# STATEMENT

Synopsis of Research Report 166, Parts 1–4

HEALTH EFFECTS INSTITUTE

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

## INTRODUCTION

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

## BACKGROUND

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

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

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

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

## APPROACH

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

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Drs. Jacob D. McDonald of the Lovelace Respiratory Research Institute, Albuquerque, New Mexico, Jeffrey C. Bemis of Litron Laboratories, Rochester, New York, and Lance M. Hallberg of the University of Texas Medical Branch, Galveston, Texas, and their colleagues, and Daniel J. Conklin and Maiying Kong of the University of Louisville, Louisville, Kentucky. The complete report, *Advanced Collaborative Emissions Study (ACES) Subchronic Exposure Results: Biologic Responses in Rats and Mice and Assessment of Genotoxicity* (© 2012 Health Effects Institute), can be obtained from HEI or our Web site (see last page).

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

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

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

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

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

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

## RESULTS AND CONCLUSIONS

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

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

In their extensive analysis of the physical and chemical composition of the emissions, McDonald and colleagues found that the most abundant pollutants were carbon dioxide, carbon monoxide (CO), NO, and $NO_2$, whereas concentrations of particulate matter, sulfur dioxide, and semivolatile and volatile organic species were very low. These findings confirm that the components of emissions from the 2007-compliant engine differ strikingly from those of older engines, in which particulate matter concentrations are much higher. The multiple

standardized toxicity endpoints evaluated in this study — including histology, serum chemistry, and respiratory function — were appropriate for evaluating the hypothesis. The panel agreed with McDonald and colleagues that there were no changes in health endpoints for the majority of biologic tests conducted in rats and mice. When results were compared at 1 and 3 months across the species, the few changes observed were reported more often in rats than mice and almost exclusively with exposure to high-level diesel exhaust.

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

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

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

The panel concluded that the ancillary studies assessed generally well-accepted markers of both genotoxicity — micronuclei formation in reticulocytes (in the report by Bemis et al.) and DNA damage and lipid peroxidation (in the report by Hallberg et al.) — and systemic inflammation and thrombosis (in the report by Conklin and Kong) and that they were valuable extensions to the ACES core study. The panel agreed with the investigators that no genotoxic effects could be detected that were associated with exposure for up to 3 months to any level of diesel exhaust from the 2007-compliant engine. The small group size (only 5 animals of each sex in each exposure group) and the assessment of genotoxic endpoints that, although well validated, are relatively short term (lasting one month or less) slightly reduced confidence in the utility of these negative findings. Most of the thrombotic and inflammatory endpoints measured in plasma in Conklin and Kong's study were not affected by exposure to diesel exhaust, but some scattered changes — including changes in rat levels of cholesterol and high density lipoprotein — were detected. The pathophysiologic significance of these scattered changes was uncertain, however, because they were seen at only one of the two exposure time points in each sex, and the direction of the change (positive or negative) differed at the different exposure time points. Similarly, inconsistent and scattered changes in levels of IgE were seen, but these changes did not support the interpretation that new-technology diesel emissions acted as an

adjuvant — an enhancer of specific immune responses — for the allergic response, as has been suggested for the effects of diesel exhaust particles in some prior animal and human studies.

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

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

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

Jacob D. McDonald et al.

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

Jeffrey C. Bemis et al.

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

Lance M. Hallberg et al.

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

Daniel J. Conklin and Maiying Kong

**COMMENTARY** *by the HEI ACES Review Panel*

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