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The subchronic toxicity of higher olefins in Han Wistar rats

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Abstract

Higher olefins (HO) are a category of unsaturated hydrocarbons widely used in industry applications to make products essential for daily human life. Establishing safe exposure limits requires a solid data matrix that facilitates understanding of their toxicological profile. This in turn allows for data to be read across to other members of the category, which are structurally similar and have predictable physico-chemical properties. Five independent subchronic oral toxicity studies were conducted in Wistar rats with Oct-1-ene, Nonene, branched, Octadec-1-ene, Octadecene and hydrocarbon C12-30, olefin-rich, ethylene polymn. by product, at doses ranging from 20 to 1000 mg/kg bw. These HO were selected considering gut absorption, carbon chain length, double-bond position and carbon backbone structural variations. Generally, limited and non-adverse toxicity effects were observed at the end of the treatment for short carbon chain HO. For instance, alpha 2u-globulin nephropathy in the male rats and liver hypertrophy. No clear trend in systemic toxicity was linked to the double-bond position. Key factors for hazard assessment include absorption, carbon chain length, and branching, with Nonene, branched, identified as the worst-case substance. Taken together, the no observed adverse effect level (NOAEL) of each HO in these subchronic studies was set at the highest dose tested.

Keywords Subchronic toxicity, OECD 408, No observed adverse effect level (NOAEL), Higher olefins, Unknown or variable composition, Complex reaction products and biological materials (UVCBs)

Introduction

Higher olefins (HO) are used as building blocks for other chemicals and are key raw materials for producing a wide range of products, ranging from detergents, cleaning products and sun creams to plastics, lubricants and drilling fluids [8]. HO, which have the general formula C_nH_{2n}, belong to the family of unsaturated hydrocarbons, and are structurally similar to paraffins but contain two fewer hydrogen atoms resulting in one double-bond between adjacent carbon atoms [5]. HO can be produced from refinery streams or synthesized from oligomerization of either ethylene or propylene with carbon ranges from C6 to C54 [1]. Refinery olefins are highly branched, while synthetic olefins are predominantly linear [26]. Therefore, depending on the position of the double-bond, or

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the degree of branching, there are four types of HO [27, 28]: linear α olefins (straight chain with a single double bond in the first, or "α" position), linear internal olefins (straight chain molecules with a single double bond in an internal position), branched olefins (isomerized olefins with a single double-bond in the α position), branched internal olefins (isomerized olefins with a single double-bond in an internal position).

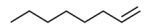
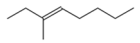
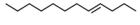
To address regulatory requirements according to the REACH legislation in the EU, the Higher Olefins and Poly Alpha Olefins REACH Consortium (HOPA) was formed in 2009. Initially, 9 members founded the consortium, however current membership stands at 6 member entities. The consortium is responsible for creating and maintaining regulatory dossiers for 32 HO in a bespoke category. To address the information requirements of Annexes IX and X of REACH, HOPA developed a testing strategy based upon the compositions of the HO. Firstly, HO were characterised by the percentage weight of different olefin types, from which substances with the highest concentration of a particular olefin type were selected to be test materials in subsequent studies. By identifying the extreme corners of the category, these data can be read across to the "corners" of the category. HOPA then outlined the endpoints that needed to be addressed, which included repeated-dose, reproductive and developmental toxicity. These endpoints are key requirements in the higher-tiers of the REACH legislation (Annexes IX and X), which registrants manufacturing or importing over 100 tonnes per annum must address in their regulatory dossiers. In addition to this, HOPA also commissioned non-GLP mechanistic studies to determine how bioavailable higher olefins are likely to be when dosed orally, using an everted intestinal-sac model and performed selective supporting OECD 422 studies (Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) to aid read- across between the HOs. These data have been reported in separate publications.

The results of in vitro absorption and metabolism studies suggested that certain types of HO are more bioavailable than others. A number of HO (carbon range from C6 to C27) were investigated in the in vitro rat everted small intestinal sac method to determine and rank the intestinal absorption potential [27]. The results showed that the HO with carbon range from C6 to C10 were readily absorbed into the intestinal sacs, whereas olefins above 14 carbons were either very poorly absorbed or not absorbed at all. Furthermore, marked inter-compound differences were observed, with the amount of absorption generally decreasing with increasing carbon number. In vitro metabolism studies suggest that the position of the double-bond as well as the degree of substitution influences metabolism, with alpha olefins appearing more biologically reactive relative to internal and/or branched olefins [9, 15, 17, 23]. This conclusion is corroborated by the work of Leibman et al. [15] who demonstrated that olefins are oxidized by hepatic microsomal enzymes to glycols (diols) via an epoxide intermediate. The subsequent hydrolysis of the oxirane ring by epoxides hydrolase is rapid for alpha-olefins whereas glycol formation for internal- and branched chain olefins is less efficient due to steric hindrance by the alkyl substituents in the region of the oxirane ring. In addition, epoxide formation via cytochrome P-450 dependent processes is similarly subject to steric hindrance, with ready accessibility of the alpha double-bond in linear olefins contrasting with the relative inaccessibility of the double-bond in internal olefins [17].

Regarding the safety assessment of HO, most of the human health data are publicly available. The majority of data addresses the toxicological endpoints described in REACH Annex VIII (predominantly, the requirement to address subchronic toxicity). In general, some HO showed mild skin, eye and respiratory irritation (e.g. Hex-1-ene, Oct-1-ene, and Dec-1-ene) [14]. Regarding the systemic toxicity, there are 11 Annex VIII repeated-dose toxicity tests (i.e. OECD 407 and/or OECD 422 studies) covering a carbon range from C6 to C24, including all four types of HO [2, 22, 28]. Although some common effects have been observed during these subacute repeated-dose toxicity tests, the effects were concluded to be adaptive changes of low toxicological concern or of limited relevance to human toxicity. For instances, kidneys and liver enlargement, and alpha 2u-globulin nephropathy in male rats were reported in our previous studies [28]. Hence, HO in general do not show any significant adverse effects up to the limit dose (i.e. the no-observed-adverse-effect-level (NOAEL) is 1000 mg/kg bw/day). Still, these animals were only dosed for 28 days. To ensure a comprehensive evaluation of the toxicological effects of HO as mandated by REACH Annexes IX and X, a longer dosing time is needed.

In the current investigation, we have undertaken a series of five 90-day oral gavage repeated-dose toxicity experiments involving representative subclasses of HO category displaying diverse structural characteristics and carbon chain length, as outlined in Table 1. The findings derived from these comprehensive studies have culminated in a robust dataset elucidating subchronic repeated-dose systemic toxicity. This dataset subsequently serves as foundational knowledge for facilitating read-across assessments to extrapolate and make informed inferences regarding the toxicological effects of other HO in the category.

