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Health assessment of gasoline and fuel oxygenate vapors: Subchronic inhalation toxicity

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ABSTRACT

Sprague Dawley rats were exposed via inhalation to vapor condensates of either gasoline or gasoline combined with various fuel oxygenates to assess whether their use in gasoline influences the hazard of evaporative emissions. Test substances included vapor condensates prepared from an EPA described "baseline gasoline" (BGVC), or gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA). Target concentrations were 0, 2000, 10,000 or 20,000 mg/m^3 and exposures were for 6 h/day, 5 days/week for 13 weeks. A portion of the animals were maintained for a four week recovery period to determine the reversibility of potential adverse effects. Increased kidney weight and light hydrocarbon nephropathy (LHN) were observed in treated male rats in all studies which were reversible or nearly reversible after 4 weeks recovery. LHN is unique to male rats and is not relevant to human toxicity. The no observed effect level (NOAEL) in all studies was 10,000 mg/m^3, except for G/MTBE (<2000) and G/TBA (2000). The results provide evidence that use of the studied oxygenates are unlikely to increase the hazard of evaporative emissions during refueling, compared to those from gasoline alone.

1. Introduction

The 1990 amendments to the Clean Air Act (CAA) mandated the use of oxygenates in motor gasoline. In 1994, the U.S. Environmental Protection Agency (EPA) issued a final rule under the Act which added new health effects information and testing requirements to the Agency's existing registration requirements. As described in more detail in a companion paper (Henley et al., 2014), requirements include inhalation exposures to evaporative emissions of the gasoline or additive in question. The health endpoints include assessments for standard subchronic toxicity, neurotoxicity, genotoxicity, immunotoxicity, developmental and reproductive toxicity, and chronic toxicity/carcinogenicity. The results of chronic toxicity testing of gasoline and gasoline combined with MTBE have already been reported (Benson et al., 2011) and reported elsewhere in this issue are the findings for genotoxicity (Schreiner et al., 2014), neurotoxicity (O'Callaghan et al., 2014), immunotoxicity (White et al., 2014), reproductive toxicity (Gray et al., 2014), and developmental toxicity testing in mice and rats (Roberts et al., 2014a,b). This paper describes the results of subchronic toxicity testing submitted to EPA.

Test substances evaluated in the 13 week toxicity studies included vapor condensates prepared from an EPA defined "baseline gasoline" (BGVC), as well as gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA). The goal of the studies was to provide information on the extent to which the use of oxygenates in gasoline might alter the hazard of evaporative emissions that are encountered during refueling of vehicles, compared to those from gasoline alone.

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2. Materials and methods

2.1. Test substance preparation and characterization

Gasoline and gasoline/oxygenate vapor condensates were prepared and supplied in 100 gallon gas cylinders by Chevron Research and Technology Center (Richmond, CA). Since only 5-gallon cylinders were practical for use in exposure operations, the test substance was dispensed as needed at the testing facility from the 100 gallon cylinders into 5-gallon cylinders using nitrogen pressurization. The methodology for preparation and analytical characterization of the samples is described in a companion paper (Henley et al., 2014). Test substances included vapor condensates prepared from an EPA described "baseline gasoline" (BGVC), identified as API Lot 99-01, and gasoline combined with methyl tertiary butyl ether (G/MTBE), ethyl t-butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), ethanol (G/EtOH), or t-butyl alcohol (G/TBA).

2.2. Experimental design

Seven separate 13-week inhalation toxicity studies were conducted of gasoline and gasoline/oxygenate vapor condensates at Huntingdon Life Sciences (East Millstone, NJ). In each of these studies, a total of 120 animals were used in the main and recovery phases (Table 1) and an additional 160 animals were used in satellite studies to more closely evaluate neurotoxicity, immunotoxicity and genotoxicity (Table 2). The exposure initiation dates for the studies were: BGVC – September 13, 2000; G/MTBE – February 6, 2001; G/EtOH – April 17, 2001; G/TAME – June 26, 2001; G/ETBE – October 23, 2001; G/DIPE – February 12, 2002; and G/TBA – June 25, 2002.

After 13 weeks of exposure, 20 control and 20 high dose animals were kept unexposed for an additional 4 weeks to serve as a recovery group. Clinical chemistry, coagulation and hematology evaluations were conducted on ten males and females per treatment group after 4 and 13 weeks of exposure as well as on the recovery group at the end of the recovery period. Necropsies were conducted on 10 male and 10 female animals per exposure group at the end of 13 weeks, and on all of the recovery group animals consisting of 10 male and 10 female animals in the control and high concentration groups.

2.3. Animal selection, assignment and care

CD (Sprague–Dawley derived) [Crl: CD@ IGS BR] albino rats (approximately 6 weeks old) were received from Charles River Laboratories (Kingston, NY) for each study. Animals were acclimated for at least 16 days after receipt and examined to confirm suitability for study.

Each rat was assigned a temporary number upon receipt and then identified with a metal ear tag bearing its assigned animal number. The assigned animal number plus the study number comprised the unique animal number for each animal. In addition, each cage was provided with a cage card, which was color-coded for exposure level identification and contained study number and animal number information.

Animals considered suitable for study on the basis of pretest physical examinations, body weight data and pretest ophthalmology evaluations were randomly assigned, by sex, to control or treated groups in an attempt to equalize mean group body weights. Individual weights of animals placed on test were within ±20% of the mean weight for each sex for each study. Currently acceptable practices of good animal husbandry were followed (National Academy of Sciences, 1996). Huntingdon Life Sciences, East Millstone, NJ is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.4. Diet and drinking water

Certified Rodent Diet, No. 5002; (Meal) (PMI Nutrition International, St. Louis, MO) was available without restriction, except during exposures. Fresh feed was presented weekly. Analysis of each feed lot used during this study was performed by the manufacturer. There were no known contaminants in the feed which were expected to interfere with the results of this study. Water was available without restriction via an automated watering system, except during exposures. Monthly water analyses are conducted by Elizabethtown Water Company, Westfield, NJ (Raritan-Millstone Plant). In addition, water samples were collected biannually from representative rooms in the testing facility for microbiological analyses by a subcontract laboratory. There were no known contaminants in the water which were expected to interfere with the results of this study.

