COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT)

Potential toxicological risks from electronic nicotine (and non-nicotine) delivery systems (E(N)NDS – e-cigarettes). Paper 12: Toxicological review of nicotine.

Background

1. As part of the review on the potential toxicity of electronic nicotine delivery systems (ENDS) and electronic non-nicotine delivery systems (ENNDS) (collectively abbreviated to E(N)NDS), the COT has been reviewing potential toxicity of exposure to nicotine from these products. Previous discussion papers on this topic have included TOX/2018/25 ('Preliminary overview of nicotine toxicity'), TOX/2018/45 ('A review of data relating to developmental toxicity in offspring following parental exposure to nicotine'), and TOX/2019/01 ('Additional information on toxicity in adolescent and young adult users'). In addition, studies that have reported on potential effects of nicotine in the context of ENDS aerosol mixtures have been reviewed in discussion papers TOX/2018/24 ('Toxicological and epidemiological evaluations of E(N)NDS aerosol exposures') and TOX/2018/46 ('Additional information on developmental toxicity studies of E(N)NDS aerosols').

2. Following on from the above-mentioned discussion papers, the Committee requested that a toxicological review of nicotine be carried out, with the aim to identify study data of use for establishing a health-based guidance value (HBGV) for exposure to nicotine from ENDS products. This topic is addressed in the current paper.

Introduction

3. Nicotine (3-[(2S)-1-methylpyrrolidin-2-yl]pyridine; CAS 54-11-5) is an alkaloid that is present in leaves of the tobacco plant (*Nicotiana tabacum*). Nicotine is used as an insecticide in some countries, but use of nicotine-containing plant protection products is no longer permitted in the EU. The major form of exposure to nicotine globally is through the use of tobacco products (smoking conventional cigarettes (CC), cigars and pipes, using snuff, or chewing tobacco). Therapeutically, nicotine is used for the relief of nicotine withdrawal symptoms and as an aid to smoking cessation, in forms such as transdermal patches, lozenges, chewing gums, and nasal sprays. Less common therapeutic uses include treatment of ulcerative colitis and trials of nicotine therapy to alleviate cognitive symptoms in Parkinson's and Alzheimer's diseases. Nicotine is present in ENDS liquids, generally at concentrations in the range of up to around 20 mg/mL, although products with higher

nicotine concentrations may be available in some countries. In the UK, the Tobacco and Related Products Regulations 2016 states that "nicotine-containing liquid which is presented for retail sale in an electronic cigarette or refill container must not contain nicotine in excess of 20 milligrams per millilitre" (Part 6, section 36(4)). Some studies have found that nicotine concentrations in ENDS liquids are not always true-to-label. In studies that measured concentrations of nicotine in ENDS aerosols, levels were in the approximate range of <1–100 µg/puff (reviewed in TOX/2019/39, being presented at the current meeting). In areas of moderate ENDS used (for example, a single user in a standard sized room or a vehicle), levels of nicotine in ambient air have been reported to range from undetectable to around 10 µg/m^3, while some data have indicated levels > 100 µg/m^3 in ambient air in specific situations such as 'vaping conventions' where there is a high level of ENDS product use, particularly if room ventilation is poor (reviewed in TOX/2019/11).

Search strategies

4. Searches of the PubMed and Scopus databases for publications relating to nicotine toxicity were performed for the period 01/01/2008 to 29/04/2019, as described in Annex A. Literature prior to 2008 was identified from published toxicological reviews of nicotine, which are noted in paragraph 6, below. In addition, the text and reference lists of literature papers that was reviewed were inspected for any further citations of relevance.

Toxicity evaluation

Authoritative reviews and reference values

5. Nicotine has been registered under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations. It is classified as acutely toxic (category 2) by oral, dermal, and inhalation exposure and has hazard statements H300: fatal if swallowed, H310: fatal in contact with skin, and H330: fatal if inhaled.

6. Several authoritative bodies have evaluated the toxicity of nicotine, including The Criteria Group for Occupational Standards of the Swedish National Institute for Working Life (NIWL 2005), Health Council of the Netherlands Committee on Updating of Occupational Exposure Limits (HCN 2005), UK Rapporteur for the EU peer review process for pesticides (UK-DAR 2007), the United States Environmental Protection Agency (EPA 2008), Agence Française de Sécurité Sanitaire des Aliments (AFSSA 2009), The German Federal Institute for Risk Assessment (BfR 2009), European Food Safety Authority (EFSA 2009), and a European Chemicals Agency (ECHA) harmonised classification and labelling (CLH) report prepared by RIVM (RIVM 2015).

7. As part of an evaluation of potential risks for public health due to the presence of nicotine in wild mushrooms, EFSA[1] established an oral acute reference dose

[1] The report by EFSA (2009) can be found at https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2009.286r (accessed 06/05/2019)

(ARfD) of 0.0008 mg/kg/bw/day for nicotine. This was established using data from a study by Lindgren et al. (1999) (see paragraph 30 for details of this study), from which a lowest observed adverse effect level (LOAEL) of 0.0035 mg/kg bw/day was determined based on slight, transient increased heart rate in human CC smokers on intravenous (i.v.) infusion of nicotine. The ARfD was set by applying an overall uncertainty factor (UF)[2] of 10 and a correction factor of 0.44 for oral bioavailability of nicotine (extrapolation from i.v. to oral route) (EFSA 2009). Given that nicotine has a short biological half-life and does not accumulate in the body, and that the most sensitive effect was considered to be the pharmacological effect on the cardiovascular system, EFSA considered that the value set for the ARfD would be suitable to protect from chronic effects and could also be applied as the acceptable daily intake (ADI). The German Federal Institute for Risk Assessment[3] also established an ARfD for nicotine of 0.0008 mg/kg bw/day based on the study of Lindgren et al. (1999) (BfR 2009).

8. The US EPA[4] evaluated the occupational risk of short- and intermediate-term use of nicotine by certified applicators. Nicotine was evaluated for use as a pesticide, in the format of smoke-generating canisters, on ornamental plants in greenhouses (only) for a re-registration application eligibility decision. A no observed adverse effect level (NOAEL) of 1.25 mg/kg bw/day was identified for hepatotoxicity (mild fatty change, mild focal necrosis, mild dark cell change, with effects on the mitochondria) in a 10-day rat drinking-water study (Yuen et al. 1995) (see paragraph 46 for details of this study). EPA considered that a margin of exposure (MOE) of 1000 would be protective of human health (10 for inter-species extrapolation, 10 for intra-species variability, 10 for database uncertainty). The major potential source of risk for exposure was considered to be inhalation, with relatively less exposure dermally (EPA 2008).

9. Under the EU peer review process for pesticides, in 2007, a draft assessment report (DAR) by a UK Rapporteur proposed reference values for nicotine, in the form of a fumigation formulation on vegetables grown in glasshouses. A value of 0.0001 mg/kg bw/day was proposed for the ARfD, ADI, and systemic acceptable operator exposure level (AOEL). This was based on an estimated lowest observed effect level (LOEL) of 0.01 mg/kg bw/day for clinical signs of toxicity in children exposed dermally, as described by Woolf et al. (1997) (see paragraph 29 for details of this study), with application of a UF of 100 (10 for intra-species variability and 10 for use of a limited data set) (UK-DAR 2007). The French Food Safety Agency (AFSSA) endorsed the ADI and ARfD of 0.0001 mg/kg bw/day proposed by the UK in 2007 (AFSSA 2009). Based on the DAR, the Standing Committee on the Food

[2] EFSA noted that "The LOAEL is considered to be close to the NOAEL and the overall uncertainty factor of 10 would be sufficient to cover not only human variability but the extrapolation from the LOAEL to NOAEL for the pharmacological effect observed at the LOAEL."
[3] The BfR opinion can be found at https://www.bfr.bund.de/cm/349/nicotine_in_dried_boletus_mushrooms_causes_for_contamination_must_be_determined.pdf (accessed 06/05/2019).
[4] The report by EPA (2008) can be found at https://archive.epa.gov/pesticides/reregistration/web/pdf/nicotine_red.pdf (accessed 01/03/2019).

Chain and Animal Health concluded that existing evidence was not sufficient to demonstrate a safe use of nicotine as a plant protection product with respect to operators, workers, bystanders, and consumers, thus evaluation under Directive 91/414/EEC resulted in the withdrawal of plant protection products containing nicotine from 8 June 2009, with requirement for removal of all existing stocks by 8 June 2010 (EC 2008).

10. The Criteria Group of the Swedish National Institute for Working Life (NIWL) conducted a toxicological review of nicotine in 2004[5], finding that the overall scientific material was not sufficient to identify a critical effect following occupational exposure to nicotine (NIWL 2005). However, they concluded that judging from animal experiments, the critical effect of nicotine exposure is its effect on reproduction, with effects on development of the nervous system in the young. They noted that changes in motor behaviour were observed in mice treated from PND 10–15 with 66 µg nicotine/kg bw/day by subcutaneous (s.c.) injection (as 2 injections/day). This dose was recalculated by NIWL, for the purpose of job-related inhalation exposure, to correspond to an air level of around 0.1 mg/m^3. The NIWL Group also noted that symptoms of acute poisoning in tobacco harvesters have been observed at serum cotinine levels corresponding to inhalation exposure of around 2 mg/m^3, that nicotine is addictive although a threshold for this effect is not known, and that nicotine has acute cardiovascular effects.

11. The Health Council of the Netherlands (HCN) Committee on Updating of Occupational Exposure Limits published a toxicological review of nicotine[6] as part of a health-based re-assessment of administrative and occupational exposure limits. They concluded that "Despite a rather rich toxicological database, the committee considers the toxicological database on nicotine too poor to justify recommendation of a health-based occupational exposure limit" (HCN 2005). In assessing the health hazard to workers via occupational and dermal exposure, the HCN Committee noted that in humans, case studies have shown local and systemic skin reactions and skin sensitisation to nicotine patches, and that many cases of acute fatal poisoning have been reported in the older literature. Studies of groups with occupational exposure, or of users of smokeless tobacco, did not provide sufficiently reliable exposure data to ascertain long-term effects, although smokeless tobacco use had been associated with increased risk of cardiovascular disease. Studies of addiction suggested a 5 mg/day threshold for CC smoking, but that daily oral doses of 25 mg for up to 1 year did not lead to addiction. The Committee concluded that in humans, nicotine may contribute to adverse reproductive outcomes via mechanisms that may include reduction in uteroplacental blood flow and direct effects on the developing fetal brain.

[5] The full text of the report can be found at http://www.inchem.org/documents/kemi/kemi/ah2005_07.pdf (pp 84–105, Consensus Report for Nicotine) (accessed 06/05/2019).
[6] The full text of the report can be found at https://www.healthcouncil.nl/documents/advisory-reports/2004/03/30/nicotine (accessed

12. The Harmonised Classification and Labelling (CLH) report[7] reviewed acute toxicity data for nicotine, and proposed the following harmonised classifications: Acute Tox 1, H300, based on an acute toxicity estimate (ATE) of 3.3 mg/kg bw for acute oral toxicity; Acute Tox 1, H310, based on an ATE of 50 mg/kg bw for acute dermal toxicity; Acute Tox 2, H330, based on an ATE of 0.25 mg/L for acute inhalation toxicity (RIVM 2015).

Toxicokinetics

13. Unless otherwise stated, information in the following paragraphs, 14–21, is taken from review articles by Hukkanen, Jacob and Benowitz (2005), Benowitz, Hukkanen and Jacob (2009), and the Royal College of Physicians (RCP 2016).