Table 1 Overview of the HO substances in sub-chronic toxicity study (OECD 408) in Wistar Han rats

HO substances	CAS number	EC Number	Type of substance	Dose levels (mg/kg bw/day)	Carbon number	Composition (%)					Representative chemical structure
						Alpha (Vinyl)	Methyladene (Vinylidene)	Internal Di-sub (cis/trans)	Tri-sub	Tetra-sub	
Arachis Oil BP (vehicle control)	NA	NA	Mixture	0	NA	NA	NA	NA	NA	NA	NA
Oct-1-ene	111-66-0	203-893-7	MC	100 300 1000	8	**~90**	< 5	<5	0	0	
Nonene, branched*	97280-95-0	306-492-6	UVCB	20 100 500	9	0	<5	~30	**~65**	<1	
Octadec-1-ene	112-88-9	204-012-9	UVCB	20 100 500	18	~60	**~35**	~5	0	0	
Octadecene	27070-58-2	248-205-6	UVCB	100 300 1000	18	<5	<5	~60	~30	0	
Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by-product**	68911-05-7	272-762-4	UVCB	100 300 1000	23.6	<5	<5	**~70**	~10	**~10**	

The highest concentration of each structural element among these 5 substances is marked in **bold**

NA Not Applicable, *MC* Monoconstituent, *UVCB* Unknown or Variable composition, Complex reaction products or Biological materials

*Note that after a review of substance naming in 2014, the UVCB Nonene was renamed Nonene, branched with the new CAS and EC number as stated in the table above

**Also known as Alkenes, C19-23, this substance ceased manufacture in 2018

Materials and methods

Test materials

Details and representative structures of the five test materials are summarized in Table 1. Oct-1-ene (CAS No. 111-66-0), Octadec-1-ene (CAS No. 112-88-9), and Octadecene (CAS No. 27070-58-2) were supplied by INEOS. Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by-product (CAS No. 68911-05-7) was supplied by Shell. Nonene, branched (CAS No. 97280-95-0) was supplied by Braskem. All test items were prepared as solutions in Arachis oil BP (Evans Ltd., Liverpool, UK) at appropriate concentrations, ensuring formulations were within 90–110% of the nominal concentration to confirm their suitability and accuracy.

Animal and conditions

The studies followed the OECD Testing Guideline 408 (Subchronic Oral Toxicity—Rodent: 90 Day Study, Adopted 21 September 1998) under Good Laboratory Practice (GLP) [21]. Male and female Wistar Han™:RccHan™:WIST strain rats were obtained from Harlan Laboratories U.K. Ltd., Oxon, UK. Upon receipt, animals were checked for signs of ill-health or injury and acclimatized for nine days, during which their health status was assessed. At the start of treatment, males weighed 190–252 g, and females weighed 141–193 g, being approximately six to eight weeks old. Animals were housed in groups of three or four by sex in polypropylene cages with stainless steel mesh lids and softwood flake bedding (Datesand Ltd., Cheshire, UK). They had free access to a pelleted diet (Rodent 2014 C Teklad Global Certified Diet, Harlan Laboratories U.K. Ltd., Oxon, UK) and drinking water from polycarbonate bottles. Wooden chew blocks and cardboard tunnels (Datesand Ltd., Cheshire, UK) were provided for enrichment. The diet, water, bedding, and enrichment materials were contaminant-free. Animals were housed in an air-conditioned room at Harlan Laboratories Ltd., Shardlow, UK Barrier Maintained Rodent Facility, with at least fifteen air changes per hour and a 12-h light/dark cycle. Environmental conditions, including temperature (22±3 °C) and humidity (50±20%), were continuously monitored to meet study plan targets.

Group assignment and dosing

Study animals were divided into groups (ten males and ten females per group) receiving either vehicle or the test materials at doses specified in Table 1. Rats were randomly allocated to treatment groups using a stratified body weight randomization procedure to ensure group mean body weights were similar across treatment groups. Dose levels were chosen based on previous 28-day repeated-dose toxicity studies with the reproductive/developmental toxicity screening test (OECD 422) [28] and fourteen-day dose range-finding studies (data not shown). For Oct-1-ene, Octadecene, and Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by-product, the high, intermediate, and low dose levels were 1000, 300, and 100 mg/kg bw/day, respectively. For Nonene, branched and Octadec-1-ene, due to treatment-related findings at 1000 mg/kg bw/day in previous OECD 422 studies, the dose levels were 500, 100, and 20 mg/kg bw/day. The test items were administered daily for ninety consecutive days by gavage using a stainless steel cannula attached to a disposable plastic syringe. Control animals received 4 ml/kg of Arachis oil BP in an identical manner. The volume administered was based on the most recent body weight and adjusted weekly.

Observations and examinations

All animals were monitored daily for abnormalities and mortality. Observations included behavioural assessments, functional performance tests (motor activity and forelimb/hindlimb grip strength), sensory reactivity, and ophthalmoscopic examinations. Body weight, water, and food consumption were recorded weekly throughout the study.

Hematologyl and blood chemistry

Hematological and blood chemical investigations were conducted on all animals from each test and control group at the end of the study (Day 90). Blood samples were obtained from the lateral tail vein and, if necessary, repeat samples were taken by cardiac puncture prior to necropsy on Day 91. Animals were not fasted prior to blood sampling. Hematological parameters measured included: hemoglobin (Hb), erythrocyte count (RBC), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (WBC), neutrophils (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophils (Eos), basophils (Bas), platelet count (PLT), and reticulocyte count (Retic). Plasma parameters measured included: urea, inorganic phosphorus (P), glucose, aspartate aminotransferase (ASAT), total protein (Tot.Prot.), alanine aminotransferase (ALAT), albumin, alkaline phosphatase (AP), albumin/globulin (A/G) ratio (by calculation), creatinine (Creat), sodium (Na^+), total cholesterol (Chol), potassium (K^+), total bilirubin (Bili), chloride (Cl^-), bile acids, and calcium (Ca^{++}).

Gross finding at necropsy and organ weights

At the end of the dosing period, all animals were euthanized by intravenous overdose of a barbiturate agent followed by exsanguination. A full external and internal examination was conducted, and any macroscopic abnormalities were recorded. The following organs were

dissected free from fat and weighed before fixation: adrenals, ovaries, brain, spleen, epididymides, testes, heart, thymus, kidneys, uterus, and liver.