2.5. Housing and environmental conditions

Animals were housed individually in suspended stainless steel wire mesh cages. During exposure periods, animals were individually housed in stainless steel, wire mesh cages within a 1000 L stainless steel and glass whole-body exposure chamber. A twelve hour light/dark cycle controlled via an automatic timer was provided. Temperature and relative humidity were monitored in accordance with Testing Facility SOPs and maintained within the specified range (18–26 degrees C, and 30–70%, respectively) to the maximum extent possible. Excursions outside the specified range were not considered to have affected the integrity of the study.

During exposure periods chamber static pressure was recorded every half-hour. Chamber temperature and relative humidity were

Table 1
Main and recovery study design.

Main study		Main and recovery study animals					
		Number of animals at initiation of exposure[a]		Necropsy and microscopic pathology[b]			
				Terminal (13 weeks)		After 4 weeks recovery	
Exposure group	Target concentration (mg/m^3)	M	F	M	F	M	F
Control	0 (air only)	20	20	10	10	10	10
Low	2000	10	10	10	10	–	–
Middle	10,000	10	10	10	10	–	–
High	20,000	20	20	10	10	10	10

[a] Exposures were 6 h/day, generally 5 days/week for 13 weeks, for at least 65 exposures.
[b] Complete postmortem evaluations were performed on animals.

Table 2
Satellite studies conducted.

Satellite studies		Number of animals at initiation of exposure[a]		Number of animals used in satellite studies							
Exposure group	Target concentration (mg/m^3)			Neuropathology[a]		GFAP[a]		Immunotoxicity[b]		Genotoxicity[b]	
		M	F	M	F	M	F	M	F		
Control	0 (air only)	15	25	5	5	5	5	0	10	5	5
Low	2000	15	25	5	5	5	5	0	10	5	5
Middle	10,000	15	25	5	5	5	5	0	10	5	5
High	20,000	15	25	5	5	5	5	0	10	5	5

[a] Exposures were 6 h/day, 5 days/week for 13 weeks, for at least 65 exposures for Neuropathology and GFAP studies. Neurobehavioral studies (functional observational battery and motor activity) were performed on the animals in these studies.
[b] Exposures were 6 h/day, generally 5 days/week for 4 weeks for 20 exposure for Genotoxicity and Immunotoxicity studies.

recorded every half-hour during exposure and maintained, to the maximum extent possible, within the target ranges (20–24 degrees C and 40–60%, respectively). Excursions outside the specified range were not considered to have affected the integrity of the study. Chamber oxygen levels (maintained at least 19%) were measured pretest and at the beginning, middle and end of the study. Air samples were taken in the vapor generation area pretest and at the beginning, middle and end of the study. Light (maintained approximately 30–40 foot-candles at 1.0 m above the floor) and noise levels (maintained below 85 decibels) in the exposure room were measured pretest and at the beginning, middle and end of the study.

2.6. Test substance administration

The test substance was administered as a vapor in the breathing air of the animals. The test atmosphere was generated by an appropriate procedure determined during pre-study trials. The trials were performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels and maintain uniform conditions throughout the exposure chambers. Two chambers were used for each exposure level, in general one for male animals and one for female animals, with the placement of the animals in the whole-body exposure chambers rotated weekly to ensure uniform exposure of the animals.

The test substance was administered for 6 h/day, generally 5 days per week for 13 weeks for at least 65 exposures for main study and recovery animals. Test substance administration continued through the day prior to necropsy for all animals except the animals in the control and high concentration groups designated for recovery which were held untreated for at least 4 additional weeks prior to necropsy.

2.7. Exposure chamber operation

Unexposed control group – Houseline nitrogen was delivered from a regulator with a backpressure gauge via 1/4″ tubing to a flow meter regulated by a metering valve (Fig. 1). This nitrogen flow was then split via a 1/4″ stainless steel "T" to both chamber turrets of the 1 cubic meter glass and stainless steel exposure chamber where it was mixed with room air as it was drawn into the chamber.

Low, middle and high exposure groups – Houseline nitrogen was delivered from a regulator with a backpressure gauge through a stainless steel fitting to create three flow systems: the test substance pressurization flow, the purge flow and the volatilization flow.

The nitrogen for the test substance pressurization flow was directed through a metering valve, attached to a back pressure gauge, into the vapor inlet valve of the test substance cylinder. The metering valve was used to adjust and maintain the pressure within the cylinder. From the pressurized cylinder, the test substance flowed from the liquid outlet valve through a disconnect fitting (equipped with a toggle valve) and through a filter to prevent equipment contamination. From the filter, the test substance flowed to a liquid flow meter via 1/8″ tubing. The outlet of the flow meter was regulated by a metering valve. From this metering valve, the test substance flowed via 1/8″ tubing onto the glass helix of a counter current volatilization chamber. This glass helix was heated by a nichrome wire which was controlled by a variable autotransformer and inserted in the center of the glass tube that supported the helix external to the volatilization chamber.

The nitrogen for the purge flow system was directed, via 1/4″ tubing to a flow meter regulated by a metering valve. The purge nitrogen was delivered via 1/8″ tubing to the bottom of the tube containing the nichrome wire. This nitrogen flow continuously purged the area surrounding the nichrome wire within the tube, thereby, protecting the wire from oxidation.

The nitrogen for the volatilization system was directed via 1/4″ tubing to a flow meter regulated by a metering valve. From the flow meter, the volatilization nitrogen flowed via 1/4″ tubing to a ball and socket joint at the bottom of the volatilization chamber. This nitrogen flowed up through the volatilization chamber passing over the coil and volatilizing the test substance. The pressure within the counter-current volatilization chamber was maintained slightly negative to the room and was monitored with a pressure gauge.

This test substance laden nitrogen exited the top of the volatilization chamber through a stainless steel "T" which divided the flow, via 1/2″ tubing, to the turrets of two 1 cubic meter glass and stainless steel exposure chambers. As the test substance laden nitrogen was drawn into each of the chambers, it was mixed with room air.

The whole-body exposure chambers each had a volume of approximately 1000 L (l m3). Each chamber was operated at a minimum flow rate of 200 L/min. The final airflow was set to provide at least one air change (calculated by dividing the chamber volume by the airflow rate) in 5.0 min (12 air changes/h) and a T99 equilibrium time (calculated by multiplying the air change by the exponential factor 4.6) of at most 23 min.

