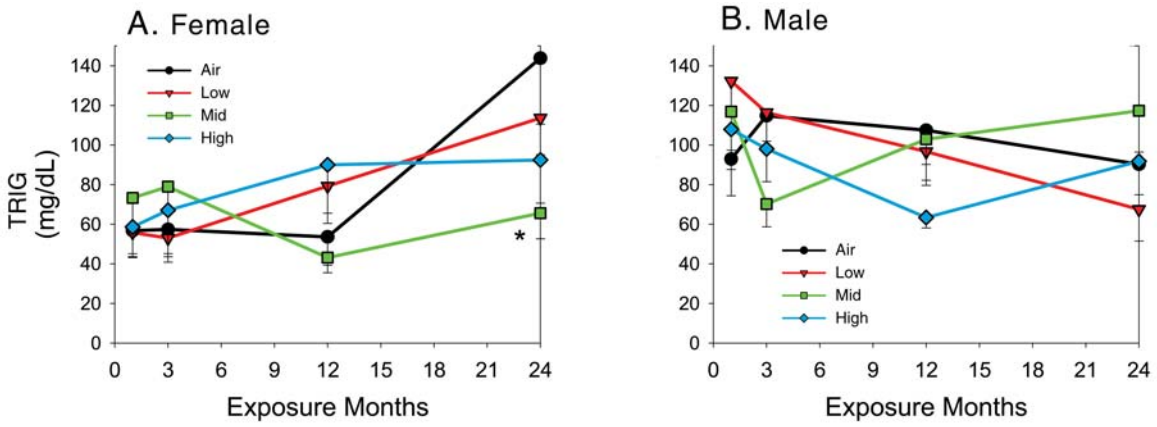

Figure 3. Plasma TRIG levels in female (A) and male (B) rats over the 24 months of exposure to filtered air or DE at the low, mid, or high level. Values are the mean ± SE. *N* = 9 to 10 rats/group (see Table 1 and Additional Materials 1). *indicates a significant difference between the air control and DE group.

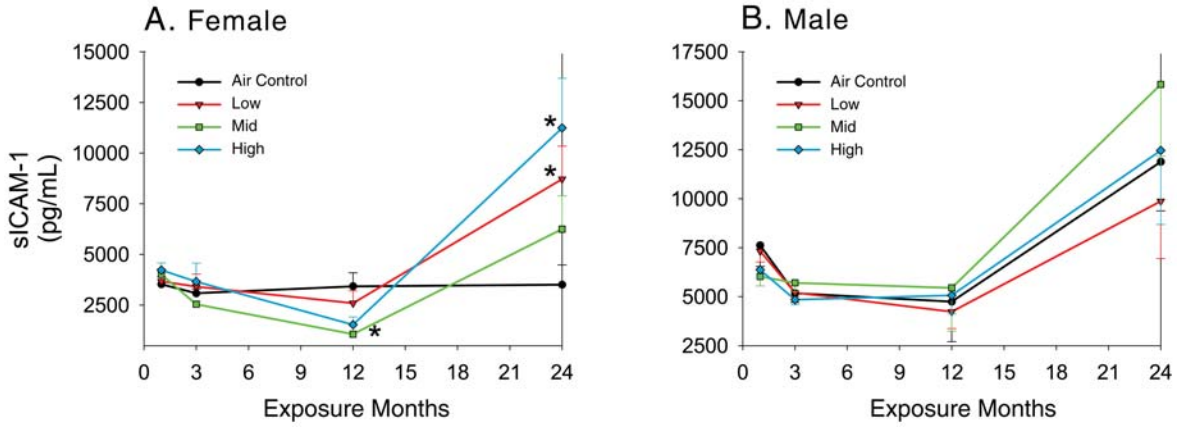

Figure 4. Plasma sICAM-1 levels in female (A) and male (B) rats over the 24 months of exposure to filtered air or to DE at the low, mid, or high level. Values are the mean ± SE. *N* = 6 to 10 rats/group (see Table 2 and Additional Materials 1). *indicates a significant difference between the air control and DE group.

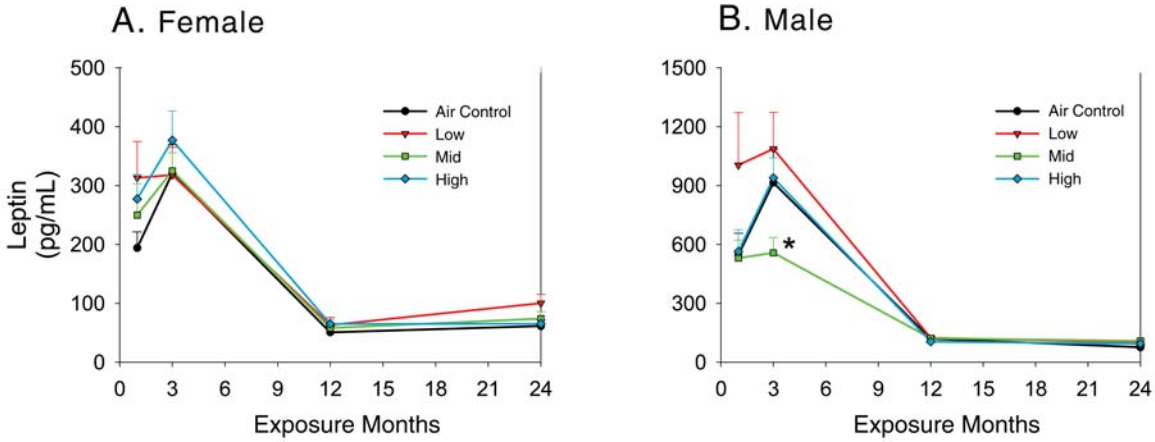

Figure 5. Plasma leptin levels in female (A) and male (B) rats over the 24 months of exposure to filtered air or to DE at the low, mid, or high level. Values are the mean ± SE. *N* = 6 to 10 rats/group (see Table 2 and Additional Materials 1). *indicates a significant difference between the air control and DE group.

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Table 6. Cardiac Fibrosis and Aorta Morphometry Data (Mean ± SE) in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1, 3, 12, or 24 Months[a]

	% Total Left Ventricle Fibrosis	% Vascular Fibrosis	% Avascular Fibrosis	Luminal Area ($\mu m^2 \times 10^6$)
1-Month Exposure: Male Rats				
Air	nd	nd	nd	1.28 ± 0.18 (6)
Low				1.28 ± 0.18 (5)
Mid				1.22 ± 0.13 (5)
High				1.08 ± 0.12
1-Month Exposure: Female Rats				
Air	nd	nd	nd	0.87 ± 0.08
Low				0.86 ± 0.07 (6)
Mid				1.06 ± 0.10
High				0.96 ± 0.12
3-Month Exposure: Male Rats				
Air	nd	nd	nd	1.67 ± 0.12 (6)
Low				1.50 ± 0.23
Mid				1.77 ± 0.15
High				1.41 ± 0.17
3-Month Exposure: Female Rats				
Air	nd	nd	nd	1.06 ± 0.12 (6)
Low				1.03 ± 0.12
Mid				1.00 ± 0.12 (5)
High				1.03 ± 0.09
12-Month Exposure: Male Rats				
Air	1.98 ± 0.32	23.47 ± 4.81	1.98 ± 0.31	1.97 ± 0.16 (5)
Low	2.12 ± 0.36	34.57 ± 5.46	1.78 ± 0.32	1.90 ± 0.12
Mid	2.20 ± 0.23 (6)	40.96 ± 3.89 (6)	1.78 ± 0.21 (6)	2.03 ± 0.15 (5)
High	1.61 ± 0.41 (5)	38.93 ± 7.41 (4)	1.33 ± 0.38 (5)	1.71 ± 0.14 (5)
12-Month Exposure: Female Rats				
Air	1.28 ± 0.20 (6)	33.22 ± 6.96 (5)	1.13 ± 0.15 (6)	1.33 ± 0.152 (3)
Low	1.64 ± 0.23	29.33 ± 1.14 (6)	1.48 ± 0.21	0.81 ± 0.15
Mid	1.88 ± 0.24	31.54 ± 6.80 (6)	1.71 ± 0.21	1.35 ± 0.16 (6)
High	1.75 ± 0.17	32.64 ± 5.43 (6)	1.58 ± 0.19	1.21 ± 0.15
24-Month Exposure: Male Rats				
Air	3.10 ± 0.12 (5)	32.60 ± 4.01 (4)	2.29 ± 0.31 (4)	2.72 ± 0.28 (5)
Low	2.93 ± 0.51	27.82 ± 2.94	2.57 ± 0.50	2.22 ± 0.13
Mid	3.40 ± 0.39 (5)	29.78 ± 2.90 (5)	2.95 ± 0.36 (5)	2.01 ± 0.11 (6)
High	3.37 ± 0.31	35.58 ± 5.03	3.02 ± 0.27	2.15 ± 0.17
24-Month Exposure: Female Rats				
Air	2.25 ± 0.21 (6)	32.02 ± 5.12 (5)	2.21 ± 0.34 (6)	1.59 ± 0.10 (6)
Low	2.80 ± 0.38	32.36 ± 2.75 (6)	2.36 ± 0.32	1.84 ± 0.22
Mid	2.48 ± 0.27 (6)	34.30 ± 4.52 (6)	1.98 ± 0.35 (6)	1.68 ± 0.10 (6)
High	2.46 ± 0.33	27.54 ± 3.39 (6)	2.11 ± 0.23	1.57 ± 0.12 (5)

(Table continues next page)

[a] The sample size was 7 per group unless otherwise indicated by the number in the parentheses (*n*) after the value. "nd" means not determined.

Table 6 (*Continued*). Cardiac Fibrosis and Aorta Morphometry Data (Mean ± SE) in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1, 3, 12, or 24 Months[a]

	Wall (Area = $\mu m^2 \times 10^6$)	WA/LA	Adventitia (Area = $\mu m^2 \times 10^6$)	Media (Area = $\mu m^2 \times 10^6$)
1-Month Exposure: Male Rats				
Air	0.74 ± 0.05 (6)	0.65 ± 0.10 (6)	0.18 ± 0.01 (6)	0.56 ± 0.04 (6)
Low	0.76 ± 0.034 (5)	0.66 ± 0.09 (5)	0.21 ± 0.01 (5)	0.55 ± 0.03 (5)
Mid	0.82 ± 0.06 (6)	0.75 ± 0.07 (5)	0.22 ± 0.01 (6)	0.59 ± 0.04 (6)
High	0.78 ± 0.06	0.80 ± 0.11	0.25 ± 0.03	0.53 ± 0.05
1-Month Exposure: Female Rats				
Air	0.61 ± 0.03	0.94 ± 0.30	0.18 ± 0.02	0.44 ± 0.02
Low	0.65 ± 0.03 (6)	0.85 ± 0.09 (6)	0.19 ± 0.01 (6)	0.46 ± 0.03 (6)
Mid	0.62 ± 0.03	0.67 ± 0.13	0.16 ± 0.01	0.45 ± 0.02
High	0.69 ± 0.04	0.85 ± 0.16	0.19 ± 0.01	0.50 ± 0.03
3-Month Exposure: Male Rats				
Air	0.99 ± 0.04 (6)	0.61 ± 0.06 (6)	0.23 ± 0.02 (6)	0.75 ± 0.03 (6)
Low	0.78 ± 0.14	0.73 ± 0.21	0.33 ± 0.09	0.69 ± 0.04
Mid	0.96 ± 0.05	0.57 ± 0.07	0.24 ± 0.02	0.73 ± 0.03
High	0.85 ± 0.02	0.94 ± 0.36	0.20 ± 0.01	0.65 ± 0.02
3-Month Exposure: Female Rats				
Air	0.66 ± 0.04 (6)	0.66 ± 0.06 (6)	0.15 ± 0.01 (6)	0.51 ± 0.03 (6)
Low	0.76 ± 0.05	0.83 ± 0.12	0.18 ± 0.01	0.58 ± 0.04
Mid	0.74 ± 0.02 (5)	0.77 ± 0.09 (5)	0.18 ± 0.01 (5)	0.55 ± 0.02 (5)
High	0.78 ± 0.09	0.80 ± 0.13	0.19 ± 0.02	0.58 ± 0.08
12-Month Exposure: Male Rats				
Air	1.06 ± 0.04 (5)	0.55 ± 0.03 (5)	0.27 ± 0.02 (5)	0.80 ± 0.04 (5)
Low	1.13 ± 0.10	0.59 ± 0.03	0.29 ± 0.02	0.84 ± 0.08
Mid	1.12 ± 0.10 (6)	0.56 ± 0.05 (5)	0.27 ± 0.02 (6)	0.84 ± 0.09 (6)
High	0.96 ± 0.03 (5)	0.58 ± 0.04 (5)	0.23 ± 0.02 (5)	0.72 ± 0.02 (5)
12-Month Exposure: Female Rats				
Air	0.75 ± 0.05 (3)	0.57 ± 0.04 (3)	0.16 ± 0.02 (3)	0.58 ± 0.04 (3)
Low	0.75 ± 0.04	1.19 ± 0.25	0.16 ± 0.01	0.58 ± 0.04
Mid	0.83 ± 0.06 (6)	0.68 ± 0.08 (6)	0.21 ± 0.02 (6)	0.62 ± 0.04 (6)
High	0.80 ± 0.04	0.72 ± 0.09	0.20 ± 0.02	0.60 ± 0.02
24-Month Exposure: Male Rats				
Air	1.50 ± 0.22 (5)	0.54 ± 0.03 (5)	0.29 ± 0.05 (5)	1.21 ± 0.18 (5)
Low	1.19 ± 0.04	0.55 ± 0.03	0.21 ± 0.01	0.98 ± 0.04
Mid	1.36 ± 0.04 (6)	0.69 ± 0.04 (6)	0.25 ± 0.02 (6)	1.11 ± 0.04 (6)
High	1.22 ± 0.08	0.57 ± 0.02	0.26 ± 0.02	0.96 ± 0.08
24-Month Exposure: Female Rats				
Air	0.90 ± 0.05 (6)	0.57 ± 0.03 (6)	0.16 ± 0.02 (6)	0.74 ± 0.04 (6)
Low	1.09 ± 0.11	0.61 ± 0.03	0.20 ± 0.02	0.89 ± 0.09
Mid	1.06 ± 0.08 (6)	0.63 ± 0.01 (6)	0.17 ± 0.01 (6)	0.89 ± 0.07 (6)
High	0.98 ± 0.11 (5)	0.62 ± 0.05 (5)	0.20 ± 0.02 (5)	0.78 ± 0.09 (5)

(Table continues next page)

[a] The sample size was 7 per group unless otherwise indicated by the number in the parentheses (*n*) after the value. "nd" means not determined.

Table 6 (*Continued*). Cardiac Fibrosis and Aorta Morphometry Data (Mean ± SE) in Male and Female Rats Exposed to Air or Low-, Mid-, or High-Level DE for 1, 3, 12, or 24 Months[a]

	Mean Media Thickness (μm)	Mean Wall Thickness (μm)	Mean Adventitia Thickness (μm)	Media Thickness/Adventitia Thickness
1-Month Exposure: Male Rats				
Air	108.42 ± 5.20 (6)	144.60 ± 7.11 (6)	36.18 ± 2.68 (6)	3.09 ± 0.14 (6)
Low	106.29 ± 4.07 (5)	145.03 ± 4.15 (5)	38.74 ± 2.48 (5)	2.83 ± 0.26 (5)
Mid	116.73 ± 7.42 (6)	159.47 ± 8.06 (6)	42.75 ± 1.84 (6)	2.79 ± 0.17 (6)
High	109.27 ± 5.08	153.63 ± 5.99	44.35 ± 3.84	2.68 ± 0.24
1-Month Exposure: Female Rats				
Air	98.69 ± 3.03	136.50 ± 4.47	37.82 ± 2.38	2.68 ± 0.16
Low	101.80 ± 3.63 (6)	139.58 ± 4.10 (6)	37.79 ± 4.16 (6)	2.93 ± 0.34 (6)
Mid	100.34 ± 2.07	135.46 ± 2.66	35.13 ± 1.38	2.95 ± 0.11
High	106.88 ± 5.94	145.11 ± 6.64	38.23 ± 1.87	2.83 ± 0.19
3-Month Exposure: Male Rats				
Air	129.15 ± 4.69 (6)	172.98 ± 7.58 (6)	43.82 ± 3.88 (6)	3.07 ± 0.21 (6)
Low	122.93 ± 6.96	160.18 ± 7.44	37.25 ± 4.74	3.70 ± 0.55
Mid	124.30 ± 4.08	160.96 ± 5.63	36.66 ± 3.83	3.82 ± 0.48
High	119.60 ± 3.23	152.47 ± 2.50	32.86 ± 2.07	3.88 ± 0.29
3-Month Exposure: Female Rats				
Air	104.47 ± 3.48 (6)	136.43 ± 3.63 (6)	31.96 ± 2.15 (6)	3.42 ± 0.27 (6)
Low	117.25 ± 4.93	146.90 ± 6.96	29.65 ± 2.67	4.20 ± 0.30
Mid	112.33 ± 2.83 (5)	144.44 ± 3.75 (5)	32.11 ± 1.25 (5)	3.54 ± 0.09 (5)
High	113.22 ± 5.72	147.36 ± 6.25	34.14 ± 2.46	3.48 ± 0.27
12-Month Exposure: Male Rats				
Air	136.89 ± 4.86 (5)	174.25 ± 2.65 (5)	37.36 ± 4.09 (5)	3.98 ± 0.63 (5)
Low	141.31 ± 8.54	183.53 ± 11.12	42.22 ± 2.78	3.37 ± 0.11
Mid	142.19 ± 13.38 (6)	185.45 ± 16.32 (6)	43.25 ± 4.06 (6)	3.38 ± 0.28 (6)
High	128.65 ± 4.52 (5)	165.86 ± 5.64 (5)	37.20 ± 1.66 (5)	3.48 ± 0.14 (5)
12-Month Exposure: Female Rats				
Air	115.05 ± 9.50 (3)	148.08 ± 8.27 (3)	33.03 ± 1.78 (3)	3.56 ± 0.47 (3)
Low	122.00 ± 4.47	152.67 ± 5.68	30.67 ± 3.11	4.28 ± 0.50
Mid	125.40 ± 6.52 (6)	159.37 ± 10.86 (6)	33.97 ± 4.92 (6)	4.13 ± 0.64 (6)
High	122.53 ± 3.58	156.04 ± 5.95	33.51 ± 3.06	3.92 ± 0.32
24-Month Exposure: Male Rats				
Air	186.38 ± 14.35 (6)	227.90 ± 16.23 (6)	41.51 ± 2.26 (6)	4.76 ± 0.30 (6)
Low	154.89 ± 6.19	189.02 ± 5.58	34.13 ± 1.33	4.83 ± 0.37
Mid	168.46 ± 7.15 (6)	207.11 ± 5.32 (6)	38.65 ± 3.03 (6)	4.59 ± 0.49 (6)
High	156.62 ± 8.37	194.94 ± 8.07	38.32 ± 1.48	4.18 ± 0.34
24-Month Exposure: Female Rats				
Air	138.48 ± 5.57 (6)	171.11 ± 7.30 (6)	32.63 ± 3.47 (6)	4.50 ± 0.40 (6)
Low	153.34 ± 7.54	187.27 ± 8.93	33.93 ± 2.67	4.73 ± 0.48
Mid	162.85 ± 9.12 (6)	192.16 ± 9.26 (6)	29.31 ± 0.58 (6)	5.78 ± 0.44 (6)
High	142.35 ± 10.48 (5)	177.12 ± 10.27 (5)	34.76 ± 2.17 (5)	4.22 ± 0.47 (5)

[a] The sample size was 7 per group unless otherwise indicated by the number in the parentheses (*n*) after the value. "nd" means not determined.