14. Absorption of nicotine across biological membranes is pH dependent. Nicotine is a weak base with pKa 8.0 and is not well absorbed in the ionised state, in acidic conditions. Absorption in the mouth is thus dependent on the pH of the smoke or aerosol inhaled. Nicotine is quickly absorbed in the small airways and alveoli where the pH of lung fluid is 7.4, leading to a rapid rise in blood concentrations and delivery to the brain within 10–20 s of inhalation. Nicotine absorption from chewing tobacco or snuff occurs more slowly, with blood concentration peaking at around 30 min after use. Nicotine replacement therapies (NRT) (transdermal patches, nasal sprays, inhalers, sublingual tablets, lozenges) are buffered to alkaline pH, nevertheless absorption is slower than from CC smoking, with nasal spray providing the most rapid form of absorption. Nicotine undergoes first-pass metabolism following ingestion, reducing its bioavailability. Nicotine is well absorbed through skin, with a time lag of approximately 1 h between application of a transdermal patch and appearance of nicotine in the blood. Percentage bioavailability for nicotine administered as single doses by various routes was reported as follows: smoking 1 CC (80-90%); i.v. approximately 5.1 mg (100%); nasal spray 1 mg (60-80%); gum 2-4 mg (55-78%); inhaler 4 mg (51-56%); lozenge 2-4 mg (50-79%); transdermal patch 14-21 mg/24 h (68-100%); s.c. injection 2.4 mg (100%); oral capsule 3-4 mg (44%); oral solution approximately 3 mg (20%); enema approximately 3.5 mg (15-25%).

15. Absorbed nicotine is distributed extensively to body tissues, with the highest affinity to liver, kidney, spleen, and lung, and lowest affinity in adipose tissue. Nicotine has high affinity for brain tissue. Nicotine accumulates in gastric juice, saliva, breast milk, crosses the placental barrier, and accumulates in fetal serum and amniotic fluid. Nicotine arterial blood concentrations after smoking one CC are reported to range generally between 20–60 ng/mL, with an arterial/venous ratio around 2.3–2.8. Levels fall rapidly during 20 min after smoking.

16. Approximately 70-80% of nicotine is metabolised to cotinine, of which 90% is mediated by hepatic cytochrome P450 (CYP) 2A6 enzyme. Cotinine is then

[7] The report can be found at https://echa.europa.eu/documents/10162/56df129c-42d3-4533-a828-e5d26a58bc63 (accessed 15/05/2019).

metabolised exclusively by CYP2A6 to 3'-hydroxycotinine. Nicotine and metabolites are excreted in the urine. The nicotine metabolite ratio (NMR), 3'-hydroxycotinine: cotinine, is an indicator of CYP2A6 nicotine clearance. CYP2A6 genotypic variation is associated with variable rates of nicotine metabolism, with slow metabolism associated with higher rates of abstinence from CC smoking and higher success rates of quitting, either unaided or with NRT. Other enzymes that have a minor role in nicotine metabolism include flavin-containing monooxygenase (FMO)3, uridine diphosphate glucuronyltransferase (UGT)2B10 and UGT2B17, which also show polymorphic variation.

17. The physiologic and pharmacologic effects of nicotine are mediated via binding to nicotinic acetylcholine receptors (nAChRs), which are expressed throughout the body, including the central nervous system and peripheral nervous system. nAChRs are ligand-gated ion channels comprising differing combinations of five transmembrane subunit proteins, α_2-α_{10} and β_2-β_4 subunits around a central pore. Receptor subtypes differ in aspects such as nicotine affinity, sensitivity to upregulation, and desensitisation. The expression of different subtypes shows a pattern of distribution within the brain, with $\alpha_4\beta_2$ being the most common. Tolerance to the stimulant effects of nicotine develops rapidly (days), leading to withdrawal symptoms, and chronic exposure leads to neuroadaptations, including desensitisation and upregulated expression of nAChRs. Binding of nicotine to nAChRs leads to the release of other neurotransmitters, including dopamine, serotonin, γ-aminobutyric acid (GABA), glutamate, noradrenaline, acetylcholine, and endorphins. Pairing of reward/reinforcing effects (e.g. dopamine release) with CC smoking-related environmental stimuli is thought to be linked to the development and maintenance of smoking-related cravings and relapse.

18. Nicotine clearance is dependent on hepatic blood flow and thus increases with eating. Clearance decreases with older age, is generally higher in women than men, and is increased by oral contraceptive use and in pregnancy. Nicotine metabolism is diminished in neonates, with a half-life of three- to four-fold that in adults.

19. Nicotine is excreted by glomerular filtration and tubular secretion, with reabsorption depending on urinary pH (higher reabsorption at higher pH).

20. Plasma nicotine half-life on i.v. infusion is around 2 h, with terminal half-life of 11 h. Blood plasma nicotine levels in CC smokers generally range from 10 to 50 ng/mL, with typical daily trough concentrations of 10 to 37 ng/mL and peaks of 19 to 50 ng/mL, and a mean nicotine boost per 1 CC smoked of 10.9 ng/mL. *Ad libitum* use of NRT products generally provides a plasma nicotine concentration approximately one-third to two-thirds of that achieved by CC smoking. Steady-state plasma nicotine concentrations from transdermal patches are in the range of 10–20 ng/mL, with a range of 5–15 ng/mL from gum, inhaler, sublingual tablet, and nasal spray. Systemic doses delivered from different nicotine delivery systems are reported as follows: smoking 1 CC, 1–1.5 mg; nicotine gum, 2 mg from one 4-mg

gum; transdermal patch, 5–21 mg per day; nasal spray, 0.7 mg per 1-mg dose of 1 spray in each nostril; inhaler, 2 mg for a 4-mg dose released from the 10-mg inhaler; lozenge, 1 mg for a 2 mg lozenge; oral snuff, 3.6 mg for 2.5 g held in the mouth for 30 min; chewing tobacco, 4.5 mg for 7.9 g chewed for 30 min.

21. Cotinine is used as a biomarker for nicotine exposure and established methods are available to estimate nicotine dose based on plasma, urinary, or salivary cotinine measurement. Average blood cotinine concentrations in CC smokers are 250–300 ng/mL. A review based on 32 publications providing sensitivity/specificity cut-off data for cotinine levels in smokers and nonsmokers indicated values of 10–25 ng/mL (saliva), 10–20 ng/mL (serum), and 50–200 ng/mL (urine) based on self-reported status, with lower cut-off values of 3 ng/mL (serum) and 12 ng/mL (saliva) (Kim 2016).

Acute toxicity

22. Nicotine is described as acutely toxic to humans by all routes of exposure (inhalation, dermal, oral). Nevertheless, most reports of fatalities appear to relate to oral or dermal exposure. Symptoms of nicotine toxicity manifest as effects on the gastrointestinal, central nervous, neuromuscular, cardiovascular, respiratory, and glandular systems. Common symptoms of moderate intoxication include nausea, vomiting, abdominal pain, diarrhoea, headache, sweating, fatigue, and palpitations. More severe symptoms include faintness, dizziness, weakness, and confusion, progressing to muscle weakness, collapse, and respiratory arrest. Large doses lead to rapid onset of symptoms, with death resulting from paralysis of respiratory muscles due to peripheral neuromuscular blockade and cardiovascular collapse (HCN 2005).

23. In humans, a wide range of tolerance to the toxic effects of nicotine between individuals is reported. The lethal dose in adults is generally cited as 40–60 mg (0.6–1.0 mg/kg bw), while doses as low as 2–5 mg (0.03–0.08 mg/kg bw) are reported to cause toxic effects in some individuals (EPA 2008, EFSA 2009). A review article published in 2014 questioned the range for fatal dose of nicotine in humans that is generally cited (Mayer 2014). Based on correlations with blood and plasma nicotine levels measured in cases of fatal nicotine intoxication, the review by Mayer suggested that the lower limit for fatality is more likely to be in the range of 500–1000 mg ingested nicotine (6.5–13 mg/kg bw), corresponding to a lower limit of lethal nicotine concentration of 4 mg/L in plasma. In conducting this review, the author had traced back the history of reporting lethal doses for nicotine in humans, noting that a commonly cited original reference for these data (Lazutka, Vasiliauskene and Gefen Sh 1969)[8] is in fact a report of animal (but not human) data, which has subsequently been repeatedly, but incorrectly, cited. The original citation of a lethal dose of 60 mg

[8] Article in Russian

in humans was identified as likely to be a publication from 1906 (Kobert 1906)[9], for which a clear interpretation of the data described would be difficult to achieve.

24. A number of publications have described case reports or case series of poisoning incidents from ENDS liquids containing nicotine. Seo et al. (2016) reported a case in Korea in which a 12 kg, 15-month-old child died after ingesting 50 mg nicotine (5 mL ENDS liquid containing 10 mg/mL nicotine), despite vomiting immediately after ingestion. Urinary cotinine level 12 h after admission to intensive care was 1716 ng/mL. Park and Min (2018) described 2 cases of attempted suicide by ingestion of ENDS liquid, with estimated nicotine exposures of 23 mg/kg bw (27-year-old male) and 30 mg/kg bw (17-year-old female). Plasma nicotine levels were not measured. Both patients presented with seizure-like movement and cardiac arrest, and both had metabolic acidosis and transient cardiomyopathy. They were ultimately discharged from hospital with cerebral performance categories of 2 ('Cerebral disability but sufficient function for independent activities of daily living') and 4 ('Coma'), respectively. A report by Sommerfeld et al. (2016) also described 2 cases of acute nicotine poisoning resulting from intentional use of ENDS liquids in attempted suicide (1 oral, 1 i.v.). In the case of oral exposure, symptoms of nicotine exposure without convulsions occurred in association with serum nicotine and cotinine concentrations of 0.096 mg/L and 4.4 mg/L, respectively. The subject with i.v. exposure showed no classic signs of nicotine toxicity except for unconsciousness and slow respiration, with serum nicotine and cotinine of 0.8 and 1.3 mg/L, respectively. Belkoniene et al. (2019) reported a case of attempted suicide in which a 51-year-old man injected 1000 mg nicotine i.v. (10 mL ENDS liquid containing 100 mg/mL nicotine in PG). At 18 h post injection, plasma nicotine and cotinine concentrations were 0.012 mg/L and 3.2 mg/L, respectively. The patient presented with agitation, followed by coma and bradypnoea requiring mechanical ventilation. Transitory neurological impairment was followed by complete recovery.

25. Hua and Talbot (2016) summarised a series of case reports concerning potential health effects of E(N)NDS exposure in 26 individuals, identified from literature published between April 2012–January 2016. Of these, 12 described nicotine poisoning by accidental or deliberate exposure to ENDS liquids (3 paediatric, 9 adult). Further details of these 12 cases are given in Table 1, below.

[9] Article in German

Table 1. Case reports involving health effects of E(N)NDS. Data reproduced (modified) from Table 1 of Hua & Talbot (2016).

Patient Demographics (Age, Sex, Country); Citation	**Pre-existing Medical History**	**(1) Smoking History (2) E(N)NDS Device/ Refill Fluid Info**	**(1) Presentation/Signs (2) Diagnosis**	**(1) Treatment (2) Health Outcome**
Nicotine Poisonings				
Accidental Poisonings				
10 months, male, USA; Basset et al (2014)	N/A	(1) N/A (2) N/A; accidental ingestion of Wintergreen E(N)NDS refill fluid containing 18 mg/mL nicotine; unknown PG, glycerol and flavouring concentrations	(1) Vomiting, tachycardia, grunting respiration, truncal ataxia developed after ingestion of E(N)NDS fluid. (2) Nicotine poisoning	(1) Unspecified medical treatment. (2) Recovered based line health 6 h after ingestion of fluid.
30 months, female, UK; Gupta et al. (2014)	N/A	(1) N/A (2) N/A; accidental ingestion of E(N)NDS refill fluid. No brand or content details available.	(1) Vomiting (2) Nicotine poisoning	(1) No treatment; observed to be systemically well. (2) Resolved without intervention.
2 y, female, Canada; Gill et al. (2015)	N/A	(1) N/A (2) N/A; accidental ingestion of E(N)NDS refill fluid. One 60 mL bottle of grape-flavoured fluid containing 24 mg/mL nicotine suspected, brand not specified.	(1) Sudden onset of vomiting and irritability resolving at time of presentation. (2) Nicotine poisoning: potential mild toxicity effects.	(2) No treatment: observed to be at patient baseline for 2 h before discharge. (2) Resolved without intervention.
Poisonings caused by intentional misuse and/or abuse				
22 y, female, Italy; Cervellin et al. (2013)	N/A	(1) N/A for CC smoking. Opioid addiction. (2) Brand or fluid not specified. Mixed residual content of E(N)NDS fluid (10 mL of 0.8% solution) with 0.8% methadone. Injected 2 mL and ingested 60 mL?	(1) Tachycardia, flushing, salivation, and nausea. (2) Nicotine poisoning by intentional ingestion and abuse of nicotine/drugs.	(1) Unspecified medical treatment (if any). Patient observed and discharged after psychiatric counselling and directed to addiction services. (2) No follow-up details.
Suicide attempts				
36 y, female, Denmark	N/A	(1) N/A (2) No E(N)NDS brand specified. Ingested 20 mL refill fluid containing 18 mg nicotine.	(1) No symptoms (2) N/A	(1) Admitted to emergency ward; treated with activated charcoal. (2) No follow-up details.

