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Biased Dismissal of Epigenetic Evidence for "Clean-Diesel" Carcinogenicity and Genotoxicity

Shrader-Frechette K* and McQuestion C
Department of Biological Sciences, University of Notre Dame, USA

***Corresponding author:** Shrader-Frechette K, Department of Biological Sciences, 100 Malloy Hall, University of Notre Dame, Notre Dame, IN 46556, USA

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Abstract

In 2012, the International Agency for Research on Cancer (IARC) named Traditional Diesel Exhaust (TDE) a "known human carcinogen". Most western nations agreed, passing new regulations. Yet the US denies TDE is a known carcinogen, says scientific data are uncertain, and does not regulate TDE emissions of 80 percent of US-diesel vehicles. It did require post-2007, US-heavy-duty-diesel vehicles to have "clean-diesel" or New-Technology-Diesel Exhaust (NTDE) --- filters/engine improvements to reduce emissions. A major reason the US disagrees with the rest of the world is its reliance on the 10-year series of US studies on NTDE, the 2015 Advanced Collaborative Emissions Study (ACES). Co-funded by US Environmental Protection Agency, ACES was overseen by the Health Effects Institute, a research group that the US National Academies of Science earlier praised. Who is right on diesel carcinogenicity, the hundreds of IARC, or the ACES, studies? This review article concludes IARC is correct. It (1) surveys the role of epigenetics in assessing TDE and NTDE carcinogenicity and genotoxicity. Next it (2) shows how, despite some ACES strengths, it ignored much epigenetic evidence for NTDE carcinogenicity and genotoxicity because of wrong/incomplete tests, trimming the data, using incorrect assessment-time frames, making value judgments instead of empirically-confirmed judgments, begging key questions---then invalidly concluding that NTDE causes "only a few mild effects on the lungs," no cancer or serious ailments, and "no…gene-damaging effects." Finally the article (3) suggests why these scientific errors occurred in prominent studies and (4) answers objections to its criticisms of ACES.

Keywords: ACES; Air pollution; Diesel; IARC; Particulate matter

Introduction

The World Health Organization estimates that about 7 million people die prematurely every year as a result of air pollution. It says fine and ultrafine particles, like those from diesel engines, are the single most lethal form of air pollution because of their carcinogenicity, cytotoxicity, embryotoxicity, genotoxicity, and reproductive toxicity [1-5].

In 2012, the International Agency for Research on Cancer (IARC), part of the World Health Organization, (WHO) named diesel exhaust, especially diesel exhaust, especially DPM a "known human carcinogen" and called for tighter regulations. Most western nations and medical associations agreed, and Europe passed new regulations. The US, however, denies that DPM is a known human carcinogen. Because it says DPM is merely "likely to be carcinogenic to humans," it has failed to name DPM a "hazardous air pollutant". Yet it admits that the cancer risks from USTDE vehicles, mostly from DPM, are seven times greater than the combined risk of all 187 air toxics that the U.S. Environmental Protection Agency (US-EPA) tracks [6-8].

US-EPA appears to have rejected the IARC and WHO findings of known diesel carcinogenicity for two main reasons. First, (1) it says that because it has no precise, quantitative, cancer dose-response curve for diesel, only a quantitative range within the possible response lies, US-EPA cannot estimate DPM cancer potency; second (2) US-EPA says it has no reliable mechanism of action for DPM induction of cancer [7,9-12].

However, the preceding US-EPA response (1) may be questionable because although the entire range of DPM risk, spanning two orders of magnitudes, is not quantitatively precise, it is entirely above the level at which government says regulation must occur. Thus, even if the lowest part of the range of DPM risk is correct, this risk appears to require more regulation, in part because it is on the order of the risk of dioxin, one of the most potent carcinogens ever known. Besides, one can know that something is carcinogenic without knowing precisely how part of its dose-response curve is shaped. Scientists are still arguing about the precise shape of the low-dose end of dose-response curve for many known humans' carcinogens, including ionizing radiation, yet that does not jeopardize the "known human carcinogen" status of these carcinogens.US-EPA response (2) likewise appears questionable because knowing that something is carcinogenic is independent of knowing precisely how, through what mechanisms, it is carcinogenic. After scientists find a carcinogenic cause-effect relationship, it often takes decades to determine the precise mechanisms of that causal relationship. The same is true for DPM carcinogenicity and genotoxicity and their partially epigenetic mechanisms, such as partly heritable changes in methylation that lead to a cascade of inflammation, including creation of reactive oxygen species [13-19].