This chamber size and airflow rate was considered adequate to maintain the oxygen level at least 19% and the animal loading factor below 5%. At the end of each exposure, all animals remained in the chamber for a minimum of 30 min. During this time, each chamber was operated at approximately the same flow rate using clean air only. The chambers were exhausted through the in house filtering system, which consisted of a coarse filter, a HEPA filter, activated charcoal and then through a fume incinerator.

2.8. Exposure concentration determination

A nominal exposure concentration was calculated. The flow of air through the chamber was monitored using appropriate calibrated equipment. The test substance consumed (weight difference of the 5 gallon cylinder) during the exposure (mg) was

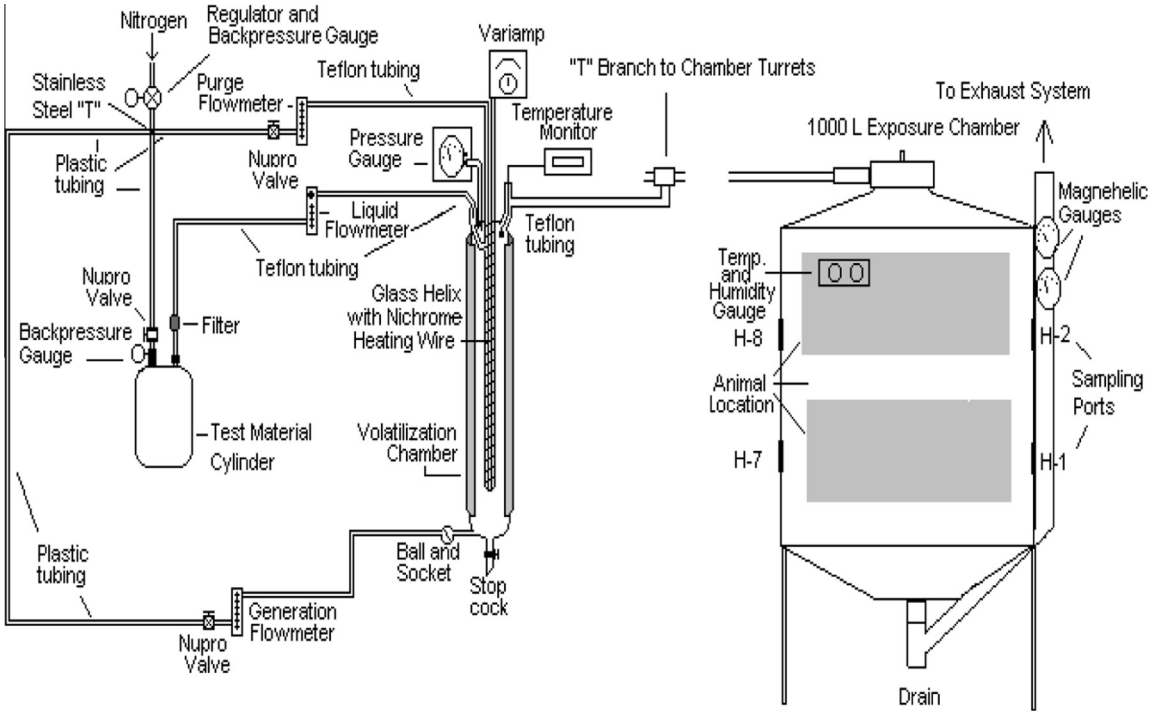

Fig. 1. Exposure and air sampling methodology: two chambers (designated chambers A and B) were used for each exposure level, in general one for male animals and one for female animals. During each exposure, measurements of airborne concentrations were performed in the animals' breathing zone at least 4 times using an infrared (IR) spectrophotometric analytical procedure. Also, one charcoal tube sample was collected per chamber per week and analyzed by gas chromatography (GC) to characterize at least 10 major components (comprising at least 80% by weight of the test substance) to show test substance stability and comparison between the neat liquid test substance and the vaporized test atmospheres.

divided by the total volume of air (m^3) passing through the chamber (volumetric combined flow rate for the 2 chambers times total exposure time) to calculate the nominal concentration mg/m^3).

During each exposure, measurements of airborne concentrations were performed in the animals' breathing zone at least 4 times using an appropriate sampling procedure and infrared (IR) spectrophotometric analytical procedure. Also, one charcoal tube sample was collected per chamber per week and analyzed by gas chromatography (GC) to characterize at least 18 major components (comprising at least 80% by weight of the test substance) to show test substance stability and comparison between the neat liquid test substance and the vaporized test atmospheres.

During each week of exposure, particle size determinations were performed using a TSI Aerodynamic Particle Sizer to characterize the aerodynamic particle size distribution of any aerosol present. The samples were drawn for 20 s at a flow rate of 5.00 L/min. The mass median aerodynamic diameter, geometric standard deviation and total mass concentration were calculated based on the amount of particles collected.

2.9. Experimental observations and evaluations

Animals were observed in their cages twice daily for mortality and signs of severe toxic or pharmacologic effects. Animals in extremely poor health or in a possible moribund condition were identified for further monitoring and possible euthanasia. All animals were observed as a group at least once during each exposure. Each animal was removed from its cage and examined twice pretest and once weekly during the study period. Examinations included observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration, palpation for tissue masses, circulatory effects, autonomic effects, central nervous system effects, changes in motor activity, and reactivity to handling or sensory stimuli. Animals were also removed from their cages and weighed twice pretest, weekly during treatment and terminally (after fasting). Terminal, fasted body weights were obtained just prior to necropsy.

Food consumption was measured (weighed) during the week prior to treatment initiation and weekly throughout the study. Feed was available without restriction 7 days/week except during inhalation exposures and when fasting prior to blood collection. Animals were presented with weighed feeders at the scheduled intervals. After 7 days (4 days during the 4″ week of the recovery period), the feeders were reweighed and the resulting weight subtracted from the initial feeder weight to obtain the grams of feed consumed per animal per week. The grams consumed per kilogram of body weight per day was then calculated for each animal.