Patient Demographics (Age, Sex, Country); Citation	**Pre-existing Medical History**	**(1) Smoking History (2) E(N)NDS Device/ Refill Fluid Info**	**(1) Presentation/Signs (2) Diagnosis**	**(1) Treatment (2) Health Outcome**
(2 x suicide attempts, same patient); Christensen et al. (2013)		(1) N/A (2) No E(N)NDS brand specified. Ingested 50 mL refill fluid containing 30 mg nicotine.	(1) Two hours. after ingestion abdominal pain, nausea, vomiting. (2) Nicotine poisoning by intentional ingestion.	(1) Treated with activated charcoal and 6 h observation. (2) No follow-up details.
13 y, male, Denmark; Christensen et al. (2013)	N/A	(1) N/A (2) No E(N)NDS brand specified. Ingested 3 mL refill fluid, no details on contents.	(1) Nausea and shivering. (2) Nicotine poisoning by intentional ingestion; mild toxicity.	(1) Activated charcoal treatment. (2) No follow-up details.
24 y, male, Germany; Eberline et al. (2014)	Depression; sexual identity issues.	(1) N/A. (2) No E(N)NDS brand specified. Ingested 1 liquid capsule containing 180 mg nicotine.	(1) 10 minutes after ingestion, patient vomited. Nausea persisted for a few hours. Dizziness. (2) Nicotine poisoning by intentional ingestion.	(1) Activated charcoal treatment. (2) Symptoms cleared, unspecified outcome.
22 y, male, USA; Valento (2013)	N/A	(1) N/A (2) No E(N)NDS brand specified.	(1) Dizziness, nausea, mild tremor and brachycardia. Ingested 30 mL E(N)NDS liquid containing 24 mg/mL nicotine. Rubbed additional 30 mL dermally. (2) Nicotine poisoning by intentional ingestion.	(1) Skin decontamination prior to emergency room visit; unspecified medical treatment. (2) Complete recovery.
29 y, male, USA; Thornton et al. (2014)	History of depression.	(1) Previous CC smoker; history not detailed. (2) N/A; No E(N)NDS brand or refill fluid specified. Fatal intravenous injection using E(N)NDS refill fluid.	(1) Cardiovascular resuscitation; full body seizure. (2) Death caused by intentional intravenous injection of E(N)NDS refill fluid. Declared brain dead on day 5.	(1) N/A. (2) Death.
27 y, male, Netherlands; Schipper et al. (2014)	Borderline personality disorder.	(1) N/A (2) N/A; No E(N)NDS brand specified. Ingested 5 E(N)NDS 'fillings' containing a total of 420 mg nicotine.	(1) Before emergency room arrival, patient vomited 3 times. (2) Nicotine poisoning by intentional ingestion.	(1) Activated charcoal (3x); 24 h observation period. (2) Discharged after 30 h observational period with no adverse events and with psychiatric consultation.

Patient Demographics (Age, Sex, Country); Citation	**Pre-existing Medical History**	**(1) Smoking History** **(2) E(N)NDS Device/ Refill Fluid Info**	**(1) Presentation/Signs** **(2) Diagnosis**	**(1) Treatment** **(2) Health Outcome**
34 y, male, Germany; Bartschat et al. (2015)	Psychosis	(1) N/A (2) N/A; No E(N)NDS brand specified. Suspected liquid containing 72 mg/mL nicotine. Mother of patient also found 5 'Titanium Ice' nicotine solution vials, of which 3 were empty.	(1) Medical autopsy revealed fatal and significant nicotine concentrations in femoral blood (5.5 mg/L), lungs (89.5 mg/kg), and in kidney tissue (42.6 mg/kg). (2) Fatal E(N)NDS refill fluid ingestion.	(1) N/A. (2) Death.
24 y, female, USA; Chen et al. (2015)	Unspecified psychiatric disorder	(1) N/A. (2) N/A; E(N)NDS brand not specified. 15 mL vials containing 100 mg/mL nicotine found.	(1) Medical autopsy revealed plasma nicotine concentration > 1000 ng/mL. (2) Fatal E(N)NDS refill fluid ingestion.	(1) N/A. (2) Death; multiple acute infarcts; severe anoxic brain injury. Died 3 days post ingestion.

N/A, not available

26. Vardavas et al. (2017) gathered data on ENDS liquid poisoning incidents reported to poison centres in 28 European Union Member States over the period 2012–2015. A total of 343 incidents were reported, of which 277 provided data for analysis (42.7% in children, 57.2% in adults). In 39.4% of cases no medical effects were reported, while the remaining cases listed minor (53.8%), moderate (6.3%), or major (0.5%) medical effects. No deaths occurred. Of the 60.6% of cases that listed a medical outcome, 54.8% were associated with ingestion, 28.6% with inhalation, 9.5% ocular, and 7.9% dermal exposure. The most common clinical symptoms were vomiting (20.3%), dizziness (14.5%), and nausea (13.8%). However, no data were provided on the exposures other than route.

27. 'Green-tobacco sickness' (GTS) is a non-fatal, acute toxicity that has been reported in tobacco workers in the field who have direct skin contact with tobacco plants, particularly in wet conditions. Symptoms of intoxication include nausea, vomiting, weakness, and dizziness. This condition has also been reported in tobacco factory workers with inhalation exposure to nicotine-containing dust (HCN 2005). The Nordic Expert Group toxicological review of nicotine noted that in studies of field tobacco workers, cotinine levels were highest (average 890 µg/L) in those who picked tobacco leaves, with one study reporting that 25% of these workers had symptoms of GTS (dizziness and nausea) (NIWL 2005).

28. In an analysis of data from randomised controlled trials of nicotine replacement therapy (NRT) for quitting or reducing CC smoking, Tonstad et al. (2014)[10] found that for the approximately 10% of participants (n = 746 of 7120 total) in whom cotinine levels increased > 50% over baseline, typical symptoms of nicotine overdose (nausea, vomiting, palpitations, dizziness, headache) were rare.

29. In 2007, a DAR for nicotine as a fumigation formulation proposed ARfD and ADI values that were established using data from a report by Woolf et al. (1997) (see paragraph 9). Woolf described a post-marketing surveillance study that included data collected at US poison centres on 36 children aged 0–15 y (mean, 3 y) who were exposed to transdermal nicotine patches. Eighteen children were dermally exposed and 18 were orally exposed (biting, chewing or swallowing the patch). Twenty-two children were asymptomatic, while 14 (9 dermal exposure, 5 oral exposure) had symptoms, which included nausea, vomiting, abdominal pain, weakness, dizziness, and localised rashes. Symptoms from oral exposure were transient, while those from patches were longer lasting, and symptoms were more likely to occur with newly opened patches than from used patches. Nicotine exposures were calculated for the cases of dermal exposure, based on known dose/delivery rates of the patches, and verified body weights. Symptoms were present in 4/4, 1/2, and 2/7 children at estimated doses of 0.1 mg/kg bw, 0.01 mg/kg bw, and < 0.01 mg/kg bw nicotine, respectively. The mean estimated nicotine dose for symptomatic children was 0.18 mg/kg bw, compared with 0.006 mg/kg bw for non-symptomatic children.

[10] Declaration of interests: "GG, EK, JMW, ÅW [authors] are employees of McNeil AB, a company developing and manufacturing smoking cessation medication."

30. In 2009, EFSA established an oral ARfD for nicotine, based on a study by Lindgren et al. (1999) (EFSA 2009) (see paragraph 7). In the study by Lindgren, dose-response relationships for electroencephalographic (EEG) parameters and heart-rate frequency over a range of nicotine doses were evaluated in 14 regular CC smokers. Participants abstained from nicotine for 12 h (baseline plasma nicotine <4.0 ng/mL), after which they were given i.v. infusions of 0, 0.0035, 0.007, 0.014, and 0.028 mg/kg bw nicotine over a 10 min period in a single-blind, randomised crossover design with 2 test sessions per day. Recordings were made for EEG (6 segments), auditory P300, and heart rate. Nicotine infusions increased heart rate in a dose- and time-dependent manner. For EEG, linear dose-related decreases of delta and theta power were found, along with increases in alpha power and alpha peak frequency. $Alpha_1$, beta, and P300 parameters were unaffected.

31. The toxicological reviews by Swedish National Institute for Working Life Nordic Council (NIWL 2005) and Health Council of the Netherlands (HCN 2005) identified one study describing acute effects of inhalation exposure to nicotine in humans. In this study, reported by Hansson et al. (1994), healthy, nonsmoking subjects (3 men, 2 women) took a single-breath of 0.01 mL nebulised nicotine solution at times 0 and 10 min. No cardiovascular effects were noted at concentrations up to 64 mg/mL. Effects were also evaluated in 8 nonsmoking volunteers (4 men, 4 women) who inhaled nicotine solutions of 0, 2, 4, or 8 mg/mL on 4 different days. A single breath was taken every 15 s up to 5 min (total 21 inhalations), giving a total dose of 0, 0.4, 0.8, or 1.7 mg nicotine per 5 min. Heart rate and systolic blood pressure, observed over the subsequent 30 min, were significantly increased at all doses, in a dose-related manner, compared with the vehicle-exposed controls. Maximal responses were seen within 3 min after nicotine inhalation, and the responses lasted between 6 and 10 min. There were no significant changes in diastolic blood pressure. Nicotine caused a decrease in skin temperature, with maximal response at 5 min. Seven of the subjects complained of headache, which reached a maximum at 5–6 min and lasted for 20 min. None of the subjects noticed a tremor or nausea. Coughing was triggered by a single inhalation at a dose of 0.04 mg nicotine.

32. Animals are reported to tolerate higher doses of nicotine than humans (UK-DAR 2007). Toxicological reviews note that nicotine is acutely toxic in laboratory animals by all routes of exposure (oral, dermal, and inhalation). However, data on toxicity via inhalation are scarce. The Netherlands Health Council report summarised LD_{50} values in laboratory studies, covering intratracheal, dermal, oral, intraperitoneal (i.p.), and i.v. exposure in mice, rats, rabbits, and dogs, ranging from 3.3 mg/kg bw (oral, mouse) to 188 mg/kg bw (oral, rat) (HCN 2005). The REACH dossier for nicotine reported an acute dermal LD_{50} of 70.4 mg/kg bw in New Zealand White rabbits (OECD 402) (Unnamed study report, 2015[11]) and an acute oral LD_{50} of 77.83 mg/kg bw in NMRI mice (OECD 425) (Unnamed study report, 2015[12]).

[11] The original study report was not seen.
[12] The original study report was not seen.