Histopathological examination

Samples of the following tissues were collected from animals in the high dose group and control and preserved in buffered 10% formalin, except where stated: adrenals, ovaries, aorta (thoracic), pancreas, bone & bone marrow (femur including stifle joint), pituitary, bone & bone marrow (sternum), prostate, brain (including cerebrum, cerebellum, and pons), rectum, caecum, salivary glands (submaxillary), colon, sciatic nerve, duodenum, seminal vesicles (including coagulating gland), epididymides, esophagus, skin, eyes, spinal cord (cervical, mid-thoracic, and lumbar), gross lesions, heart, spleen, ileum (including Peyer's patches), stomach, jejunum, testes, kidneys, thymus, liver, thyroid/parathyroid, lungs (with bronchi), tongue, lymph nodes (mandibular and mesenteric), trachea, mammary glands, urinary bladder, muscle (skeletal), uterus (with cervix), and vagina.

Histopathological examination was extended to include similarly prepared sections from animals in the low and intermediate dose groups upon indications of treatment-related changes in the high dose group. For example, kidney sections from males in each treatment and control group were stained immunohistochemically for alpha-2μ-globulin and examined.

Statistical analysis

The statistical analysis was performed using the most appropriate methods. Homogeneity of variance from mean values was analysed using Bartlett's test. Intergroup variances were assessed using suitable ANOVA or, if required, ANCOVA with appropriate covariates. Transformed data were analysed to identify the lowest treatment level showing a significant effect using the Williams Test for parametric data or the Shirley Test for nonparametric data. If no dose response was found but the data showed non-homogeneity of means, the data were analysed by a stepwise Dunnett's (parametric) or Steel (non-parametric) test to determine significant differences from the control group. For unsuitable data, pair-wise tests were conducted using the Student *t*-test (parametric) or the Mann-Whitney U test (non-parametric). Statistical significance was considered at $p<0.05$. GraphPad Prism (version 8.02) was used for generating figures.

Results and discussion

Clinical signs and observations

Throughout the 90-day study period, none of the test materials caused any signs of morbidity or mortality at any dose level. Hydrocarbon C12-30 did not induce any clinical signs at any dose level. However, the other test materials, including Oct-1-ene, Nonene (branched), Decene, Hexadecene, and Octadec-1-ene, caused episodes of increased salivation during the treatment period in both male and female animals at high and intermediate dose levels. No such effects were noted at the low dose level. This observation is consistent with our previous OECD 422 studies, where increased post-dosing salivation was seen in both sexes at doses of 300 and 1000 mg/kg bw/day for these test materials [28]. The increased salivation is likely due to the unpalatability or irritative nature of the test formulations rather than systemic toxicity. This aligns with findings from HO studies on substances with carbon numbers ranging from C6 to C18, which showed mild skin and eye irritation [22]. Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product, showed no irritation in New Zealand white rabbits in OECD 404 studies [29], and thus no salivation was observed with this substance in the current study.

Additionally, there were no treatment-related changes in behavioural assessments, functional performance tests, or sensory reactivity assessments for any test material at any dose level (Table 2). Other observations, including body weights (Fig. 1), food consumption (Fig. 2), water consumption, and ophthalmological effects (Table 2), recorded weekly throughout the 13-week exposure period, showed no significant effects in either sex for any dose group for all test materials.

Hematology and blood chemistry

Hematological and blood chemistry parameters, critical indicators of chemical toxicity, revealed several statistically significant changes across different dose groups and test materials. For Oct-1-ene, males in all dose groups exhibited a statistically significant increase in MCHC ($p<0.05$). (Appendix Table 1). Nonene, branched caused a reduction in Hb and MCHC in males at 500 mg/kg bw/day ($p<0.05$ and $p<0.01$, respectively) (Appendix Table 2). Octadec-1-ene led to a statistically significant reduction in prothrombin time and activated partial thromboplastin time in males at 500 mg/kg bw/day (Appendix Table 3). Octadecene lowered MCHC in both sexes at all doses, except for females at 100 mg/kg bw/day (Appendix Table 4). Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product resulted in statistically significantly lower MCHC in males at 1000 mg/kg bw/day and in females at 100 mg/kg bw/day, with females at 1000 mg/kg bw/day also showing lower RBC counts. (Appendix Table 5). Despite these statistically significant changes, the values remained within the normal background range; lacked a dose-related response, and lacked any associated histopathological correlates, and were thus considered not toxicologically significant.

In terms of blood chemistry, Oct-1-ene showed a statistically significant increase in ALAT in males treated with

Table 2 Overview of endpoints conducted in OECD 408 studies for Oct-1-ene, Nonene, branched, Octadec-1-ene, Octadecene, and hydrocarbons, C12-30, olefin-rich, ethylene polymn. by-product

Substances	Oct-1-ene	Nonene, branched	Octadec-1-ene	Octadecene	Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by product
Dose level (mg/kg)	0, 100, 300 and 1000	0, 20, 100 and 500	0, 20, 100 and 500	0, 100, 300 and 1000	0, 100, 300 and 1000
Mortality	No unscheduled deaths	No unscheduled deaths	No unscheduled deaths	No unscheduled deaths	No unscheduled deaths
Clinical observations	Either sex treated with 1000 and 300 mg/kg bw/day showed episodes of increased salivation during the treatment period.	Either sex treated with 500 and 100 mg/kg bw/day showed episodes of increased salivation during the treatment period.	Either sex treated with 500 mg/kg bw/day showed episodes of increased salivation between Days 27 and 50 for males and between Days 27 and 90 for females.	Either sex treated with 1000 mg/kg bw/day showed episodes of increased salivation during the treatment period.	No clinical signs observed
Behavioral assessment	No treatment-related changes	No treatment-related changes	No treatment-related changes	No treatment-related changes	No treatment-related changes
Functional performance	No toxicologically significant changes	No toxicologically significant changes	No toxicologically significant changes	No toxicologically significant changes	No toxicologically significant changes
Sensory reactivity assessments	No treatment-related changes	No treatment-related changes	No treatment-related changes	No treatment-related changes	No treatment-related changes
Body weight	No effect on body weight performance	No effect on body weight performance	No effect on body weight performance	No effect on body weight performance	No effect on body weight performance
Food consumption	No treatment-related effects on food consumption or food conversion efficiency	No treatment-related effects on food consumption or food conversion efficiency	No treatment-related effects on food consumption or food conversion efficiency	No treatment-related effects on food consumption or food conversion efficiency	No treatment-related effects on food consumption or food conversion efficiency
Water consumption	No treatment-related effect	No treatment-related effect	No treatment-related effect	No treatment-related effect	No treatment-related effect
Ophthalmoscopy	No treatment-related ocular effects	No treatment-related ocular effects	No treatment-related ocular effects	No treatment-related ocular effects	No treatment-related ocular effects
Hematology	No toxicologically significant effects	No toxicologically significant effects	Males treated with 500 mg/kg bw/day showed a statistically significant reduction in prothrombin time and activated partial thromboplastin time	No toxicologically significant effects	No toxicologically significant effects
Blood chemistry	No toxicologically significant effects	No toxicologically significant effects	No toxicologically significant effects	No toxicologically significant effects	No toxicologically significant effects