Table 7. Mean Squares and *P* Values Resulting from the Final Three-Way ANOVA Model for Cardiac Fibrosis and Aorta Morphometry Measured in Male and Female Rats Exposed to Air or DE for 1, 3, 12 and 24 Months[a,b]

	% Total Left Ventricle Fibrosis[c] Original Scale		% Vascular Fibrosis[c] Original Scale		% Avascular Fibrosis[c] Box–Cox Transformation		Luminal Area (μm^2) Original Scale	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	0.495	0.507	104.04	0.502	0.068	0.778	2.49E+11	0.140
Sex	6.819	**0.001**	32.041	0.623	1.036	**0.021**	1.49E+13	**< 0.001**
Duration	26.392	**< 0.001**	42.898	0.569	5.547	**< 0.001**	7.01E+12	**< 0.001**
Exposure level:Sex	0.260	0.745	161.219	0.305	0.087	0.707	2.25E+11	0.176
Exposure level:Duration	0.109	0.915	77.024	0.626	0.099	0.661	1.25E+11	0.505
Sex:Duration	0.731	0.285	72.311	0.460	0.097	0.471	4.87E+11	**0.015**
Exposure level:Sex:Duration	0.599	0.421	166.4	0.292	0.472	0.062	1.79E+11	0.225
Residuals	0.632	NA	131.342	NA	0.186	NA	1.35E+11	NA

	Wall Area (μm^2) Rank Transformation		WA/LA Rank Transformation		Adventitia Area Rank Transformation		Media Area Rank Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	1925.505	0.190	3084.953	0.381	2995.096	0.227	1972.347	0.145
Sex	146,583.6	**< 0.001**	33,968.73	**0.001**	203,775.8	**< 0.001**	116,398.5	**< 0.001**
Duration	88,755.74	**< 0.001**	6816.043	0.082	7386.377	**0.015**	107,097.5	**< 0.001**
Exposure level:Sex	2780.038	0.077	2813.701	0.423	2420.68	0.318	2632.106	0.067
Exposure level:Duration	395.571	0.964	2575.346	0.562	1454.101	0.699	408.509	0.944
Sex:Duration	2843.048	0.072	3947.823	0.270	3791.569	0.140	2740.68	0.059
Exposure level:Sex:Duration	974.616	0.605	3246.687	0.377	4472.984	**0.025**	450.569	0.925
Residuals	1198.923	NA	2994.454	NA	2047.884	NA	1080.672	NA

	Mean Media Thickness Box–Cox Transformation		Mean Wall Thickness (μm) Box–Cox Transformation		Mean Adventitia Thickness (μm) Original Scale		Media Thickness/ Adventitia Thickness Box–Cox Transformation	
Sources of Variation	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value	Mean Square	*P* Value
Exposure level	2.64E-06	0.171	5.10E-07	0.473	26.746	0.688	0.057	0.208
Sex	6.24E-05	**< 0.001**	3.34E-05	**< 0.001**	1395.706	**< 0.001**	0.045	0.272
Duration	1.49E-04	**< 0.001**	3.70E-05	**< 0.001**	182.678	**0.020**	1.501	**< 0.001**
Exposure level:Sex	4.71E-06	**0.031**	1.46E-06	0.069	43.312	0.497	0.046	0.298
Exposure level:Duration	7.11E-07	0.902	3.23E-07	0.849	46.618	0.563	0.026	0.706
Sex:Duration	3.97E-07	0.858	3.85E-07	0.594	37.011	0.564	0.016	0.730
Exposure level:Sex:Duration	9.72E-07	0.776	6.33E-07	0.408	72.646	0.221	0.032	0.577
Residuals	1.56E-06	NA	6.07E-07	NA	54.274	NA	0.037	NA

[a] NA indicates not available.

[b] *P* values ≤ 0.05 are in **bold**, showing statistical significance.

[c] Includes data from 12 and 24 months only, and thus, the degree of freedom for duration is 1 instead of 3.

DISCUSSION

The etiology or pathogenesis of cardiovascular diseases such as atherosclerosis and heart failure is complex and multigenic. Induction and acceleration of these disease states by environmental exposures (both acute and chronic) have been established in both epidemiologic and animal exposure studies. Moreover, air pollution has been indicated as an important contributor to all-cause and CVD-related mortality and morbidity. To better understand how air pollution, and specifically DE emissions, affect increased CVD risk under chronic exposure conditions, female and male rats were exposed to DE for up to 2 years. A variety of endpoints were measured after 1 and 2 years of DE exposure to assess potential evidence of chronic cardiovascular injury.

As outlined above, three distinctive yet overlapping mechanisms are likely causal in cardiovascular morbidity and mortality associated with air pollutant exposure. These paths include (1) thrombogenicity, (2) vascular inflammation, and (3) APR (acute systemic inflammation). Thus, we measured plasma markers representative of inflammation (vascular and systemic), thrombosis, and APR along with a panel of general toxicity markers to assess potential ongoing injury as a result of the activation of a coordinated stress–response pathway (e.g., increased CRP in the APR) or of specific organ injury (e.g., ALT or CK leakage into the blood from injured hepatocytes or striated muscle, respectively). Even though we evaluated more than 20 plasma markers, we observed few exposure-dependent changes, and in both sexes no plasma marker was altered by DE at all exposure durations. This was consistent with findings reported previously for rats and mice exposed for 1 and 3 months to DE (Conklin and Kong 2012).

Importantly, we complemented plasma measures by assessing morphologic changes in cardiac and aorta structures (i.e., measuring cardiac fibrosis and the architecture of the thoracic aorta wall) to detect whether DE exposure stimulated a remodeling of the heart and aorta that is reflective of chronic cardiovascular injury. Similar to the results obtained with plasma markers, DE exposures did not alter the expected growth- and age-dependent changes in cardiac fibrosis and aortic wall morphometry in rats over the 2 years of exposure. However, the animal used in this study, the Wistar Han rat, does not develop atherosclerotic lesions, hypertension, or heart failure, as do susceptible rat models, such as the hypertensive rat (e.g., SHR) or one prone to heart failure. Nonetheless, changes in heart and aorta architecture may reflect ongoing inflammation or could arise as secondary changes resulting from DE-induced effects on hemodynamics, such as hypertension. For example, acute (4-hour) DE exposure (500 $\mu g/m^3$ $PM_{2.5}$) stimulated cardiac bradyarrhythmias and contractile changes in aged, heart-failure-prone rats (16 months old); this emphasizes both the utility of studying susceptible rats to detect DE effects (Carll et al. 2013) and the importance of monitoring endpoints appropriate to the time course under study. Although the data in this study collectively indicate that DE exposures (at levels studied) of up to 2 years (i.e., 16 hr/d $\times$ 7 d/wk $\times$ 52 wk/yr) had limited effect on the rat cardiovascular system, it is possible (albeit unlikely) that a more subtle form of injury occurred, yet went undetected.

PLASMA MARKERS: SHORT-TERM VERSUS CHRONIC EXPOSURES

As summarized in the Results section, only a handful of plasma markers were altered in the current study by 1- to 24-month DE exposures, and no consistent or obvious changes or patterns emerged. In previous studies, plasma lipids (e.g., CHOL) also have been extensively examined in female and male rats exposed to DE subchronically (Conklin and Kong 2012; Reed et al. 2004, 2005, 2006a; Seagrave et al. 2005). Although we have reported significant changes at 1 month in plasma levels of total and HDL cholesterol in male rats exposed to mid- and high-level DE (Conklin and Kong 2012), these changes were not significant in the context of plasma cholesterol changes over 24 months of DE exposure. After statistical analysis of the collective lipids data, we found only three lipids were statistically significantly changed (CHOL, non-HDL, and TRIG) in female rats over 24 months (see Table 5). For example, we observed significant decreases in both total and non-HDL cholesterol levels in female rats exposed to high-level DE for 24 months (see Table 5 and Figure 2). Similarly, previous reports have shown that chronic DE exposure — albeit at $PM_{2.5}$ levels over 50 times higher than in our current ACES investigation — decreased plasma cholesterol level in rats (Reed et al. 2004, 2005, 2006a), and we reported that mid-level DE exposure for 3 months decreased cholesterol levels in female rats (see Additional Materials 1 for this report [available on the HEI Web site]) (Conklin and Kong 2012; McDonald et al. 2012). In the current study, TRIG levels were slightly decreased in female rats at the mid-level DE exposure at 24 months, but overall, plasma TRIG levels were highly variable with no consistent pattern with respect to DE exposure over the course of the 24 months (Table 1). Because rats were not fasted prior to euthanization and blood collection, variable lipid levels, especially in TRIG, were expected, and thus, an unfasted condition confounded lipid measurements.

This issue was addressed in more detail in our initial ACES subchronic report (Conklin and Kong 2012); however, some of the variation in levels of lipids and other plasma markers also could be a consequence of age or other factors associated with chronic exposure (e.g., noise and single housing) (Peng et al. 1989; Perez et al. 1997; Perkins and Lipman 1996). Whether circulating cholesterol level is a sensitive marker of DE exposure in rats is still unclear. Thus, although we found evidence that DE exposure decreases total and non-HDL cholesterol levels in female rats, the biologic significance of these changes is unknown.

Whereas rapidly elevated levels of blood lipids are hallmarks of a coordinated APR, elevated blood levels of chemokines and certain cytokines are evidence of inflammation. We measured a variety of cytokines and chemokines in the 2-year exposure study, and only three of these were significantly altered by DE exposure compared with matched air controls (Tables 2 and 4). Significant changes in plasma IL-6 (three changes), sICAM-1 (three changes), and leptin (one change) were modest and variable with respect to direction of change and DE exposure. Of the seven changes, six occurred at either 12 or 24 months of DE exposure; three of seven changes were at the highest DE exposure level; and four of seven changes were in female rats. In all cases, no single marker was altered by DE exposure in a consistent pattern. For example, plasma sICAM-1 levels in female rats fluctuated widely after 12 months (significantly decreased) and 24 months (significantly increased) of exposure (see Table 2 and Figure 4A). Similarly, plasma leptin levels at 12 and 24 months were substantially reduced from baseline levels in young rats (Figure 5). These data establish that aging in rats in this study was the single most important determinant of baseline levels of several of our critical plasma markers.

Although these data are insufficient to indicate an age- and sex-dependent sensitivity of the endothelium to DE exposure, an activated endothelium increases the expression of adhesion molecules, including ICAM-1, and subsequent shedding of the extracellular receptor portion of those molecules leads to increased circulating levels of sICAM-1 (Nakashima et al. 1998). It is known that some toxins, such as acrolein, can increase or decrease plasma sICAM-1 levels depending on treatment conditions and rodent species (Conklin et al. 2011a, 2011b); so, in practice, a single agent can stimulate opposing effects. It should be noted, however, that the single greatest factor contributing to a significant DE effect in female rats at 12 and 24 months is the consistent baseline level of sICAM-1 in the air control group. This contrasts with baseline sICAM-1 levels in male rats that differed widely at 12 and 24 months (compare Figures 4A and 4B, and see Table 2). Thus, we speculate that DE exposure in females may slightly accelerate what is an apparent, inevitable, aging-related activation of the endothelium that has already occurred by 2 years of age in the air control male group. Interestingly, plasma IL-6 levels significantly increased in female rats exposed to DE for 24 months (mid- and high-level exposure), and perhaps, these changes in IL-6 levels are linked with changes in sICAM-1. Because vascular inflammation and endothelium injury can increase expression of both IL-6 and sICAM-1 in endothelial cells (Chen et al. 2006; Dayal et al. 2002), the relationship between these two markers may provide a stronger signal of pollutant-induced endothelium injury than either marker alone. Unfortunately, measurement of plasma markers as was done in this study did not provide definitive evidence of the specific cell, tissue, or organ source of the marker, so this hypothesis will require further study.

As in our initial study (Conklin and Kong 2012), we measured numerous plasma markers of inflammation and general toxicity, and assays were performed at the University of Louisville or by a commercial vendor using well-established assays, calibration standards, and positive "technical" 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 samples (i.e., the 12- and 24-month exposures) were processed at the same time, and thus, freezing and thawing was minimized. For lipid and protein analyses performed at the University of Louisville, samples were thawed only at the time of the analysis, and at that time a separate aliquot was refrozen and then 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 values obtained previously using the same control samples (Conklin and Kong 2012; see also Additional Materials 3 of this report). Although the data presented here and those provided by McDonald and colleagues (McDonald et al. 2012 and Part 1 of this volume) on rat sera chemistry provided external assay validation for some analytes, uncertainty remained about the quality and robustness of assays for other analytes, especially some of those measured via commercial vendor (e.g., rat SAA) (Marhaug and Dowton 1994). Because of this specific uncertainty, rat SAA was not measured in the current study. Yet fibrinogen, a marker of thrombosis and APR, and CRP, also an APR reactant, were measured, and these analytes, which varied relatively little, provided some of the most consistent data sets measured

(see Table 2). Notably, these markers (i.e., pro-APR, prothrombotic, proinflammatory) were not altered by DE exposures. We conclude that the assay conditions and protocols used were appropriate for the endpoints measured in this study.

AGE-RELATED CHANGES IN CARDIOVASCULAR ORGANS

Because of the importance of structural organ remodeling in chronic CVDs such as atherosclerosis and heart failure, we added endpoints to assess changes in the aging thoracic aorta and heart. As expected, obvious age-dependent changes in aorta morphometry (e.g., in the LA and in WT) and cardiac fibrosis were observed in both female and male rats. Despite documenting what appears to be a robust dynamic range of change in the measured parameters over time (e.g., 1% change in cardiac fibrosis over 12 months), we did not observe any statistically significant changes related to DE exposure. Wold and colleagues (2012) reported that exposing mice to CAPs for 9 months led to increased cardiac dysfunction and increased fibrosis. CAPs exposure levels used in the study by Wold and colleagues were approximately 85 $\mu g/m^3$ (i.e., about 5–6 times greater than the highest DE $PM_{2.5}$ level used in our current study). However, their exposures were only for 5 to 6 hours per day and only 5 days per week, and the estimated continuous $PM_{2.5}$ exposure integrated over the 24-hour day was in the range of 10 to 20 $\mu g/m^3$, which is similar to the highest DE exposure level in ACES (McDonald et al. 2012). Moreover, a 50-day exposure of apolipoprotein E-null mice to DE emissions led to particle-dependent alterations in atherosclerosis composition (Campen et al. 2010) indicating the potential for DE to induce aortic remodeling. Although no statistically significant changes were observed in rat aorta in the present study, nearly all the mean values for the DE-exposed groups for the aorta LA, aorta WA, and in MT were greater than (females) or less than (males) the mean values of matched air control groups (see Figure B.2 in Additional Materials 2, available on the HEI Web site). It is possible then that in this study the rudimentary morphometry methods employed and the limited sample size ($N \leq 7$ group) compromised the statistical robustness of group comparisons of these endpoints.

CONCLUSIONS

Although air pollution increases CVD risk in humans, the specific components and mechanisms responsible for this increased risk are not completely known. Likewise, many exposure studies in animals have shown that DE alters CVD risk factors or directly modifies the cardiovascular disease process and outcome. Because of the long-term (2-year) exposure period, we hypothesized that DE exposure could lead to alterations in plasma markers of CVD risk, general toxicity, or cardiovascular organ remodeling. However, evidence of any DE-induced effects was limited. For example, only in female rats exposed to DE for 2 years did we find elevated plasma levels of sICAM-1 and IL-6 along with decreased plasma total and non-HDL cholesterol. Collectively, this constellation of changes may reflect an enhanced sensitivity of the aging female cardiovascular system to chronic DE exposure (Chen et al. 2005). This conclusion likely is best interpreted within the context of the exposure conditions and methodologic shortcomings of this study: (1) rats were singly housed for the whole exposure duration, (2) tissue was collected from unfasted rats, and (3) aged female rats lose reproductive and cardioprotective status. Although we did not measure reproductive status in our current study, the literature describes amply how CVD risk increases in postmenopausal women as a result of loss of the protective action of estrogen (Maxwell 1998; Reckelhoff 2001; Schwartz et al. 1995). A previous study, done with young and aged female and male rats, provides supportive data about endothelium function and general sensitivity to a common component of DE, phenanthraquinone (Prisby et al. 2008). The data indicated that 2-year-old female rats were less protected than younger counterparts. If the DE emissions used in this study accelerated postestrous (postmenopausal) onset, then increased CVD injury (reflected by elevated plasma risk factors) is a plausible outcome as predicted by previous rat studies assessing susceptibility in aging or ovariectomized females to CVD outcomes (Castillo et al. 2005; Lee et al. 2008; Mercier et al. 2002; Mori et al. 2011). It is important to note that it is difficult to compare the data of the current study, which tested the emissions of new and emerging technologies (e.g., 2007-compliant engines, low-sulfur fuel), with those of previously published studies of DE exposures that were performed under very different conditions (e.g., different engine loads, exposure durations, fuels, and $PM_{2.5}$ levels) (Campen et al. 2003; Cherng et al. 2009; Maresh et al. 2011). Nonetheless, the results from the present study suggest that exposure of rats to DE emissions from 2007-compliant diesel engines for up to 2 years under the conditions of the study has limited effect on the aging cardiovascular system. A few major limitations related to this conclusion are worth mentioning: (1) we made a large number of statistical comparisons, which increases the likelihood of a Type I error; (2) we did not measure cardiovascular function; had we done so, we might have found direct evidence of potential DE-induced cardiovascular injury (e.g., measuring blood pressure could have revealed hypertension

and measuring vascular function could have revealed endothelium dysfunction) (Campen 2009); and (3) we did not use a susceptible-animal model that may have better reflected enhanced sensitivity (e.g., to heart failure) of more susceptible humans. Future studies could address these limitations.

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REFERENCES

Annoni G, Luvara G, Arosio B, Gagliano N, Fiordaliso F, Santambrogio D, et al. 1998. Age-dependent expression of fibrosis-related genes and collagen deposition in the rat myocardium. Mech Ageing Dev 101(1–2):57–72.

Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, et al. 2008. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. Circ Res 102(5):589–596.

Arimoto T, Takano H, Inoue K, Yanagisawa R, Yoshino S, Yamaki K, et al. 2007. Pulmonary exposure to diesel exhaust particle components enhances circulatory chemokines during lung inflammation. Int J Immunopathol Pharmacol 20(1):197–201.

Bateman RM, Tokunaga C, Kareco T, Dorscheid DR, Walley KR. 2007. Myocardial hypoxia-inducible HIF-1α, VEGF, and GLUT1 gene expression is associated with microvascular and ICAM-1 heterogeneity during endotoxemia. Am J Physiol Heart Circ PhysSiol 293(1):H448–456.

Brook RD. 2008. Potential health risks of air pollution beyond triggering acute cardiopulmonary events. JAMA 299(18):2194–2196.

Brook RD, Rajagopalan S, Pope CA III, Brook JR, Bhatnagar A, Diez-Roux AV, et al. 2010. Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. Circulation 121(21):2331–2378.

Brunekreef B, Russell A. 2009. Particulate matter and human health focus issue. Environ Sci Technol 43(13): 4613–4614.

Cabana VG, Reardon CA, Wei B, Lukens JR, Getz GS. 1999. SAA-only HDL formed during the acute phase response in apoA-I$^{+/+}$ and apoA-I$^{-/-}$ mice. J Lipid Res 40(6):1090–1103.

Campen MJ. 2009. Nitric oxide synthase: "enzyme zero" in air pollution-induced vascular toxicity. Toxicol Sci 110(1):1–3.

Campen MJ, Lund AK, Knuckles TL, Conklin DJ, Bishop B, Young D, et al. 2010. Inhaled diesel emissions alter atherosclerotic plaque composition in ApoE(−/−) mice. Toxicol Appl Pharmacol 242(3):310–317.

Campen MJ, McDonald JD, Gigliotti AP, Seilkop SK, Reed MD, Benson JM. 2003. Cardiovascular effects of inhaled diesel exhaust in spontaneously hypertensive rats. Cardiovasc Toxicol 3(4):353–361.

Carll AP, Lust RM, Hazari MS, Perez CM, Krantz QT, King CJ, et al. 2013. Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats. Toxicol Sci 131(2): 583–595.

Castillo C, Ariznavarreta MC, Lahera V, Cachofeiro V, Gil-Loyzaga P, Tresguerres JA. 2005. Effects of ovariectomy and growth hormone administration on body composition and vascular function and structure in old female rats. Biogerontology 6(1):49–60.

Channell MM, Paffett ML, Devlin RB, Madden MC, Campen MJ. 2012. Circulating factors induce coronary endothelial cell activation following exposure to inhaled diesel exhaust and nitrogen dioxide in humans: evidence from a novel translational in vitro model. Toxicol Sci 127(1):179–186.

Chen LH, Knutsen SF, Shavlik D, Beeson WL, Petersen F, Ghamsary M, et al. 2005. The association between fatal coronary heart disease and ambient particulate air pollution: are females at greater risk? Environ Health Perspect 113(12):1723–1729.

Chen Q, Fisher DT, Clancy KA, Gauguet JM, Wang WC, Unger E, et al. 2006. Fever-range thermal stress promotes lymphocyte trafficking across high endothelial venules via an interleukin 6 trans-signaling mechanism. Nat Immunol 7(12):1299–1308.