33. Shao et al. (2013) carried out tests using a nicotine delivery method targeting respirable aerosol to the alveolar region of rodents. Acute inhalation toxicity was evaluated using an Up and Down procedure, according to EPA guideline. The study used nicotine freebase dissolved in water or saline solution, pH adjusted with HCl, nebulized to aerosol with a mass median aerodynamic diameter (MMAD) of approximately 2.5 µm (geometric standard deviation (GSD) 1.8). Male Sprague-Dawley rats were nose-only exposed to aerosols at a fixed pressure of 40 psi, for 20 min, at progressive concentrations in the range of 5–56% nicotine, pH 8.0. An LC_{50} value (20 min) of approximately 32% nicotine in the nebulizer was identified, which was calculated as a concentration of 2.3 mg/L [2300 mg/m^3] in breathing air (confidence interval, 1.24–4.07 mg/L). Testing at lower pH values indicated similar findings at pH 7.4, while at pH 6.8 the LC_{50} was higher (estimated to be > 4.1 mg/L), which the authors considered was probably due to reduced absorption and/or bioavailability of acidified nicotine in the lungs. The study also investigated nicotine pharmacokinetics in arterial and venous blood. Rats were exposed to 1% nicotine for 2 min and plasma nicotine and arterial and venous cotinine were evaluated over 40 min. Maximal arterial nicotine (43.2 ng/mL) occurred at 1–4 min, which declined to 16.0 ng/mL after 20 min. Venous plasma nicotine increased to 21.0 ng/mL at 3.5 min and was between 15–25 ng/mL over the following 36 min. These findings were noted to be similar to the pharmacokinetics of nicotine in humans via smoking CC. The authors concluded that the magnitude and rapid rise in arterial nicotine concentration were consistent with the hypothesis that nicotine aerosol reaches and deposits in the alveolar region where it is quickly absorbed into the pulmonary circulation during aerosol inhalation. Based on the study of Shao et al. (2013), an LC_{50} (20 min) of 2.3 mg/L [2300 mg/m^3] air for nicotine (aerosol in water) in Sprague-Dawley rats was reported in the REACH dossier for acute inhalation toxicity, with conversion using Haber's law, according to point 3.1.2.1. (c) of the CLP Regulation (Cn x t = constant), to an LC_{50} (4 h) of 0.19 mg/L [190 mg/m^3].

34. A study to evaluate acute effects of exposure to nicotine in saline solution via a nose-only nebulizer system in rats was reported by Ahmad et al. (2019). Anaesthetized male Sprague-Dawley rats were exposed for 15 min to aerosol produced from a 0% (control), 5%, or 10% solution of nicotine in sterile saline, mixed with air and delivered at a rate of 6 L/min (1 L/min of aerosol). For the 5% nicotine solution, the measured air nicotine concentration was 24.47 mg/m^3, and aerosol particle MMAD was 1.12 µm (GSD 2.39), considered to be consistent with the size characteristics of ENDS aerosols. A dose-dependent increase in plasma cotinine, collected 6 h after exposure, was observed, with levels in the range of approximately 10 ng/mL and 60 ng/mL from the 5% and 10% nicotine solutions, respectively. The authors considered these levels to be in the range of those measured in 'light' CC smokers or ENDS users. Heart rate, breathing rate, and oxygen saturation were monitored at various time points during 24 h after exposure. At both of the nicotine exposure levels, heart rate decreased during the first 4 h then increased back towards baseline levels by 24 h. Breathing rate and tissue oxygen saturation did not change (results not provided). In bronchoalveolar fluid lavage fluid (BALF) at 6 h post exposure, IgM levels were significantly increased in the 5% and 10% nicotine groups

compared with controls, while protein was significantly increased in the 10% nicotine group compared with controls. The authors considered these findings to indicate disruption of the alveolar-capillary barrier. Wet-to-dry lung weight ratio at 6 h (10% group) and 24 h (5% and 10% groups) post exposure, taken as an indicator of impaired fluid clearance and pulmonary damage, was also significantly increased compared with controls. Immunohistochemical analysis of lung tissue at 24 h post exposure showed extensive congestion in the vessels in the 10% group, with evidence of neutrophils in air spaces. Circulatory complete blood counts showed that neutrophil, white blood cell, eosinophil, and basophil counts were significantly increased in the 5% nicotine group at 3, 6, and 24 h post exposure compared with controls. Levels were not increased in the 10% nicotine group, except for neutrophils and white blood cells at 6 h. Parallel studies conducted using *in vitro* air-liquid interface cultures showed a dose-dependent loss of barrier and increase in epithelial cell death associated with nicotine exposure at levels approximately 1000-fold lower than those used in the *in vivo* studies. The authors concluded that these studies indicated that exposure to nicotine from ENDS may have adverse pulmonary and systemic effects.

Irritation and corrosion

35. Local and systemic skin reactions to nicotine patches have been reported in humans, and placebo-controlled trials have indicated statistically significant increases in local skin reactions in groups treated with nicotine-containing patches. Occupational dermatitis has been described in people who work in tobacco processing or nicotine production (HCN 2005).

36. A meta-analysis of data from randomised controlled trials (RCTs) listed in the Cochrane Tobacco Addiction Group trials register as of July 2017 evaluated the efficacy of NRT for smoking cessation (Hartmann-Boyce et al. 2018). As part of the analysis, adverse events related to use of NRT product were evaluated, with the following summary:

> "The only adverse event that appears to interfere with use of the patch is skin sensitivity and local skin irritation; this may affect up to 54% of patch users, but it is usually mild and rarely leads to withdrawal of patch use (Fiore 1992). The major adverse events reported with the nicotine inhalator and nasal and oral sprays are related to local irritation at the site of administration (mouth and nose respectively). For example, symptoms such as throat irritation, coughing, and oral burning were reported significantly more frequently with participants allocated to the nicotine inhalator than to placebo control (Schneider 1996); none of the experiences, however, were reported as severe. With the nasal spray, nasal irritation and runny nose are the most commonly reported adverse events. In the study of oral spray, hiccoughs and throat irritation were the most commonly reported adverse events (Tønnesen 2012). Nicotine sublingual tablets have been reported to cause hiccoughs, burning and

smarting sensation in the mouth, sore throat, coughing, dry lips and mouth ulcers (Wallstrom 1999). Adolescents report similar adverse events to adults (Bailey 2012)."

37. The REACH dossier noted that nicotine caused skin irritation but not corrosion on the treatment site of rabbits in both sexes in an acute dermal toxicity study (OECD 402), with erythema fully reversible within 3 (females) or 14 (males) days (Unnamed study 2015[13]). No other dermal effects were noted. The result was interpreted as Category 2 (irritant). Nicotine was highly corrosive to the eye in rats. Irreversible redness (grade 1), haemorrhage (grade 2) and opacity (grade 2) were observed in an *in vivo* study of high quality (OECD 405) (Unnamed study 2018[14]). Irritating effects of tissues were also observed *in vitro* (OECD 492) after 30 minutes. Data were interpreted as Category 1 (irreversible effects on the eye). All studies were GLP compliant and considered to be of high quality (Klimisch 1).

Sensitisation

Skin

38. The HCN (2005) review of nicotine toxicity listed a study in which sensitisation responses to pure nicotine were studied in 10 men and 4 women who had previous adverse skin reactions from the use of nicotine patches. Aqueous solutions of 1%, 10%, and 50% nicotine base or 5% nicotine sulphate were applied under occlusion for 2 to 3 days. Positive allergic patch test reactions were noted in 1/14, 4/14, and 5/14 subjects at nicotine base concentrations of 1%, 10%, or 50%, respectively, and in 1/14 subjects tested with 5% nicotine sulphate. At the highest concentration, irritant reactions from occlusion occurred in the remaining 9 subjects (Bircher et al. 1991, *cited in* HCN 2005). The HCN Committee concluded that nicotine is a skin irritant and sensitizer.

39. The REACH dossier for nicotine noted one skin sensitisation study *in vivo* (local lymph node assay, LLNA) (OECD 429), which was GLP compliant and of high quality (Klimisch 1). Nicotine was not sensitising under the conditions of the study (Unnamed study 2014[15]).

Respiratory

40. The HCN (2005) review listed a study in which respiratory effects of nicotine were investigated in 13 nonsmoking subjects. Each participant inhaled a single breath of 0.01 mL nebulised nicotine solution at concentrations of 1, 2, 4, 8, 16, 32, or 64 mg/mL, with a 15-minute time interval between inhalations, either on one day or on three separate days. A concentration-dependent cough response and airway obstruction was produced, which was reproducible over three different days.

[13] The original study report was not seen.
[14] The original study report was not seen.
[15] The original study report was not seen.

According to the authors, these effects were due to stimulation of afferent nerve endings in the bronchial mucosa and mediated by parasympathetic cholinergic pathways (Hansson et al. 1994).

41. No data were identified for respiratory sensitisation in animals.

Repeat dose toxicity

Inhalation

42. No data were identified on toxicity of repeated or long-term inhalation exposure to nicotine *per se* in humans.

43. Phillips et al. (2015)[16] reported a 28-day (OECD 412) inhalation toxicity study of nebulised nicotine, alone or in combination with pyruvic acid, to evaluate local and systemic effects in SD rats. In addition to classic endpoints, transcriptomic and lipidomic profiling was also performed on a subset of animals. Rats (n=10/sex/group, plus n=8 males/group for omics studies) were exposed for 6 h/day, 5 days/week, in a nose-only chamber, to either filtered air (Sham) or aerosols of phosphate-buffered saline vehicle (PBS) at physiologic pH; 50 µg/L [50 mg/m^3] nicotine (Nic); 33.9 µg/L pyruvate (Pyr); 18 µg/L nicotine/9.8 µg/L pyruvate, 25 µg/L nicotine/13.6 µg/L pyruvate, or 50 µg/L nicotine/27.1 µg/L pyruvate (Nic/Pyr groups). Aerosol MMADs were in the range of 1.4–2.0 µm (GSD 1.7–2.2). The authors calculated that a nicotine concentration of 50 µg/L represented a delivered dose (DD) to the rat of 13.6 mg/kg bw[17], and a human equivalent dose (HED) of 2.2 mg/kg bw[18]. This was considered to be equivalent to a daily dose of nicotine equivalent to smoking approximately 130 CC (132 mg for a 60 kg person). Liver-related effects assessed were liver weight, histopathology, transcriptomics and lipidomics. Rats exposed to Nic or Nic/Pyr had decreased body weight gains and concentration-dependent increases in liver weight. Blood neutrophil counts were increased and lymphocyte counts decreased in rats exposed to nicotine; activities of alkaline phosphatase and alanine aminotransferase were increased, and levels of cholesterol and glucose decreased. In non-respiratory-tract organs, the only histopathologic finding was increased liver vacuolation and glycogen content. In the respiratory tract, some nicotine exposure-related changes in the larynx were observed, which were considered by the authors to be adaptive changes. Gene expression changes in the lung and liver were described by the authors as ‘very weak’. Nic and Nic/Pyr was associated with significant upregulation of Cyp1a1 gene. Other changes were predominantly related to energy metabolism and fatty acid metabolism but were not considered to indicate an obvious toxicity-related response. Nicotine exposure

[16] Study funded by Philip Morris Products S.A.. Author affiliations listed as Philip Morris International Research Laboratories Pte Ltd, Science Park II, Singapore, and Philip Morris International R&D, Philip Morris Products S.A., Neuchatel, Switzerland

[17] Calculated by the authors as follows: DD = (C X RMV X D) / BW, where DD = delivered dose (mg/kg); C = concentration of substance in air (mg/L); RMV = respiratory minute volume (L/min); D = duration of exposure (min); and BW = body weight (kg), [DD = (0.05 mg/L X 0.194 L/min X 360 min) / (0.25 kg) = 13.6 mg/(kg BW), or 3.4 mg/rat (250 g BW)].

[18] Calculated as follows: HED = DD / 6.6 (from CDER 2005).

lowered plasma lipids, including cholesteryl ester and free cholesterol and, in the liver, phospholipids and sphingolipids. Nic, Pyr and Nic/Pyr decreased hepatic triacylglycerol and cholesteryl ester. In the lung, Nic and Nic/Pyr increased cholesteryl ester levels. The authors concluded that these data suggested that only minor biologic effects related to inhalation of Nic or Nic/Pyr aerosols were observed in this 28-day study.