Table 2 (continued)

Substances	Oct-1-ene	Nonene, branched	Octadec-1-ene	Octadecene	Hydrocarbons, C12-30, olefin-rich, ethylene polymn. by product
Necropsy	No toxicologically significant macroscopic abnormalities	Three males treated with 500 mg/kg bw/day had enlarged kidneys. Two further males treated with 500 mg/kg bw/day had pale kidneys and one of these males also had mottled kidneys. All males treated with 500 mg/kg bw/day and three males treated with 100 mg/kg bw/day had an enlarged liver.	No toxicologically significant macroscopic abnormalities	Neither the type, incidence nor distribution of findings observed at terminal necropsy indicated any obvious effect of treatment	Neither the type, incidence nor distribution of findings observed at terminal necropsy indicated any obvious effect of treatment
Organ weights	Males treated 1000 mg/kg bw/day and females from all treatment groups showed a statistically significant increase in liver weight both absolute and relative to terminal body weight Males treated with 1000 mg/kg bw/day showed a statistically significant reduction in epididymides weight both absolute and relative to terminal body weight	Males treated with 500 and 100 mg/kg bw/day showed a statistically significant increase in kidney and liver weights both absolute and relative to terminal body weight. Females treated with 500 mg/kg bw/day also showed a statistically significant increase in absolute and relative liver weight. Females from all treatment groups showed a statistically significant increase in absolute and relative kidney weight. Males treated with 100 and 20 mg/kg bw/day showed a statistically significant reduction in spleen weight both absolute and relative to terminal body weight	Males from all treatment groups showed a statistically significant reduction in spleen weight both absolute and relative to terminal body weight. Males treated with 500 mg/kg bw/day showed a statistically significant increase in absolute and relative thymus weight. Females treated with 500 mg/kg bw/day showed a statistically significant increase in absolute and relative liver weight.	No effects in the organ weights	No effects in the organ weights
Histopathology	Kidneys: increased incidence and severity of hyaline droplets and granular casts were evident in males from all treatment groups. Stomach: Acanthosis, hyperkeratosis, ulceration and submucosal inflammation was evident in the forestomach of animals of either sex treated with 1000 and 300 mg/kg bw/day. Lungs: increased incidence and severity of alveolar macrophages were evident in animals of either sex treated with 1000 mg/kg bw/day and in females treated with 100 mg/kg bw/day.	Kidneys: hyaline droplets and tubular basophilia was evident in males from all treatment groups. Granular casts were also evident in males treated with 500 and 100 mg/kg bw/day. Liver: centrilobular hypertrophy was evident in animals of either sex treated with 500 and 100 mg/kg bw/day. Thyroid: hypertrophy/hyperplasia of follicular cells was evident in animals of either sex treated with 500 and 100 mg/kg bw/day. Stomach: acanthosis of the forestomach was evident at extremely low severity in males treated with 500 mg/kg bw/day.	Mesenteric Lymph nodes: A minimal or mild inflammatory cell infiltrate was present in the periglandular fat surrounding the mesenteric lymph nodes of eight males and nine females treated with 500 mg/kg bw/day. Adrenals: Minimal diffuse hypertrophy of the adrenal cortex was present in two females treated with 500 mg/kg bw/day.	No abnormal findings observed during microscopic examination	No abnormal findings observed during microscopic examination
No Observed Effect Level (NOEL)	Male: Not established Female: 100 mg/kg bw/day	Male: Not established Female: 20 mg/kg bw/day	100 mg/kg bw/day for both sexes	1000 mg/kg bw/day for both sexes	1000 mg/kg bw/day for both sexes
No Observed Adverse Effect Level (NOAEL)	1000 mg/kg bw/day for both sexes	500 mg/kg bw/day for both sexes	500 mg/kg bw/day for both sexes	1000 mg/kg bw/day for both sexes	1000 mg/kg bw/day for both sexes