Chen TL, Liao JW, Chan WH, Hsu CY, Yang JD, Ueng TH. 2013. Induction of cardiac fibrosis and transforming growth factor-β1 by motorcycle exhaust in rats. Inhal Toxicol 25(9):525–535.

Cherng TW, Campen MJ, Knuckles TL, Gonzalez Bosc L, Kanagy NL. 2009. Impairment of coronary endothelial cell

ET(B) receptor function after short-term inhalation exposure to whole diesel emissions. Am J Physiol Regul Integr Comp Physiol 297(3):R640–647.

Conklin DJ, Barski OA, Lesgards JF, Juvan P, Rezen T, Rozman D, et al. 2010. Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. Toxicol Appl Pharmacol 243(1):1–12.

Conklin DJ, Haberzettl P, Lee J, Srivastava S. 2011a. Aldehydes and cardiovascular disease. In: Environmental Cardiology: Pollution and Heart Disease, Vol. 8. (Bhatnagar A, ed). London:Royal Society of Chemistry, 390.

Conklin DJ, Kong M. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute, 189–223.

Conklin DJ, Prough RA, Juvan P, Rezen T, Rozman D, Haberzettl P, et al. 2011b. Acrolein-induced dyslipidemia and acute-phase response are independent of HMG-CoA reductase. Mol Nutr Food Res 55(9):1411–1422.

Cornwell GG III, Thomas BP, Snyder DL. 1991. Myocardial fibrosis in aging germ-free and conventional Lobund-Wistar rats: the protective effect of diet restriction. J Gerontol 46(5):B167–170.

Dayal SD, Hasko G, Lu Q, Xu DZ, Caruso JM, Sambol JT, et al. 2002. Trauma/hemorrhagic shock mesenteric lymph upregulates adhesion molecule expression and IL-6 production in human umbilical vein endothelial cells. Shock 17(6):491–495.

Erdely A, Liston A, Salmen-Muniz R, Hulderman T, Young SH, Zeidler-Erdely PC, et al. 2011. Identification of systemic markers from a pulmonary carbon nanotube exposure. J Occup Environ Med 53(6 Suppl):S80–86.

Esmon CT, Fukudome K, Mather T, Bode W, Regan LM, Stearns-Kurosawa DJ, et al. 1999. Inflammation, sepsis, and coagulation. Haematologica 84(3):254–259.

Fantuzzi G, Faggioni R. 2000. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol 68(4):437–446.

Faraway J. 2005. Linear Models. New York:CRC Press.

Fujimaki H, Kurokawa Y, Yamamoto S, Satoh M. 2006. Distinct requirements for interleukin-6 in airway inflammation induced by diesel exhaust in mice. Immunopharmacol Immunotoxicol 28(4):703–714.

Gervois P, Kleemann R, Pilon A, Percevault F, Koenig W, Staels B, et al. 2004. Global suppression of IL-6-induced acute phase response gene expression after chronic in vivo treatment with the peroxisome proliferator-activated receptor-alpha activator fenofibrate. J Biol Chem 279(16): 16154–16160.

Ghio AJ, Sobus JR, Pleil JD, Madden MC. 2012. Controlled human exposures to diesel exhaust. Swiss Med Wkly 142: w13597.

Grad S, Ertel W, Keel M, Infanger M, Vonderschmitt DJ, Maly FE. 1998. Strongly enhanced serum levels of vascular endothelial growth factor (VEGF) after polytrauma and burn. Clin Chem Lab Med 36(6):379–383.

Haberzettl P, Lee J, Duggineni D, McCracken J, Bolanowski D, O'Toole TE, et al. 2012. Exposure to ambient air fine particulate matter prevents VEGF-induced mobilization of endothelial progenitor cells from the bone marrow. Environ Health Perspect 120(6):848–856.

Harris HW, Gosnell JE, Kumwenda ZL. 2000. The lipemia of sepsis: triglyceride-rich lipoproteins as agents of innate immunity. J Endotoxin Res 6(6):421–430.

Hiramatsu K, Azuma A, Kudoh S, Desaki M, Takizawa H, Sugawara I. 2003. Inhalation of diesel exhaust for three months affects major cytokine expression and induces bronchus-associated lymphoid tissue formation in murine lungs. Exp Lung Res 29(8):607–622.

Hirano S, Furuyama A, Koike E, Kobayashi T. 2003. Oxidative-stress potency of organic extracts of diesel exhaust and urban fine particles in rat heart microvessel endothelial cells. Toxicology 187(2–3):161–170.

Hukkanen RR, Liggitt HD, Murnane RD, Frevert CW. 2009. Systemic inflammatory response syndrome in nonhuman primates culminating in multiple organ failure, acute lung injury, and disseminated intravascular coagulation. Toxicol Pathol 37(6):799–804.

Ikeda M, Shitashige M, Yamasaki H, Sagai M, Tomita T. 1995a. Oxidative modification of low density lipoprotein by diesel exhaust particles. Biol Pharm Bull 18(6):866–871.

Ikeda M, Suzuki M, Watarai K, Sagai M, Tomita T. 1995b. Impairment of endothelium-dependent relaxation by diesel exhaust particles in rat thoracic aorta. Jpn J Pharmacol 68(2):183–189.

Inoue K, Takano H, Sakurai M, Oda T, Tamura H, Yanagisawa R, et al. 2006. Pulmonary exposure to diesel exhaust particles enhances coagulatory disturbance with endothelial damage and systemic inflammation related to lung inflammation. Exp Biol Med (Maywood) 231(10):1626–1632.

Kindy MS, de Beer MC, Yu J, de Beer FC. 2000. Expression of mouse acute-phase (SAA1.1) and constitutive (SAA4) serum amyloid A isotypes: influence on lipoprotein profiles. Arterioscler Thromb Vasc Biol 20(6):1543–1550.

Kitagawa K, Matsumoto M, Sasaki T, Hashimoto H, Kuwabara K, Ohtsuki T, et al. 2002. Involvement of ICAM-1 in the progression of atherosclerosis in APOE-knockout mice. Atherosclerosis 160(2):305–310.

Kodavanti UP, Thomas R, Ledbetter AD, Schladweiler MC, Shannahan JH, Wallenborn JG, et al. 2011. Vascular and cardiac impairments in rats inhaling ozone and diesel exhaust particles. Environ Health Perspect 119(3):312–318.

Lee SD, Kuo WW, Ho YJ, Lin AC, Tsai CH, Wang HF, et al. 2008. Cardiac Fas-dependent and mitochondria-dependent apoptosis in ovariectomized rats. Maturitas 61(3): 268–277.

Li YJ, Kawada T, Matsumoto A, Azuma A, Kudoh S, Takizawa H, et al. 2007. Airway inflammatory responses to oxidative stress induced by low-dose diesel exhaust particle exposure differ between mouse strains. Exp Lung Res 33(5):227–244.

Maresh JG, Campen MJ, Reed MD, Darrow AL, Shohet RV. 2011. Hypercholesterolemia potentiates aortic endothelial response to inhaled diesel exhaust. Inhal Toxicol 23(1):1–10.

Marhaug G, Dowton SB. 1994. Serum amyloid A: an acute phase apolipoprotein and precursor of AA amyloid. Baillieres Clin Rheumatol 8(3):553–573.

Maxwell SR. 1998. Women and heart disease. Basic Res Cardiol 93 Suppl 2:79–84.

McDonald JD, Doyle-Eisele M, Gigliotti A, Miller RA, Seilkop S, Mauderly JL, et al. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute, 9–120.

Mercier I, Pham-Dang M, Clement R, Gosselin H, Colombo F, Rouleau JL, et al. 2002. Elevated mean arterial pressure in the ovariectomized rat was normalized by ET(A) receptor antagonist therapy: absence of cardiac hypertrophy and fibrosis. Br J Pharmacol 136(5):685–692.

Mori T, Kai H, Kajimoto H, Koga M, Kudo H, Takayama N, et al. 2011. Enhanced cardiac inflammation and fibrosis in ovariectomized hypertensive rats: a possible mechanism of diastolic dysfunction in postmenopausal women. Hypertens Res 34(4):496–502.

Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R. 1998. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler Thromb Vasc Biol 18(5):842–851.

Nolan A, Weiden MD, Thurston G, Gold JA. 2004. Vascular endothelial growth factor blockade reduces plasma cytokines in a murine model of polymicrobial sepsis. Inflammation 28(5):271–278.

Peng X, Lang CM, Drozdowicz CK, Ohlsson-Wilhelm BM. 1989. Effect of cage population density on plasma corticosterone and peripheral lymphocyte populations of laboratory mice. Lab Anim 23(4):302–306.

Perez C, Canal JR, Dominguez E, Campillo JE, Guillen M, Torres MD. 1997. Individual housing influences certain biochemical parameters in the rat. Lab Anim 31(4):357–361.

Perkins SE, Lipman NS. 1996. Evaluation of microenvironmental conditions and noise generation in three individually ventilated rodent caging systems and static isolator cages. Contemp Top Lab Anim Sci 35(2):61–65.

Peters A, Fröhlich M, Döring A, Immervoll T, Wichmann HE, Hutchinson WL, et al. 2001. Particulate air pollution is associated with an acute phase response in men; results from the MONICA-Augsburg Study. EurHeart J 22(14): 1198–1204.

Pickkers P, Sprong T, Eijk L, Hoeven H, Smits P, Deuren M. 2005. Vascular endothelial growth factor is increased during the first 48 hours of human septic shock and correlates with vascular permeability. Shock 24(6):508–512.

Pope CA II, Dockery DW. 2006. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 56(6):709–742.

Pope CA III, Hansen ML, Long RW, Nielsen KR, Eatough NL, Wilson WE, et al. 2004. Ambient particulate air pollution, heart rate variability, and blood markers of inflammation in a panel of elderly subjects. Environ Health Perspect 112(3):339–345.

Prisby RD, Muller-Delp J, Delp MD, Nurkiewicz TR. 2008. Age, gender, and hormonal status modulate the vascular toxicity of the diesel exhaust extract phenanthraquinone. J Toxicol Environ Health A 71(7):464–470.

Reckelhoff JF. 2001. Gender differences in the regulation of blood pressure. Hypertension 37(5):1199–1208.

Reed MD, Blair LF, Burling K, Daly I, Gigliotti AP, Gudi R, et al. 2005. Health effects of subchronic exposure to diesel-water emulsion emission. Inhal Toxicol 17(14):851–870.

Reed MD, Blair LF, Burling K, Daly I, Gigliotti AP, Gudi R, et al. 2006a. Health effects of subchronic exposure to diesel-water-methanol emulsion emission. Toxicol Ind Health 22(2):65–85.

Reed MD, Campen MJ, Gigliotti AP, Harrod KS, McDonald JD, Seagrave JC, et al. 2006b. Health effects of subchronic exposure to environmental levels of hardwood smoke. Inhal Toxicol 18(8):523–539.

Reed MD, Gigliotti AP, McDonald JD, Seagrave JC, Seilkop SK, Mauderly JL. 2004. Health effects of subchronic exposure to environmental levels of diesel exhaust. Inhal Toxicol 16(4):177–193.

Reinhart WH. 2003. Fibrinogen — marker or mediator of vascular disease? Vasc Med 8(3):211–216.

Rosenbloom JI, Wilker EH, Mukamal KJ, Schwartz J, Mittleman MA. 2012. Residential proximity to major roadway and 10-year all-cause mortality after myocardial infarction. Circulation 125(18):2197–2203.

Saber AT, Bornholdt J, Dybdahl M, Sharma AK, Loft S, Vogel U, et al. 2005. Tumor necrosis factor is not required for particle-induced genotoxicity and pulmonary inflammation. Arch Toxicol 79(3):177–182.

Saber AT, Jacobsen NR, Bornholdt J, Kjaer SL, Dybdahl M, Risom L, et al. 2006. Cytokine expression in mice exposed to diesel exhaust particles by inhalation. Role of tumor necrosis factor. Part Fibre Toxicol 3:4.

Saito Y, Azuma A, Kudo S, Takizawa H, Sugawara I. 2002. Long-term inhalation of diesel exhaust affects cytokine expression in murine lung tissues: comparison between low- and high-dose diesel exhaust exposure. Exp Lung Res 28(6):493–506.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7): 671–675.

Schwartz J, Freeman R, Frishman W. 1995. Clinical pharmacology of estrogens: cardiovascular actions and cardioprotective benefits of replacement therapy in postmenopausal women. J Clin Pharmacol 35(1):1–16.

Seagrave J, McDonald JD, Reed MD, Seilkop SK, Mauderly JL. 2005. Responses to subchronic inhalation of low concentrations of diesel exhaust and hardwood smoke measured in rat bronchoalveolar lavage fluid. Inhal Toxicol 17(12):657–670.

Shao B, Fu X, McDonald TO, Green PS, Uchida K, O'Brien KD, et al. 2005a. Acrolein impairs ATP binding cassette transporter A1-dependent cholesterol export from cells through site-specific modification of apolipoprotein A-I. J Biol Chem 280(43):36386–36396.

Shao B, O'Brien KD, McDonald TO, Fu X, Oram JF, Uchida K, et al. 2005b. Acrolein modifies apolipoprotein A-I in the human artery wall. Ann N Y Acad Sci 1043:396–403.

Sithu SD, Srivastava S, Siddiqui MA, Vladykovskaya E, Riggs DW, Conklin DJ, et al. 2010. Exposure to acrolein by inhalation causes platelet activation. Toxicol Appl Pharmacol 248(2):100–110.

Srivastava S, Sithu SD, Vladykovskaya E, Haberzettl P, Hoetker DJ, Siddiqui MA, et al. 2011. Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. Atherosclerosis 215(2):301–308.

Tsakadze NL, Sen U, Zhao Z, Sithu SD, English WR, D'Souza SE. 2004. Signals mediating cleavage of intercellular adhesion molecule-1. Am J Physiol Cell Physiol 287(1):C55–63.

Venables W, Ripley B. 2002. Modern Applied Statistics with S. New York:Springer.

Wheat LA, Haberzettl P, Hellmann J, Baba SP, Bertke M, Lee J, et al. 2011. Acrolein inhalation prevents vascular endothelial growth factor-induced mobilization of flk-1+/sca-1+ cells in mice. Arterioscler Thromb Vasc Biol 31(7):1598–1606.

Wold LE, Ying Z, Hutchinson KR, Velten M, Gorr MW, Velten C, et al. 2012. Cardiovascular remodeling in response to long-term exposure to fine particulate matter air pollution. Circ Heart Fail 5(4):452–461.

Yano K, Liaw PC, Mullington JM, Shih SC, Okada H, Bodyak N, et al. 2006. Vascular endothelial growth factor

is an important determinant of sepsis morbidity and mortality. J Exp Med 203(6):1447–1458.

Ying Z, Yue P, Xu X, Zhong M, Sun Q, Mikolaj M, et al. 2009. Air pollution and cardiac remodeling: a role for RhoA/Rho-kinase. Am J Physiol Heart Circ Physiol 296(5): H1540–1550.

Zambon A, Gervois P, Pauletto P, Fruchart JC, Staels B. 2006. Modulation of hepatic inflammatory risk markers of cardiovascular diseases by PPAR-alpha activators: clinical and experimental evidence. Arterioscler Thromb Vasc Biol 26(5):977–986.

ADDITIONAL MATERIALS AVAILABLE ON THE WEB

Additional Materials contain supplemental material not included in the printed report. They are available on the HEI Web site at *http://pubs.healtheffects.org*.

Additional Materials 1. Summary Data Tables of Plasma Markers Measured in Rats Exposed to Filtered Air or Diesel Exhaust Emissions at 1 or 3 Months of Exposure

Additional Materials 2. Aorta Morphometry in Rats Over 24 Months of Exposure to Filtered Air or Diesel Exhaust Emissions

Additional Materials 3. Plasma Levels of Markers Measured in Rats Exposed to Acrolein as Positive Technical Controls

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OTHER PUBLICATION RESULTING FROM THIS RESEARCH

Conklin DJ, Kong M. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA: Health Effects Institute.

ABBREVIATIONS AND OTHER TERMS

ALB	albumin
ALT	alanine aminotransferase
ANOVA	analysis of variance
APR	acute phase response
AST	aspartate aminotransferase
CAPs	concentrated ambient particles
CHOL	total cholesterol
CK	creatine kinase
CREA	creatinine
CRP	C-reactive protein
CVD	cardiovascular disease
DE	diesel exhaust
ELISA	enzyme-linked immunosorbent assay
HDL	high-density lipoprotein
GM-CSF	granulocyte-macrophage colony-stimulating factor
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-10	interleukin-10
KC	keratinocyte-derived chemoattractant
LA	luminal area
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LRRI	Lovelace Respiratory Research Institute
MCP-1	monocyte chemotactic protein 1
MT	media thickness
NAP	non-albumin protein
NO_2	nitrogen dioxide
non-HDL	non-high-density lipoprotein (cholesterol)
OEL	outer elastic lamina
$PM_{2.5}$	particulate matter ≤ 2.5 μm in aerodynamic diameter
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
VEGF	vascular endothelial growth factor
WA	wall area
WT	wall thickness

COMMENTARY

HEI ACES Review Panel

Research Report 184, *Advanced Collaborative Emissions Study (ACES): Lifetime Cancer and Non-Cancer Assessment in Rats Exposed to New-Technology Diesel Exhaust,* 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 the four studies conducted as Phase 3B of the multipart Advanced Collaborative Emissions Study (ACES*) program. HEI's evaluation was conducted by a specially convened ACES Review Panel.

These studies investigated the health effects of exposures of rats over their lifetime (up to 30 months) and of mice for 3 months to diesel exhaust emissions from a heavy-duty diesel engine system compliant with U.S. 2007 regulations. These emissions are referred to as *new-technology diesel exhaust* (NTDE) to distinguish them from the emissions of older, pre-2007 engine systems that did not use these technologies, referred to as *traditional-technology diesel exhaust* (TDE).

The core study was led by Dr. Jacob McDonald, of Lovelace Respiratory Research Institute (LRRI) in Albuquerque, New Mexico, where the exposures were carried out. Other ancillary studies were led by 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. These ancillary studies evaluated endpoints not examined in the core study. Included in this volume is the report from McDonald and colleagues, with results from the final exposure time point — 28 months (males) and 30 months (females) — as well as interim results from 1, 3, 12 and 24 months, and the reports from Bemis, Hallberg, and Conklin and their colleagues, with data from 1-, 3-, 12- and 24-month exposure times. A previous HEI Research Report (ACES 2012) presented results from the subchronic exposure time points of 1 and 3 months (with some 12-month results from McDonald and colleagues' study).

This Commentary, prepared by the Panel, is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of these studies and by placing them in a scientific and regulatory perspective.

BACKGROUND TO THE ACES PROGRAM

In light of concerns identified over many decades about the potential health effects of diesel emissions, the U.S. EPA and the California Air Resources Board adopted stringent new regulations for heavy-duty highway diesel engines, which were required to meet a new standard for PM by 2007. A tighter standard for nitrogen oxides (NO_x, 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.

Engines needed to meet a new standard for PM (light-duty engines by 2006 and heavy-duty engines by 2007) and for NO_x (by 2010). Engine manufacturers and other industries developed advanced engine technologies 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 fuel (< 15 ppm) was essential for proper functioning of the aftertreatment systems. Introduction of these changes was expected to result in substantially

Dr. McDonald's 4-year study, "Development of a Diesel Exhaust Exposure Facility and Conduct of a Chronic Inhalation Bioassay in Rats and Mice," began in February 2010. Total expenditures were $8,423,802. The draft Investigators' Report from Dr. McDonald and colleagues was received for review in January 2014. A revised report, received in June 2014, was accepted for publication in July 2014.

Dr. Bemis's 2.5-year study, "Genotoxicity of Inhaled Diesel Exhaust: Examination of Rodent Blood for Micronucleus Formation," began in February 2010. Total expenditures were $28,340. The draft Investigators' Report from Dr. Bemis and colleagues was received for review in March 2013. A revised report, received in July 2013, was accepted for publication in August 2013.

Dr. Hallberg's 2.5-year study, "Assessment of the Genotoxicity of Diesel Exhaust/Diesel Exhaust Particulates from Improved Diesel Engines," began in May 2010. Total expenditures were $63,380. The draft Investigators' Report from Dr. Hallberg and colleagues was received for review in March 2013. A revised report, received in December 2013, was accepted for publication in April 2014.