All animals received ophthalmoscopic examinations pretest and at study termination. Eyelids, lacrimal apparatus and conjunctiva were examined grossly; cornea, anterior chamber, lens, vitreous humor, retina and optic disc were examined by indirect ophthalmoscopy. The eyes were examined after instillation of a mydriatic solution (Mydriacyl 1%).

2.10. Clinical pathology

Clinical pathology procedures and parameters were based on those recommended in guidelines published by the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing (Weigand et al., 1996). Blood was obtained from lightly anesthetized (carbon dioxide/oxygen, 60/40%) animals via puncture of the orbital sinus (retrobulbar). Rats were fasted overnight prior to blood collection. The Main Study animals were bled at the Interim (−4 weeks) and Terminal intervals.

2.11. Hematology

Blood for hematology studies was collected (approximately 0.25 mL) into tubes containing EDTA anticoagulant. Blood samples were analyzed for: hemoglobin concentration, hematocrit, erythrocyte count, platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total leukocyte count, reticulocyte count, differential leukocyte count, and erythrocyte morphology.

2.12. Coagulation studies

Blood for coagulation studies was collected (approximately 1.0 mL) into tubes containing sodium citrate anticoagulant. Blood samples were analyzed with Mechanical clot detection system (STA compacta, Diagnostica Stago Products) for prothrombin time and activated partial thromboplastin time.

2.13. Clinical chemistry

Blood for clinical chemistry studies was collected (approximately 1.5 mL except −1.0 mL for the female animals at the 4th week interval) into tubes with no anticoagulant, allowed to clot, and centrifuged to obtain serum. Blood samples were analyzed with a Hitachi 717 automatic analyzer (Roche Corporation) for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, sorbitol dehydrogenase, blood urea nitrogen, creatinine, glucose, creatine kinase, cholesterol, albumin, total bilirubin, direct bilirubin, sodium, potassium, chloride, calcium, inorganic phosphorus, gamma-glutamyl transferase, bile acids, globulin, albumen/globulin ratio, and indirect bilirubin.

2.14. Postmortem evaluations

Animals were fasted overnight prior to necropsy and euthanized by exsanguination following carbon dioxide inhalation for main study and recovery animals. A necropsy schedule was established to ensure that approximately equal numbers of males and females were examined on each day of necropsy and that examination of animals of both sexes were performed at similar times of the day throughout the necropsy period.

2.15. Macroscopic examinations

Katherine M. Whitney, DVM, PhD was the pathologist for all of the studies, and had oversight responsibility for the gross and microscopic pathology aspects of the studies. Complete macroscopic postmortem examinations were performed on all main study and recovery animals. Animals that were humanely sacrificed or were found dead in satellite studies also received a macroscopic examination, which included examination of the external surface and all orifices; the external surfaces of the brain and spinal cord; the organs and tissues of the cranial, thoracic, abdominal and pelvic cavities and neck; and the remainder of the carcass for the presence of macroscopic morphologic abnormalities.

The organs indicated in Table 3 were weighed for all main study and recovery animals during the scheduled necropsy. Prior to recording weights, the organs were carefully dissected and properly trimmed to remove adipose and other contiguous tissues in a uniform manner. Organs were weighed as soon as possible after dissection in order to avoid drying. Paired organs were weighed together.

2.16. Histopathologic examinations

Tissues shown in Table 3 were excised at the scheduled sacrifice intervals and preserved for all "main" and "recovery study" animals. In addition, slides of the tissues were prepared and examined microscopically for all main study animals. Any abnormalities not noted during macroscopic examinations which were seen during histology processing were recorded. Screening for alpha 2u-globulin nephropathy: A preliminary diagnosis was made on hematoxylin and eosin stained slides of the kidney and/or based on elevated kidney weights at the terminal sacrifice. The sections were then stained with Mallory Heidenhain stain (Sheehan and Hrapchak, 1987) since the following lesions were seen with H&E: angular hyaline inclusions in the proximal convoluted tubule, tubular degeneration and regeneration, and/or globular casts at the cortico-medullary junction in the BGVC and G/MTBE studies.

All retained tissues were preserved in 10% neutral buffered formalin. Eyes were placed in glutaraldehyde/paraformaldehyde initially and then retained in 10% formalin. Testes and epididymides were placed in Modified Davidson's solution and then retained in 10% neutral buffered formalin. Lungs and urinary bladders were infused with formalin prior to their immersion into a larger volume of the same fixative. After fixation, the tissues and organs from all animals were routinely processed, embedded in paraffin, cut at a microtome setting of 4–7 microns, mounted on glass slides, stained with hematoxylin and eosin and examined by light microscopy. The bones were decalcified in Decalcifier™.

2.17. Statistical analysis

Statistical analyses were conducted on mean body weight values and body weight changes (from pretest), mean feed consumption values (presented as grams of feed/kg of body weight per day), mean clinical laboratory values, mean organ weights, organ-to-body weight ratios, and organ-to-brain weight ratios. Mean values of all exposure groups were compared to the mean value for the control group at each time interval.

Evaluation of equality of group means was made by the appropriate statistical method, followed by a multiple comparison test if needed. Bartlett's test (Bartlett, 1937; Sokal and Rohlf, 1995) was performed to determine if groups had equal variances. For all parameters except organ weights, if the variances were equal, parametric procedures were used; if not, nonparametric procedures were used. Organ weight data was analyzed only by parametric methods. The parametric method was the standard one-way analysis of variance (ANOVA) using the F ratio to assess significance (Armitage, 1971; Dunlap and Duffy, 1975). If significant differences among the means were indicated, additional tests were used to determine which means were significantly different from the control: Dunnett's (Dunlap et al., 1981; Dunnett, 1955,1964), Williams (Williams, 1971, 1972), or Cochran and Cox's modified t-test (Cochran and Cox, 1959).

The nonparametric method was the Kruskal–Wallis test (Kruskal and Wallis, 1952, 1953) and if differences were indicated, Shirley's test (Shirley, 1977), Dunn's test (Dunn, 1964), Steel's test (Steel, 1959) or Pairwise Comparison with Bonferroni Correction (Games and Howell, 1976) were used to determine which means differed from control. Bartlett's test for equality of variance was conducted at the 1% significance level; all other statistical tests were conducted at the 5% and 1% significance levels.