44. A short-term, repeat dose inhalation toxicity study (OECD 422 – Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test) was listed in the REACH dossier for nicotine (Unnamed study, 2018[19]). The study was GLP compliant and was considered to be of good quality (Klimisch 1). Sprague-Dawley CD rats (10/sex/group) were exposed by nose-only inhalation to target nicotine vapour/aerosol in a vehicle of water at concentrations of 0 (sham-exposed controls exposed to room air), 10, 15 or 20 µg/L[20], for 6 h/d, 7 d/wk, for up to 5 weeks (males) or 10 weeks (females). The F_0 males were exposed during the pre-cohabitation, cohabitation and post-mating periods (approximately 35 days) and then euthanized and necropsied. The F_0 females were exposed during the pre-cohabitation and cohabitation periods and during gestation (gestation day (GD) 0 to GD 19) and lactation (lactation day (LD) 5 to LD 13) for approximately 70 days and then euthanized and necropsied. Adverse effects were noted at all exposure levels in adult males and females for clinical signs (including mortality), body weights and food consumption. Hepatocellular necrosis and/or hydropic degeneration occurred at ≥ 10 µg/L in unscheduled decedents and animals surviving to terminal sacrifice. Although the findings did not exhibit a clear dose-response relationship, they were considered to be related (directly or indirectly) to the test item, because they did not occur in the control groups and were present (at up to marked severity) in most of the unscheduled decedents at 15 µg/L. Microscopic changes secondary to nicotine-associated chronic stress occurred at ≥ 10 µg/L in the adrenal gland (cortical hypertrophy), thymus (atrophy), and uterus (atrophy) and correlated with stress-associated organ weight changes. Stress-associated microscopic changes were generally greater in incidence and/or severity in females than males and clinical findings resulting in welfare euthanasia occurred only in females. This was considered likely to reflect the longer duration of the test item exposure period for females. Pathology findings were not by themselves considered adverse except when viewed in conjunction with the mortality and other adverse effects seen in the study. The male and female parental systemic NOAEL ($NOAEL_{systemic}$, F_0) could not be determined because adverse effects were seen at all exposure levels in the parental animals, thus a LOAEL ($LOAEL_{systemic}$, F_0) was determined to be 10 µg/L [10 mg/m^3].

45. The reports by the NIWL (2005) and HCN (2005) groups noted a study in which female Sprague-Dawley rats were exposed by inhalation to 0.5 mg/m^3 nicotine for 2 years. In this study, reported by Waldum et al. (1996), rats were exposed to

[19] The original study report was not seen.
[20] Mean measured nicotine vapour/aerosol concentrations were 0, 10.87, 14.44, and 20.29 µg/L.

nicotine in 2 chambers (n=34 rats/chamber), both receiving the same supply of medical-grade air containing an average concentration of 0.5 mg/m^3 nicotine (range 0.40–0.65 mg/m^3), for 20 h/day, 5 d/week, for up to 103 weeks. Control animals (n=34) were placed in a separate chamber without nicotine. Mean nicotine concentrations in plasma, measured after 5 days and at the end of the study, were 108 ng/mL and 130 ng/mL in nicotine-exposed rats (considered to represent a plasma concentration found in heavy smokers), and < LOD (< 2 ng/mL) in controls. Macroscopic and microscopic examination at 6, 12, 18 months, and at the end of the study, revealed no statistically significant increases in incidences of fibroadenomas of the mammary gland, of adenomas of the pituitary gland, and of adenocarcinomas of the ovary, compared with the control group. Neither lung tumours nor any increase in pulmonary neuroendocrine cells were detected. The median absolute heart weights of exposed and control animals were not statistically significantly different, and no increase in atherosclerotic lesions was found. Macroscopic or microscopic examination of other tissues (brain, gastrointestinal tract, liver, kidneys) did not reveal treatment-related abnormalities. Body weights of nicotine-exposed rats were described as consistently approximately 5% lower than those of controls.

Non-inhalation

46. In an evaluation of nicotine as a pesticide in the format of smoke-generating canisters in greenhouses, US EPA (2008) selected a study in which rats were exposed for 10 days to nicotine in drinking-water study (Yuen et al. 1995) as the key study for the evaluation. Groups of non-pregnant (n=24/group) or pregnant (GD10, n=16/group) female Sprague-Dawley rats were given drinking water containing 0, 54, or 108 µmol/L nicotine hydrogen tartrate for 10 days. At termination, livers were fixed and examined histologically for fatty change, focal necrosis, confluent necrosis, and dark cell change. In non-pregnant rats, statistically significant increases in all 4 parameters were observed in the higher dose group compared with controls, and in all parameters, except fatty change, compared with the lower dose group. Effects were less pronounced in pregnant rats, with the higher dose being associated with a statistically significant increase in dark cell change (compared with control) and confluent necrosis (compared with control and the low-dose group). The US EPA evaluation considered the two nicotine doses tested to be equivalent to 1.25 mg/kg bw/day (NOAEL) and 2.5 mg/kg bw/day (LOAEL).

47. A study reported by Kim et al. (2010) investigated tissue distribution, urinary excretion, and effects on serum biochemical parameters of orally administered nicotine. Male Sprague-Dawley rats were treated for 3 weeks with doses of 1, 5, or 10 mg/kg bw/day nicotine (base) dissolved in water and administered orally once per day. During treatment, 24-h urine collections were made once per week. The numbers of animals per group were not reported, nor any details of treatment for the control group. Blood, heart, liver, kidney, brain, and lung tissues were collected 24 h after final dosing. In serum, a significant, dose-dependent increase in α-glutathione-S-transferase (α-GST) was noted. Aspartate aminotransferase (AST) and blood urea

nitrogen (BUN) were significantly increased at 10 mg/kg bw/day[21]. There were no significant differences between groups for other serum markers. Reduced body weight gain was reported in all treatment groups, with the decrease statistically significant at 4 weeks for 10 mg/kg bw/day nicotine compared with controls. Liver/body weight ratio at 4 weeks decreased significantly in all treatment groups compared with controls, but the ratio was not altered for other organs except for reduced kidney weight in the 10 mg/kg bw/day group. Excreted urine volume decreased significantly in the 5 and 10 mg/kg bw/day nicotine groups compared with controls. The authors considered that these observations were indicative of some liver and kidney damage associated with nicotine treatment. However, histopathological analyses of the tissues collected did not indicate any changes. Cotinine/nicotine/hydroxycotinine measurements in urine at 1 and 4 weeks indicated saturation of nicotine-metabolising enzymes at all nicotine doses, occurring more rapidly at the higher doses. Tissue analyses generally showed increased cotinine levels with nicotine dose, with changes being statistically significant for the 5 mg/kg bw/day compared with 1 mg/kg bw/day nicotine group in heart, kidney, and lung. Overall, the authors of this report concluded that decreased body weight and urine volume indicated toxicity that was slight at 1 mg/kg bw/day nicotine and more pronounced at higher doses, organ weight indicated primarily effects on the liver, but also the kidneys, and that increased serum α-GST, AST, and BUN were consistent with liver and kidney toxicity.

48. The HCN (2005) review of nicotine toxicity highlighted some studies in which rats had been exposed to nicotine via drinking water (Wenzel and Richards 1970, Welzl et al. 1988, Gaddnas, Pietila and Ahtee 2000). In the study of Gaddnas et al. (2000), 5-week-old rats given 60-65 mg/kg bw/day nicotine in drinking water for 50 days showed increased locomotor activity at day 50 compared with controls. No difference between exposed and control animals was observed 12–14 h after nicotine withdrawal. Concentrations of brain monoamines were increased in nicotine-exposed animals at day 50, but 23-25 h after nicotine withdrawal, only hypothalamic dopamine remained elevated. In the study of Welzl et al. (1988), 'young' and 'old' rats given 0, 20, or 50 mg/L[22] nicotine in drinking water for 131 days had significantly decreased body weight and increased locomotor activity compared with controls, while exploratory efficiency was attenuated in 'young' rats. Habituation and memory tasks were not affected by nicotine exposure. In the study of Wenzel and Richards (1970), rats were given 1.14 or 4.56 mg/kg bw/day nicotine for 34 weeks, after which half of each group was exposed to 6% oxygen for 12 hours. Mortality, gross cardiac lesions, haematocrits, and the activities of several heart enzymes, selected as potential indices of early cellular injury, were examined. The only effects reported in nicotine-only-treated animals were a statistically significant increase in the activity of myocardial enzymes isocitric dehydrogenase and acid phosphatase and a statistically significant decrease in the activity of ß-glucuronidase at the high dose.

[21] Results of serum measurements were listed for the control and 3 nicotine-treatment groups, but details of statistical analyses or which group-comparisons were performed were not given.
[22] Calculated in the HCN (2005) report as possibly equivalent to 1.5–3.8 mg/kg bw/day for 'young' rats, and 1.1–2.8 mg/kg bw/day for 'old' rats.

HCN (2005) also mentioned one study in which rats were exposed to nicotine via subcutaneous osmotic minipump (Singh et al. 2000). In this study, exposure to 1 mg/kg bw/day for 3 weeks inhibited ConA-induced proliferation of peripheral blood cells and spleen cells, with effects persisting 2 weeks after removal of nicotine.

Cardiovascular effects

49. Lee and Fariss (2017)[23] conducted a systematic review and meta-analysis of serious adverse health effects (SAHEs) associated with use of pharmaceutical[24] NRT products, including data from both RCTs and population-based epidemiological studies. The main aim of the meta-analysis was, where possible, to use published study data to evaluate outcomes for NRT use compared with no NRT use, in smokers. A total of 11 studies were identified relating to CVD and/or stroke. Overall, these studies were considered to be of poor-to-fair quality, with weaknesses including few relevant events, short follow-up periods, and lack of control for confounders, including smoking habits after starting NRT use. Analysis was carried out based on acute myocardial infarction (AMI), mortality and stroke.

50. Acute myocardial infarction. Studies were based on small numbers of cases. Four studies (Joseph et al. 1996, Kimmel et al. 2001, Mohiuddin et al. 2007, Woolf et al. 2012) showed a relative risk (RR) < 1.00 for NRT vs. no NRT, but outcomes were not statistically significant. One uncontrolled study showed an increased RR of 5.63 (95% confidence interval (CI) 1.07–29.64) for AMI in HIV-infected patients provided NRT vs. no NRT, although the NRT group had a higher rate of coronary heart disease at baseline (Elzi et al. 2006). One population study indicated relative incidences for AMI of 5.55 (95% CI 4.42–6.98) in the 56 days prior to NRT prescription and 1.27 (95% CI 0.82–1.97) in the 56 days after NRT prescription, suggesting an effect of AMI on subsequent prescription of NRT (Hubbard et al. 2005). Lee and Fariss (2017) calculated a combined random-effect meta-analysis estimate for all studies for AMI of 0.97 (95% CI 0.55–1.71).

51. Mortality (deaths considered to be likely to have occurred from CVD). RRs were mostly < 1.00 for NRT vs. no NRT, with one study showing a significant effect of NRT on lower mortality (RR = 0.23, 95% CI 0.07–0.73) (Mohiuddin et al. 2007), although another study showed increased RR (6.06, 95% CI 1.65–22.21) in a very small number of patients undergoing coronary artery bypass surgery (Paciullo et al. 2009). The random-effect meta-analysis based on all studies showed no significant overall effect, with an RR of 0.81 (95% CI 0.41–1.60) (Lee and Fariss 2017).

52. Stroke. Three studies were identified (Hubbard et al. 2005, Joseph et al. 1996, Panos et al. 2010). The population study of Hubbard et al. (2005) showed a relative incidence of 3.59 (95% CI 2.56–5.03) for stroke in the 56 days prior to NRT prescription and a value of 1.30 (95% CI 0.77–2.19) in the 56 days after NRT

[23] Work funded by Altria Client Services LLC

[24] Thus excluding smokeless tobacco products and ENDS, which are not currently approved and licensed as drugs or medicines to aid smoking cessation (Royal College of Physicians, 2016; US Food and Drug Administration, 2016, *cited by* Lee and Fariss, 2017)

prescription, which Lee and Fariss suggested to indicate reverse causation of NRT prescription and stroke. The other 2 studies showed no evidence of risk of stroke but were considered to be inadequate to make clear conclusions (Lee and Fariss 2017).