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US-EPA's denial of DPM's "known human carcinogenicity" on grounds (2) above is especially puzzling because it admits the existence of epigenetic mechanisms for cancer but says they are "poorly understood". It recognizes that "the three best known epigenetic mechanisms involve DNA methylation, histone modification, and alteration of the expression of micro-RNAs." It likewise recognizes that "the level of intensity of DNA methylation is [carcinogen-] dose-dependent," that cancer-and-epigenetics "results are reproducible from experiment to experiment, and from laboratory to laboratory". It says that just as genome-wide association studies have successfully identified diseases associated with specific genetic variants, so also epigenome-wide association studies, based on knowledge of tissue-specific epigenetic modifications are now becoming successful in associating environmental exposures to diseases like cancer. US-EPA likewise admits that both epidemiological and animal studies support the view that exposure to environmental contaminants like DPM increases susceptibility to multiple diseases, including cancer, and that because "epigenetic modifications can occur both before and after overt disease is evident," they likely play a role in cancer initiation and progression [20].

The Critical Role of Epigenetics in DPM Carcinogenicity

Thus apart from their disagreement over whether DPM is a "known" or a "likely" human carcinogen, IARC, WHO, and US-EPA all agree that "epigenetic changes have emerged as key mechanisms in cancer development, including genotoxic-cancer development. All critical changes in cancer cells, such as silencing of tumor suppressor genes, activation of oncogenes, and defects in DNA repair, can be caused not only by genetic but also by epigenetic mechanisms such as methylation" [21-23]. The main diesel-carcinogenesis difference between IARC and US-EPA appears to lie in the level of knowledge of epigenetic mechanisms---that each thinks is necessary before claiming diesel is a known, rather than merely a likely, carcinogen [13].

Epidemiological studies have identified factors such as DPM, associated with lung and other cancers, while animal tests and human studies have identified the epigenetic mechanisms and molecular pathways that tie specific factors to different cancers. All three types of studies provide evidence for a sequence of epigenetic and genetic effects as malignancy progresses. Loss of DNA methylation was one of the first epigenetic changes described in human cancer, and soon scientists showed that overall 5mC content was inversely associated with tumor progression [24]. In the 30 years since publication of these classic epigenetics findings, almost every type of cancer has been shown to have an overall deficiency of 5mC compared with normal tissue, something that increases genomic instability and promotes the progression of tumorigenesis [23]. Depending on the different cancers, abnormal patterns of methylation arise, causing both hypomethylation of distal regulatory regions and repetitive elements---and hypermethylation of CpG islands [25,26], so that tumors from different sites display distinct CpG methylation profiles [27] and distinct pathways of carcinogenesis within different tumor sites [23,28,29].

Flawed Science and Failure to Reliably Assess NTDE Carcinogenicity and Genotoxicity

Given established scientific consensus that epigenetic changes are likely key mechanisms in carcinogenicity and genotoxicity, it is puzzling that the 2015 Advanced Collaborative Emissions Study (ACES) of NTDE health effects, co-sponsored by the US Environmental Protection Agency and the oil-and-auto industries, ignored most of the epigenetic evidence for NTDE carcinogenicity and genotoxicity. Yet ACES describes itself as the "most comprehensive" study of health effects of NTDE ever done. Having surveyed the critical role of epigenetics in assessing TDE and NTDE carcinogenicity and genotoxicity, the article now shows how, despite some ACES strengths, ACES studies ignored much epigenetic evidence for NTDE carcinogenicity and genotoxicity because of doing wrong or incomplete tests, trimming the data, using incorrect assessment-time frames, making value judgments instead of empirically-confirmed judgments, begging key questions – then invalidly concluding that NTDE causes "only a few mild effects on the lungs," no cancer or serious ailments, and "no…gene-damaging effects".