2.18. Compliance

These studies were conducted in accordance with the United States Environmental Protection Agency's (EPA) Good Laboratory

Table 3
Tissues examined microscopically.

Tissue[a]	Weighed	Tissues examined microscopically	
		Exposure groups	Exposure groups
		Control and high	Low and middle
Adrenal glands	X	X	
Aorta (thoracic)		X	
Bone (sternum/femur with articular surface)		X	
Bone marrow (femur)[b]		X	
Brain (medulla/pons, cerebrum)	X	X	
Epididymides	X	X	
Esophagus		X	
Eyes with optic nerve			
Heart	X	X	
Kidneys	X	X[f]	X[f]
Lacrimal glands			
Large intestine (cecum, colon and rectum		X	
Larynx[c]		X	
Liver	X	X	
Lungs (with mainstem bronchi)	X	X	X
Lymph nodes (mesenteric and thoracic)		X	
Mammary gland			
Muscle (biceps femoris)			
Nasopharyngeal tissue[d]		X	
Nerve (sciatic)		X	
Ovaries	X	X	
Pancreas		X	
Pituitary			
Prostate	X	X	
Salivary glands with submandibular lymph node		X	
Seminal vesicles	X	X	
Skin			
Small intestine (duodenum, ileum, jejunum)		X	
Spinal cord (cervical, thoracic, lumbar)		X	
Spleen	X	X	
Stomach		X	
Testes	X	X	
Thymus	X	X	
Thyroid (with parathyroids)		X	
Trachea		X	
Urinary bladder		X	
Uterus (body/horns with cervix)	X	X	
Zymbal's gland			
Gross lesions and tissue masses		X	X
Target organs[e] (kidneys – main and recovery male rats only)		X	X

[a] These tissues were examined for animals in low and mid-dose groups which died prior to study termination.
[b] Qualitative examination (no differential count). Bone marrow smears were prepared and archived.
[c] The laryngeal mucosa was examined. Sections of the larynx examined included the epithelium covering the base of the epiglottis, the ventral pouch and the medial surfaces of the vocal processes of the arytenoid cartilages.
[d] Four sections of the nasopharyngeal tissue were examined. This included sections through the nasal cavity and examinations of the squamous, transitional, respiratory and olfactory epithelia.
[e] Target organs were designated by the Study Director, Pathologist and/or Sponsor based on experimental findings.
[f] The kidneys of all male animals in groups II and III were only examined in the BGVC and G/MTBE studies. The kidneys of male animals in the other five studies were expected to have shown similar effects as those examined in the BGVC and G/MTBE studies.

Table 4
Exposure chamber concentrations.

Experimental group	Exposure chamber concentrations – mg/m^3 ± standard deviation[a]							
	TARGET	BGVC	G/MTBE	G/EtOH	G/TAME	G/ETBE	G/DIPE	G/TBA
Group I (control)	0	0	0	0	0	0	0	0
Group II	2000	2050 ± 154	2029 ± 192	2066 ± 191	2031 ± 176	2027 ± 193	1995 ± 132	2013 ± 168
Group III	10,000	10,148 ± 739	10,099 ± 834	10,310 ± 768	10,320 ± 890	10,060 ± 691	10,030 ± 692	10,088 ± 585
Group IV	20,000	20,324 ± 1183	20,242 ± 1256	20,400 ± 1102	20,150 ± 1105	19,930 ± 1031	20,120 ± 869	20,051 ± 871
Molecular weight[b]		73.8	73.7	65.9	77.2	77.1	76.5	72.1

[a] Total hydrocarbon concentrations as determined by infrared spectroscopy.
[b] Average molecular weight of hydrocarbons in condensate samples.

Practice Standards (US EPA, 1994a), and complied with all appropriate parts of the Animal Welfare Act Regulations (USDA, 1989, 1991). The study also met the requirements of EPA's guidelines for subchronic toxicity testing (US EPA, 1994b).

3. Results

Due to the large amount of data generated in these studies only the most significant findings are presented here. Actual laboratory

reports for these studies can be found in the Federal Docket Management System (FDMS) at EPA-HQ-OAR-2003-0065.

3.1. Chamber monitoring

Chamber distribution analyses conducted by the laboratory during the conduct of the seven studies showed that the test substance was evenly distributed within each chamber. The mean measured concentrations (analytical and nominal) were reasonably close to the targeted exposure levels for each of the gasoline oxygenate studies. Those determined analytically are summarized in Table 4. Also shown in the table are the average molecular weights of the hydrocarbons contained in the different condensate samples which can be used to calculate the approximate part per million concentrations. The differences between measured and nominal concentrations were typical for this type of vapor exposure where a ratio of 1:1 is expected. In the TBA study, the differences between measured and nominal concentrations varied somewhat (up to 5%) from the expected 1:1. The exact cause of the differences was not determined but was probably a result of a combination of slight inaccuracies in the calibration of the chamber airflows and the calibration of the IR monitor.

Measurement of the mass median aerodynamic diameter of the exposure atmospheres did not reveal significant differences from the air control chambers indicating that the atmospheres were essentially vapor. Some particles are invariably present in chamber atmospheres, representing background air. It is unlikely that gasoline vapor condensed on these particles given the volatility of these light end vapor components and the high level of consistency in constituent proportions demonstrated analytically from week to week.

An estimate of the concentrations of the 18 reference hydrocarbons in the test substances which were generated for use in the studies is shown in Table 5. These values, which were derived pre-study, are expressed as area percent and intended to facilitate comparison between studies (Henley et al., 2014). Analysis of the reference hydrocarbons in the test atmospheres showed a close agreement with the neat test substance, with the exception of ethanol.

The levels of ethanol measured in the test atmospheres were about 20% less than measured in the neat test substance. This was partially attributed, after the study was completed, to an inefficiency in extracting ethanol from charcoal tubes using carbon disulfide. This data demonstrated that the test animals were exposed, as expected, to all of the major components of the test substance in their proper proportion. The data was also consistent from week-to-week during the study indicating stability of the test substance and the atmosphere generation techniques (data not shown).