53. As part of a meta-analysis of NRT for smoking cessation, using data from RCTs listed in the Cochrane Tobacco Addiction Group trials register as of July 2017, Hartmann-Boyce et al. (2018) also evaluated incidence of adverse events, including cardiovascular events. The authors noted that some studies have shown association of NRT use with increased incidence of chest pains and palpitations, but not with more severe adverse cardiovascular events. They cited 2 meta-analyses published by Mills and colleagues that had evaluated adverse events associated with NRT. The first of these meta-analyses, which included 92 RCTs and 28 observational studies, calculated an odds ratio (OR) of 2.06 (95% CI 1.51–2.82) across 12 studies for reported chest pains and heart palpitations among NRT users compared with placebo groups (Mills et al. 2010). A subsequent meta-analysis of cardiovascular events associated with smoking cessation pharmacotherapies, which included 21 RCTs, indicated a statistically significant increased rate of cardiovascular events with NRT compared with placebo (RR = 2.29, 95% CI 1.39–3.82) (Mills et al. 2014). However, analysis was restricted to serious adverse cardiac events (myocardial infarction, stroke, and cardiovascular death) and had not shown a statistically significant difference (RR = 1.95, 95% CI 0.26–4.30). A sensitivity analysis indicated that the increased risk was associated with cardiovascular events such as tachycardia and arrhythmia. Hartmann-Boyce et al. (2018) used a similar data set to Mills et al. (2010), including data from a total of 11,074 participants in 15 trials. The OR for chest pains or palpitations for any form of NRT relative to control was 1.88 (95% CI 1.37–2.57), and the overall frequencies of these events were low (2.5% and 1.4% in NRT and placebo groups, respectively). Finally, on the basis of their review of the available literature, Hartmann-Boyce et al. (2018) considered that studies focussing specifically on NRT use in CC smokers with pre-existing cardiac disease have generally indicated no difference in serious adverse cardiovascular events associated with NRT use.

54. The NIWL (2005) review of nicotine toxicity noted some animal studies that had been carried out to assess cardiovascular effects of nicotine, generally using high doses of nicotine. Male rats given nicotine in drinking water at 0, 1.14, or 4.56 mg/kg bw/day for 34 weeks had elevated mortality in response to hypoxia at the highest dose, which was reported to be similar to that from heavy CC smoking (>20 CC/day). There was no effect on haematocrit (Wenzel and Richards 1970). In other studies, high-dose intramuscular (i.m.) or s.c. injections of nicotine led to changes in plasma lipoproteins (Fisher et al. 1973, Strohschneider et al. 1994, *cited in* NIWL 2005). Rabbits with hypercholesterolemia given high dose (11 mg/kg bw/day) of nicotine by i.m. or s.c. injection developed atherosclerosis (Strohschneider et al. 1994, *cited in* NIWL 2005). Lower doses of nicotine (1 mg/kg bw/day) and a low-cholesterol diet had no effect (Fischer et al. 1973 *cited in* NIWL 2005). In the study of Waldum et al. (1996) (described in paragraph 45), rats exposed by inhalation to 0.5 mg/m^3 nicotine for up to 2 years showed no higher

incidence of mortality or atherosclerosis than controls. The NIWL Group concluded that at plasma levels equivalent to those found in CC smokers and users of non-combustible tobacco (plasma nicotine > 10–20 µg/L), nicotine stimulates the sympathetic nervous system, accelerates heart rate, and raises blood pressure, and can also affect lipid metabolism and cause endothelial damage related to the development of arteriosclerosis, but without convincing evidence of effects at lower exposure levels.

55. Literature searches identified some additional studies that indicated potential atherogenic effects of nicotine. Treatment of low-density lipoprotein (LDL) -/- mice fed an atherogenic diet with a 90-day time-release nicotine pellet (5 mg, implanted s.c.) (steady blood nicotine concentration of approximately 57 ng/mL, considered to be in the range of levels in heavy CC smokers) led to increased aortic lesion size compared with mice implanted with a placebo pellet, with upregulation of atherogenic mediators in the nicotine-treated animals (Lau et al. 2006). Zhou et al. (2013) reported that treatment of atherosclerosis-prone apoE-/- mice for 15 weeks with high-fat diet and nicotine (100 mg/L in drinking water, producing a plasma cotinine level of around 15 ng/mL) markedly exacerbated inflammatory monocyte levels and atherosclerotic plaque accumulation in apoE-/- mice, while these effects were not seen in $CD36^{-/-}$ $apoE^{-/-}$ mice, indicating a role of enhanced CD36 expression.

56. Further studies were identified that indicated effects of nicotine on cerebral ischaemia.

57. Paulson et al. (2010) evaluated effects of nicotine treatment, at a level considered to represent that to which heavy CC smokers are exposed, on response to middle cerebral artery occlusion (MCAO) in mice. Nicotine treatment by i.p. injection, calculated as equivalent to 4.5 mg/kg bw/day, at 1, 3, or 5 h prior to MCAO resulted in significant increase in oedema ratio (ipsilateral/contralateral hemisphere slice) and infarct ratio (infarct area/brain slice area) in comparison with saline-treated controls. Subsequent experiments in which nicotine exposure was given via s.c. osmotic minipump at 4.5 mg/kg bw/day for 1 day, 1 week, or 3 weeks (with associated plasma levels, respectively, of 45.0, 71.7, or 60.42 ng/mL (nicotine) and 269.2, 261.6, or 240.8 ng/mL (cotinine)) showed similar findings for oedema ratio but no effect of nicotine treatment on infarct ratio. Locomotor activity measurements showed no effect of 1 h nicotine treatment alone, but significantly greater reduction in parameters of locomotor activity after MCAO in nicotine-treated compared with saline-treated mice. However, no significant effect of nicotine to enhance MCAO-associated, reduced locomotor activity was seen with the longer term (3 week) nicotine treatment. The authors suggested that these findings indicated the potential of nicotine exposure to worsen stroke outcome by increased oedema formation.

58. Bradford et al. (2011) investigated the effect of nicotine on cerebrovascular endothelium in ischaemia/reperfusion injury. Initially, nicotine was administered to C56BL/6 mice by s.c. osmotic minipump at 0, 0.5, 2.0, or 5.0 mg/kg bw/day for 14 days. Plasma nicotine and cotinine levels, respectively, at day 13 were 4.5 and

10.2 ng/mL (0.5 mg/kg bw/day), 99 and 169 ng/mL (2.0 mg/kg bw/day), and 184 and 523 ng/mL (5.0 mg/kg bw/day), which were considered to reflect concentrations in 'moderate, average and heavy smokers'. All nicotine-treated mice showed reduced body weight from day 10 onwards. Analysis of isolated brain microvessels after 14 days in the 2 mg/kg bw/day nicotine-treated mice indicated higher expression of inflammatory mediators, cytokines, chemokines, and adhesion molecules. After ischaemia/reperfusion injury (MCAO), nicotine showed dose-dependent effects, with increased brain infarct size, worse neurological deficits, and a higher mortality rate. Nicotine exposure was also associated with enhanced leukocyte infiltration into brain after transient MCAO, while this was not noted in the absence of MCAO. The authors considered that these experiments highlighted nicotine regulation of brain endothelial cell phenotype and postischaemic inflammatory response at the brain-vascular interface.

59. In an evaluation of the effect of nicotine on cerebral microvessel thrombosis, mice (strain TO) were treated by daily i.p. injection with 1 mg/kg bw/day nicotine or saline control for 21 days. Plasma levels of superoxide dismutase (SOD), lactate dehydrogenase (LDH), liver enzymes, creatinine and blood urea nitrogen (BUN) were measured and histopathological studies were carried out. Nicotine was associated with significantly decreased plasma SOD, increased LDH, increased liver aspartate aminotransferase (AST) and alanine aminotransferase (ALT). BUN was not altered by nicotine treatment. Histological analysis of lung tissue showed intravascular thrombosis with necrosis, and macrophage and neutrophil infiltration in nicotine-treated but not control mice. Histological analysis of liver showed foci of intravascular thrombosis and portal inflammation. Heart and kidney tissues showed no histopathological changes in either control or nicotine-treated mice. After bolus i.v. fluorescein injection, a photo insult of cerebral microvessel was performed and platelet aggregation in microvessels was video-recorded and analysed. A significant prothrombotic effect was associated with nicotine exposure. The authors concluded that this 21-day nicotine exposure caused an increase in thrombosis in cerebral microvessels and systemic, hepatic, and pulmonary toxicity (Fahim et al. 2014).

Reproductive and developmental toxicity

60. The reproductive and developmental toxicity of nicotine has been reviewed in COT discussion papers TOX/2018/45 (gestational or early neonatal exposure) and TOX/2019/01 (adolescents and young adults). These papers noted that the majority of human data relate to nicotine exposure in the context of tobacco products (CC or smokeless tobacco), with some information also available from studies of the use of NRT during pregnancy. A large body of data was available regarding studies of developmental effects of nicotine in animal models, but this did not include standard 1- or 2-generation reproduction studies or standard developmental toxicity studies. Studies have used routes of exposure including subcutaneous osmotic minipump, i.v. or i.p. injection, and oral exposure via drinking water. Main findings described in TOX/2018/45 and TOX/2019/01are summarised in the following paragraphs, along

with any further data identified from literature search for the present discussion paper.

Inhalation

61. In humans, maternal CC smoking during pregnancy is causally associated with adverse pregnancy outcomes, while exposure to CC smoke *in utero* has been associated with adverse effects on development of the nervous and respiratory systems. However, no data were identified on reproductive or developmental effects of inhalation exposure to nicotine *per se* in humans.

62. A combined repeated dose study with reproduction/developmental toxicity in rats (OECD 422) was listed in the REACH dossier for nicotine (Unnamed study report 2018[25]). The study was GLP compliant and was considered to be of good quality (Klimisch 1). Sprague-Dawley CD rats (10/sex/group) were exposed by nose-only inhalation to target nicotine vapour/aerosol at concentrations of 0 (sham-exposed controls exposed to room air), 10, 15 or 20 µg/L [26], for 6 h/d, 7 d/wk, up to 5 weeks (males) or 10 weeks (females). Adverse effects at all exposure levels were noted in adult males and females. These data are described in paragraph 44. Test item-related effects on parental female oestrous cycling were noted at all exposure levels. Specifically, the percentages of irregular cycles ranged from 22 to 40% for the test item-treated females. The mean cycle lengths increased for all test item-treated females from 2.1 to 2.8 x control. Despite the test item-related effect on oestrous cycle number and duration, there was no impact on mating and fertility and therefore these effects were considered non-adverse. There were decreases in pup body weights on PND 7 (F_1 females), and 11 and 13 (F_1 males and females) and in body weight change for intervals PND 4 to 7, 7 to 11, and 11 to 13 (F_1 males and females) at all exposure levels. By PND 13 at 20 µg/L [20 mg/m^3], mean nicotine-exposed pup body weights were 22.6% and 27.2% less than the control pup body weights for males and females, respectively. The decreases in pup body weights occurred at the same intervals when the dams in the treated groups had decreased body weights, decreased body weight gain and decreased food consumption, therefore the decreased pup body weights were considered secondary to the maternal systemic toxicity. A parental reproductive NOAEL ($NOAEL_{reproductive}$, F_0) was determined to be ≥ 20 µg/L nicotine based on no adverse effects on no adverse effects at the highest dose. The F_1 NOAEL for growth and developmental toxicity ($NOAEL_{developmental}$, F_1) was determined to be ≥ 20 µg/L nicotine based on no adverse effects at the top dose. Authors concluded that there were no item-related adverse effects observed in the repeated dose toxicity study in reproductive organs or tissues or any adverse effects in the screening study in rats for reproductive toxicity by the inhalation route of exposure.

Non-inhalation

[25] Original study report not seen.
[26] Mean measured nicotine vapour/aerosol concentrations were 0, 10.87, 14.44, and 20.29 µg/L.

63. In humans, use of smokeless tobacco is associated with increased risk of preterm delivery, stillbirth, and orofacial clefts, but with little effect on fetal growth restriction.

64. Some data on the developmental effects in humans following maternal exposure to nicotine from non-tobacco sources are available from published studies on NRT use during pregnancy, including RCTs and population-based epidemiological studies. Overall, outcomes reported from studies of nicotine exposure via NRT use during pregnancy have been variable. Limiting factors include generally low rates of adherence to NRT use and of smoking cessation in RCTs, and a lack of data on the extent of CC smoking in association with NRT use and the small proportion of women in the NRT groups in epidemiological studies, leading to limited power. Studies have mostly evaluated short-term endpoints such as birth outcomes, thus with limited capability to identify adverse outcomes such as neurobehavioral effects which may only become apparent later in life. These data have been reviewed in detail in TOX/2018/45, and a summary of main findings is given in paragraphs 65–67, below.