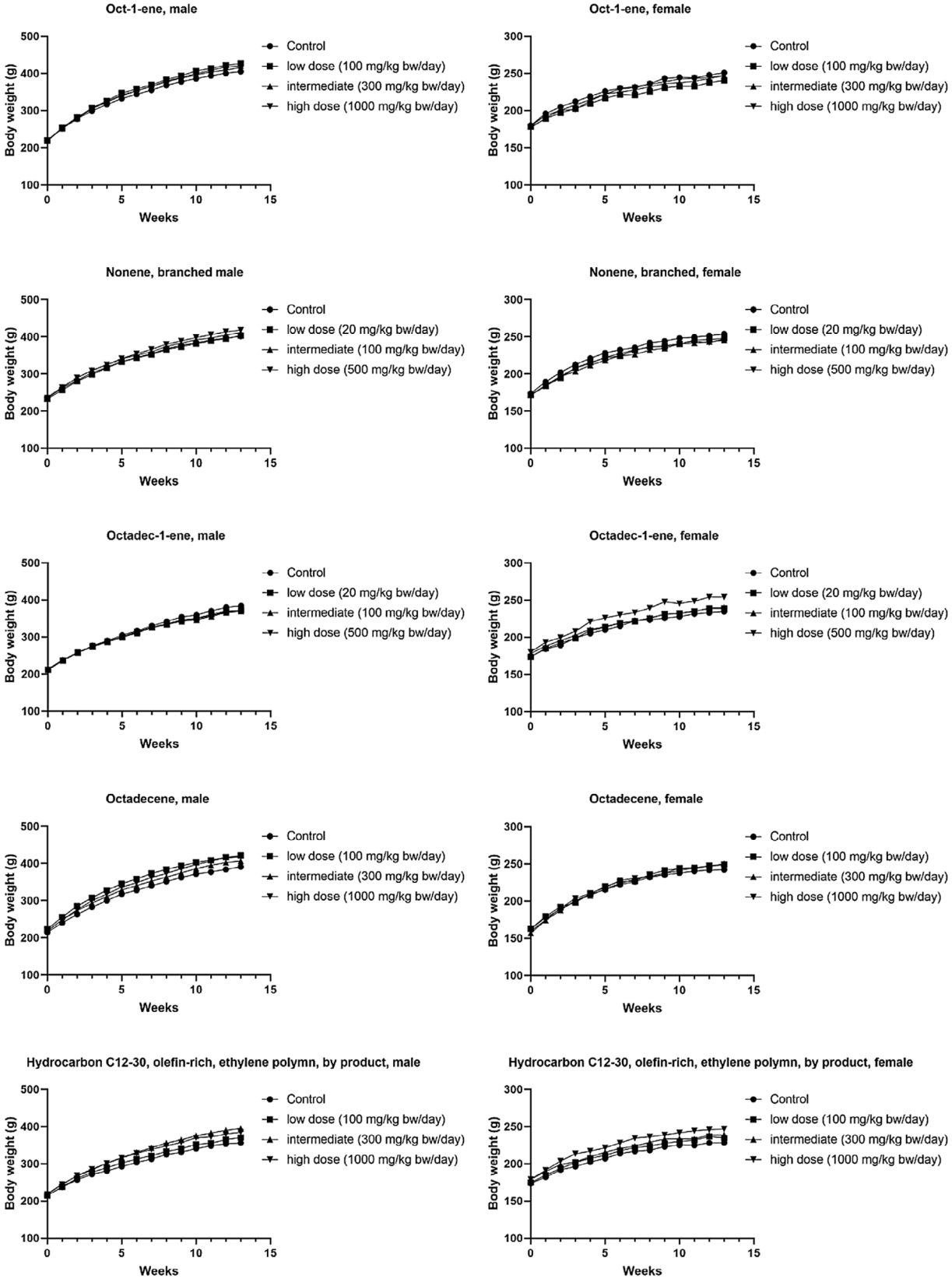


Fig. 1 Mean weekly body weights (g) of rats (male and female) administrated with Oct-1-ene, Nonene, branched, Octadec-1-ene, Octadecene, and Hydrocarbon C12-30, olefin-rich, ethylene polymn. by product

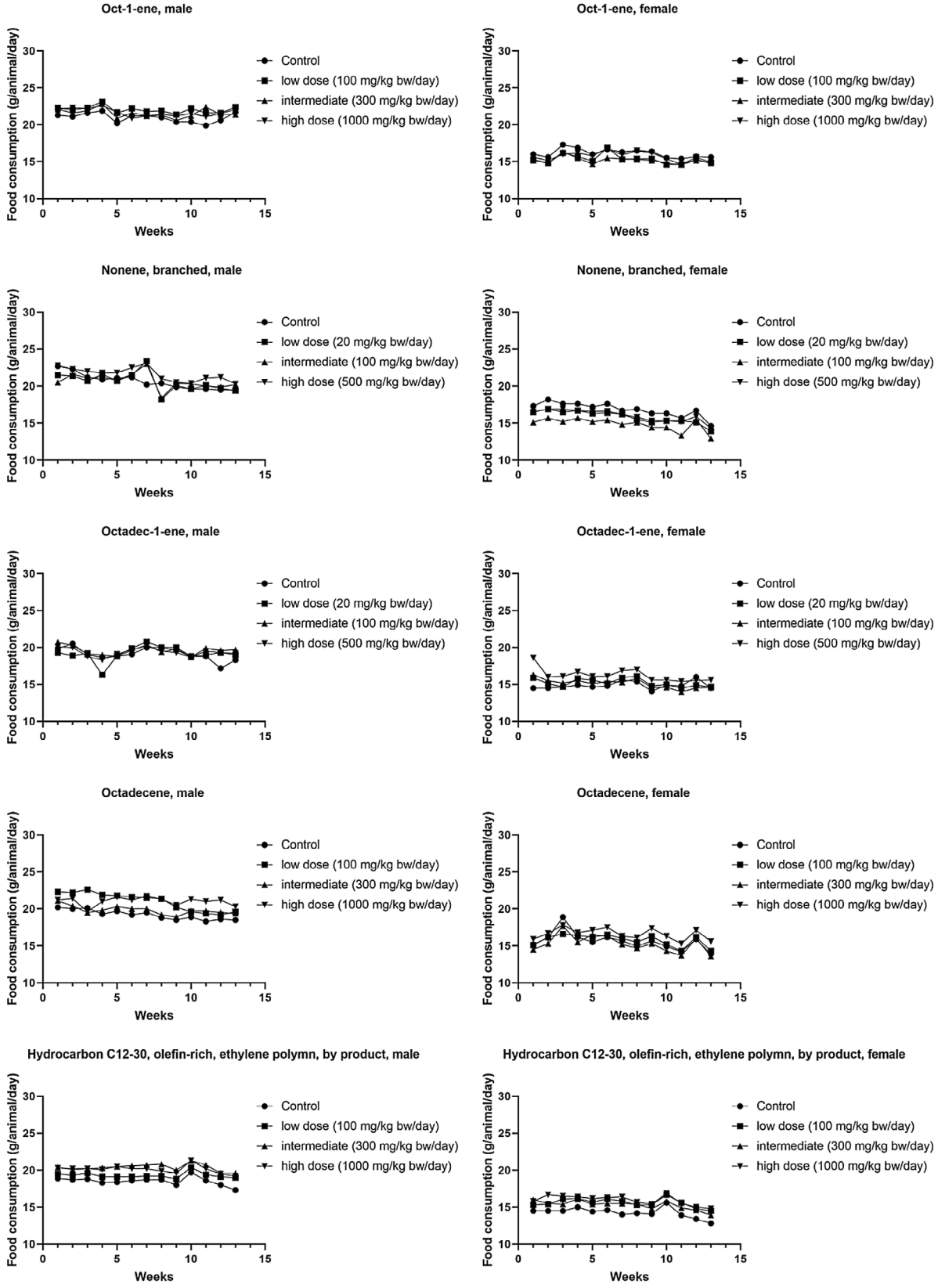


Fig. 2 Mean weekly food consumption of rats (male and female) administrated with Oct-1-ene, Nonene, branched, Octadec-1-ene, Octadecene and Hydrocarbon C12-30, olefin-rich, ethylene polymn. by product