Drs. Conklin and Kong's 2.5-year study, "Effects of Diesel Emissions on Vascular Inflammation and Thrombosis," began in February 2010. Total expenditures were $91,920. The draft Investigators' Report from Conklin and Kong was received for review in March 2013. A revised report, received in September 2013, was accepted for publication in October 2013.

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.

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

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 — estimated at about a 90% reduction compared with emissions from pre-2007 engine systems — would have substantial public health benefits. HEI's industry 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 reduce 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 goals of ACES were to characterize the exhaust emissions of these new advanced heavy-duty diesel (Class 8) engines with aftertreatment systems 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 report, ACES was designed in three phases. Phases 1 and 2 focused on emissions characterization from 2007- and 2010-compliant engines, respectively; Phase 3 focused on an evaluation of the health effects of emissions from a 2007-compliant engine. In view of its expertise in emissions characterization and its existing expert committee and oversight structure for planning and monitoring 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 non-cancer effects in rats and mice exposed to emissions from a 2007-compliant engine system, or NTDE. The study was to be conducted in two subphases: *Phase 3A,* the establishment of an exposure facility, as well as the characterization and optimization of engine exposure conditions before the start of the inhalation study (for a description of this substudy and the results, see Mauderly and McDonald 2012), and *Phase 3B*, the evaluation of health outcomes in animals exposed to NTDE for up to 24 months or longer, depending on the number of animals surviving.

In response, Dr. Joe L. Mauderly and a team at LRRI 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 staff, the ACES Oversight Committee, and additional experts, Dr. Mauderly's proposal was selected, and the study was approved by the HEI Board of Directors in February 2007. When Dr. Mauderly retired in 2010, 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 longer daily exposure period 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 and vascular markers of inflammation and damage — that were not included in the core chronic inhalation bioassay funded under RFP 06-1. As described in the Preface, HEI selected five investigator teams to conduct these Phase 3B ancillary studies. Three teams completed their studies.

REVIEW OF THE ACES PHASE 3B REPORTS

ACES Phase 3B was designed to examine the effects of chronic exposure to NTDE 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, as well as at the end of the study. Thus, each investigator team prepared an Investigators' Report with results from subchronic exposures of rats and mice (1- and 3-month

time points) and submitted it to HEI. Then, at the end of the study, the investigator teams submitted reports with data from the chronic exposures of rats.

To review the reports from the ACES Phase 3B core and ancillary studies, HEI set up a special ACES Review Panel (see the list of Panel members at the beginning of this volume). 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. The ACES Review Panel reviewed the investigators' reports and made suggestions to the investigators to modify their reports by revising them, predominantly to enhance clarity, to explain further some of the statistical analyses, and to consider alternative interpretations of the data. The investigators submitted revised reports that the Panel subsequently reviewed and approved for publication by HEI. The Panel summarized its review of the reports of the subchronic exposures in the Commentary accompanying the reports published in HEI Research Report 166 (ACES 2012). The current Commentary summarizes the Panel's review of the reports submitted at the end of the study and containing data from all exposure time points.

HEI set up an Exposure Characterization Review Panel (see the list of members at the front of this volume) to review the exposure characterization report submitted by McDonald and colleagues (Appendix I of Part 1; available on the Web at *http://pubs.healtheffects.org*). This panel reviewed a draft report and recommended revisions, which the investigators addressed in a revised report submitted shortly before the issue of this volume. The panel was not able to review the revised version in time for publication.

COMMENTARY ON THE CORE STUDY OF BIOLOGIC EFFECTS BY MCDONALD AND COLLEAGUES: ASSESSMENT OF CARCINOGENICITY AND BIOLOGIC RESPONSES IN RATS AFTER LIFETIME INHALATION OF NEW-TECHNOLOGY DIESEL EXHAUST IN THE ACES BIOASSAY

SCIENTIFIC BACKGROUND

Long-Term Inhalation Exposure to "Traditional Diesel" in Rats

Before the current study of NTDE emissions from a 2007-compliant engine, several studies had investigated the effects of long-term inhalation exposure in multiple animal species to TDE (reviewed in Hesterberg et al. 2005; International Agency for Research on Cancer [IARC] 2012) — emissions derived from pre-2007 diesel engines (i.e., before the new technologies were introduced and the 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 TDE concentrations (> 2 mg/m^3 PM) 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. 1987b, 1994; Nikula et al. 1995). In contrast to the effects in rats, few studies conducted in mice (or hamsters) found excess tumors resulting from long-term inhalation of TDE 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 TDE concentrations that induced tumors in rats.

The importance of the particulate fraction of TDE in causing tumors in rats in these early studies was supported by studies in which TDE particles had been removed from the exhaust by filters; in the absence of TDE 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). Findings from the study by Heinrich and colleagues (1986) are particularly relevant because they were conducted in female Wistar Han rats (after 24 months of exposure), the same strain as was used in the current study. As in the current study, PM emissions in the filtered TDE were very low, as was the ratio of PM to NO_2. The NO_2 level (1.1 ppm) in the 1986 study by Heinrich and colleagues was comparable to the lowest level of NTDE exposure in the current study, but the NO_2:NO_x ratio in the Heinrich study was somewhat lower than the ratio found in NTDE at the highest level of exposure in the current study. In a later long-term study (1995) of exposure to TDE from which particles had been removed, Heinrich and colleagues reported levels of PM and NO_2 and ratios of NO_2:NO_x and of PM:NO_2 that were similar to their 1986 findings (see Results section of the current study).

Pathways of Induction of Tumors in Rats Exposed to DE

Several early studies identified organic components of DE as carcinogenic when applied to the skin or implanted in the lungs of rodents (Grimmer et al. 1987; Nesnow et al. 1982) 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 TDE (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 1989). Thus, a major hypothesis that emerged from these studies to explain the carcinogenicity of TDE was that organic components of DE damaged deoxyribonucleic acid (DNA) — for example, by inducing the formation of adducts —leading to an increase in the number of mutations (see Beland 1995).

However, long-term inhalation studies in rats that compared the effects of exposure to high concentrations of TDE (2.5 mg/m^3 PM or higher) 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 high concentrations of TDE 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 (Mauderly and McCunney 1996; Nikula et al. 1995). Particle overload was hypothesized to result in the generation of reactive oxygen species (ROS) and oxidative damage (e.g., 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 TDE enhances damage to DNA, such as by 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 of F344 rats in the study by Nikula and colleagues (1995), did not find an increase in DNA adducts. In contrast, Iwai and colleagues (2000) found increases in levels of DNA adducts and 8-hydroxy-deoxyguanosine (8-OHdG) in the lung tissue of F344 female rats over the course of a 12-month exposure to 3.5 mg/m^3 TDE. The investigators associated the development of tumors in these rats at the end of the study (30 months) to the earlier effects of oxidative damage from TDE. Reed and colleagues (2004) at LRRI exposed male and female A/J mice and male and female F344 rats to different concentrations of TDE (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 TDE did not affect the number of micronucleated reticulocytes (MN-RETs) or the incidence of lung tumors (adenomas) 6 months after the end of the exposure.

In 2002 the U.S. EPA discussed the possible mode of action of DE in its *Health Assessment Document for Diesel Engine Exhaust* (2002) and again considered the hypothesis that tumor induction in rats exposed to high TDE concentrations was related to the overloading of normal lung clearance mechanisms, the accumulation of particles, lung inflammation, and cell damage. The EPA also postulated that other mechanisms may predominate at lower DE concentrations; for example, production of ROS generated from organic compounds may cause DNA damage at concentrations below those required to produce particle overload. Moreover, organic compounds, especially PAHs, can bind to and damage DNA and may be involved in the induction of tumors.

Based in part on these toxicology results, IARC (1989) classified diesel exhaust as a Group 2A "probable human carcinogen." In 2012, while the current study was underway, IARC convened a panel to evaluate the full body of scientific literature on the effects of DE. Included in the review were two recently published epidemiologic studies of historical exposures to diesel exhaust in occupational settings, the Diesel Exhaust in Miners Study (Attfield et al. 2012) and the Truckers Study (Garshick et al. 2012). Both studies had been specifically designed to address shortcomings identified in earlier epidemiologic studies of diesel exhaust and lung cancer. As a consequence of this review, IARC reclassified diesel exhaust as a Group 1 carcinogen ("carcinogenic to humans") (IARC 2012). McDonald and colleagues' report (ACES 2012) of subchronic ACES findings of the effects of NTDE in mice and rats, accompanied by the HEI ACES Review Panel's Commentary, was evaluated and included in the IARC report. However, although IARC recognized that NTDE differed from TDE, this first report of the effects of NTDE was not discussed at length in the IARC review.

Long-Term Exposure to NO_2

In previous TDE animal exposure studies, dilution levels were based on PM concentrations. However, the HEI ACES Oversight Committee, which designed the current study, recognized that this approach would not be viable in ACES because of the low particle concentrations in the exhaust of the 2007-compliant diesel engines and control systems (Khalek et al. 2009, 2011). They decided to use NO_2 as the target component in NTDE for determining the dilution ratios for animal exposures. Their reasons for choosing NO_2 were that it is a major gaseous component of both TDE and NTDE and that it is known to have respiratory effects in

rodents (see below in this section). Thus, as explained in the Preface, the highest DE exposure level in the current study was set with a concentration of NO_2 that was expected to have some biologic effects.

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. 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/day, 5 day/wk = 332.5 ppm • hr/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 suggested that, at the exposure levels used, NO_2 induced mild cytotoxicity and oxidative stress. There was no increase in inflammatory cells in BALF.

In their second study, Mauderly and colleagues (1989) used NO_2 exposure conditions identical to those used in their 1987 study for a 24-month NO_2-exposure study in rats. 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 and 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 (specifically, increased 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 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 NTDE to 4.2 ppm, using a 16-hour exposure cycle for 5 day/wk (for a total of 336 ppm • hour/week). 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. This issue is discussed further in the next subsection "Technical Approach" in this Commentary.

Kubota et al. (1987) conducted a morphometric study of Wistar Han rats — the strain used in the current study — exposed continuously to 0.04, 0.4, and 4.0 ppm NO_2 for up to 27 months. They found no effects of 0.04 ppm throughout the study, but rats exposed to 0.4 ppm NO_2 showed small alterations to the epithelium after 27 months. Exposure to 4 ppm NO_2 resulted in proliferation of the bronchial epithelium at 9 months, which progressed further at 18 months; fibrosis was noted at 27 months. Exposure to 4 ppm NO_2 also showed an increase of the arithmetic mean of the thickness of the alveolar wall at 9 months, which was significant after 18 months. The arithmetic mean of the thickness was slightly decreased at 27 months compared with 18 months, which the investigators interpreted as a "recovery of alveolar epithelium and decreased amount of septal edema, which in turn led to fibrosis."

Previous reports have also evaluated the effects of longer-term NO_2 exposure on some of the same biochemical endpoints examined in the current core and ancillary ACES reports. For example, male Wistar Han rats exposed to 4.0 ppm NO_2 for 9 months or longer had increases in a marker of oxidative stress — lipid peroxidation, as measured by an increase in thiobarbituric acid reactive substances (TBARS) (Sagai et al. 1984). In the same study, long-term exposure (18 months) to 0.4 or 4.0 ppm NO_2 also decreased the activity of enzymes that regulate levels of the antioxidant GSH.

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 long-term exposure to NO_2 has not been linked to carcinogenicity in animal studies (U.S. EPA 2008). That assessment also concluded that acute exposure studies have shed light on potential mechanisms of NO_2 effects in the airways: 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 resulted in acute inflammatory effects in the airways, such as increased numbers of polymorphonuclear (PMN) leukocytes in BALF, within hours after exposure (e.g., Frampton et al. 2002; Solomon et al. 2000). 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.

TECHNICAL APPROACH

As discussed in the Preface, before the Phase 3B studies began, the ACES Oversight Committee and the ACES

investigators at LRRI decided on several key design features, as follows:

- Of the four heavy heavy-duty diesel engines (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 tested in ACES Phase 1, one would be selected for health effects testing. As described below, a second, backup engine was also used for different periods in the study.
- Wistar Han rats and C57BL/6 mice would be evaluated.
- The duration of exposure would be 24 months for rats, with evaluations at intermediate time points; the duration might be extended up to 30 months, depending on survival. For mice, the exposure duration would be 3 months.
- Three concentrations of NTDE, based on NO_2 concentrations, and a control atmosphere (filtered air) would be evaluated.

Objectives and Hypothesis

The goal of the ACES Phase 3B exposure studies was to evaluate the effects of lifetime inhalation exposure (up to 30 months) of NTDE in rats and in mice (exposed for up to 3 months).

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.

As indicated above, this Commentary and accompanying reports focus on the results of chronic exposure (up to 30 months) of rats to NTDE; results from 3-month exposures of mice can be found in their entirety in HEI Research Report 166 (ACES 2012) and are discussed in the Commentary accompanying that report.

Study Design

Engine Selection ACES Phase 1 involved the extensive characterization at the Southwest Research Institute (SwRI) of gaseous and particulate emissions from the four heavy-duty diesel engines (model year 2007) made available by different engine manufacturers (Khalek et al 2009). The engine selection process for Phase 3B was guided by a group comprising a subset of members of the CRC ACES Panel (described in the Preface) and the HEI ACES Oversight Committee. In brief, after a review of the emissions indicated similar generation of gaseous and particulate-phase compounds from all four engines, engine B was randomly selected for the health study. After the selection had been made, the manufacturer of engine B (Detroit Diesel, a subsidiary of Daimler Corp.) provided a backup engine (referred to as B′). Engine B′ had the same technical specifications and was equipped with the same emission controls as engine B, but was of model year 2008. Engine B′ underwent testing at SwRI for regulated emission characterization and was then shipped to the LRRI investigators for further testing, in parallel with engine B, as part of ACES Phase 3A. The results of these tests were published in HEI Communication 17 by Mauderly and McDonald (2012).

Generation and Characterization of NTDE Comprehensive information about the setup of the emissions dilution 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 I (available on the Web at *http://pubs.healtheffects.org*) of the report by McDonald and colleagues, and a summary of the composition of NTDE in the study is provided in Table 3 of that report (Part 1 of the current volume).

Generation During the study McDonald and colleagues generated exhaust from two similar 2007-compliant heavy-duty diesel engines, termed B and B′, as described above. A decision was made to start the animal exposures using engine B′ because it was a slightly newer model that was thought to better represent the engines in the marketplace at that time (Donald Keski-Hynnila, Detroit Diesel, personal communication, 8 September 2009). During the study, engine B′ was used from February 2010 to September 2011. Because of engine maintenance requirements, this engine was replaced with engine B, which continued in operation until May 2012. Engine B′ was reinstalled in June 2012 and operated until the end of the study (December 2012).

Both engines were fueled with ultra-low-sulfur diesel fuel meeting current on-road specifications and were operated with a dynamometer. The engines 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 engines were run on a unique and strenuous 16-hour cycle specifically designed for the ACES program (described in the Preface) to represent more closely the real-world operations of modern engines than the operating cycles used in older long-term studies of TDE.

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 animal inhalation chamber. Three dilution ratios were determined 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 NTDE 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 NTDE exposure level (1.5–2°C higher than lower exposure levels and control).

Exposure Characterization A detailed description of exposure characterization of the animal chambers is found in Appendix I of Part 1 of this volume (McDonald et al.), available on the Web at *http://pubs.healtheffects.org*. Note that Appendix I contains data from both the 30-month rat exposure study and the 3-month mouse exposure study, which was completed before the lifetime rat exposures started.

In brief, 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), particle mass (using a Dekati Mass Monitor) and particle size distribution (using aerodynamic and fast mobility particle sizers), and black carbon (using a photoacoustic spectrometer) were also measured continuously in the high-exposure chamber on a daily basis. During periodic intensive characterizations (at 2.5, 11.5, and 23.5 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 the 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 140 male and 140 female 6-week-old Wistar Han rats per exposure group were entered in the study (see Table 1 of the report by McDonald and colleagues, Part 1 of this volume) and exposed at LRRI to filtered air as a control or one of the three targeted NTDE dilutions — 4.2 (high), 0.8 (mid), or 0.1 (low) ppm NO_2. Exposures were conducted 16 hours per day (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).

Groups of 10 male and 10 female rats (per exposure group) were euthanized after 1, 3, 12, and 24 months of exposure. LRRI investigators harvested blood and tissues at these time points and analyzed them as described below. The LRRI investigators also sent aliquots of blood and tissue samples from these animals at these time points to the ACES Phase 3B ancillary studies investigators. At 3, 12, and 24 months, the LRRI investigators also measured the rats' pulmonary function before euthanizing them.

The remaining rats at each time point were assigned to the chronic inhalation groups for assessment of cancer and pre-cancerous endpoints at the end of the exposure period. Thus, 100 animals per sex and per exposure level were assigned to the terminal sacrifice for these evaluations. In effect, as explained in Table 1 of McDonald et al., the total pool of animals evaluated for the chronic study was slightly more than 140, because the investigators had purchased a few extra animals in anticipation of losses during shipping from the breeder. All of these animals were examined histologically.

Most of the animals developed a pododermatitis (ulcerative foot lesions) by 12 months into the study. This condition was treated and changes were made to the cages so that the animals were not resting on the wire-mesh floors.

If the condition became severe, the animal was euthanized. This occurred in 183 out of over 1000 animals in the study, predominantly in the (heavier) male rats.

As specified in the original project plan, the duration of the exposure was scheduled for 24 months, unless survival in the control group decreased to less than 20%, in which case the exposures would be stopped and the terminal sacrifice conducted. Rats were to be evaluated after 23 months of exposure for survival rates and general health status, and a decision was to be made whether to sacrifice all surviving rats at 24 months or to keep them for an additional 6 months or until survival in the control group decreased to less than 20%. Based on these pre-established criteria, the final sacrifice of all surviving male rats was conducted after 28 months of exposure and after 30 months of exposure for surviving female rats.

Biologic Assays Table 2 in the report by McDonald et al. shows all the biologic endpoints examined. The endpoints fell into the following broad categories:

- *Hematology* — counts of multiple cell types, plus coagulation endpoints;
- *Serum chemistry* — including triglyceride and protein components;
- *Lung lavage endpoints* — numbers of cells, level of total protein (μTP, as a marker of tissue injury), multiple cytokines, and markers of oxidative stress;
- *Pulmonary function* — measures of total lung capacity, peak expiratory flow (PEF), quasistatic lung compliance, CO diffusing capacity (DL_{CO}) and alveolar volume, forced expiratory flow (FEF), mean mid-expiratory flow (MMEF), and others (measurements performed immediately preceding necropsy at the 3-, 12-, and 24-month exposure time points); and
- *Other clinical observations* — 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 by McDonald and colleagues (Part 1 of this volume). 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).

Sections of target tissues (lung, nasal turbinates, larynx, trachea, kidney, liver, heart, brain, ovaries, and testes) were examined from all animals. Other tissues (such as pancreas, stomach, adrenal glands, and pituitary gland) were examined from all animals only in the control and high-level exposure groups. Non-target tissues were examined in animals from intermediate-level exposure groups only when the tissues exhibited gross abnormalities or when there was evidence of an exposure effect in that tissue in the high-level exposure group.

Slides from rats were read by the two study pathologists, 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. The 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 (National Institute of Environmental Health Sciences), who were engaged by HEI to review pathology results at all interim time points.

Pathology Working Group In May 2013 HEI convened a group of five pathologists to meet in Chantilly, Virginia, to review the pathology findings from rats at the terminal sacrifice. The composition of this independent Pathology Working Group (PWG), the details of the slides reviewed and how they were selected, and the study findings and conclusions are described in the PWG report, found in this volume. One member of the PWG (Dr. Harkema) was also a member of the ACES Review Panel. The PWG did not review slides from the interim sacrifices (at 1, 3, 12, and 24 months) "with the exception of several proliferative lesions of the lung and nasal cavity identified by the study pathologist at the 12- and 24-month interim sacrifices."

In an additional report (the Pathology Working Group Comparison Statement, found in this volume), the PWG compared the lung histology findings in slides from the terminal sacrifice (at 28 and 30 months) in the current study with slides from previous long-term inhalation exposure rat studies — specifically, the NTP 30-month ozone study; a 24-month study conducted at LRRI with TDE (Mauderly et al. 1994), using 2.5–6.5 mg/m^3 PM; a 24-month study conducted at LRRI with NO_2 (Mauderly et al. 1989) with up to 9.5 ppm NO_2; and a 30-month TDE study conducted at the Fraunhofer Institute in Germany (Heinrich et al. 1986), with 4.2 mg/m^3 PM. These slides are shown in the PWG Comparison Statement, as well as in Figure 5 of the report by McDonald et al. (Part 1 of this volume).