65. RCTs of NRT use in pregnancy have been carried out in Europe (UK, Denmark, France), and the USA. The largest of these studies was the 'SNAP' trial for smoking cessation in pregnancy, carried out by Coleman and colleagues at the University of Nottingham, UK. This study included 1050 pregnant smokers randomised to NRT (15 mg/day over 16 hours) or placebo transdermal patches for periods of up to 8 weeks, starting between 12–24 weeks of pregnancy. No differences were noted in birth outcomes between NRT and placebo groups, other than a significantly increased rate of birth by caesarean section in the NRT group (OR = 1.45, 95% CI 1.05–2.01 for NRT compared with placebo). However, overall adherence to patch use and smoking cessation rates was poor (Coleman et al. 2012). A 2-year follow up of SNAP data suggested that 'survival without impairment' was higher in infants from the NRT compared with placebo group (OR = 1.40, 95% CI 1.05–1.86, p = 0.023) (Cooper et al. 2014).

66. Several epidemiological studies have used data from national databases to assess parameters of potential NRT use/exposure during pregnancy and their correlation with various pregnancy and infant/childhood developmental outcomes. Researchers at the University of Nottingham used data from 192,498 births registered between 2001–2012 in the UK 'THIN' general practitioner database. Women who had been prescribed NRT during a period from 4 weeks before conception to the end of the 1st trimester of pregnancy were identified and their smoking status was ascertained. Analyses of birth outcomes were carried out based on these data. Overall, approximately 1.4% of women had been prescribed NRT, 5.2% were smokers not prescribed NRT, and 93.4 % were nonsmokers who were not prescribed NRT. Evaluation of system-specific major congenital anomalies (MCAs) in infants born to these women, indexed by ICD-10 codes, showed no statistically significant difference between groups except for a significant increase in respiratory system anomalies in infants born to women prescribed NRT in

comparison with both non-NRT/nonsmokers (OR = 4.65, 99% CI 1.76–12.25, p < 0.001) and non-NRT/smokers (OR = 3.49, 99% CI 1.05–11.62, p = 0.007). The authors commented that the overall study findings had been limited by the low rate of NRT prescription during pregnancy and by the fact that certain morbidity rates were different between comparison groups. Notably the rate of asthma was higher in the NRT group (14.5%) compared with the no-NRT/smoker (10.4%) and no-NRT/nonsmoker (8.2%) groups (Dhalwani et al. 2015). A follow-up study of stillbirth in the same cohort found no effect of NRT prescription during the whole of pregnancy or the preceding 4 weeks on rate of stillbirth (Dhalwani et al. 2018).

67. Several studies have been carried out using data from the Danish National Birth Cohort (DNBC), which contains data from around 100,000 pregnancies in Denmark during 1996–2002. Studies of this cohort reported that NRT use during pregnancy was associated with increased rates of congenital malformations (RPR = 1.6, 95% CI 1.01–2.58 for nonsmokers who used NRT compared with nonsmokers who did not use NRT) (Morales-Suarez-Varela et al. 2006) and infantile colic (AOR = 1.6, 95% CI 1.0–2.5 for nonsmoker NRT users compared with nonsmokers who did not use NRT, and AOR = 1.5, 95% CI 1.3–1.8 for smokers using NRT compared with nonsmokers who did not use NRT) (Milidou et al. 2012). Use of NRT by women during pregnancy was also associated with an increased risk of ADHD in the child, but the findings were only statistically significant for women with nonsmoking partners (HR = 2.28, 95% CI 1.48–3.51 for NRT use/partner a nonsmoker; HR = 1.28, 95% CI 0.57–2.89 for NRT use/partner a smoker compared with children whose parents were nonsmokers and the mother did not use NRT) (Zhu et al. 2014).

68. Animal studies of nicotine exposure have shown adverse effects on the development of several organ systems, with a large part of the literature focussed on effects on development of the neurological and respiratory systems. The majority of studies administered nicotine during gestation via continuous subcutaneous osmotic minipump in order to avoid the acute high plasma nicotine levels associated with bolus injections, which may cause uteroplacental vasoconstriction and fetal hypoxia (Slotkin 2004). Nicotine (free base) doses in the range of approximately 1–6 mg/kg bw/day have been used in rodents, with 2 mg/kg bw/day and 6 mg/kg bw/day reported to reproduce plasma nicotine levels observed with moderate (10–20 CC/day) and high (20–40 CC/day) levels of smoking, respectively (Slotkin 2004).

69. The higher dose of 6 mg/kg bw/day has been associated with reduced maternal weight gain and fetal resorption, but these effects are generally not observed at 2 mg/kg bw/day. Studies in rodents, mostly rats, have shown that nicotine exposure *in utero* adversely affects structural brain development via interaction with nAChRs, leading to subtle structural alterations in the CNS that persist after birth. These alterations correspond with adverse effects on cognitive and behavioural development in offspring, with effects persisting through infancy, to adolescence, and into adulthood. It has been emphasized that effects are subtle and seen at exposures that do not lead to other secondary effects, indicating that growth

retardation *per se* is not an adequate measure for developmental neurotoxicity of nicotine (Slotkin 2008, England et al. 2017).

70. Further studies in this area have indicated that neurodevelopmental effects of nicotine vary between male and female offspring. One study modelled the adverse neurodevelopmental effects of nicotine at an exposure concentration equivalent to that in secondhand CC smoke (0.2 mg/kg bw/day) compared with tobacco smoke extract (TSE) containing an approximately equivalent nicotine concentration, and with a 10-fold higher nicotine exposure (2 mg/kg bw/day, representing exposure to 'moderate' direct CC smoking). There were no effects on maternal weight gain or toxicity, and only a small decrease in offspring postnatal weight gain in the TSE group. Various adverse effects were identified on cholinergic and serotonergic signalling systems in different brain regions of offspring in all exposure groups, with some sex-specific effects. Overall, the authors calculated that for male offspring, the effects of 0.2 mg/kg bw/day nicotine were significantly correlated with those of TSE and accounted for 36% of the TSE effects, while 2.0 mg/kg bw/day nicotine accounted for 46%. For female offspring, nicotine accounted for 13% of the TSE effects at 0.2 mg/kg bw/day and 7% at 2.0 mg/kg bw/day (Slotkin et al. 2015).

71. A follow-up study with the same exposure regimes found that all exposures led to disruptions of cognitive and behavioural function, with hyperactivity, working memory deficits, and impairments in emotional processing. Effects were greater with TSE exposure than with either dose of nicotine alone, with the magnitude of effects in the TSE-exposed group more in line with 2.0 mg/kg bw/day nicotine, and a lower level of effects seen at 0.2 mg/kg bw/day. This indicated that exposure to an approximately equivalent level of nicotine incurs a greater magnitude of adverse effects on cognitive and behavioural development in combination with other components of TSE than alone (Hall et al. 2016).

72. In the conclusions of the review of nicotine toxicity, NIWL (2005) highlighted a study by Ankarberg, Fredriksson and Eriksson (2001) that showed effects on memory and learning in 7-month-old mice treated by s.c. injection from PND 10–15 with 0.132 mg/kg bw/day nicotine. Changes in nicotine-induced motor behaviour at 4 months were observed at a dose of 0.066 mg/kg bw/day, while no effects were seen at 0.007 mg/kg bw/day. NIWL (2005) calculated that these two lower doses would be equivalent to air concentrations breathed by adult humans of 0.13 mg/m^3 and 0.014 mg/m^3, and considered them to represent LOAEL and NOAEL values, respectively. The Group concluded that although scientific material was not sufficient to identify a critical effect for nicotine, animal experiments indicated that the effect on reproduction, specifically development of the nervous system in the young, was likely to be the critical effect.

73. A summary of studies in rodents on the effects of prenatal or early postnatal nicotine exposure on cognitive and behavioural development in offspring, with doses and routes of exposure, is given in Table 2, below.

Table 2. Studies of the effects of prenatal or early postnatal nicotine exposure on cognitive and behavioural development in rodent offspring.

Study	**Nicotine dose reported** (mg/kg bw/day) – maternal dose unless otherwise stated	**Route of administration**	**Duration of administration**	**Outcome** (offspring, unless otherwise stated)
Nakauchi et al. (2015) [mouse]	21 (to nursing dams)	Subcutaneous osmotic minipump	PND1 - PND15	Deficits in long-term memory for object location and increased anxiety (male offspring tested at adolescence)
Vaglenova et al. (2004) [rat]	6	Subcutaneous osmotic minipump	GD3 - delivery	Reduced pup body weight; Alterations in locomotion, adaptation, anxiety, and cognitive behaviours, from birth into adulthood; effects more severe in females than males
Sorenson, Raskin and Suh (1991) [rat]	6.0 (approximately)	Drinking water	15 days prior to mating and throughout gestation	Reduced pup birth weight; Impaired ability to learn an 8-arm radial maze (both sexes, tested at PND45-65)
Sobrian et al. (1995) [rat]	5.0 (+ daily subcutaneous injections of saline)	Subcutaneous osmotic minipump	GD8 - GD21	Transient increase in locomotor activity that occurred within a 30-min delay following acute challenge with apomorphine on PND20-PND22
Sobrian, Marr and Ressman (2003) [rat]	5.0, 2.5 (+ daily subcutaneous injections of saline)	Subcutaneous osmotic minipump	GD8 - GD21	Increased risk taking behaviour in offspring at 12-14 months age at 5.0 mg/kg bw/day
Schneider et al. (2011) [rat]	4.61 ± 0.54	Drinking water	3 weeks prior to mating – delivery	Reduced pup birth weight and delayed sensorineural development; Deficits in tests of attention and impulsivity and altered learning ability in adulthood
Cutler et al. (1996) [rat]	4	Subcutaneous osmotic minipump	GD4 - GD21	Reduced maternal weight gain and pup birth weight; Altered cognitive function in response to adrenergic challenge in radial arm maze in adulthood

Study	**Nicotine dose reported** (mg/kg bw/day) – maternal dose unless otherwise stated	**Route of administration**	**Duration of administration**	**Outcome** (offspring, unless otherwise stated)
Paz et al. (2007) [mouse]	3.5 ± 0.4 (average)	Drinking water	2 weeks prior to mating, during gestation, with gradual step-down postnatally	Alterations in spontaneous locomotion, preference for cocaine-associated place, learned helplessness, increased learning of trace-conditioned, fear-associated cues in adult offspring
Yanai et al. (1992) [mouse]	3.0 (1.5, 2x per day) (pregnant dams) 1.5 (pups)	Subcutaneous injection	GD9 - GD18 (dams) or PND2 - PND21 (pups)	Reduced birthweight/body weight associated with nicotine treatments; weights had normalised before behavioural testing was carried out. Deficit in hippocampus-related behaviours in pups tested at PND50 in association with both maternal or postnatal exposure
Franke et al. (2008) [rat]	3	Subcutaneous osmotic minipump	GD4 - GD18	Alterations in natural and drug-induced reinforcement in adolescent male offspring
Levin et al. (1993) [rat]	2	Subcutaneous osmotic minipump	GD4 - GD20	'Subtle alterations' in various tests of cognitive performance that were magnified by challenges on nicotinic and adrenergic systems in adult offspring
Levin et al. (1996) [rat]	2	Subcutaneous osmotic minipump	GD4 - GD20	'Subtle changes' in cognitive function; choice accuracy, response to behavioural challenge and response to effect of drug challenge in adult offspring; sex dependent
Liang et al. (2006) [rat]	1.4 (to pups) (0.7, 2x per day)	Subcutaneous injection	PND8 - PND12	Impaired nicotinic enhancement of central auditory processing and auditory learning in adulthood
Eppolito et al. (2010)	0.96, 2.0	Subcutaneous osmotic minipump	GD4 - PND10	Decreased preweaning pup weight gain, with recovery after weaning; Subtle cognitive deficits and increased anxiogenic behaviours in offspring in adulthood but not adolescence; some sex differences noted

Study	**Nicotine dose reported** (mg/kg bw/day) – maternal dose unless otherwise stated	**Route of administration**	**Duration of administration**	**Outcome** (offspring, unless otherwise stated)
Eppolito and Smith (2006) [rat]	0.96	Subcutaneous osmotic minipump	GD4 - PND10	Significant reduction in weight gain of female offspring starting at puberty; Mild deficits in spatial learning and memory in females tested at PND60; Slower swim speed in male offspring
Hall et al. (2016) [rat]	0.2, 2.0, or tobacco-smoke extract (TSE) corresponding to 0.18 mg/kg bw/day nicotine)	Subcutaneous osmotic minipump	3 days prior to mating - PND12	Early mild reduction in pup body weight, with recovery at later ages; Effects of both nicotine doses and TSE on behavioural test outcomes in adolescence; magnitude of effects of TSE considered to correlate with those of the 2.0 mg/kg bw/day nicotine treatment
Lacy, Mactutus and Harrod (2011) [rat]	0.15 (0.05, 3x per day)	Intravenous injection	GD8 - GD21	Long-lasting alterations in sensorimotor gating, with sex-specific differences in adulthood
Ankarberg et al. (2001) [mouse]	0.007, 0.066, 0.132 (to pups) (0.0033, 0.033, 0.066, 2x per day)	Subcutaneous injection	PND 10 - 15	Changes in nicotine-induced motor behaviour at 4 months in 0.066 and 0.132 mg/kg bw/day groups, but not 0.007 mg/kg bw/day group. Alterations in learning and memory at 7, but not 4 months, in 0.132 mg/kg bw/day group (other doses not tested).