1000 mg/kg bw/day and in Urea in females treated with 1000 mg/kg bw/day ($p<0.01$) (Appendix Table 6). In addition, males from all treatment groups showed statistically significant reductions in Na+ ($p<0.05$), K+ ($p<0.05$) and Cl- concentrations ($p<0.05$) and a statistically significant increase in P level ($p<0.05$). Males treated with 100 mg/kg bw/day also showed a statistically significant increase in Bili ($p<0.01$). Females treated with 1000 and 300 mg/kg bw/day showed a statistically significant increase in glucose ($p<0.05$). For Nonene, branched treatment (Appendix Table 7), males treated with 500 mg/kg bw/day showed a statistically significant increase in albumin and P levels ($p<0.05$ and $p<0.01$; respectively). At 100 mg/kg bw/day, males also showed a statistically significant increase in albumin ($p<0.05$) and males treated with 20 mg/kg bw/day showed a statistically significant increase in A/G ratio ($p<0.05$) when compared to controls. In addition, females treated with 500 mg/kg bw/day Nonene, branched showed a statistically significant increase in chol ($p<0.05$) when compared to controls. For Octadec-1-ene treatment (Appendix Table 8), males treated with 20 mg/kg bw/day showed a statistically significant increase ($p<0.05$) in urea whilst females treated with 100 mg/kg bw/day showed a statistically significant increase ($p<0.05$) in Tot.Prot. Octadecene showed a higher albumin and Ca++ levels in males at 1000 mg/kg bw/day when compared to control (Appendix Table 9). In addition, lower bile acid levels for males at 1000 mg/kg bw/day of Octadecene also attained statistical significance. For females at 1000 mg/kg bw/day, higher ALAT attained statistical significance when compared with control. Additionally, for females at 1000 mg/kg bw/day, lower urea and creatinine levels also attained statistical significance. For females at all dosage levels, lower bile acid values attained statistical significance. Higher P levels for females at 300 mg/kg bw/day and lower glucose levels for females at 100 mg/kg bw/day also attained statistical significance when compared with control. For Hydrocarbon C12-30 treatment (Appendix Table 10), males at 1000 mg/kw bw/day showed a statistical significance in lower albumin, total protein and A/G ratio. In the assessment of blood chemistry parameters, a multitude of effects that reached statistical significance were discerned across one or more dosage groups for each HO compound. Nevertheless, it is noteworthy that the substantial majority of individual values corresponding to these effects fell well within the established normal range for rats of the specified strain and age, and exhibited no discernible dose-response correlation. Furthermore, the absence of any concurrent histopathological alterations in all instances strongly suggests that these observations were likely the result of random occurrences. As a result, the observed alterations in blood chemistry parameters were deemed to lack toxicological significance.

Gross findings at necropsy

In the necropsy examination, no gross findings indicative of treatment-related effects were observed at any dose level for Octadecene and Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product (Table 2). However, after oral administration of Oct-1-ene, one female at 1000 mg/kg bw/day had a mass surrounding the heart and lungs, and a fluid-filled vagina. Microscopic examination revealed an abscess in the heart, granuloma in the lungs, and a dilated lumen in the uterus, confirming these findings. Additionally, reddened lungs were noted in one control female, one female treated with 100 mg/kg bw/day, two females and one male treated with 300 mg/kg bw/day, and four females treated with 1000 mg/kg bw/day. Microscopic examination showed lung congestion in the treated animals, but these findings were considered incidental and unrelated to the treatment, as similar findings were present in the control group. The occurrence of reddened lungs and lung congestion across all dose groups, including controls, was considered within the normal background range for untreated animals of the same age and strain. Notably, HO with carbon ranges from C6 to C24 are classified as aspiration toxicity Category 1 according to EU CLP criteria [6], suggesting that the lung findings could be due to aspiration toxicity rather than specific effects of Oct-1-ene.

Upon administration of Nonene, branched, all males treated with 500 mg/kg bw/day and three males treated with 100 mg/kg bw/day had enlarged livers at necropsy. Three males treated with 500 mg/kg bw/day had enlarged kidneys, with one of these males also exhibiting mottled kidneys, increased pelvic space, and fluid-filled kidneys. Additionally, two males treated with 500 mg/kg bw/day had pale kidneys, with one also displaying mottled kidneys. These macroscopic organ findings (i.e., enlarged livers and kidneys) correlated with microscopic findings discussed in the histopathology section "Histopathological Examination". Other observations, such as one male with small epididymides and six females (including control females and those treated with various doses) with reddened lungs, were considered non-toxicologically significant due to the absence of associated histopathology.

Following Octadec-1-ene treatment, reddened lungs were observed in one control female, four females treated with 20 mg/kg bw/day, one female treated with 100 mg/kg bw/day, and one female treated with 500 mg/kg bw/day at necropsy. The lack of any concurrent histopathological correlations led to the conclusion that these differences held no toxicological significance.

Organ weight

Octadecene and Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product showed no treatment effects on organ weights in either sex at all dose levels (i.e. 100, 300,

and 1000 mg/kg bw/day) (Table 2). However, exposure to Oct-1-ene, males treated 1000 mg/kg bw/day and females from all dose groups resulted in a statistically significant increase in absolute and relative liver weight $p<0.01$ for males, $p<0.05$ for females) (Appendix Table 11). In addition, exposure to Oct-1-ene at 1000 mg/kg bw/day led to a statistically significant reduction in absolute and relative epididymides weight in males ($p<0.05$). Nonene, branched treatment led to statistically significant increases in kidney and liver weights both absolute and relative to terminal body weight in males treated with 500 and 100 mg/kg bw/day ($p<0.05$ for 100 mg/kg bw/day and $p<0.01$ for 500 mg/kg bw/day, respectively), and a reduction in absolute and relative spleen weight in males treated with 100 and 20 mg/kg bw/day ($p<0.05$) (Appendix Table 12). Additionally, females treated with 500 mg/kg bw/day showed a statistically significant increase in absolute and relative liver weight ($p<0.01$), and all treated female groups showed increased kidney weight both absolute and relative to terminal body weight ($p<0.05$ for 20 and 100 mg/kg bw/day, $p<0.01$ for 500 mg/kg bw/day). For Octadec-1-ene (Appendix Table 13), males from all treatment groups exhibited a statistically significant reduction in absolute and relative spleen weight ($p<0.05$) and an increase in absolute and relative thymus weight at 500 mg/kg bw/day ($p<0.05$). Females treated with 500 mg/kg bw/day also had a statistically significant increase in absolute and relative liver weight ($p<0.01$).

While several organ weight changes were observed following Oct-1-ene and Octadec-1-ene administration, these were considered not toxicologically relevant as the values fell within the normal range for the strain and age of the rats used, with no dose-related response or associated histopathological findings. Conversely, Nonene, branched caused statistically significant increases in liver and kidney weights in both sexes, correlating with histopathological findings, which will be discussed further in section "Histopathological Examination" of this manuscript.