Statistical Approaches The authors used the Poly-3 test for the statistical analysis of neoplastic and non-neoplastic lesions in all the tissues examined (Bailer and Portier 1988). The Poly-3 test adjusts the denominator of the observed overall tumor or lesion incidence to take into account the survival of each individual animal in that group. These "adjusted rates" for each lesion type and each exposure group are given in Tables H.1 and H.2 of Appendix H of the report by McDonald et al. in this volume (available on the Web at *http://pubs.healtheffects.org*). They were calculated as follows: An animal with a lesion or an animal surviving until the end of the study was given full weight (i.e., assigned a weight of $n = 1$). A lesion-free animal dying during the course of the study was assigned a weight corresponding to the fraction of the total duration of time it survived in the study raised to the third power. Thus, for example, an animal without a lesion dying halfway through the study was assigned a weight of $n = (½)^3$, or 0.125. These n's were then summed, and this total replaced the denominator of the observed overall lesion rate. The statistical tests (both trend and pairwise comparisons) were then applied to these Poly-3–adjusted lesion rates.

This survival-adjusted test is also currently used by the NTP in its evaluation of rodent tumor data. For a more detailed description of the method and its application by the NTP, see recent NTP technical reports linked to the following Web site: *http://ntp.niehs.nih.gov/results/pubs/longterm/reports/longterm/index.html*. As described above, for non-target organs, animals in the low- and mid-level exposure groups were examined histopathologically for tumors only if a gross lesion was observed at necropsy in that organ that suggested the presence of a tumor. In such cases, consistent with the NTP's practice, the Poly-3 test was not applied because of the resulting "gross lesion sampling bias" when computing the overall tumor incidence.

For the continuous variables — including the endpoints in blood, BALF, and lung tissue — Analysis of Variance (ANOVA) procedures were used to assess the overall effects of exposure, sex, and/or duration, as well as the interactions between these factors. The significance of a linear trend in response was also assessed. For some variables, variance-stabilizing logarithmic transformation was applied before statistical analysis. Pairwise comparisons were made by the Dunnett test.

Endpoints that had 10 or fewer distinct values (e.g., cellular count data) were analyzed with an ANOVA and trend analysis approach for categorical data based on weighted least squares (Grizzle et al. 1969). Since there is no standard multiple comparison method for categorical data that is analogous to the Dunnett test, pairwise comparisons from the categorical ANOVA were adjusted using the Bonferroni correction.

THE ACES REVIEW PANEL'S EVALUATION OF THE REPORT BY MCDONALD ET AL.

GENERAL COMMENTS

The HEI ACES Review Panel, which conducted an independent, detailed review of the report submitted by the investigators, thought that the study addressed an important issue: the health effects of inhalation of DE generated by a heavy-duty engine compliant with 2007 regulations, which significantly reduced particulate emissions. The Panel's review highlighted several strengths of the study. It was carefully designed and executed and is the first, and so far the only, in vivo evaluation in rats of the health effects of a range of exposures of NTDE over a lifetime from the new generation of heavy-duty diesel engines. On a daily basis, these exposures were much longer (16 hours/day) than traditional NTP exposures (typically 6 hours/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 an exposure period lasting more than 30 months, McDonald and colleagues maintained the NTDE exposure levels (high, mid, and low) at close to the target levels.

The evaluation of tumor incidence was a critical outcome of this lifetime study. The independent histopathologic review conducted by the PWG of the findings reported by the investigators confirmed the histologic findings from the study. As described in detail below, the Panel agreed with the investigators that exposure of rats to NTDE for up to 30 months did not induce tumors or preneoplastic changes in the lung, and did not increase tumors that were considered to be related to NTDE in any other tissue.

The standardized toxicity endpoints measured in this study were also appropriate for evaluating the study hypothesis, namely, that the emissions would not cause an increase in tumor formation or substantial toxic health effects in rats, although some biologic effects, might occur. The Panel also agreed with the investigators that exposure to NTDE induced few biologic effects, and those that were detected were mild and occurred mostly in rats exposed to the highest NTDE level evaluated. The Panel considered that the statistical methods described in the report were appropriate for data analysis and were applied correctly by the authors. The Panel noted some minor limitations to the interpretation — particularly, the lack of internal controls for some of the biochemical assays.

KEY RESULTS AND INTERPRETATION

Exposure Characterization

The Panel considered the following to be the key findings regarding the characterization of the NTDE:

- Over the course of the study, average NO_2 exposure levels within 20% of the target were achieved: 4.4, 0.9, and 0.1 ppm for high-, mid-, and low-level NTDE exposures for the entire study (see Table 3 of McDonald et al.), and 4.2, 0.8, and 0.1 ppm for the 30-month rat exposure period (see Appendix I, Table 3B).
- The most abundant pollutants by mass were CO_2, CO, NO, and NO_2.
- NTDE particle concentrations were very low over the course of the exposures. At the chamber inlet, they ranged from 2.5 to approximately 8 $\mu g/m^3$ over the three exposure levels. Inside the chamber, engine-generated PM was $\leq$ 10.4 $\mu g/m^3$. Particle number concentrations ranged from 2 to 8 $\times$ 10^5 particle/cm^3 over the three exposure levels. Continuous measurements of particle number indicated that most of the particles were generated only during regeneration of the diesel filter, which occurred once or twice in a 16-hour exposure period (Khalek et al. 2009). These combustion-derived particles 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).

  Thus, the ratios of PM mass to NO_2 (and NO_2 to NO_x) in NTDE in this study were substantially different from those found in TDE (see Commentary Table 1): PM mass:NO_2 was much lower (approximately 30-fold) and NO_2:NO_x was much higher (approximately 3-fold) than in the earlier studies (Mauderly et al. 1994; Heinrich et al. 1995). As the table also indicates, PM mass:NO_2 and NO_2:NO_x ratios were comparable in the current study to those found in the study of Heinrich and colleagues (1995) in which particles were removed from TDE.

The PM mass:NO_2 ratio changed slightly over the course of the current study, which McDonald and colleagues attributed to a decrease in the efficiency of the diesel particle filter's conversion of NO to NO_2. However, the Panel considered that the report did not provide sufficient evidence to justify that conclusion and thought the change in ratio could also have resulted from differences in performance between the two engines used in the study. Concentrations of some NTDE components, including VOCs such as benzene, appeared to change over the course of the study (see

Commentary Table 1. Concentrations of PM, NO_2, and NO_x in the Current Study (Mouse and Rat Exposures) and in Studies of Older Diesel Engines Used in Rat Exposure Studies[a]

Study / Exposure Level (dilution)	PM Mass[b] (mg/m^3)	NO_2 (ppm)	NO_x (ppm)	PM/NO_2	NO_2/NO_x (%)
Current Study[c]					
High exposure (25:1 dilution)	0.0273	4.4	11.0	0.006	40
Mauderly et al. 1994[d]					
High exposure (dilution ratio NA)	6.33	3.8	23.5	1.7	16
Heinrich et al. 1995[e]					
High exposure (9:1 dilution)	6.98	3.8	33.1	1.8	11
Filtered exhaust[f] (15:1 dilution)	0.010	2.9	23.9	0.003	12

[a] All data collected in inhalation chambers with rats present.

[b] Measured gravimetrically inside the exposure chamber.

[c] 2007 14L heavy-duty diesel engine and ultra-low-sulfur fuel.

[d] 1988 6.2L light-duty diesel engine and high-sulfur fuel.

[e] 1.6L light-duty diesel engine and high-sulfur fuel (age of engine not given).

[f] Particles removed from emissions by filters.

NA = data not available.

data in Appendix I of Part 1 of this volume; available on the Web at *http://pubs.healtheffects.org*). However, the Panel noted that the concentrations measured were derived from engine B at the end of the study (2012), but were derived from engine B′ in the earlier characterizations (2010 and 2011).

- Concentrations of volatile and semivolatile organic compounds (VOCs and SVOCs) were very low. High-molecular-weight alkanes and polar compounds (derivatives of benzoic acid) were the major SVOC, with lower levels of PAHs.
- Sulfur dioxide (SO_2) levels were also very low.
- Approximately 50% of the mass of the particles measured in the chamber when the animals were present was carbon at the high exposure level; the proportion of organic carbon in the total carbon varied slightly at different DE exposure levels. Most of the remainder of the mass consisted of nitrate, ammonium, sulfate, and the elements zinc, manganese, copper, and iron.
- Animals in the exposure chambers generated larger particles (3.5–4 μm), which were major contributors to the total particle mass. Additionally, the animals contributed to the VOCs measured and to the composition of the PM through reaction of ammonia with inorganic ions/gases to form inorganic salts.

More detailed descriptions of the exposure atmosphere characterization are provided in Appendix I of the report by McDonald and colleagues. Commentary Table 1 shows a comparison of results from the current study with those of earlier studies.

Biologic Effects

The investigators tested a wide variety of biologic endpoints; most of those tests, including tumor incidence, did not show any exposure-related effects. A few statistically significant changes were observed at one or more exposure time points and almost exclusively at the highest exposure level. Commentary Table 2 summarizes these effects and the negative findings in tumor cell incidence. The sections below summarize the Panel's conclusions about these findings.

Histology

Tumor Incidence Inhalation exposure of NTDE for up to 30 months (female) and 28 months (male) rats at the levels used resulted in no lung tumors and did not increase tumors that were considered to be related to NTDE in any other tissue. The Panel was in broad agreement with the investigators and the PWG about the major histologic findings and their interpretation: see the Pathology Working Group's Report in a separate section of this volume, which focused exclusively on the histopathologic findings in the core study, and the Pathology Working Group's Comparison Statement (also in this volume), which compared histopathologic findings in the current study with findings from other relevant long-term rat exposures — specifically to TDE, ozone, and NO_2.

NTDE-exposed female — but not male — rats showed a significantly greater number of thyroid gland tumors (follicular cell adenomas and combined follicular cell adenomas and carcinomas) than control animals (see Table H.1 in Appendix H of the report by McDonald and colleagues; available on the Web at *http://pubs.healtheffects.org*). Although the possibility that this finding represented an endocrine disrupter effect was considered, the Panel, in broad agreement with the investigators and the PWG, concluded for several reasons that these thyroid tumors did not represent a response to NTDE exposures. The main reason was because the female controls in this study had an unusually low incidence of thyroid follicular cell tumors (1%), that is, lower than the tumor incidence in control groups of 24-month bioassays in the same strain of rat (Giknis and Clifford 2003; RCC 2003). In addition, the PWG's detailed review of slides from all NTDE-exposed and control groups of female and male rats at the terminal sacrifice (28 and 30 months, respectively) found (a) no exposure-associated dose–response in the incidence of follicular cell tumors among females; (b) no corresponding evidence of an increase in thyroid follicular cell hyperplasia, the relevant pre-neoplastic change; and (c) no evidence of an increase in thyroid tumor incidence in male rats between controls and any group of NTDE-exposed rats.

Non-Neoplastic Lesions in the Respiratory Tract Four types of exposure-associated non-neoplastic lesions, restricted to the lung, were detected in male and female rats at all time points beyond 1 month (see Table 6 and Figure 3 of the report by McDonald and colleagues). Lesions were detected only with exposure to high-level NTDE. Overall, these lesions progressed slightly from 3 to 12 months, without further progression between 12 months and up to the final sacrifice (28 or 30 months). These lesions are summarized in Commentary Table 2 and described below.

Periacinar epithelial hyperplasia (an increased number of airway epithelial cells) affected only a portion of the centriacinar regions, lining pre-terminal and terminal bronchioles, alveolar ducts and alveoli close to the terminal bronchioles, and alveolar ducts. The hyperplasia was minimal to mild. In some examples of "mild lesions" (see Figure 3 of McDonald et al. in this volume), epithelial cell proliferation was manifest as small papillary-like projections into the lumen of preterminal and terminal bronchioles, sometimes with a central stalk. The Panel agreed with the investigators and the PWG that this was not a

Commentary Table 2. Summary of (1) Histopathologic Findings (Showing Lack of NTDE-Associated Tumor Incidence) and (2) Statistically Significant Biologic Effects by Sex, Exposure Duration, and NTDE Level in McDonald et al.[a]

Evaluation	Results
Histology	
Tumors	*Evaluated in all animals, at all exposure time points as well as in animals dying during the study.* No tumors in lungs; no tumor increases considered to be related to NTDE in any other tissue.
Non-neoplastic lesions	*Evaluated in all animals, at all exposure time points as well as in animals dying during the study.* **In lung,** periacinar epithelial hyperplasia; bronchiolization; accumulation of macrophages; periacinar interstitial fibrosis, mild, in both sexes *at high NTDE.* Slight progression from 3 to 12 months, but no further progression from 12 months on. No lesions associated with NTDE exposure found in any other tissue.
Pulmonary Function	
	FEF (multiple markers): ↓ in females (at *3 and 24 months*) and males (at *24 months*), mostly at *high NTDE*; greater in females than in males.
	DL_{CO}: ↓ trend *across all exposure groups* in combined males and females at *3, 12, and 24 months.*
Biochemical Markers	
Lung tissue	HO-1: ↑ in both sexes, predominantly at *high NTDE* at *3, 12, and 24 months*.
	IL-6: ↑ trend *across all exposure groups* in females (at *24 months*) and combined males and females (at *12 and 24 months*).
	KC: ↑ trend *across all exposure groups* in females (at *24 months*) and combined males and females (at *12 months*).
BALF	µTP: ↑ *high NTDE* in combined sexes at *3, 12, and 24 months*.
	ALP: ↑ trend (males, females, and combined sexes) and ↑ at *high NTDE* (combined sexes) at *24 months.*
	Albumin: ↑ trend for females at *12 months*.
	Mean corpuscular hemoglobin: ↑ in males (*mid-level NTDE*), ↑ females (trend), and ↑ combined sexes (*mid- and high-level NTDE*; trend) at *24 months*.
	Total white blood cells and absolute macrophage count: ↑ trend in females at *24 months*.
	TEAC and GSSG: inconsistent responses (↑ at some time points; ↓ at others).
Blood	Albumin: ↑ trend in males at *24 months*.
	ALP: ↓ *high NTDE* (and trend) at *12 months* in both sexes.

[a] ↑ = increase; ↓ = decrease; ALP = alkaline phosphatase; DL_{co} = lung diffusing capacity of carbon monoxide; FEF = forced expiratory flow; GSSG = oxidized glutathione; HO-1 = heme oxygenase 1; IL-6 = interleukin 6; KC = keratinocyte-derived chemokine; µTP = micro–total protein; TEAC = Trolox equivalent antioxidant capacity.

pre-neoplastic lesion — that is, it would not lead to the development of tumors — but was a response to the inhalation of NTDE.

Although epithelial cell hyperplasia was detected histologically in the lung, the investigators did not find evidence of exposure-associated epithelial cell proliferation in an assay that used the expression of Ki67 (a nuclear protein) as a measure of dividing cells; that is, there were no differences in any group in the number of cells expressing Ki67. The Panel agreed with the investigators that this assay was unlikely to have detected changes in epithelial cell proliferation because the histologic findings indicated that the hyperplasia was restricted to only a very limited area of the lung, the central acinus, and effects in this area would probably have been diluted out in this assay, which sampled the whole lung. The Panel considered that although the interpretation of focal hyperplasia is likely correct, the authors might have been able to corroborate this conclusion by re-evaluating the labeling indices, specifically in the serial sections in those small focal locations in which epithelial hyperplasia was documented.

Bronchiolization (in which normally flat epithelial cells change to a cuboidal shape, similar to cells lining the terminal bronchioles) was found in some animals. The cells in question were generally ciliated and were found extending into alveolar ducts and centriacinar alveoli.

An accumulation of macrophages accompanied bronchiolization in some samples, as noted by both the PWG and the Panel. However, as the PWG also noted (see the Pathology Working Group Report earlier in this volume), "a remarkable finding was the general lack of inflammatory cells," which contrasted with findings from TDE-exposed rats.

Periacinar interstitial fibrosis (so named by the investigators because the area of fibrosis was restricted to the periacinar region, to distinguish it from the more general "interstitial fibrosis" used in the subchronic exposure findings [ACES 2012]) involved the wall at the terminal bronchiole and proximal alveolar ducts in the centriacinar areas underlying the epithelial changes (see Figure 4 of McDonald et al.). It was found at 3 months and after longer exposures; incidence was high at 12 months (80% in males and 100% in females) and continued to be high throughout the rest of the study. The severity of fibrosis was usually minimal at all the time points at which it was found, including terminal sacrifice.

Nasal Cavity Areas of the nasal cavity (nasal turbinates 1–3) showed some effects, usually minimal to mild, at multiple time points in many groups of male and female rats. The PWG reviewed the nasal lesions and concluded that there was no clear difference in either incidence or severity of lesions between control and animals exposed to high-level NTDE. In addition, the lesion distribution in the nasal cavity of NTDE-exposed rats did not fit a site-specific pattern characteristic of inhaled nasal toxicants (Harkema et al. 2006). Thus, the PWG concluded that these lesions were not exposure related.

Pulmonary Function

As indicated in Commentary Table 2, the investigators noted exposure-associated decreases in multiple related markers of FEF rates over the lung volume range — FEF_{75} (i.e., FEF at 75% of the forced vital capacity [FVC]), FEF_{50}, FEF_{25}, and FEF_{10}, as well as PEFR and MMEF. Decreases were seen both in females (3 and 24 months) and in males (24 months) and were greater in females than in males. With the exception of a reduction in FEF_{25}/FVC in males at 24 months at the mid-level exposure, most effects were seen only at high NTDE. McDonald and colleagues concluded that these findings suggested that the exposures affected airways with smaller diameters more than those with larger diameters.

In some analyses, DL_{CO}, a measure of alveolar–capillary gas exchange, showed a small effect of exposure to NTDE — specifically, a small decreasing trend across all exposure groups in combined males and females at all the time points examined (3, 12, and 24 months). McDonald and colleagues concluded that "the small effect of exposure on DL_{CO} at 24 months was in part a small reduction of lung size relative to control. Accordingly, the impact on the efficiency of gas transfer across the alveolar–capillary membrane, if any, must have been very slight." Alternatively, the Panel considered that, while the reductions in DL_{CO} were relatively small, they were not completely accounted for by reductions in lung volume. The Panel considered that the small reductions in DL_{CO} were consistent with the histopathologic findings of mild histologic changes in the gas-exchange regions of the lung, indicating that the histologic changes detected may have had functional effects.

Biochemical Markers

The great majority of the biologic tests performed by the investigators showed no effect. The Panel agreed with the investigators that the few changes observed (shown in Commentary Table 2) — including small increases in levels of HO-1, IL-6, KC, μTP, total white blood cells, and macrophages in the lung

— were consistent with mild pulmonary oxidative stress and inflammation and mild tissue injury.

KEY CONSIDERATIONS IN THE INTERPRETATION OF RESULTS

Although the Panel generally agreed with McDonald and colleagues' interpretations of the data, it noted that some topics, described below, also needed to be considered.

The Engine Systems

At different times in this study, two engine systems with similar, but not identical, emissions profiles were used (see Mauderly and McDonald 2012; also see Appendix I of McDonald et al., available on the Web at *http://pubs.healtheffects.org*). Although the investigators concluded that the change, mentioned earlier, in the ratio of NO:NO_2 over the course of the study was due to a decrease in efficiency of the diesel particle filter, the Panel did not think there was any evidence to conclude this. The Panel noted that engine B produced slightly more NO_2 than did engine B′, suggesting that the components of the emissions produced by the two engines may also have varied somewhat at different times during the study. In addition, over the course of the study, the investigators maintained the average NO_2 concentrations in the chamber within the range (± 20%) specified for the target NO_2 concentrations. However, the Panel noted that the NO_2 concentration varied widely within the 16-hour cycle (see Appendix I). Nonetheless, given the overall lack of toxicity of the emissions, the Panel did not consider that these variations in NO_2 concentration during the study had an overall impact on the results.

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 program, the ACES Oversight Committee had well understood the desirability of such a comparison, but it was clear 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 Review Panel concurred with the Oversight Committee that such a side-by-side comparison could have enhanced the study.

Emissions Temperature

Because a heavy-duty engine can generate more power and heat than light- and medium-duty engines that have been used in previous studies, the potential to generate high temperature emissions in the exposure chambers was recognized during the design phase of the study. Throughout the study, the investigators successfully maintained the temperature in the exposure chambers within the specified range of 18 to 26°C. Because the dilution of the NTDE from the engine was kept relatively low in order to reach the desired exposure concentrations, however, the hot exhaust was not diluted with cool air to the same extent as in previous studies. Thus, animals in the high-level NTDE exposure group in the current study were more often exposed to higher temperatures than those in other groups. Whether and how these differences in temperature among the exposure groups might have affected the testing of the primary hypothesis of this study is not clear.