3.2. Mortality

All test substance-exposed main study and recovery animals in the G/MTBE, G/EtOH, G/TAME, G/ETBE G/DIPE, and G/TBA studies survived throughout the exposure and recovery phases. One mid-concentration male in the BGVC study was humanely sacrificed on day 69 of the exposure phase. Serology evaluation found no infectious viral diseases; macroscopic findings were swollen fore and hind paws, and bilateral slight discoloration of eyes and histopathology revealed bilateral keratitis, unilateral anterior uveitis, and tenosynovitis affecting all distal extremities. These gross and histologic changes were not observed in other animals in the study and were considered unrelated to test substance exposure. One control male in the G/TBA study was found dead on day 71.

3.3. Clinical observations

No significant changes were observed in the animals during the daily in chamber observations. In the weekly observations during non-exposure periods, scattered observations such as staining of the snout, red nasal discharge, chromodacryorrhea and lacrimation were noted. The incidences of red nasal discharge were more

Table 5
Hydrocarbons detected in exposure atmospheres.

	Hydrocarbons detected in test material (area percent)[a]						
	BGVC	G/MTBE	G/EtOH	G/TAME	G/ETBE	G/DIPE	G/TBA
Isobutane	3.6	2.2	2.2	1.9	2.0	2.0	3.0
n-Butane	15.2	11.1	11.6	10.4	10.6	11.5	9.9
Isopentane	35.1	31.0	34.0	33.6	32.5	32.2	25.2
n-Pentane	13.2	9.1	10.2	10.3	9.8	9.6	11.6
Trans-2-pentene	2.5	2.0	2.1	2.3	2.1	2.1	2.1
2-Methyl-2-butene	3.8	2.9	3.1	3.4	3.2	3.1	3.2
2,3-Dimethylbutane	1.6	0.9	2.2	1.5	1.4	1.3	1.6
2-Methylpentane	6.3	4.5	5.1	5.6	5.1	4.5	6.1
3-Methylpentane	3.6	2.6	2.9	3.2	2.9	2.7	3.8
n-Hexane	3.0	2.1	2.4	2.6	2.4	1.8	3.4
Methylcyclopentane	1.5	1.1	1.2	1.4	1.3	1.0	1.6
2,4-Dimethylpentane	1.0	0.9	1.0	1.2	1.0	1.0	1.0
Benzene	2.1	1.5	1.6	2.0	1.8	1.8	2.0
2-Methylhexane	1.1	1.0	1.1	1.2	1.1	1.1	1.3
2,3-Dimethylpentane	1.1	1.0	1.1	1.3	1.1	1.1	1.3
3-Methylhexane	1.3	1.1	1.2	1.5	1.3	1.3	1.5
Isooctane	1.3	1.2	1.3	1.5	1.4	1.4	1.5
Toluene	3.0	2.5	2.4	3.2	2.7	2.6	3.4
MTBE		21.3					
EtOH			13.3				
TAME				11.9			
ETBE					16.3		
DIPE						17.8	
TBA							16.5

[a] Values (mean concentrations) for these 18 reference hydrocarbons were derived pre-study (Henley et al., 2014). A total of 131 peaks were separated and identified for the BGVC study. The reference hydrocarbons comprised over 81% of the total mass but are normalized to 100% to ease comparison between labs.

pronounced in high concentration BGVC and G/TBA males and females and in high concentration males in the G/DIPE study. Significant increases in the frequency of yellow anogenital staining were seen in the high concentration G/TBA and G/TAME exposed females and of chromodacryorrhea in high concentration G/TAME exposed females; most of these had subsided during the recovery period. Otherwise, the test animals were generally unremarkable during the non-exposure periods (afternoon evaluations).

3.4. Ophthalmic examinations

There were no toxicologically significant differences in ocular findings in most of the animals exposed to vapors of gasoline or gasoline/oxygenate mixtures compared to the air control animals. A slight increase in the incidence of focal retinopathy was seen in the 20,000 mg/m^3 G/ETOH exposed male animals (3 of 20) after 13 weeks of exposure, compared to air controls (0 of 20). Ocular examination after the 4 week recovery period showed a similar incidence in the two groups (1 of 10). The affected animal at recovery was one of the three animals observed with focal retinopathy after 13 weeks of exposure. The other two animals were terminated at week thirteen. In the opinion of the attending veterinary ophthalmologist, these abnormalities were not related to test substance exposure. A slight increase in the incidence of focal retinopathy also was seen in the 20,000 mg/m^3 G/ETBE-exposed female animals (1 of 20) after 13 weeks of exposure, compared to air controls (0 of 20). Similar to retinopathy findings observed in the G/EtOH study, the attending veterinary ophthalmologist concluded the finding was not likely caused by an ocular toxicant.

3.5. Body weights

There were no toxicologically significant differences (defined as 10% lower than controls) in absolute body weights in any of the animals exposed to vapors of gasoline or gasoline/oxygenate mixtures compared to the air control animals. There were exposure related differences in body weight gains in the high concentration males and females exposed to G/EtOH. Male rats gained significantly less weight than air control animals during the initial 3 weeks of exposure. Thereafter, weekly weight gains were comparable to air controls but were never sufficiently greater than air controls to make up the difference from the early weeks of exposure. These weights were not statistically significantly lower than air controls. A similar trend was seen in high concentration females from the 2nd to the 13th week of exposure. The observed lower body weights and weight gain for both sexes were indicators that a maximum tolerated concentration had been reached in the study.

Exposure related decreases in body weight gains were also observed in high concentration G/ETBE animals between intervals 0–4 and 0–13 weeks. All differences abated prior to or during the recovery period. Fewer statistically significant differences were noted at the weekly intervals in the other gasoline/oxygenate exposure studies. None of these demonstrated a treatment-related pattern and so were not considered toxicologically significant.

3.6. Feed consumption

There were no toxicologically significant differences in feed consumption in any of the studies. Statistically significant differences (increases and decreases of 15% or less from control values) were noted at several of the weekly measurement intervals. Overall, these differences were not considered to have been in a treatment-related pattern and were not considered attributable to test substance exposure.

3.7. Hematology and coagulation

There were no significant treatment-related differences in hematology and coagulation values except in the animals exposed to G/ETBE and G/DIPE. In the G/ETBE study, there were decreased reticulocyte counts in high concentration male and females at the 4 week interval (which were statistically significant in females). These differences were not seen at the terminal interval. A few other statistically significant differences (such as increased MCV in mid and high concentration males) were observed but not in treatment related pattern, or absolute differences were minimal.