For the last 20 years, scientists have shown that even two-hour exposure to typical TDE causes changes in methylation at about 2,800 different points on DNA, affecting about 400 genes, sometimes increasing methylation, sometimes decreasing it. For instance, genotoxic DPM methylates and inactivates the p16INK4a gene in 50% of lung tumors, while it inactivates the ER gene in 15% of the lung tumors. This methylation inhibits gene transcription by a factor of 30-60 times, and methylation frequency is a function of exposure. Repeated experiments in rats show that tumors induced by TDE PM Carcinogens arise in part by methylation, by inactivation of genes like the p16 and ER, and that this process generates oxidative stress and inflammation that can nick DNA, cause single-strand breaks, and thus contribute to lung cancer. DPM thus induces a chronic inflammatory response, causes DNA adducts in lung tissue, and thus induces cancer through epigenetic mechanisms such as gene inactivation and hypermethylation of regulatory genes. After DPM exposure, tumors in rats arise on average after 18 months and at all exposure levels. Moreover both in vivo and in vitro experimental studies provide strong evidence that DPM is not only carcinogenic but also genotoxic [4,30-41].

Ignoring Epigenetic Evidence for Diesel-Caused Lung Cancers

Despite the science of the preceding 20 years, two of the most questionable and question-begging claims that the ACES authors make regarding carcinogenicity are that "the [study] effects that were observed with NTDE were limited to the respiratory tract" and were "mild and generally seen at only the highest exposure level" [42]. They also claim that NTDE "did not induce tumors or pre-cancerous changes in the lung, [and that only]….a few mild changes were seen in the lungs" [43].

The preceding ACES claims arguably err because DPM pollution, the deadliest part of NTDE---which contains 200,000 to 800,000 DPM particles per cubic centimeter [42] --- can cause lung cancer and genotoxicity, partly through epigenetic mechanisms. ACES directly tested none of these epigenetic mechanisms, yet denied any lung cancer from NTDE. The 198-page ACES scientific reports contain no mention either of "epigenetic" or "methylation," despite the fact that epigenetic changes are key cancer mechanisms that typically appear prior to disease. DPM, in particular, is associated with changes in DNA methylation, at genes such as GSTP1, that are known to be

associated with inflammation, oxidative stress, and higher risk for lung cancer and asthma [44]. Epigenetic assessment likewise implicates inactivation of the tumor-suppressor genes APC, p16, p53, and RASSF1, especially by hypermethylation, as a contributing factor to development of lung cancer associated with exposure to the particulate carcinogens such as DPM [45]. Methylation changes in p16, for instance, correlate directly with loss of gene transcription, and it appears in roughly 60 percent of diesel-induced tumors [46]. DPM exposure also changes the miRNA expression profile in human airway epithelial cells, so that a majority miRNAs (e.g., 197 of 313 miRNAs or 62.9%) can be either up-regulated or down regulated by nearly a factor of 2. For the 12 most altered miRNAs, DPM exposure is associated with inflammatory-response pathways and "a strong tumorigenic disease signature" [47].

Ignoring Evidence for Diesel-Caused Genotoxic Effects

Just as ACES denies its study revealed any precancerous effects, it also denies that NTDE causes any genotoxic effects [43]. Yet because this ACES claim is based on studies that are short-term, low-powered, insensitive analyses, without either positive controls or any studies of the lung, at least 6 reasons suggest that it is a false-negative conclusion.

First, the ACES claim that NTDE causes no genotoxic effects is questionable because ACES researchers did only very short-term tests to assess genotoxicity and no assay to assess accumulation of mutations. For instance, ACES attempted to assess genotoxic effects by evaluating the blood of NTDE-exposed animals. ACES checked the number of immature red blood cells (reticulocytes, or RETs) that contained Micronuclei (MN). If NTDE had caused double-strand breaks or disrupted chromosome segregation during cell division, the scientists concluded that an increase in MN frequency would result. The problem, however, is that the MN endpoint is only a short-term indicator of genotoxicity. It assesses damage only over the last 3 days of exposure. Thus contrary to what ACES claimed, this study does not access "lifetime" effects of NTDE. Besides, given DNA repair, the number of MN-RETs does not measure cumulative exposure, even over the study's 24-month time-frame, unless the animals had compromised responses to damage, such as deficient DNA repair capacity or detoxification systems---for which ACES provides no evidence. Given these problematic biological endpoints, ACES likely did not measure genotoxicity, but only DNA repair capacity or detoxification systems. ACES genotoxocity studies were too short, and they used the wrong endpoints. Instead ACES should have used assays to assess mutations, such as mutations in the *hprt*gene in peripheral blood cells, and accumulation of mutations, if it wanted information on the longer-lasting effects of NTDE exposures [42].