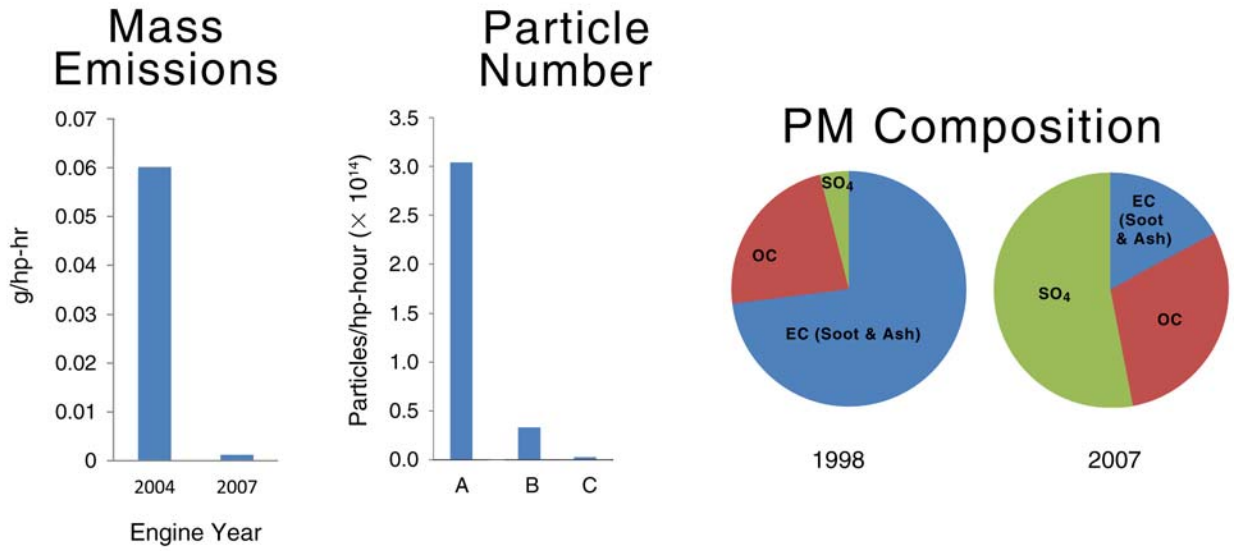
Biochemical Assay Controls

Most of the biochemical assays employed in this study 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 whether the lack of effects might 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 (e.g., Weldy et al. 2011; da Cunha et al. 2011).

Toxicity of NTDE vs. TDE

The Commentary Figure shows how much lower the PM mass and particle numbers in emissions from 2007-compliant engines fitted with particle-reduction technologies are than in emissions from pre-2007 engines. As shown, the ACES Phase I study (Khalek et al. 2009) found that emissions from 2007-compliant engines were reduced more than 90% compared with those from a 2004 engine. Commentary Table 3 shows that emissions of hydrocarbons and other air toxics by 2007-compliant engines were also lower by more than 80% than those of older engines. Furthermore, the Commentary Figure indicates how the composition of particles in NTDE and TDE differs — with sulfate, rather than elemental carbon, being the major component of NTDE mass.

Regarding the question of whether the *toxicity per unit mass* of the PM emitted from the 2007-compliant engines was changed compared with older engines, the Panel pointed out that ACES was not designed to investigate this question. Consequently, the most straightforward inference would be that the steep drop in particle mass and



Commentary Figure. Comparison of PM in traditional-technology diesel exhaust (pre-2007) and new-technology diesel exhaust (2007-compliant) by mass, particle number, and composition. The particle number graph shows the results as measured using the Federal Test Procedure from **(A)** a 2004 engine with no diesel particulate filter (DPF); **(B)** a 2007-compliant engine with a DPF and with active regeneration; **(C)** a 2007-compliant engine with a DPF and without active regeneration. (EC = elemental carbon; OC = organic carbon; SO_4 = sulfate.) (Data from Clark et al. 2007; Khalek and Merritt 2009; personal communication, Imad Khalek, 22 March 2012.)

levels of organic components in NTDE significantly *decreased* the overall toxicity of NTDE compared with the toxicity of TDE.

Commentary Table 3. Reductions in Unregulated Components of Emissions from 2007-Compliant Diesel Engines[a,b]

Compounds	% Reduction Relative to 2004-Technology Engine
Single-ring aromatics	82
PAHs	79
Nitro-PAHs	81
Alkanes	85
Polar	81
Hopanes/steranes	99
Carbonyls	98
Inorganic ions	38
Metals and elements	98
Organic carbon	96
Elemental carbon	99
Dioxins/furans[c]	99

[a] Source: Coordinating Research Council (Khalek et al. 2009).

[b] 16-hour cycle.

[c] Relative to 1998-technology engine.

The beneficial effects of filtering out particles from TDE have also been reported in controlled short-term human exposures: In healthy male volunteers exposed via inhalation for 1 hour to TDE (derived from a 1991 Volvo engine), Lucking et al (2011) found impairment of cardiovascular endpoints, namely, reduced vasodilatation and increased ex vivo thrombus formation. Emissions from the same engine equipped with a diesel particulate filter showed reduced particle mass and number, and exposure of the volunteers for the same length of time was associated with increased vasodilatation and reduced thrombus formation (Lucking et al. 2011).

Comparison of Histopathologic Effects of NTDE with Other Pollutants

The Panel agreed with the PWG's conclusion in the Comparison Statement Report that "the most dramatic differences were between the new 2007-technology diesel exhaust study, in which few, and mostly minimal, lesions were found, and the old-technology diesel exhaust studies in which the lungs were loaded with pigment, inflammatory changes, hyperplasia, and cancer." The contrast in findings between TDE and NTDE studies is illustrated in the slides shown in the PWG Comparison Statement (earlier in this volume) and in Figure 5 of McDonald and colleagues' report (Part 1 of the current volume). None of the histologic changes in the lung associated with chronic

exposure to high levels of TDE were found in the current study of exposure to NTDE, which was conducted at much lower PM levels.

The small histologic changes in the lung that were found in the current study, particularly the changes in the preterminal bronchiole, were similar to those found in male F344 rats exposed to 9.5 ppm NO_2 for 24 months in an earlier study by Mauderly and colleagues (1989) (see PWG Comparison Statement and Figure 5E of the study by McDonald and colleagues, both in this volume). In addition, as noted by the investigators and the PWG, the minimal to mild periacinar lesions in the lungs of rats exposed to high-level NTDE in the current study were similar to those previously reported by Kubota et al. (1987) in Wistar Han rats exposed continuously for 24 hours/day to 4 ppm NO_2 for 27 months. The Panel also agreed with the investigators that NTDE from engine systems compliant with regulations introduced in 2010, which mandate a decrease in emissions of NO_2, is likely to result in even fewer lesions in the respiratory tract.

Histopathologic changes in an earlier NTP study (1994) in the rat lung after long-term exposure to ozone (344/N rats exposed for 30 months to 1 ppm ozone), including changes in bronchiolar epithelial cells in the centriacinar area and an increase in alveolar macrophages, were also similar to those found in the current study (see the PWG Comparison Statement and Figure 5F of the study by McDonald and colleagues, both in this volume). Although ozone was not present in NTDE, these findings lend support to the notion that exposure to an oxidant gas results in the changes in the lung comparable to those seen in the current study.

Both the investigators and the Panel noted that although engine-generated PM mass was greatly reduced (to ≤ 11 µg/m^3 inside the chamber) in this study, substantial numbers of particles (between 2 and 8×10^5/cm^3) in the ultrafine range (20 to 40 nm in diameter) were detected. These levels are in the range of (or somewhat higher than) those found on or near major roads in urban areas and in environments in which diesel-powered traffic dominates (e.g., McCreanor et al. 2007; Morawska et al. 2008; Zhang et al. 2009). It is possible that components of NTDE other than NO_2 may have contributed to the effects reported, but the low levels of other components suggest that they would not be primarily responsible.

Choice of Rat Strain

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 the inhalation chambers). (See also the Preface to this report and Additional Materials 1 of McDonald et al., available on the Web at *http://pubs.healtheffects.org*.)

One possible concern discussed during the study design phase was that a proportion of Wistar Han rats have a mutation in the aryl hydrocarbon receptor (AhR) gene that may alter their responses to dioxin and PAHs, which also bind to the AhR. Because PAHs are present in DE and some have known carcinogenic properties, there was a question as to whether the AhR mutation might make the Wistar Han strain more resistant than strains with a wild-type 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 TDE, as well as to other particles (Heinrich et al. 1986, 1995; Karagianes et al. 1981).

SUMMARY

- This study is the first to address the issue of whether lifetime inhalation exposure of male and female rats to a range of concentrations of new-technology diesel exhaust (NTDE) emissions, compliant with 2007 regulations, has the same biologic effects as exposure to traditional-technology diesel exhaust (TDE).
- Lifetime exposure (28 months for males, 30 months for females) of rats to NTDE, which has greatly reduced particle emissions compared with TDE, did not induce tumors or pre-cancerous changes in the lung and did not increase tumors considered to be related to NTDE in any other tissue.
- Some mild histologic changes were observed in the lung, consistent with long-term exposure to NO_2, a major component of NTDE from 2007-compliant engines.
- Of more than 100 different biologic endpoints evaluated in lung and blood, only a few showed NTDE-associated changes, consistent with mild pulmonary inflammation and oxidative stress. There were also small decreases in some measures of respiratory function,
- The effects of chronic exposure to NTDE differed markedly from the effects of chronic exposure to TDE, which include tumor incidence and the presence of multiple pre-neoplastic lesions in the lungs.

A more detailed Summary and Conclusions section covering all the ACES 3B studies is included at the end of this Commentary.

COMMENTARY ON ANCILLARY STUDIES: EFFECTS ON GENOTOXICITY (BEMIS ET AL. AND HALLBERG ET AL.) AND VASCULAR MARKERS (CONKLIN AND KONG)

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 and vascular markers of inflammation and damage. Consequently, as described in the Preface, HEI funded several additional studies to complement the core study of McDonald and colleagues.

The three completed ancillary studies are included in the current Research Report. The principal investigators, study proposal titles, and study goals were as follows:

- Dr. Jeffrey C. Bemis, Litron Laboratories, Rochester, New York: "Genotoxicity of inhaled diesel exhaust: examination of rodent blood for micronucleus formation" (to evaluate the frequency of reticulocytes containing micronuclei in the blood of NTDE-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 NTDE-exposed animals); and
- Dr. Daniel J. Conklin, University of Louisville, Louisville, Kentucky: "Effects of diesel engine emissions on vascular inflammation and thrombosis" (to measure effects on multiple vascular markers).

THE ACES GENOTOXIC STUDIES

The ACES genotoxic studies made use of samples from rats exposed by inhalation to NTDE in the core study at LRRI (McDonald et al. in this volume).

Scientific Background

As described in the Scientific Background section above on the report by McDonald and colleagues, a few studies (Bond et al. 1990; Iwai et al. 2000; Randerath et al. 1995) have evaluated whether long-term exposure of rodents to TDE affects genotoxic endpoints, specifically, causing damage to DNA, such as DNA adduct formation, and oxidative damage in different tissues. Bond and colleagues (1990) and Randerath and colleagues (1995) did not find any changes in adduct formation. Iwai and colleagues (2000) reported increased levels of DNA adducts and 8-OHdG in the lung tissue of F344 female rats exposed for 12 months to 3.5 mg/m^3 TDE. Iwai and colleagues reported an association between the development of tumors in these rats at the end of the study (30 months) to the earlier effects of oxidative damage effects from TDE. Reed and colleagues (2004) found that exposure of mice to TDE (up to 1 mg/m^3 for 7 days per week for 6 months) had no effect on another genotoxic endpoint, the number of micronucleated reticulocytes (MN-RETs), or the incidence of lung tumors (adenomas) 6 months after the end of the exposure.

The current study is the first to evaluate the potential effects of exposure of rats to NTDE on these well-accepted genotoxic endpoints.

Exposure to NTDE

The generation of NTDE and the exposure of rats to high, mid, and low levels of NTDE are described in the report by McDonald and colleagues and summarized in the preceding sections of this Commentary.

After euthanizing sets of rats at 1, 3, 12, and 24 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 study by McDonald and colleagues. Note that samples for the ancillary studies were collected only at these four exposure time points; no samples were collected at the time of terminal sacrifice (28–30 months) in the core study.

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 NTDE-exposed animals the number of immature red blood cells (reticulocytes, or RETs) that contained micronuclei (MN), an indicator of genotoxic changes. Conditions that cause double strand breaks or disrupt the proper segregation of chromosomes during cell division result in an increase in MN frequency.

Samples

Peripheral blood (100 µL) from between 4 and 6 rats of each sex per exposure group euthanized after each exposure

time point was collected at LRRI in a special anticoagulant and then fixed according to established procedures. Samples were shipped frozen to Bemis and his team.

Methods

Pilot Study Before the main study started, Bemis and colleagues conducted a pilot study to determine whether MN could be detected in older rats after exposure to a known genotoxic agent, cyclophosphamide (see Additional Materials 2 of the report by Bemis et al., Part 2 of this volume; available on the Web at *http://pubs.healtheffects.org*). After first establishing a baseline level of MN-RETs for each animal by collecting blood from the tail, the investigators treated 3 males and 2 female rats aged between 10 and 21 months with cyclophosphamide (15 mg/kg body weight administered at 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 In the current study, the investigators used flow cytometry to differentially identify and count three types of red blood cells in each blood sample: RET with or without MN (RET and MN-RET), and mature normochromatic erythrocytes (NCEs). (The investigators did not collect MN-NCE data in this study because MN-NCEs in rat blood are not reliable indicators of chromosomal damage — the action of the rat spleen removes these cells from the circulation.) Figure 3 of the report by Bemis and colleagues (Part 2 of the current volume) 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 quadrants of the flow-cytometric readout.

Statistical Approaches The means and SEM were calculated for each group of rats, combining males and females (so that there were up to 10 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, the arcsin transformation did not achieve a suitably normal distribution for the %RET data, so the values for %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. All data were analyzed using a three-way ANOVA that included the factors of sex, exposure level, and exposure duration. Significance was set at $P \leq 0.05$.

Key Results

- Exposure of rats for up to 24 months to NTDE did *not* increase the frequency of MN-RET in peripheral blood.
- In the pilot study, 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.

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 circulating reticulocytes — to determine whether exposure to NTDE had genotoxic effects. The data were analyzed with appropriate statistical approaches, and the conclusions drawn were largely appropriate — in particular, that there were no exposure-related changes in the numbers of MN-RETs measured in the blood of rats exposed for up to 24 months to NTDE. Data from a pilot study indicated that an increase in MN-RETs could be detected after treating rats with cyclophosphamide, establishing that changes in the endpoint in ACES samples could have been detected by the assay used.

One limitation to the study was that the MN endpoint is only a short-term indicator of genotoxicity; that is, it assesses damage that has occurred only over approximately the last 3 days of exposure — the length of time taken for a red blood cell precursor in bone marrow to differentiate into an immature $CD71^+$ erythrocyte (RET), and then differentiate further into a mature $CD71^-$ erythrocyte (NCE). Thus, the number of MN-RETs does not measure cumulative exposure over the entire 24-month period, or a substantial fraction of it, unless the animals' damage responses, such as DNA repair capacity or detoxification systems, have been compromised by the exposure — for which there is no evidence in the current study. Thus, the value of using the MN assay as a marker of cumulative genotoxicity in this long-term exposure is not clear.

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

Investigators at LRRI took samples (50 µL of plasma, the right accessory lung lobe, and half the brain with the olfactory bulb) from male and female rats euthanized at each of the exposure time points and shipped them frozen to Hallberg and colleagues. The goal was to evaluate samples from 5 male and 5 female rats at each time point, but as the report (Part 3 of the current volume) indicates, some groups in the 12- and 24-month exposures had between 3 and 5 animals.

Methods

Comet Assay This assay detects damage to DNA by immobilizing cells on a slide coated with agarose gel and then lysing the cells, followed by electrophoresis. Under the influence of 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. DNA damage is quantified by measuring the displacement between DNA in the nucleus ("comet head") and the resulting "comet tail." The measurements made were *%DNA in tail*; *tail length* (the distance of DNA migration in the tail); *tail moment* (the product of the tail length and the fraction of total DNA in the tail); and *olive moment* (the product of tail %DNA and tail moment length — 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 (which are missing a nucleotide) and sites at which excision repair is occurring.

Positive Technical Controls The investigators showed that they could detect genetic damage in the comet assay through an earlier experiment in which exposure of control cells to the DNA-damaging agent bleomycin resulted in exposure-dependent increases in tail length and tail moment (see Appendix C to the report by Hallberg and colleagues, available on the Web at *http://pubs.healtheffects.org*).

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 enzyme-linked immunosorbent assay (ELISA) 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/mg protein.

TBARS Lipid peroxidation is a manifestation 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 by-product, TBARS, which 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 Data Differences among exposure groups, differences between sexes, and the interaction between these two factors at specific time points were assessed using ANOVA procedures. Post-hoc comparisons, made only when the overall ANOVA was significant, used a Bonferroni correction, although the specific test used for the post-hoc comparisons was not identified. Tests were carried out at the $P < 0.05$ level.

OHdG and TBARS Data Differences among exposure groups, differences between sexes, and the interaction between these two factors at specific time points were assessed using ANOVA procedures, if the data were normally distributed. If the data did not meet normality or variance criteria, the Student–Newman–Keuls nonparametric method was used to examine the exposure effects, controlling for sex and stratifying by duration. The statistical method used for post-hoc comparisons was not identified, no mention was made of the Bonferroni correction, which was used in the comet data analysis, and no mention was made of the level of significance used for the statistical tests.

Power Analysis Before the start of the study, the investigators calculated the number of animals they would need to have in each group to detect statistically significant differences in olive tail moment data in the comet assay at different exposure levels. The power calculations were based on mouse data from a previous study (Hallberg et al. 2012) and assumed a specific set of underlying parameter values (namely, controls, 1.0; low level, 1.75; mid level, 2.5; high level, 3.25; effect size, 0.99; level of significance: $P < 0.05$; power > 0.92; and underlying SD, 0.85 [see Table A.1 in Appendix A of the Hallberg report in this volume; available on the Web at *http://pubs.healtheffects.org*]). The justification

for these parameter choices and the relevance of power calculations based on previous mouse data to the current rat study are unclear from the investigators' report.

Under these conditions, the authors estimated that four animals per group would be sufficient to detect differences between exposure levels (see Table A.1 in Appendix A), but they increased the number to five animals per group of each sex to look for sex differences. Later in the study, the investigators performed similar power calculations for other comet data — tail length, %DNA in tail, and tail moment — based on data from rats exposed for 12 months to various concentrations of NTDE. These results are also presented in Appendix A.

Key Findings

Exposure of rats for up to 24 months to NTDE resulted in the following:

- No DNA damage in lung cells, as measured by %DNA in tail, tail length, tail moment, or olive moment in the alkali modification of the comet assay was observed. (A few small and scattered effects were noted after 1- and 3-month exposures, but no effects were seen at 12 and 24 months.)
- No oxidative damage (as measured by 8-OHdG levels in serum) or lipid peroxidation in the hippocampus (as measured by TBARS) was observed. (A few small and scattered effects were noted in 8-OHdG levels in some NTDE-exposed groups at 12 months, but these were not exposure-dependent and were not seen at other exposure times.)

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 NTDE had genotoxic effects. 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, the TBARS assay does not provide information on a larger spectrum of possible changes in lipid peroxidation.

In addition, although overall the statistical approaches using ANOVA for the comet, 8-OHdG, and TBARS data were appropriate, there were inconsistencies and lack of clarity in the description of the statistical approaches. For example, the Panel could not evaluate the statistical methods used for post-hoc pairwise comparisons in all the assays, because they were not identified in the report. Moreover, these methods were inconsistently applied across the assays — the Bonferroni correction was used only for the comet data, but not for the 8-OHdG and TBARS data, even though the same general ANOVA approach was used for all of these variables. Furthermore, the authors did not explain why it was necessary for the overall ANOVA to be significant before making post-hoc comparisons for the comet data, but not for the 8-OHdG or TBARS data.