In the G/DIPE study, there were statistically significant decreases (21–23%) in reticulocyte counts for the high concentration males which occurred at both exposure period intervals (4th week and terminal) and at termination of the recovery interval. Statistically significant (13–17%) decreases also occurred in reticulocyte counts for the low and mid- concentration exposed males but only at the 4th week interval. Similar differences did not occur in female animals at any exposure level or during any interval but there were statistically significant (35–36%) decreases in absolute monocytes for the high concentration females but only at the 4th week and recovery intervals. Other differences (such as increases in platelets in all exposed groups of males at the terminal interval) were seen but not in an exposure-level related pattern and were attributed to normal variability.

In the G/TBA study, a trend toward reductions in lymphocyte counts, reflected in reduced total leukocyte counts relative to control values was seen in high concentration males at 4 and 13 weeks of exposure and for all exposed female groups at 13 weeks. However values were close to control ranges and differences were not exposure level-related. In the absence of evidence of immunotoxicity (White et al., 2014) or microscopic pathology changes, these slight differences did not appear to represent an effect of the test substance.

3.7.1. Coagulation

There were no toxicologically significant treatment-related differences in coagulation values.

3.8. Clinical chemistry

There were no significant treatment-related differences in clinical chemistry findings in the majority of the studies, with the exception of animals exposed to G/ETBE and G/TBA. During the 4th week of exposure in the G/ETBE study, decreased aspartate aminotransferase values in high concentration males and females, decreased lactate dehydrogenase values in all exposed males and females, and decreased creatine kinase values in high concentration females were observed. These differences were not seen at the terminal interval.

In the G/TBA study, slight to moderate increases in serum cholesterol in the mid- and high-concentration males (21–34% higher after 4 and/or 13 weeks of exposure) were observed. After 4 weeks of recovery the values were similar to controls (within 12%) and no liver pathology was seen. Statistically significant decreases were also seen in lactate dehydrogenase, sorbitol dehydrogenase and creatine kinase values in one or more exposed groups. No corresponding microscopic pathology changes were seen.

3.9. Organ weights

There were no toxicologically significant differences in organ weights and brain measurements in animals exposed to BGVC or G/DIPE. Significant increases in kidney weights were observed in mid- and high concentration males exposed to G/MTBE, G/EtOH,

G/TAME, and G/TBA, as well as high concentration males exposed to G/ETBE, and mid- and high concentration females exposed to G/MTBE. These kidney weights were not significantly different when measured at the end of the recovery interval except in the G/TBA exposed males. Statistically significant changes were seen in several other organ weights at various intervals in the studies. However, these were not seen in an exposure level related pattern and/or the absolute differences were minimal and there were no corresponding histopathology findings.

3.10. Pathology – macroscopic findings

No gross abnormalities related to test substance exposure were evident on necropsy examination of animals in the BGVC, G/MTBE, G/EtOH, G/DIPE, and G/TBA exposure groups. The macroscopic lesions noted were similar to those routinely encountered in animals of this age and species or were represented by individual animals and considered incidental findings. In G/TAME exposed animals, six of 20 high concentration females (3 terminal, 3 recovery) had foci of red or tan discoloration of lungs which were not seen in control females, but which were observed in several control males. Since no microscopic changes were correlated with these observations, it was concluded that they were not likely related to test substance exposure. Two mid concentration and 5 high concentration G/ETBE exposed animals had exposure dependent incidence of bilateral renal foci of red discoloration at terminal sacrifice, which was not observed in control or low concentration animals. Some individual control and low concentration females had similar foci.

3.11. Pathology – microscopic findings

Microscopic findings that were considered exposure related were found in the kidneys of male animals in all of the gasoline and gasoline/oxygenate exposure studies. In the BGVC and G/MTBE studies, male rats exposed at all exposure levels had eosinophilic hyaline granules within the cytoplasm of renal proximal convoluted tubular epithelial cells. The degree of cytoplasmic granulation varied in an exposure-level dependent manner. Most 10,000 and 20,000 mg/m^3 exposed males also had evidence of tubular regeneration (basophilic tubules) at a higher incidence and severity compared to control males. Several animals among each of these exposure groups had corticomedullary intraluminal tubular granular casts. Similar changes were not evident in females having test substance exposure or in control animals. Mallory-Heidenhain (MH) staining of renal tissue of male rats showed red staining of granular material within proximal tubular epithelium. Minimal staining was apparent in control males and slight to moderate staining was present in exposed female animals.

The 20,000 mg/m^3 exposed males in the BGVC and G/MTBE studies, sacrificed following a 4-week recovery period, had near complete resolution of the relevant histologic changes except the effect on the basophilic cortical tubules was unresolved. A single animal exposed to BGVC and two exposed to G/MTBE had granular casts and hyaline granules appreciable with routine (hematoxylin and eosin) stain. Because these renal findings were expected, and have been fully investigated during prior testing with gasoline test substances (Benson et al., 2011), no further examinations of animals from the recovery group were made in the studies of other gasoline oxygenates.

In all of the gasoline/oxygenate exposure studies, histopathologic changes similar to those seen in the BGVC and G/MTBE studies in the high exposure level males were observed. Because these changes were expected, animals at the low and middle exposure levels were not examined, however, they would have been expected to show similar effects in a treatment-related pattern, particularly where increases in kidney weights were seen.

The only other microscopic finding of toxicologic significance was in the BGVC study. Some of the high exposure level male and female rats had eosinophilic material within the nasolacrimal duct lumen at the Terminal sacrifice. This finding was considered to correlate with the increase in red nasal discharge noted previously in this group of test animals. Similar changes were not evident in control animals.

4. Discussion and conclusions

A summary of the treatment related effects noted in the seven studies are presented in Table 6 which identifies those exposure concentrations where adverse effects were noted. Thirteen weeks of inhalation exposure of rats to vapor condensates of either gasoline or gasoline combined with fuel oxygenates resulted in hydrocarbon nephropathy in male animals. The microscopic lesions observed were consistent with changes associated with

Table 6
Summary of treatment related effects.