Histopathological examination

For Octadecene and Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product, no abnormalities were observed during microscopic examination of the tissues from animals that received test materials up to 1000 mg/kg bw/day, indicating the absence of any treatment effects. Upon administration of Nonene, branched, multiple organs, including kidneys, liver, thyroid, and stomach, demonstrated microscopic abnormalities (Appendix Table 14). Specifically, hyaline droplets and tubular basophilia were evident in the kidneys of males from all treatment groups, with granular casts also present in the kidneys of males treated with 500 and 100 mg/kg bw/day. Centrilobular hypertrophy was observed in the livers of animals of either sex treated with 500 and 100 mg/kg bw/day. Additionally, hypertrophy/hyperplasia of follicular cells was evident in the thyroids of animals of either sex treated with 500 and 100 mg/kg bw/day, and acanthosis of the forestomach was observed at low severity in the stomach of males treated with 500 mg/kg bw/day.

The increased liver weight and centrilobular hypertrophy findings in animals of either sex is routinely observed and considered to be adaptive in nature in the absence of any degenerative or inflammatory changes [11, 12]. Interestingly, minimal changes in the thyroid where hypertrophy/hyperplasia of follicular cells was observed in animals of either sex treated with Nonene, branched, and consistent with the 28 day study [28]. These thyroid and liver changes maybe characteristic of hepatocellular induction as a result of enhanced hepatic metabolism. As a side effect of hepatic induction an increased liver metabolism of thyroid hormones T3 and T4 can occur. Note that in this study thyroid hormones have not been measured and neither the thyroid weight. This subsequently leads to an enhanced thyroid gland production of these hormones resulting from the negative feedback stimulation of TSH production. The appearance of thyroid follicular cell hypertrophy is considered to be a result of this process [3, 4, 7, 20, 33], and therefore, thyroid and liver changes were considered to be adaptive in nature [16, 19, 25].

The increased kidney weight was evident in females from all dose group and males from the high and intermediate dose groups. The values in females were within the range of historical control and without any histological correlates. For males, the kidneys weights are statistically significantly higher than control and with pathohistology-confirmed tubular basophilia and hyaline droplets present in males from all treated groups. These tubular findings were accompanied by granular casts in males treated with 500 and 100 mg/kg bw/day. The hyaline droplets can be directly linked to accumulation of alpha 2μ-globulin and this finding is commonly observed in adult male rats following treatment with some hydrocarbons, including HO, and is not predictive of any adverse effect in humans [10, 18, 30, 31]. The remaining kidney findings consisting of granular casts may be considered to represent an adverse effect of the test material. However, in our previous 28-days repeated-dose study with Nonene, branched [28], immunohistochemical staining of male kidney tissue confirmed the presence of alpha 2μ-globulin and that the kidney changes were associated with hyaline droplet formation. Therefore, the effects seen in male kidneys although treatment related, they are considered to be not relevant for humans.

Oct-1-ene showed microscopic abnormalities in kidneys, stomach and lungs (Appendix Table 15). For

kidneys, increased incidence and severity of hyaline droplets and granular casts were evident in males from all treatment groups. Acanthosis, hyperkeratosis, ulceration and submucosal inflammation was evident in the forestomach of animals of either sex treated with 1000 and 300 mg/kg bw/day. In addition, increased incidence and severity of alveolar macrophages in lungs were evident in animals of either sex treated with 1000 mg/kg bw/day and in females treated with 100 mg/kg bw/day. Although the kidney finding was not observed in the previous 28-days repeated dose toxicity study, some initial signs of nephropathy, such as enlarged and mottled kidneys, were evident in one male rat treated with 1000 mg/kg bw/day Oct-1-ene [28]. Moreover, the kidney effects are very similar to the observations with Nonene, branched and consistent with alpha-2μ-globulin nephropathy. In addition, the stomach changes identified at 1000 and 300 mg/kg bw/day Oct-1-ene are considered to be an adverse effect of treatment, resulting from local irritation of the test item rather than being indicative of a systemic toxic effect. In lungs, increased incidence and severity of alveolar macrophages was evident in some animals of either sex treated with 1000 mg/kg bw/day and in some females treated with 100 mg/kg bw/day. This observation is considered to be a result of local irritation following aspiration of microdroplets of the test compound as the gavage catheter was withdrawn. Such accidental aspiration of the formulation during the dosing procedure was also observed in the previous OECD 422 study where minimal to slight infiltration of inflammatory cells were observed in some rats lung treated with 1000 mg/kg bw/day of Hexadecene and Octadec-1-ene. Such effect was not reproduced in the current study with Octadec-1-ene, indicating a procedural artifact rather than systemic toxicity. In addition, the absence of the finding at 300 mg/kg bw/day is further evidence that this is a procedure-related finding and as such, it is of limited toxicological significance.

The following histopathological changes were observed in animals treated with Octadec-1-ene (Appendix Table 16): a minimal or mild inflammatory cell infiltrate in the periglandular fat surrounding the mesenteric lymph nodes of eight males and nine females treated with 500 mg/kg bw/day. The infiltrate was mixed in character, principally composed of lymphocytes and neutrophils and tended to have a perivascular or perilymphatic orientation. Although the origin of the finding is uncertain it might have resulted from local irritation of material from the gut reaching the drainage nodes, however, there was no evidence of local irritation to the gut mucosa. Interestingly, this effect on the mesenteric lymph nodes observed in the current study was also present in the OECD 422 study in animals of either sex treated with 1000 and 300 mg/kg bw/day of Octadec-1-ene [28]. This finding which is also seen with saturated hydrocarbons was considered as a nonspecific, adaptive change of low toxicological concern by the joint FAO/WHO Expert Committee on Food Additives (JECFA) [13, 24]. In addition, microscopic examination of adrenal sections did reveal minimal or mild diffuse cortical hypertrophy in two females treated with 500 mg/kg bw/day. However, in the absence of any statistically significant difference in mean organ weight this minor difference was considered of equivocal biological significance and most likely stress-related or the result of individual variation.