A second reason that ACES tests of NTDE for genotoxic effects likely produced false-negative results is that the ACES tests likely were not sensitive enough to detect most NTDE health effects. Most of the ACES assay and all of the genotoxicity studies failed to have any "positive controls" to support their ability to detect biologic effects [48]. Instead, as a positive control, ACES could have used samples from a group exposed to TDE. This would have given a better benchmark for the health effects of engineering changes associated with NTDE [49]. Even worse, to defend the ACES absence of positive controls in most of its studies and in all its genotoxicity studies, ACES used a nonscientific response. It said that adding positive controls "would have substantially increased the complexity and cost of the study and would have posed enormous logistical challenges" [42]. Obviously low-cost, non-complex studies are not the same thing as high-value or methodologically-defensible studies.

A third problem with the ACES tests of NTDE for genotoxic effects is that none of the genotoxicity researchers used lung cells. Instead one group used blood cells and another group used brain cells. They should also have used lung cells because they reveal the direct and early effects of NTDE; organs like the blood reveal indirect and later effects of NTDE. Because ACES used such short-term genotoxicity tests (e.g., three days), and because it is unclear precisely how DPM effects are mediated outside the lungs [50], arguably ACES should have assessed the early, direct effects of NTDE effects in lungs and airways [42]. Another reason for using lung cells is that researchers should not have trimmed the data by presupposing that NTDE genotoxic effects were systemic, thus detectable outside organs like the lung.

A fourth problem with the ACES tests of NTDE for genotoxic effects is that they likely produced false-negative results because of low-power studies. One of the two groups of genotoxicity researchers admitted as much, claiming they used only 5 animals, though "many more animals would have been needed to detect a difference in response among exposure groups" [42]. The same scientists also made another questionable move, stipulating effect size---standardly defined as difference in response, relative to controls----instead as the ratio of two standard deviations. Why the focus on detecting changes in standard deviations rather than in mean responses? They likewise based their power calculations on previous mouse data, not on the current rat data, arguably needed for sensitive studies. Similarly, ACES used the TBARS assay though it is neither a sensitive nor a specific assay for oxidative stress, and thus provides no information on the range of possible changes in lipid peroxidation caused by NTDE. Similarly, the assay used by Hallberg and colleagues in ACES measured only MDA, only one lipid peroxidation product---not full evaluation of both oxidative stress and its lipid peroxidation products. All these problems again suggest ACES data-trimming, likely leading to false-negative conclusions [42].

A fifth problem with ACES genotoxicity studies are their ignoring the fact that DPM is a Trojan-Horse pollutant. That is, DPM attracts other diesel-exhaust carcinogens, toxins, and metals such as arsenic, cadmium, formaldehyde, Polyaromatic Hydrocarbons (or PAHs), and zinc. They adhere to the ultrafine DPM, form fine DPM, enter the brain or lungs, and can travel to all bodily organs to cause chronic inflammation leading to many diseases, including Alzheimer's, autism, birth defects, cancer, Parkinson's, and even death [10,51-68]. PAHs, especially those that adhere to DPM, typically are genotoxic [69-71], and they comprise the major fraction of DPM components [72]. Moreover because ACES admitted that NTDE removes only 80 percent of TDE hydrocarbons [42], and NTDE PAHs remain a potential source of genotoxicity, it is puzzling that ACES genotoxicity studies failed to assess damage from PAHs [49].

A sixth scientific deficiency of ACEs genotoxicity studies, again likely leading to false-negative results, is that they considered neither all the relevant literature on genotoxicity caused by TDE and

NTDE, nor all the relevant tests. Given earlier ACES genotoxicity problems, such as wrong endpoints, low power, and absence of positive controls, ACES could have redone some of the research supporting genotoxicity, instead of merely doing new studies of questionable methodology. Top journals, assessing genotoxicity in TDE and NTDE, illustrate reliable methods that ACES could have copied. Yet ACES did assessment neither of specific genes known to be harmed by diesel exhaust, nor of cumulative mutations. Instead ACES researchers used the comet assay that, as ACES conducted it, was able to detect only a few types of known DNA damage. ACES no-genotoxicity claims thus are based on false-negative results from inadequately-sensitive tests [4,41,49,73-75].