	BGVC	G/MTBE	G/EtOH	G/TAME	G/ETBE	G/DIPE	G/TBA
Red nasal discharge	20,000 mg/m^3 M,F	WNL	WNL	WNL	WNL	20,000 mg/m^3 M,F	WNL
Decreased body weight gain	WNL	WNL	20,000 mg/m^3 M,F	WNL	20,000 mg/m^3 F	WNL	WNL
Food consumption	WNL	WNL	WNL	WNL	WNL	WNL	WNL
Functional observational battery	WNL	WNL	WNL	WNL	WNL	WNL	WNL
Motor activity	WNL	WNL	WNL	WNL	WNL	WNL	WNL
Increased cholesterol	WNL	WNL	WNL	WNL	WNL	WNL	10,000 and 20,000 mg/m^3 M
Decreased reticulocyte counts	WNL	WNL	WNL	WNL	20,000 mg/m^3 F	20,000 mg/m^3 M	WNL
Increased kidney weights	WNL	2000, 10,000 and 20,000 mg/m^3 M,F	10,000 and 20,000 mg/m^3 M[b]	20,000 mg/m^3 M	20,000 mg/m^3 M	WNL	10,000 and 20,000 mg/m^3 M
Hydrocarbon nephropathy	2000, 10,000 and 20,000 mg/m^3 M[a]	2000, 10,000 and 20,000 mg/m^3 M[a]	20,000 mg/m^3 M[b]	20,000 mg/m^3 M[b]	20,000 mg/m^3 M[b]	20,000 mg/m^3 M[b]	20,000 mg/m^3 M[b]
Neuropathology	WNL	WNL	WNL	WNL	WNL	WNL	WNL

M = males; F = females; WNL = within normal limits.
[a] Not seen at recovery interval.
[b] Terminal interval and recovery animals at lower exposure levels were not evaluated.

Table 7
Summary of no observed adverse effect levels.

	No observed adverse effect levels (NOAEL) in mg/m^3						
	BGVC	G/MTBE	G/EtOH	G/TAME	G/ETBE	G/DIPE	G/TBA
NOAEL	10,000	None	10,000	10,000	10,000	10,000	2000
Basis	Red nasal discharge	Increased kidney weights	Decreased body weight gain	Increased kidney weights	Decreased body weight gain, hematology	Hematology, red nasal discharge	Increased cholesterol

NOAEL = No observable adverse effect level based on effects other than male hydrocarbon nephropathy.

accumulation of alpha-2 microglobulin within epithelial phagolysosomes. This change has been well documented to be unique to male rats exposed gasoline hydrocarbons (Benson et al., 2011) as well as to a variety of other chemicals (Alden, 1986) and has been generally accepted not to be relevant to human risk (US EPA, 1991).

Exposure to both BGVC and G/DIPE vapors resulted in slight but reversible increases in red nasal discharge in animals exposed to 20,000 mg/m^3 of vapor. Therefore, the 10,000 mg/m^3 exposure level (excluding male rat nephropathy) was considered a no observable adverse effect level (NOAEL – i.e. the highest level at which no adverse effects were observed) (Table 7). Condensates of MTBE in gasoline resulted in slight but reversible increases in kidney weights in all female groups. No corresponding histopathologic changes were noted in kidneys. On this basis, none of the exposure levels was considered a NOAEL. There was no effect on kidney weights in Sprague Dawley rats exposed to the same concentrations of G/MTBE for 24 months (Benson et al., 2011).

Vapor condensates of G/EtOH resulted in decreased body weight gains in high concentration males and females. Since the changes in body weight gain and kidney weights were absent at the recovery interval the 10,000 mg/m^3 exposure level was considered to be the NOAEL. Decreased body weight gains were also observed in rat reproductive toxicity studies (Gray et al., 2014) and in rat developmental toxicity studies (Roberts et al., 2014b) but only at exposure levels of G/EtOH of 20,000 mg/m^3.

The NOAEL for animals in the G/TAME study was considered to be 10,000 mg/m^3, based on increased incidences of anogenital staining, red nasal discharge and chromodacryorrhrea noted in high concentration animals. There was reversible hematology and clinical chemistry parameters in high concentration G/ETBE animals and mild but reversible decreases in body weight gains in high concentration females, therefore 10,000 mg/m^3 (excluding male rat nephropathy) was considered the NOAEL for subchronic toxicology parameters.

Exposure to vapor condensates of G/DIPE resulted in increased red nasal discharge and slight, non-reversible decreases in reticulocyte values (at the 4th week and terminal and recovery intervals) in the male animals exposed to 20,000 mg/m^3 of vapor. Therefore, the 10,000 mg/m^3 exposure level (excluding male rat nephropathy) was considered a NOAEL. In the G/TBA studies, exposures caused increased yellow anogenital staining and increases in cholesterol values in male and/or female animals exposed to 20,000 mg/m^3 of vapor. The cholesterol differences were also noted in the 10,000 exposed animals. Therefore, the 2000 mg/m^3 exposure level (excluding male rat nephropathy) was considered a NOAEL.

The NOAELs for exposure to all of the gasoline/oxygenate condensates was similar to that for exposure to gasoline emissions alone (10,000 mg/m^3) with the exception of G/MTBE and G/TBA. Since the endpoints responsible for these lower NOAELs (increased kidney weights and increased cholesterol) were not considered toxicologically significant, it can be concluded that the addition of the oxygenates evaluated in this study did not significantly alter the subchronic toxicity of evaporative emissions compared to that of gasoline alone. These no effect concentrations are significantly higher than those typically encountered by humans which can reach 10–100 ppm total hydrocarbon vapor while refueling, particularly prior to the implementation of vapor recovery devices both within the automobile and the fuel pump (Zielinska et al., 2012).

Conflict of interest

Dr. Clark and Schreiner while employed at Phillips 66 Company were involved in the API technical work group that designed and oversaw the conduct of the studies. The American Petroleum Institute employed them after retirement as consultants to assist in preparing manuscripts from the original laboratory study reports. There was no influence exerted on evaluation of the scientific data and manuscript content. Dr. Gray was the API sponsor representative for the conduct of this study by the laboratory. He is now retired from API and received no compensation for his efforts in preparing this article. Drs. Hoffman and Parker have nothing to disclose.

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