Comparison of results based on carbon number and higher olefins structure

Previous OECD 422 studies showed that the carbon number rather than the position of the double bond contributes to the effects observed. To confirm this observation, olefins were included in the current study with a carbon range from C8 to C24, and representing four types of HO (Table 1): (1) linear alpha olefins (i.e. vinyl—straight chain with a single double bond in the alpha position), (2) linear internal olefins (i.e. cis/trans disubstituted—straight chain with a single double bond in an internal position), (3) branched alpha olefins (i.e. vinylidene—isomerized olefins with a single double bond in the alpha position), and (4) branched internal olefins (i.e. trisubstituted and tetrasubstituted—isomerized olefins with a single double bond in an internal position). As expected, no effect was observed for Hydrocarbon C12-30, olefin-rich, ethylene polymn. by-product and Octadecene at any dose level (i.e. NOEL is 1000 mg/kg bw/day). The observed consistency aligns with earlier findings, where HO up to a specific carbon number, above C18 in this instance, exhibited a lack of treatment-related effects. This absence of effects is substantiated by a NOEL of 1000 mg/kg bw/day in the context of repeated dose toxicity, attributable to the inherent poor bioavailability of the substance [27]. Comparing Octadec-1-ene and Octadecene, which have the same carbon numbers (i.e. C18) as well as similar bioavailability, non-adverse treatment related effects were induced by Octadec-1-ene but not Octadecene despite the fact that the highest dose of Octadec-1-ene was only half that of Octadecene. This might indicate that the alpha olefin moiety (linear or branched) plays a role in generating these effects which would be consistent with the hypothesis that alpha olefins are more biologically reactive than internal olefins [27]. In addition, when the results of Octadec-1-ene are compared to those of Oct-1-ene, both primarily comprising (linear) alpha olefins, the effects observed and/or the severity of the effects (i.e. kidney and stomach effects) of Oct-1-ene in the current study were more severe than Octadec-1-ene. This might due to the fact that the dose level (expressed in mol) of Oct-1-ene was more than

quadruple that of Octadec-1-ene, and/or Oct-1-ene having a higher bioavailability than Octadec-1-ene. Interestingly, Nonene, branched demonstrated more effects (i.e. kidney, liver and thyroid effects) than the rest of olefins. This might imply that the internal branching, in addition to the bioavailability, might induce the observed effects. From the perspective of the NOAEL, all items tested in the current studies exhibited a NOAEL at the highest dose administered, without displaying a discernible trend indicating variations in adverse effects based on HO types. In contrast, considering the NOEL perspective, bioavailability emerges as a crucial determinant. Notably, HO with lower carbon numbers (e.g., C8 and C9) manifested more effects compared to those with higher carbon numbers (e.g., C18, and C24). A comparison of HOs with similar low bioavailability, such as Octadec-1-ene versus Octadecene, suggests that the presence of a double-bond at the alpha position contributes to the observed effects. However, in the case of HOs with relatively high bioavailability (i.e. C8 and C9), the internal double-bond and branching appears to play a more prominent role in generating physiological and pathological effects due to its higher gut absorption (i.e. iso-octene) [27].

Interestingly, the impression that subchronic repeated dose toxicity studies showed variable effects with no apparent regular pattern in the systemic effects observed is inherently incorrect. As described above, the number of observed effects decreased with the increasing carbon number. Moreover, there are numerous similarities in the toxicological observations within these repeated dose studies. For example, similar target organs, such as the forestomach and kidneys, were observed across the HOs. These are associated with commonly observed rodent-specific changes, like local irritant effects on the rodent forestomach and alpha 2μ-globulin nephropathy, which have both been demonstrated not to be human-relevant regarding potential tumor formation. Other minor effects, such as increased post-dosing salivation, were observed across the majority of HOs and are often reported when a test substance is an irritant.

Comparison of results to other HO 90-days and our previous 28-days repeated dose toxicity studies

There is only one oral subchronic toxicity study available for comparison with the current studies. Four groups of 20 male and 20 female rats were dosed via oral gavage with Oct-1-ene for 90 days at 0, 5, 50, and 500 mg/kg/day [22, 32]. Changes that were considered treatment-related occurred only in the high dose group (i.e. 500 mg/kg/day). There was an increase in kidney weights in both sexes, and in males, this was accompanied by histopathological changes. This finding was consistent with our current studies in which Oct-1-ene showed histopathological renal changes in males from all dose groups. Although the authors did not indicate whether the renal changes are caused by alpha 2μ-globulin accumulation, there is a high possibility that the observed renal changes are indeed specific to male rat hydrocarbon-induced nephropathy and not relevant to humans. Hence, the NOEL and NOAEL were determined at 50 and 500 mg/kg/day, respectively. However, bearing in mind that 500 mg/kg/day is the highest dose tested in the study, the true NOAEL might be higher.

Conclusions

The current paper yields several noteworthy conclusions:

- The effects observed at the 90-day exposure mark remain consistent with those noted after a 28-day period. Consequently, screening studies such as OECD 422 adequately characterise the effects that are also seen during subchronic exposure.
- Substances with lower carbon chains (C8-C9) exhibit more pronounced effects compared to those with higher carbon chains (> C18) that were tested. Therefore, it is plausible to employ low carbon HO to represent a worst-case scenario within the category.
- Among the tested lower carbon chains, variations in potency emerge, with branched molecules demonstrating heightened effects compared to their linear counterparts with lower molecular weights. This discrepancy is likely attributed to the increased gut absorption, resulting in a higher internal dose.
- No discernible trend in systemic toxicity can be established based on the position of the double bond.
- Crucial parameters for establishing a read-across trend include absorption, carbon chain length, and branching. Consequently, Nonene, branched—a low molecular weight, branched hydroxy compound—is deemed the worst-case substance for hazard assessment within the entire category. A NOAEL of 500 mg/kg bw is considered a starting point for human health risk assessment.
- Structural differences play a pivotal role in effects at equivalent or similar carbon chain lengths.

Supplementary Information

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Supplementary Material 1

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Author contributions
Q.S., J.-C.C. and P.J.B. analyzed the data and wrote the manuscript, M.G.P., F.H., S.J. conducted the study design, A.R.V.R. and J.D. monitor the study, L.K., M.R., H.S. and Y.T. conducted the data analysis and all authors reviewed the manuscript.

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Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The studies were designed and conducted to cause the minimum suffering or distress to the animals consistent with the scientific objectives and in accordance with the Harlan Laboratories Ltd, Shardlow, UK policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedure) Act 1986 Amendment Regulations 2012. The conduct of the study may be reviewed, as part of the Harlan Laboratories Ltd, Shardlow, UK Ethical Review Process. The studies were conducted in accordance with the UK Home Office Guidance document on Regulatory Toxicology and Safety Evaluation Studies and the OECD guidance document on recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation.

Consent for publication
Not applicable.

Competing interests
The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors of this article are either employed by companies that manufacture petroleum products or consultants to those companies.

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