Regarding this sixth deficiency, why did the ACES authors largely ignore what they admitted, namely, that the scientific literature clearly shows DPM PAHs induce micronucleus formation and genotoxic damage [49]? Why did they ignore animal studies, cell-culture experiments, and cell-free systems that all suggest that diesel exposure initiates oxidative DNA damage by generating reactive oxygen species and inflammatory responses [36,76]? ACES authors ignored abundant evidence of TDE genotoxicity by making a qualitative, unsubstantiated, nonempirical judgment that newer NTDE engine designs had reduced the deadliest TDE components---DPM and PAHs---"to almost ambient levels" unlikely to cause harm, including genotoxicity [77]. Yet if one assumes NTDE PM and PAH are at "almost ambient levels," one has no testable hypothesis about NTDE harm because "almost ambient levels of a pollutant" are typically not carcinogenic or genotoxic. Because ACES begged the question at issue, there was little reason to conduct ACES studies.

Unfortunately, the preceding ACES inconsistencies in claiming both that NTDE has essentially no DPM and PAHs---versus NTDE has 10-20 percent of the DPM and PAHs in TDE---are typical of ACES. Inconsistencies, from which anything follows, characterize ACES. For instance, although it was reasonable to use the ANOVA for the comet, 8-OHdG, and TBARS data, ACES researchers used these tests/data inconsistently and never fully described their statistical approaches and why they used them. Readers cannot evaluate ACES' statistical methods for post-hoc pair wise comparisons in all the assays, because ACES did not identify them. ACES also applied the methods inconsistently across the assays. They used the Bonferroni correction 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. ACES authors also did not explain why they assumed it was necessary for the overall ANOVA (but not for the 8-OHdG or TBARS) data to be significant before making post-hoc comparisons for the comet data [42].

What Caused the ACES Scientific Errors Regarding Carcinogenicity and Genotoxocity of NTDE?

Given replicated scientific studies tying DPM exposure to genotoxic effects such as micronucleus formation, and to lung cancer via epigenetic mechanisms such as DNA methylation, how can ACES claim that exposure to DPM, the deadliest component of TDE and NTDE, causes neither genotoxic, nor cancerous, nor pre-cancerous effects? There seem to be several reasons. First, ACES used the wrong tests. It did not check for classic epigenetic precursors and mechanisms associated with DPM exposure, such as DNA methylation of specific genes and changes in the expression of micro-RNAs. As noted, it did not do the best and the most direct tests for lung cancer, cancer precursors, and genotoxicity. Instead ACES used short-term, insensitive, low-powered studies, based on the gratuitous assumption that NTDE pollutants were at "almost ambient [air] levels," [49]. Yet ACES itself admitted NTDE removed only 80 percent of TDI hydrocarbons, including PAHs [42], and only 90 percent of DPM. Thus no-safe-dose pollutants like DPM, present at 10-20 percent of their heaviest levels, arguably are not at "almost ambient [air] levels" [77].

Second, as already noted, in assuming NTDE DPM and PAHs were at "almost ambient [air] levels", ACES was begging the question, relying on unsubstantiated value judgments.

Third, ACES denied NTDE-induced lung cancer and genotoxicity by trimming the data through arbitrary interpretation of test results. Even when ACES discovered lung-cancer precursors such an inflammation, oxidative stress, or lesions, it dismissed them as atypical, claiming they were "mild" or found "predominantly at the highest exposure level". ACES used the words "mild" 49 times, and "highest" exposures 24 times, to dismiss results. Yet either there are precancerous effects, or not. There is no "mild" cancer precursor that is not a cancer precursor, any more than there is a "mild" pregnancy that is not a pregnancy. ACES also trimmed the data by contradicting itself, claiming NTDE caused no cancerous/precancerous effects, yet repeatedly admitting that subjects had serious, low-dosebronchiolization lesions [48]---well-established lung-cancer precursors [78-81]. Similarly, sometimes the same ACES researchers contradicted themselves----denying finding respiratory cancer [82], and then denying finding both respiratory cancer and precancerous lesions [82].