In addition, certain details of the power calculations were unclear. In Appendix A of the report by Hallberg and colleagues in this volume (available on the Web at *http://pubs.healtheffects.org*), the authors present power calculations for detecting various "effect sizes." An effect size is normally defined to be a difference in mean response relative to controls, possibly standardized by dividing by the standard deviation (e.g., see *http://meera.snre.umich.edu/plan-an-evaluation/related-topics/power-analysis-statistical-significance-effect-size#effect*), but the investigators defined effect size in a nonstandard way, as the ratio of two standard deviations. The Panel was not clear why the investigators' power calculations apparently focused on detecting changes in standard deviations rather than on detecting changes in mean responses.

From the power calculations they conducted during the study on comet assay endpoints, Hallberg and colleagues concluded that many more animals would have been needed to detect a difference in response among exposure groups than were used in the study. However, the Panel concluded that these power calculations may be misleading by suggesting that the study was underpowered — a large required sample size does not necessarily indicate that the study is underpowered. Rather, it may indicate that the hypothetical effect is so weak that it would be nearly impossible to detect in a study of feasible sample size. If such a weak effect were detected, its biologic significance would be questionable. For this reason, the Panel concluded that the authors' power calculations did not diminish the interpretation that the comet assay findings were negative.

GENOTOXICITY STUDIES: JOINT ASSESSMENT BY THE ACES REVIEW PANEL

Items Common to the ACES Ancillary Genotoxicity Studies

The Bemis et al. and Hallberg et al. studies are the first to show that exposure of rats to NTDE for up to 24 months does not affect well-accepted endpoints of genotoxicity. In addition, the Panel made the following observations about both studies.

Evaluation of Genotoxic Effects Outside the Airways

The initial effects of inhaled DE are expected to be in the airways, but the studies by Bemis et al. and Hallberg et al. both evaluated genotoxic effects in cells outside the airways: Bemis and colleagues assessed the incidence of MN in red blood cells, and Hallberg and colleagues assessed changes in blood and brain cells. How relevant to the effects of DE exposure are changes in non-airway cells? Several studies 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 (Crüt et al. 2008; Lucking et al. 2011; Riedl et al. 2012 [see Commentary by HEI Review Panel in that Research Report]). In addition, DNA damage has been reported in the brains of humans and dogs in areas with high levels of air pollution (Calderón-Garcidueñas et al. 2003, 2004). How the effects of inhaled particles are mediated outside the lungs is not 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 investigators in the ACES genotoxic ancillary studies anticipated finding some systemic effects attributable to inhaled particles, and thus agreed that evaluating the potential genotoxic effects of NTDE in blood cells was justifiable. Bemis and colleagues studied MN in peripheral blood red blood cells because they are a readily accessible 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 Panel thought the assessment of Hallberg and colleagues of one of their genotoxic endpoints — 8-OHdG — in blood was reasonable. However, although the Panel understood that Hallberg and colleagues chose to study lipid peroxidation in the brain because exposure to particles might affect the function of this organ, they noted that evaluating lipid peroxidation in blood might also have been useful, providing an overlap with measurements of 8-OHdG.

Lack of a Positive Control Engine The reasons for not including a "positive control," side-by-side comparison with an older pre-2007 model-year engine have been discussed earlier in this Commentary in the section on the report by McDonald and colleagues.

Use of Short-Term Assays The Panel recognized that the endpoints assessed in the Bemis et al. and Hallberg et al. studies are accepted markers of genotoxic effects and thought to be on the critical paths to the development of tumors. Nonetheless, they considered that the utility of assays of short-term effects was uncertain in assessing the effects of long-term exposure to DE. Assays to assess the accumulation of 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

- 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 the studies were valuable extensions to the ACES core study.
- No genotoxic effects associated with exposure for up to 24 months to any level of NTDE could be detected.
- The investigators' choice of genotoxic endpoints that were relatively short term was noted by the Panel as being a limitation in study design that reduced the utility of the 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 systemic markers of inflammation and thrombosis, including both blood lipids and proteins, resulting from chronic exposure to NTDE for up to 24 months. The authors' aim was to determine whether long-term exposure to NTDE would lead to alterations in plasma markers associated with increased risk of cardiovascular disease, general toxicity, or cardiovascular organ remodeling. 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 that they had previously exposed to acrolein. The investigators

also assessed the effects of NTDE on cardiac fibrosis and aortic remodeling.

The investigators presented the results of subchronic exposure (1 and 3 months) in a previous report (Conklin and Kong 2012), so the current report and this Commentary focus on the results from later exposure time points evaluated — 12 and 24 months.

Scientific Background

Cardiovascular Effects of Exposure to DE As discussed above in the Scientific Background section of the Commentary on the McDonald and colleagues report, several studies have shown that long-term inhalation exposure to TDE induced the development of lung cancer in rodents, particularly rats. In addition, short-term TDE inhalation studies in humans (e.g., Behndig et al. 2006, 2011; Mills et al. 2005; Stenfors et al. 2004) and in rats and mice (particularly in models of cardiovascular conditions) have shown effects on systemic inflammation, vascular dysfunction, and thrombosis pathways (e.g., Campen et al. 2003; Hansen et al. 2007; Mauderly et al. 1994; Reed et al. 2004, 2006). Furthermore, there is also extensive evidence based on epidemiologic studies that links exposure to ambient PM — of which diesel PM is a component — with cardiovascular mortality and morbidity (reviewed in Krewski et al. 2009). Thus, although the major focus of ACES Phase 3B was to evaluate effects of NTDE on carcinogenicity, the study also provided an excellent opportunity to examine possible changes in cardiovascular-associated markers and endpoints.

Acrolein Effects on the Liver and APR Prior to the current study, Conklin and colleagues 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 (Conklin et al. 2010, 2011). The focus on liver was because it is the site of the production of many acute phase response (APR) reactants, including C-reactive protein, fibrinogen, and serum amyloid A. The APR is a key component of the inflammatory response to an external agent (such as particles or an infectious pathogen). Cytokines produced early in the airway response to such external agents include IL-1, IL-6, and IL-8, and tumor necrosis factor-α, which enter the circulation and within hours stimulate the liver to synthesize APR reactants. Levels of some 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, levels of APR reactants can also be increased in several chronic conditions, such as obesity, cardiovascular disease, and inflammatory arthritis.

Conklin and colleagues 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 below, the data obtained after acrolein exposure provided useful comparisons with the data obtained after NTDE exposure in the current study.

Methods

Sample Collection

NTDE-Exposed Animals Peripheral blood (500 µL) from 10 male and 10 female rats at each exposure level and killed after the 1-, 3-, 12-, and 24-month exposures was collected at LRRI. Hearts and aortas (7 animals/group) were also dissected out at these time points by LRRI investigators. Samples were shipped frozen to Dr. Conklin.

Acrolein-Exposed Animals Plasma samples from 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

Plasma 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 samples from 10 rats per sex per exposure group. A commercial vendor measured plasma levels of several cytokines, chemokines, and other soluble factors in plasma: granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP-1), interleukin (IL)-1β, IL-6, IL-10, interferon-γ, keratinocyte-derived chemoattractant (KC), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF),

soluble intercellular adhesion molecule 1 (sICAM-1), fibrinogen, leptin, and C-reactive protein.

Histopathology The investigators assessed cardiac fibrosis (after 12- and 24-month exposures) and aortic remodeling (after 1-, 3-, 12- and 24-month exposures) in male and female rats (n = 7/group). The heart was isolated, cut longitudinally into dorsal and ventral halves, and formalin-fixed and paraffin-embedded for sectioning. Isolated proximal thoracic aorta was cut into as many as 3 sections and handled in the same manner as the heart for formalin fixation and paraffin embedding. Longitudinal sections of the heart (5 µm) and cross sections (5 µm) of the aorta were stained with Sirius Red and Fast Green to detect collagen fibers (red). Further details are contained in Additional Materials 2 (available on the Web at *http://pubs.healtheffects.org*) of the Conklin and Kong report (Part 4 of this volume).

The extent of cardiac fibrosis was evaluated by measuring the red-stained area as a percentage of the total area assessed. Each image was analyzed for total, vascular fibrosis (all easily identifiable blood vessels > 50 µm diameter) and avascular fibrosis (in the absence of obvious relatively large-diameter vasculature) as a percentage of the area covered. For the aorta, area (in μm^2) was measured for the lumen, wall, media, and adventitia; thickness (in µm) was measured for the wall, media, and adventitia.

Statistical Analyses For each marker, the investigators first carried out a three-way ANOVA, with exposure level, sex, and exposure duration as factors and included all two-way and three-way interactions. The normality of the residuals was assessed using the Shapiro-Wilk test. If the normality assumption was violated, the Box-Cox transformation was first applied to meet the normality assumption. Otherwise, a rank-based transformation was applied (Venables and Ripley 2002). The three-way ANOVA was applied to the transformed data, and the mean squares for each factor and their interactions and the P values for these significance tests were reported in Tables 4 and 5 of the Conklin et al. report (Part 4 of this volume).

Additionally, for each of the markers with significant exposure-level effect or significant two-way or three-way interaction with the exposure level, Conklin and Kong applied the Dunnett multiple comparison test to determine whether the measurements 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 Table 7 of the Conklin et al. report. A P value < 0.05 was considered statistically significant, and all analyses were carried out using the statistics software R (*www.r-project.org*).

Key Results

Plasma Exposure to NTDE at any concentration for up to 24 months had few effects on the more than 20 markers measured. Commentary Table 4 shows that some scattered changes were detected in one or more NTDE-exposed groups. However, most of these changes were found at only one exposure time point and in one sex, predominantly in females. Only two markers showed increases after 24 months of exposure — IL-6 and sICAM-1. However, changes in sICAM-1 levels were inconsistent over the course of the study, decreasing rather than increasing at 12 months with mid-level

Commentary Table 4. Plasma Markers with Significant Changes in Mean Levels in NTDE-Exposed Rats After 12 or 24 Months, Compared with Filtered-Air Controls[a]

Endpoint	Male (NTDE level)	Female (NTDE level)
IL-6	↑ 12 mo (high)	↑ 24 mo (mid and high)
sICAM-1		↓ 12 mo (mid), ↑ 24 mo (low and high)
Total cholesterol		↓ 24 mo (high)
Non-HDL cholesterol		↓ 24 mo (high)
Triglycerides		↓ 24 mo (mid)
Alanine aminotransferase		↓ 12 mo (mid)
Creatine kinase		↓ 12 mo (high)

[a] ↓ = decrease; ↑ = increase; HDL = high-density lipoprotein; IL-6 = interleukin 6; sICAM-1 = soluble intercellular adhesion molecule 1; NTDE = new-technology diesel exhaust (2007-compliant).

NTDE exposure. There were few changes in lipids (total cholesterol, non-HDL cholesterol, and triglycerides) over the course of the study — all were modest decreases compared with controls. Some markers, such as leptin, showed changes in levels after subchronic exposure (1 and 3 months [see Conklin and Kong 2012]) but not at 12 or 24 months.

Exposure of Sprague Dawley rats 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.

Cardiac Fibrosis NTDE exposure had no effect in either male or female rats on the age-associated increase in cardiac fibrosis that was noted in control animals (between 12 and 24 months).

Aortic Remodeling NTDE exposure had no effect on any parameter of aortic remodeling, though age-dependent increases in thoracic aorta parameters were noted, as well as differences between male and female rat aortic measurements (males larger than females) in control animals over the course of 24 months.

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 assessed a large number of plasma markers associated with inflammation and thrombosis with the goal of identifying a sensitive marker or set of markers in blood that might reflect cardiovascular effects of exposure to NTDE. 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 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. In its Commentary accompanying Conklin and Kong's subchronic NTDE exposure results (see ACES 2012), the Panel had suggested adding markers of *chronic* inflammation such as fibrosis to the longer-term exposure analyses. Thus, the Panel thought the inclusion of morphometric analyses of cardiac fibrosis and aortic remodeling in this final report was a useful addition to the study.

The Panel agreed with the investigators that NTDE exposure for up to 24 months had no effect on almost all the blood markers measured in rats. The lack of meaningful changes suggested that the 16-hours-per-day exposure to NTDE for this time period was associated with very few changes on inflammatory or thrombotic pathways; the few small changes that were detected were difficult to interpret, either because the changes were seen at only one time point or in one sex or because they were inconsistent in terms of the direction of the effect at the two exposure time points. The investigators acknowledged that because they had made a large number of statistical comparisons, it was possible that what was defined as a statistically significant difference between groups may have arisen by chance.

The Panel also agreed with the investigators that only two markers were increased by long-term exposure — IL-6 and sICAM-1 — and these were most likely indicators of endothelial cell activation, rather than of vascular injury or endothelial cell dysfunction. Because these two markers were increased in females, but not males, at the 24-month exposure time point, the Panel considered that it was reasonable for the investigators to speculate that the aged, female endothelium may be slightly more sensitive than that of males to NTDE exposure. However, as the investigators pointed out, the study did not attempt to identify the cellular source of these two markers, so it is not known whether IL-6 and sICAM-1 were produced by the endothelium. The Panel also noted that the increase in plasma IL-6 at 24 months found in the current study was consistent with the increase in lung tissue IL-6 found (in both sexes, although with a slightly greater effect in females), at the same time point in the McDonald and colleagues' study (discussed earlier in this Commentary).

The Panel agreed with Conklin and Kong that some markers — in particular, leptin and HDL levels — that were changed at 1 or 3 months by exposure to NTDE showed no effects of exposure at later time points (12 and 24 months).

Cardiac Fibrosis and Aortic Remodeling The Panel also agreed with the investigators that exposure for up to 24 months to NTDE did not affect cardiac fibrosis or aortic remodeling. The morphologic data, although assessed on a relatively small number of animals, provide some reassuring evidence of an absence of structural changes associated with long-term NTDE exposure in healthy rats. The Panel also noted that the described morphometric methodology fell short of a robust, state-of-the-art, unbiased analysis, which follows the mathematical rules of unbiased stereology (Boyce et al. 2010; Mühlfeld et al. 2010). Additionally, the

Panel noted that accurate assessment of vascular remodeling is difficult in the absence of perfusion fixation.

Interpretation of Changes in Lipid Levels The Panel agreed with the investigators that the biologic significance of the exposure-associated modest decreases in levels of cholesterol and other lipids (total cholesterol, non-HDL cholesterol, and triglycerides) at 24 months was uncertain because the animals had been fed ad libitum before sacrifice. Thus, as the investigators point out and the Panel agreed, levels of cholesterol at the time of death would most likely reflect catabolism of the food most recently eaten, rather than the effects of exposure.

Rodents as Models of Cardiovascular Effects The Panel recognized that the investigators' goal was to identify possible cardiovascular markers of effects of exposure to NTDE, with changes in thrombotic and inflammatory pathways as the key focus. However, the Panel noted that the use of "normal" rodents to evaluate cardiovascular changes of relevance to humans is not an optimal choice because humans develop atherosclerosis as the result of inflammatory processes 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 such as the Wistar Han (and C57BL/6 mice used in the subchronic ACES exposure 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 et 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).

Some special strains of rats (e.g., the spontaneously hypertensive) and 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]) do develop lipid-containing lesions or even frank atherosclerosis. Evaluating the effects of NTDE in one or more of these rodent strains was discussed during the development of the original ACES protocol. The Panel appreciated that the choice of a strain for a lifetime exposure study is complex and challenging and that the primary hypothesis of this study concerned carcinogenicity. However, because of the differences noted above in cholesterol metabolism in different species, the current study provided a weak model for testing cardiovascular effects.

Summary and Conclusions

- The Panel agreed with the investigators' conclusions that most of the endpoints measured in inflammatory and thrombotic pathways were not affected by exposure to the concentrations of NTDE tested in this study. The paucity of changes reported in the study in a large set of endpoints assessed suggests that exposure to the concentrations of NTDE used triggered few, if any, cardiovascular responses that were sustained or detectable over the 24-month exposure.
- Some scattered changes in plasma markers were detected in rats exposed to NTDE over 24 months. Of the NTDE-associated changes, only the changes in IL-6 and sICAM-1 at the longest exposure time point (24 months) were positive, and these were small and noted predominantly in females. The Panel agreed with the investigators that these observations were of uncertain pathophysiologic significance. The Panel also agreed it was reasonable for the investigators to speculate that these observations suggest the aging female rat may be more susceptible than the male rat to endothelial cell activation.
- No NTDE-exposure-associated effects on cardiac fibrosis or aortic remodelling were detected.

OVERALL CONCLUSIONS FOR THE ACES PHASE 3B REPORTS

In its independent review of the core and ancillary ACES Phase 3B reports, the HEI ACES Review Panel concluded that McDonald and colleagues' study is the first to conduct a careful, comprehensive, and well-executed evaluation in rodents of lifetime inhalation exposure to a range of emissions from a heavy-duty 2007-compliant engine, referred to as NTDE. The investigators evaluated over 100 endpoints in the broad areas of histology, serum chemistry, systemic and lung inflammation, and respiratory function. Using appropriate statistical approaches to analyse the data, the investigators in this core study confirmed the a priori hypothesis, namely, that lifetime exposure to NTDE at the concentrations studied would *not* cause an increase in tumor formation or substantial toxic health effects in rats, although some biologic effects might occur.

The investigators continuously operated for over 30 months a facility in which NTDE from a heavy-duty diesel engine compliant with 2007 regulations was generated and transported to rat exposure chambers. Diesel emissions were derived over this time period from an engine operating on a strenuous 16-hour cycle, which was intended to reproduce typical urban and rural driving conditions and

thus be more reflective of modern engine performance and characteristics than exposure cycles used in long-term studies of emissions from older pre-2007-compliant engines (referred to as TDE). The investigators also attained NTDE exposure atmospheres within 20% of the designated target levels, which were 4.2, 0.8, and 0.1 ppm NO_2 over the exposure period. These exposure concentrations were based on levels of NO_2 rather than PM, which had been used in previous rat inhalation studies of TDE effects, because PM levels in NTDE were greatly reduced compared with TDE emissions. The 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 (1987a, 1989) in which minor biologic changes — but no cancer or pre-cancerous changes — were observed in the respiratory tract.

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. Concentrations of engine-generated PM were very low (< 11 µg/m^3) at all exposure levels (in the ultrafine range of 20–40 nm in diameter), as were concentrations of sulfur dioxide and semivolatile and volatile organic species. These findings confirm that concentrations of the components of NTDE differ strikingly from those of older engines, in which the concentrations of PM, as well as volatile and PM-associated organic species, are much higher.

The major biologic finding was that most endpoints evaluated showed no NTDE-associated changes after exposure of rats for up to 28 months (males) and 30 months (females). In particular, chronic exposure to NTDE resulted in no tumors or pre-neoplastic changes in the lung and no increases in tumors that were considered to be related to NTDE in any other tissue. Some mild histologic changes were found in the lung; however, these were not "preneoplastic lesions," which have been described in previous long-term exposure studies of rats to TDE. Rather, the histologic changes — periacinar epithelial hyperplasia, bronchiolization, accumulation of macrophages, and periacinar interstitial fibrosis — were confined to a small region, the centriacinus, which is involved in gas exchange.

HEI convened a panel of expert pathologists, the Pathology Working Group (PWG), to evaluate the histopathology data collected. The PWG findings confirmed the major histopathologic observations reported by the investigators. Also, the PWG, by evaluating the findings of this study side by side with findings from prior studies, provided a context with which to compare and contrast the current study findings with those from relevant long-term studies of exposure to TDE and oxidant gases. The overall conclusions were that chronic exposure to NTDE did not produce tumors in the lung in rats and resulted in effects that differed markedly from the chronic effects of exposure to TDE observed in multiple rat studies, where lung tumors, as well as inflammation and the deposition of soot in the lung, were observed. Rather, the effects of NTDE in the lung more closely resembled changes noted after long-term exposures to gaseous pollutants, in particular NO_2, and to TDE from which particles have been filtered out. It is possible that components of NTDE other than NO_2 may have contributed to the effects reported, but the low levels of other components suggest that they would not be primarily responsible.

The multiple toxicity endpoints evaluated — including lung and serum chemistry and respiratory function — were appropriate for evaluating a wide range of possible biologic effects. There were small decreases in some respiratory endpoints, particularly in measures of expiratory flow, predominantly at the highest exposure level and more in females than males. DL_{CO} also showed a small effect of exposure to NTDE, and the Panel considered these findings consistent with the histopathologic findings of mild changes in the gas-exchange regions of the lung, indicating that the histologic changes might have resulted in functional effects. In addition, some small increases in a few markers of oxidative stress and inflammation — HO-1, IL-6, KC, µTP, total white blood cells, and macrophages — were detected in lung tissue, BALF, and blood. The Panel considered that a minor limitation to the study was that some biochemical assays lacked positive controls (to determine that each was sensitive enough to detect any changes).