Fourth, because of HEI/ACES editors' and reviewers' misrepresentation of ACES results, they contradicted ACES scientists. For instance, when ACES researchers admitted their studies were underpowered, with only 5 subjects, thus unlikely to detect genotoxicity, HEI/reviewers editors/reviewers disagreed. They put a positive "spin" on the research, denied it was underpowered, and then claimed there was no evidence of genotoxicity [42]. Likewise, though ACES researchers denied finding any lung cancer [82], ACES editors said they found neither cancer nor non-cancer outcomes such as "substantial toxic effects"; instead the editors claimed NTDE effects all were mild, and "limited to the respiratory tract" [83].

Fifth, HEI and some ACES scientists may have used flawed methods because HEI funders had financial conflicts of interests and sought to show NTDE was noncarcinogenic and nongenotoxic. HEI, the nonprofit research organization that oversaw, edited, and funded ACES, admits in the ACES foreword that half of ACES current funding comes from US-EPA and half from the "worldwide motor-vehicle" industry [84].

Answering an Objection

In response to the preceding scientific criticisms of ACES' methods, how might ACES scientists defend themselves against their ignoring most of the classic epigenetic research and methods showing TDE carcinogenicity and genotoxicity? As already mentioned, ACES scientists assume, before doing their studies, that DPM and PAHs,

the deadliest components in NTDE are at "almost ambient [air] levels". By making this assumption, they assume (rather than test whether) earlier TDE research is likely irrelevant to their NTDE studies [49]. For instance, ACES pathologists cite diesel-industry authors McClellan and Hesterberg, then claim "NTDE compared with TDE is…quite different….Studies of the health impact of TDE exposures most likely do not reflect either the hazards or the risks from NTDE" [48].

Second, as already mentioned, even ACES admits that NTDE removes only 90 percent of TDE, including DPM, and only 20 percent of the hydrocarbons---including genotoxic PAHs [42]. NTDE thus has10-20 percent of the deadliest TDE emissions, well known to have no safe dose. At any non-zero dose, these components are well known to increase carcinogenic, neurological, reproductive, cardiovascular, respiratory and other harm [13,14,67,68,85]. Yet only 10 percent of TDE pollution---less than NTDE pollution---from tens of millions of US diesel engines that now release hundreds of billions of pounds/year of DPM yearly, would still be massive. NTDE would still include at least tens of billions of pounds/year of no-safe-dose DPM [84,86]. Even ACES admits that NTDE merely reduces TDE pollution, so that NTDE still exposes people to 200,000 to 800,000 DPM particles per cubic centimeter [42]. Yet each of these particles can be inhaled directly into the brain and lungs, and then passed to the blood and all organs---to cause inflammation, oxidative stress, multiple cancer precursors, cancers, and many diseases [68].

Third, NTDE is very dirty, even compared to TDE because both are far above allowed-US-regulatory risk levels. The lung-cancer risk from the DPM in TDE, confirmed by more than 30 different studies, is about 159 times greater than EPA's acceptable cancer risk from pollution. (This is apart from the fact that DPM also increases the risk of Alzheimer's, autism, Parkinson's, stroke, and other neurological harms). If universal NTDE vehicles reduced 90 percent of these risks, the NTDE harm would still be about 16 times higher than that allowed for any US pollutants. NTDE would cause harm about on the order of dioxin risk, one of worst known pollutants [87-89].

Fourth, the ACES objection that NTDE is clean and noncarcinogenic also errs in part because NTDE creates more ultrafine PM than was present in the original TDE, and fine-ultrafine PM is the most hazardous component in DPM. This is mainly because it has much smaller particles and higher particle-number concentrations than pre-2007, non-NTDE engines. As a result, NTDE includes a higher percentage of more dangerous particles than does TDE [90-92]. NTDE also has DPM that is more dangerous because it is 50-90 percent metals, which are known neurotoxins [93-98].

Conclusion

ACES' denials of the lung-carcinogenicity and genotoxocity of NTDE are based in part on begging the question and on doing studies likely to yield false negatives. ACES errs through using short-term, low-powered, insensitive, analyses without either positive controls or any studies of lung tissue. Given little scientific justification for most ACES methods, there is little scientific justification for its conclusions that NTDE or "clean diesel" is neither carcinogenic nor mutagenic.

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