The Panel considered that the ancillary studies by Bemis et al., Hallberg et al., and Conklin and Kong were valuable extensions to the ACES core study. These generally well implemented studies took advantage of samples collected by McDonald and colleagues at several exposure time points up to 24 months to assess multiple endpoints that are not normally part of chronic inhalation bioassays. The genotoxicity studies assessed well-accepted endpoints: MN formation in RETs in the report by Bemis and colleagues, and DNA damage and lipid peroxidation in the report by Hallberg and colleagues. Conklin and Kong used a wide-ranging set of plasma markers associated with systemic inflammation and thrombosis and assays of cardiac fibrosis or aortic remodeling to identify possible cardiovascular effects of NTDE. The Panel agreed with the conclusions of Bemis and colleagues and Hallberg and colleagues that no genotoxic effects could be detected that were associated with exposure for up to 24 months to NTDE. However, the Panel's assessment that the genotoxic endpoints used are relatively short term (lasting 1 month or less) somewhat reduced the utility of these negative findings. NTDE also had few effects at up to 24 months of

exposure on the inflammatory and thrombotic pathway endpoints measured in plasma in Conklin and Kong's study. It was not clear that these scattered changes, predominantly in female rats, were of any pathophysiologic significance, however. In addition, Conklin and Kong did not find any NTDE-associated effects on cardiac fibrosis or aortic remodeling.

Overall, these results indicate that rats exposed to one of three levels of NTDE from a 2007-compliant engine for up to 30 months, for 16 hours per day, 5 days a week, with use of a strenuous operating cycle that more accurately reflected the real-world operation of a modern engine than cycles used in previous studies, showed few NTDE-exposure-related biologic effects. In contrast to the findings in rats chronically exposed to TDE, there was no induction of tumors or pre-neoplastic changes in the lung and no increase in tumors that were considered to be related to NTDE in any other tissue. The effects that were observed with NTDE were limited to the respiratory tract and were mild and generally seen at only the highest exposure level. These histologic changes in lungs were consistent with previous findings in rats after long-term exposure to NO_2 — a major component of the exposure atmosphere, which is being substantially further reduced in 2010-compliant engines.

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REFERENCES

Advanced Collaborative Emissions Study. 2012. Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Attfield MD, Schleiff PL, Lubin JH, Blair A, Stewart PA, Vermeulen R, et al. 2012. The diesel exhaust in miners study: a cohort mortality study with emphasis on lung cancer. J Natl Cancer Inst 104:869–883

Bailer AJ, Portier CJ. 1988. Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. Biometrics 44:417–31.

Barter PJ, Brewer HB Jr, Chapman MJ, Hennekens CH, Rader DJ, Tall AR. 2003. Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. Arterioscler Thromb Vasc Biol 23:160–167.

Behndig AF, Larsson N, Brown JL, Stenfors N, Helleday R, Duggan ST, et al. 2011. Proinflammatory doses of diesel exhaust in healthy subjects fail to elicit equivalent or augmented airway inflammation in subjects with asthma. Thorax 66:12–19.

Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, et al. 2006. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. Eur Respir J 27:359–365.

Beland FA. 1995. DNA Adduct Formation and T Lymphocyte Mutation Induction in F344 Rats Implanted with Tumorigenic Doses of 1,6-Dinitropyrene. Research Report 72. Cambridge,MA:Health Effects Institute.

Bond JA, Mauderly JL, Wolff RK. 1990. Concentration- and time-dependent formation of DNA adducts in lungs of rats exposed to diesel exhaust. Toxicology 60:127–135.

Boyce RW, Dorph-Petersen KA, Lyck L, Gundersen HJ. 2010. Design-based stereology: introduction to basic concepts and practical approaches for estimation of cell number. Toxicol Pathol 38:1011–1025.

Brightwell J, Fouillet X, Cassano-Zoppi AL, Gatz R, D uchosal F. 1986. Neoplastic and functional changes in rodents after chronic inhalation of engine exhaust emissions. Dev Toxicol Environ Sci 13:471–485.

Brook RD, Rajagopalan S, Pope CA 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, et al. 2010. Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. Circulation 121:2331–2378.

Brunekreef B, Beelen R, Hoek G, Schouten L, Bausch-Goldbohm S, Fischer P, et al. 2009. Effects of Long-Term Exposure to Traffic-Related Air Pollution on Respiratory

and Cardiovascular Mortality in the Netherlands: The NLCS-AIR Study. Research Report 139. Boston, MA:Health Effects Institute.

Calderón-Garcidueñas L, Maronpot RR, Torres-Jardon R, Henríquez-Roldán C, Schoonhoven R, Acuña-Ayala H, et al. 2003. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. Toxicol Pathol 31:524–538.

Calderón-Garcidueñas L, Reed W, Maronpot RR, Henríquez-Roldán C, Delgado-Chavez R, Calderón-Garcidueñas A, et al. 2004. Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. Toxicol Pathol 32:650–658.

Campen MJ, McDonald JD, Gigliotti AP, Seilkop SK, Reed MD, Benson JM. 2003. Cardiovascular effects of inhaled diesel exhaust in spontaneously hypertensive rats. Cardiovasc Toxicol 3:353–361.

Clark NN, Gautam M, Wayne WS, Lyons DW, Thompson G, Zielinska B. 2007. Heavy-Duty Vehicle Chassis Dynamometer Testing for Emissions Inventory, Air Quality Modeling, Source Apportionment, and Air Toxics Emissions Inventory. CRC Report E55/E59. Available: *http://www.crcao.org/publications/emissions/index.html* [accessed 3 December 2014].

Conklin DJ, Barski OA, Lesgards JF, Juvan P, Rezen T, Rozman D, et al. 2010. Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. Toxicol Appl Pharmacol 243:1–12.

Conklin DJ, Kong M. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Conklin DJ, Prough RA, Juvan P, Rezen T, Rozman D, Haberzettl P, et al. 2011. Acrolein-induced dyslipidemia and acute-phase response are independent of HMG-CoA reductase. Mol Nutr Food Res 55:1411–1422.

Crüts B, van Etten L, Törnqvist H, Blomberg A, Sandström T, Mills NL, Borm PJ. 2008. Exposure to diesel exhaust induces changes in EEG in human volunteers. Part Fibre Toxicol 5:4.

da Cunha AA, Ferreira AG, da Cunha MJ, Pederzolli CD, Becker DL, Coelho JG, et al. 2011. Chronic hyperhomocysteinemia induces oxidative damage in the rat lung. Mol Cell Biochem 358:153–160.

Frampton MW, Boscia J, Roberts NJ Jr, Azadniv M, Torres A, Cox C, et al. 2002. Nitrogen dioxide exposure: effects on airway and blood cells. Am J Physiol Lung Cell Mol Physiol 282:L155–L165.

Gallagher J, Hienrich U, George M, Hendee L, Phillips DH, Lewtas J. 1994. Formation of DNA adducts in rat lung following chronic inhalation of diesel emissions, carbon back and titanium dioxide particles. Carcinogenesis 15:1291–1299.

Garshick E, Laden F, Hart JE, Davis ME, Eisen EA, Smith TJ. 2012. Lung cancer and elemental carbon exposure in trucking industry workers. Environ Health Perspect 120:1301–1306

Gauderman WJ, Avol E, Gilliland F, Vora H, Thomas D, Berhane K, et al. 2004. The effect of air pollution on lung development from 10 to 18 years of age. N Engl J Med 351:1057–1067.

Gauderman WJ, Vora H, McConnell R, Berhane K, Gilliland F, Thomas D, et al. 2007. Effect of exposure to traffic on lung development from 10 to 18 years of age: a cohort study. Lancet 369:571–577.

Giknis M, Clifford C. 2003. Spontaneous neoplasms and survival in Wistar Han rats: compilation of control group data. Wilmington, MA:Charles River Laboratories.

Grimmer G, Brune H, Deutsch-Wenzel R, Dettbarn G, Jacob J, Naujack KW, et al. 1987. Contribution of polycyclic aromatic hydrocarbons and nitro-derivatives to the carcinogenic impact of diesel engine exhaust condensate evaluated by implantation into the lungs of rats. Cancer Lett 37:173–180.

Grizzle JE, Starmer CF, Koch GG. 1969. Analysis of categorical data by linear models. Biometrics 25:489–504.

Hallberg LM, Ward JB, Hernandez C, Ameredes BT, Wickliffe JK. 2012. 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. In: Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity. Research Report 166. Boston, MA:Health Effects Institute.

Hansen CS, Sheykhzade M, Møller P, Folkmann JK, Amtorp O, Jonassen T, et al. 2007. Diesel exhaust particles

induce endothelial dysfunction in apoE-/- mice. Toxicol Appl Pharmacol 219:24–32.

Harkema JR, Carey SA, Wagner JG. 2006. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicol Pathol 34:252–269.

Heinrich U, Fuhst R, Rittinghausen S, Creutzenberg O, Bellmann B, Koch W, et al. 1995. Chronic inhalation exposure of Wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inhal Toxicol 7:533–556.

Heinrich U, Muhle H, Takenaka S, Ernst H, Fuhst R, Mohr U, et al. 1986. Chronic effects on the respiratory tract of hamsters, mice and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol 6:383–395.

Hesterberg TW, Bunn WB, McClellan RO, Hart GA, Lapin CA. 2005. Carcinogenicity studies of diesel engine exhausts in laboratory animals: a review of past studies and a discussion of future research needs. Crit Rev Toxicol 35:379–411.

Huisingh J, Bradow R, Jungers R, Claxton L, Zweidinger R, Tejada S, et al. 1978. Application of bioassay to the characterization of diesel particle emissions. In: Application of Short–term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L, eds). New York:Plenum Press, 381–418.

International Agency for Research on Cancer. 1989. Diesel and gasoline engine exhausts and some nitroarenes. IARC Monogr Eval Carcinog Risk Hum 46:1–458.

International Agency for Reseach on Cancer. 2012. Diesel and gasoline engine exhausts and some nitroarenes. IARC Monogr Eval Carcinog Risk Hum 105:1–703.

Ishinishi N, Kuwabara N, Nagase S, Suzuki T, Ishiwata S, Kohno T. 1986. Long-term inhalation studies on effects of exhaust from heavy and light duty diesel engines on F344 rats. Dev Toxicol Environ Sci 13:329–348.

Iwai K, Adachi S, Takahashi M, Möller L, Udagawa T, Mizuno S, et al. 2000. Early oxidative DNA damages and late development of lung cancer in diesel exhaust-exposed rats. Environ Res 84:255–264.

Iwai K, Higuchi K, Udagawa T, Ohtomo K, Kawabata Y. 1997. Lung tumor induced by long-term inhalation or intratracheal instillation of diesel exhaust particles. Exp Toxicol Pathol 49:393–401.

Iwai K, Udagawa T, Yamagishi M, Yamada H. 1986. Long-term inhalation studies of diesel exhaust on F344 SPF rats. Incidence of lung cancer and lymphoma. Dev Toxicol Environ Sci 13:349–360.

Karagianes MT, Palmer RF, Busch RH. 1981. Effects of inhaled diesel emissions and coal dust in rats. Am Ind Hyg Assoc J 42:382–391.

Khalek IA, Bougher TL, Merritt PM. 2009. Phase 1 of the Advanced Collaborative Emissions Study. Coordinating Research Council Report. Available: *www.crcao.org/publications/emissions/index.html* [accessed 14 November 2014].

Khalek IA, Bougher TL, Merritt PM, Zielinska B. 2011. Regulated and unregulated emissions from highway heavy-duty diesel engines complying with U.S. Environmental Protection Agency 2007 emissions standards. J Air Waste Manag Assoc 61:427–442.

Krewski D, Jerrett M, Burnett RT, Ma R, Hughes E, Shi Y, et al. 2009. Extended Follow-Up and Spatial Analysis of the American Cancer Society Study Linking Particulate Air Pollution and Mortality. Research Report 140. Boston, MA:Health Effects Institute.

Kubota K, Murakami M, Takenaka S, Kawai K, Kyono H. 1987. Effects of long-term nitrogen dioxide exposure on rat lung: Morphological observations. Environ Health Perspect 73:157–169.

Li X, Liu Y, Zhang H, Ren L, Li Q, Li N. 2011. Animal models for the atherosclerosis research: a review. Protein and Cell 2:189–201.

Libby P, Aikawa M. 2002. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. Nat Med 8:1257–1262.

Lucking AJ, Lundbäck M, Barath SL, Mills NL, Sidhu MK, Langrish JP, et al. 2011. Particle traps prevent adverse vascular and prothrombotic effects of diesel engine exhaust inhalation in men. Circulation 123:1721–1728.

McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, et al. 2007. Respiratory effects of exposure to diesel traffic in persons with asthma. N Engl J Med 357:2348–2358.

Mauderly JL, Banas DA, Griffith WC, Hahn FF, Henderson RF, McClellan RO. 1996. Diesel exhaust is not a pulmonary carcinogen in CD-1 mice exposed under conditions carcinogenic to F344 rats. Fundam Appl Toxicol 30:233–242.

Mauderly JL, Bice DE, Carpenter RL, Gillett NA, Henderson RF, Pickrell JA, et al. 1987a. Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung. Research Report 8. Cambridge, MA:Health Effects Institute.

Mauderly JL, Bice DE, Cheng YS, Gillett NA, Henderson RF, Pickrell JA, et al. 1989. Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust. Research Report 30. Cambridge MA:Health Effects Institute.

Mauderly JL, Jones RK, Griffith WC, Henderson RF, McClellan RO. 1987b. Diesel exhaust is a pulmonary carcinogen in rats exposed chronically by inhalation. Fundam Appl Toxicol 9:208–221.

Mauderly JL, McCunney R. 1996. Particle Overload in the Rat Lung and Lung Cancer: Implications for Human Risk Assessment. Philadelphia, PA:Taylor & Francis.

Mauderly JL, McDonald JD. 2012. Advanced Collaborative Emissions Study (ACES) Phase 3A: Characterization of U.S. 2007-Compliant Diesel Engine and Exposure System Operation. Communication 17. Boston, MA:Health Effects Institute.

Mauderly JL, Snipes MB, Barr EB, Belinsky SA, Bond JA, Brooks AL, et al. 1994. Part I. Neoplastic and nonneoplastic lung lesions. In: Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats. Research Report 68. Cambridge, MA:Health Effects Institute.

Mills NL, Törnqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, et al. 2005. Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation 112:3930–3936.

Morawska L, Ristovski Z, Jayaratne ER, Keogh DU, Ling X. 2008. Ambient nano and ultrafine particles from motor vehicle emissions: characteristics, ambient processing and implications on human exposure. Atmos Environ 42:8113–8138.

Morton D, Sellers RS, Barale-Thomas E, Bolon B, George C, Hardisty JF, Irizarry A, et al. 2010. Recommendations for pathology peer review. Toxicol Pathol 38:1118–1127.

Mühlfeld C, Nyengaard JR, Mayhew TM. 2010. A review of state-of-the-art stereology for better quantitative 3D morphology in cardiac research. Cardiovasc Pathol 19:65–82.

Nafstad P, Håheim LL, Oftedal B, Gram F, Holme I, Hjermann I, et al. 2003. Lung cancer and air pollution: a 27 year follow up of 16,209 Norwegian men. Thorax 58:1071–1076.

National Toxicology Program. 2011. Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP). Research Triangle Park, NC:National Toxicology Program.

Nesnow S, Evans C, Stead A, Creason J, Slaga TJ, Triplett LL. 1982. Skin carcinogenesis studies of emissions extracts. Dev Toxicol Environ Sci 10:295–320.

Nikula KJ, Snipes MB, Barr EB, Griffith WC, Henderson RF, Mauderly JL. 1995. Comparative pulmonary toxicities and carcinogenicities of chronically inhaled diesel exhaust and carbon black in F344 rats. Fundam Appl Toxicol 25:80–94.

Nyberg F, Gustavsson P, Järup L, Bellander T, Berglind N, Jakobsson R, et al. 2000. Urban air pollution and lung cancer in Stockholm. Epidemiology 11:487–495.

Oftedal B, Brunekreef B, Nystad W, Madsen C, Walker SE, Nafstad P. 2008. Residential outdoor air pollution and lung function in schoolchildren. Epidemiology 19:129–137.

Okey AB, Franc MA, Moffat ID, Tijet N, Boutros PC, Korkalainen M, et al. 2005. Toxicological implications of polymorphisms in receptors for xenobiotic chemicals: the case of the aryl hydrocarbon receptor. Toxicol Appl Pharmacol 207:S43–S51.

Randerath K, Putman KL, Mauderly JL, Williams PL, Randerath E. 1995. Part II. DNA damage. In: Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats. Research Report 68. Cambridge, MA:Health Effects Institute.

RCC Ltd. 2003. Historical control data on neoplastic findings in Wistar rats (planned sacrifice after 103 weeks), compiled from 2-year bioassays. Itingen, Switzerland:RCC, Ltd.

Reed MD, Blair LF, Burling K, Daly I, Gigliotti AP, Gudi R, et al. 2006. Health effects of subchronic exposure to diesel-water-methanol emulsion emission. Toxicol Ind Health 22:65–85.

Reed MD, Gigliotti AP, McDonald JD, Seagrave JC, Seilkop SK, Mauderly JL. 2004. Health effects of subchronic exposure to environmental levels of diesel exhaust. Inhal Toxicol 16:177–193.

Riedl MA, Diaz-Sanchez D, Linn WS, Gong H Jr, Clark KW, Effros RM, et al. 2012. Allergic Inflammation in the Human Lower Respiratory Tract Affected by Exposure to Diesel

Exhaust. Research Report 165. Boston, MA:Health Effects Institute.

Rojas-Martinez R, Perez-Padilla R, Olaiz-Fernandez G, Mendoza-Alvarado L, Moreno-Macias H, Fortoul T, et al. 2007. Lung function growth in children with long-term exposure to air pollutants in Mexico City. Am J Respir Crit Care Med 176:377–384.

Sagai M, Ichinose T, Kubota K. 1984. Studies on the biochemical effects of nitrogen dioxide. IV. Relation between the change of lipid peroxidation and the antioxidative protective system in rat lungs upon life span exposure to low levels of NO_2. Toxicol Appl Pharmacol 73:444–456.

Solomon C, Christian DL, Chen LL, Welch BS, Kleinman MT, Dunham E, et al. 2000. Effect of serial-day exposure to nitrogen dioxide on airway and blood leukocytes and lymphocyte subsets. Eur Respir J 15:922–928.

Stenfors N, Nordenhäll C, Salvi SS, Mudway I, Söderberg M, Blomberg A, et al. 2004. Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. Eur Respir J 23:82–86.

U.S. Environmental Protection Agency. 2002. Health Assessment Document for Diesel Engine Exhaust. EPA/600/ 8-90/057F. U.S. Environmental Protection Agency Office of Research and Development. Washington, DC:National Center for Environmental Assessment.

U.S. Environmental Protection Agency. 2008. Integrated Science Assessment for Oxides of Nitrogen: Health Criteria EPA/600/R–08/071. Washington, DC:U.S.EPA.

Venables W, Ripley B. 2002. Modern Applied Statistics with S. New York:Springer.

Vineis P, Hoek G, Krzyzanowski M, Vigna-Taglianti F, Veglia F, Airoldi L, et al. 2006. Air pollution and risk of lung cancer in a prospective study in Europe. Int J Cancer 119:169–174.

Vostal JJ. 1986. Factors limiting the evidence for chemical carcinogenicity of diesel emissions in long-term inhalation experiments. Dev Toxicol Environ Sci 13:381–396.

Weldy CS, White CC, Wilkerson HW, Larson TV, Stewart JA, Gill SE, et al. 2011. Heterozygosity in the glutathione synthesis gene Gclm increases sensitivity to diesel exhaust particulate induced lung inflammation in mice. Inhal Toxicol 23:724–735.

Zaragoza C, Gomez-Guerrero C, Martin-Ventura JL, Blanco-Colio L, Lavin B, Mallavia B, et al. 2011. Animal models of cardiovascular diseases. J Biomed Biotech 2011:1–13.

Zhang J, McCreanor JE, Cullinan P, Chung KF, Ohman-Strickland P, Han I-K, et al. 2009. Health Effects of Real-World Exposure to Diesel Exhaust in Persons with Asthma. Research Report 138. Boston, MA:Health Effects Institute.

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