74. Adverse effects of nicotine exposure on development of the respiratory system have been demonstrated in a range of animal species, including deficits in pulmonary function that are related to alterations in the structure of the respiratory system (McEvoy and Spindel 2017). Maternal exposure in rhesus monkeys to 1.5 mg/kg bw/day nicotine base via the continuous subcutaneous osmotic minipump system throughout gestation led to alterations in pulmonary function tests in newborn offspring (Sekhon et al. 2001). Exposure to 1.0 mg/kg bw/day nicotine was associated with altered connective tissue in airways and surrounding vasculature (Sekhon et al. 1999, Sekhon et al. 2002, Sekhon et al. 2004). Follow-up studies in mice (2 mg/kg bw/day by subcutaneous osmotic minipump) were carried out to elaborate the molecular/structural alterations involved and developmental timing of the effects. Authors concluded that α7nAChRs mediate effects of nicotine on airway growth, stimulating epithelial cell growth and lung branching, leading to increased numbers of airways with small diameter (Wongtrakool et al. 2007, Wongtrakool et al. 2012). Similar effects of nicotine exposure on respiratory system development have also been reported in other species, including rats and sheep. Adverse effects in offspring were observed after maternal exposures to 0.5 mg/kg bw/day nicotine[27] via subcutaneous osmotic minipump during the 3rd trimester of gestation in sheep (Sandberg et al. 2004, Sandberg et al. 2011).

75. Animal models of nicotine exposure in adolescence have focussed on neurodevelopmental toxicity, including the potential for exposure to lead to long-lasting alterations in cognition, attention, memory, mood disorders, and gateway effects to future drug use and/or addictions. No data were available on direct effects of nicotine exposure in human adolescents, but some similar effects to those observed in animal studies have been implied from studies of adolescent CC smokers. These data have been reviewed in TOX/2019/01.

Mutagenicity/genotoxicity

76. The US Surgeon General report on the health consequences of smoking noted the following on knowledge about genotoxicity of nicotine (HHS 2014):

> "There are mixed data for a genotoxic effect of nicotine. Most studies were negative that used the Ames assay (including urine of rats exposed to nicotine), chromosomal aberration and sister chromatid exchange (SCE) assays in Chinese hamster ovary cells, and the bacterial genotoxicity luminescence test (Mizusaki et al. 1977; Riebe et al. 1982; Doolittle et al. 1991, 1995; Yim and Hee 1995). In contrast, two studies were positive for chromosomal aberration and SCEs (Riebe and Westphal 1983; Trivedi et al. 1990), one was positive for micronuclei formation that was inhibited with antioxidants (Argentin and Cicchetti 2004), one was positive for an Escherichia coli POLA+/POLA– mutation assay (Riebe et al. 1982), and another using nasal mucosal cells was positive by the Comet assay, which is inhibited by antioxidants or nicotinic receptor inhibitors (Ginzkey et al.

[27] It is not clear from this publication whether the dose refers to nicotine or nicotine bitartrate.

> 2012). One study found that cotinine, and not nicotine, was genotoxic by the bacterial genotoxicity luminescence test, but another was null for the Ames assay and SCE induction (Doolittle et al. 1995; Yim and Hee 1995). Some reports indicate that nicotine can lead to the formation of DNA adducts using the ultrasensitive technique accelerator mass spectroscopy (Cheng et al. 2003). Although cigarette smoke is highly genotoxic, a comparison of Ames mutagenicity for cigarette smoke from cigarettes with differing nicotine yields did not indicate different mutagenic potential, suggesting that there was no additional contribution by nicotine (Chen et al. 2008a)."

77. A recent systematic review of nicotine carcinogenicity (Haussmann and Fariss 2016) considered that the available data on genotoxic potential of nicotine are conflicting and have not been critically reviewed, with the following summary:

> "Genotoxicity was not observed for nicotine or its four major metabolites at concentrations of up to 1 mg/ml in the Salmonella reverse mutation assay and in a sister chromatid exchange assay in Chinese hamster ovary cells (Doolittle et al. 1995). However, in recent in vitro genotoxicity studies examining strand-breaking activity assessed by the Comet assay, chromosome aberration or micronucleus formation, nicotine was found to be active in a concentration range between 160 ng/ml and 650 mg/ml (Argentin & Cicchetti 2004; Ginzkey et al. 2012; 2013; Bavarva et al. 2014; Ginzkey et al. 2014a, 2014b). This range is beyond the systemic nicotine levels achieved by using NRT products […] but at local sites of entry, such as at respiratory tract or oral epithelia, nicotine concentrations may indeed be higher than systemic concentrations (Jarvis et al. 1984). Genotoxic effects at systemically relevant nicotine concentrations (16 ng/ml) were reported in a few studies, such as in a cytokinesis-blocked micronucleus assay (Kleinsasser et al. 2005) and in a chromosomal aberration assay (Demirhan et al. 2011). Overall, definitive studies to determine the genotoxic potential of nicotine in users of nicotine delivery systems are missing."

78. A review by Sanner and Grimsrud (2015) noted that nicotine has been shown to induce CA, SCE, single-strand DNA strand breaks, and MN *in vitro*, with the conclusion that oxidative stress is probably involved since the effects are reduced in the presence of antioxidants, and the finding that the effects decrease after co-incubation with a nAChR antagonist indicates a receptor-dependent pathway for induction of oxidative stress.

79. The REACH dossier for nicotine noted that 2 bacterial reverse mutation assays (OECD 471), one HPRT gene mutation assay (Klimisch 1) (OECD 476) and one micronucleus test (OECD 487), assessed as key studies for nicotine, indicated no aneugenic, mutagenic or clastogenic effects were observed *in vitro* in these tests.

The studies nicotine were GLP compliant and quality was overall very high (Ames tests = Klimisch 2, HPRT assay and micronucleus test, Klimisch 1).

Carcinogenicity

80. Opinions of authoritative bodies on the carcinogenicity of nicotine generally consider that the available data set is currently inadequate, but that nicotine is not likely to be a carcinogen *per se* (HHS 2014, RCP 2016, NAS 2018). Mechanistic considerations relevant to the possible effect of nicotine on carcinogenic pathways may include inhibition of apoptosis, stimulation of pathways affecting cell proliferation, stimulation of fibroblast production, and possible promotion of metastasis (HHS 2014).

81. Detailed review articles on potential carcinogenicity of nicotine have been published by Sanner and Grimsrud (2015) and Haussmann and Fariss (2016)[28]. A summary is given in the following paragraphs, 82–87.

82. Data are inadequate to evaluate the potential carcinogenic effects of nicotine in humans. Findings from one study, the Lung Health Study, indicated no association of nicotine exposure *per se* with cancer risk. In this randomised, controlled trial aiming to prevent chronic obstructive pulmonary disease, two-thirds of the 5887 participants were randomised to smoking intervention treatment, being advised to chew nicotine gum (2 mg) liberally for 6 months, with subsequent advice to stop the treatment after 2.5 years. Gum use and CC smoking were monitored for a total of 5 years and participants who were cancer-free at this point were followed for the next 7.5 years. At the end of this period, adjusted Cox proportional hazards regression analysis showed that NRT alone was not a significant predictor for lung ($p = 0.67$, gastrointestinal ($p = 0.61$), or all ($p = 0.94$) cancers, while CC smoking was a significant predictor for lung ($p = 0.03$), but not gastrointestinal ($p = 0.59$) or all ($p = 0.17$) cancers (Murray, Connett and Zapawa 2009).

83. Experimental studies in animals have suggested that nicotine is not carcinogenic *per se*, but adequate studies of long-term exposure to assess carcinogenicity are not available. Only one study of inhalation exposure was identified. In the study of Waldum et al. (1996) (described in paragraph 45), no

[28] Declaration of interest: “The employment affiliation of the authors is shown on the cover page. M. W. F. is a current employee of ALCS and H. J. H. is a former employee of Philip Morris International. H. J. H. served as a paid consultant to ALCS for preparation of thisreview. H. J. H. is an independent toxicology consultant for commercial firms including companies that manufacture and sell tobacco products. ALCS is an affiliate of Philip Morris USA Inc., U.S. Smokeless Tobacco Company LLC and NuMark LLC which are manufacturers and marketers of various tobacco products in the United States including cigarettes, smokeless tobacco and e-vapor products, respectively. The authors have not testified in litigation or represented ALCS or affiliates in meetings with FDA regarding the topic of this review. The ALCS legal department reviewed this paper solely in connection with intellectual property protection. The opinions and conclusions of the authors are their own, and do not necessarily reflect the position of ALCS or its affiliates.”

increase in tumour incidence was seen in rats exposed to 0.5 mg/m^3 nicotine[29] for up to 2 years. Commentators on this study have noted several limitations, including that the (single) dose used was too low to evaluate potential carcinogenic effects. Several other studies have reported evaluations of nicotine carcinogenicity using other exposure routes, in mice, rats, and hamsters. Overall, commentators have concluded that these studies do not suggest that nicotine is a complete carcinogen, but that the studies generally do not meet criteria of adequacy for evaluating carcinogenicity.

84. Numerous studies have assessed potential effects of nicotine to modulate carcinogenicity (chemical, physical, or transgenic initiation), although none has used inhalation exposure. Findings have been mixed, with both negative and positive study outcomes (*reviewed by* Haussmann & Fariss 2016). Studies in mice with NNK as an initiator have indicated that nicotine acts as a promoter after injection or dermal exposure, but not after oral administration (*reviewed by* Sanner & Grimsrud 2015).

85. Studies have shown that nicotine may enhance tumour growth and progression in xenograft models in mice, with nicotine exposures via s.c., oral, or dermal routes. Many of the studies showed positive effects, while those with negative findings may have been related to relatively low doses used. Although limitations to these studies have been noted, there is a general opinion that they do indicate a likely association between nicotine exposure and enhanced carcinogenesis of inoculated tumour cells (*reviewed by* Haussmann & Farris 2016).

86. It has been suggested that nicotine may inhibit antitumour immune response, and antidendritic cell effects of nicotine have been shown in animal models.

87. The systematic review by Haussmann & Fariss (2016) concluded that human data from NRT use are inadequate to assess the carcinogenicity of nicotine, and that based on animal data there is limited evidence for an association between long-term nicotine exposure and lack of a complete carcinogenic effect, inadequate data as to whether or not nicotine modulates carcinogenesis with chemical/physical/transgenic initiation, and sufficient evidence for modulation of carcinogenesis via cancer cell initiation (Haussmann and Fariss 2016).

Studies of nicotine in ENDS mixtures

88. The COT discussion papers, TOX/2018/24 and TOX/2018/46, reviewed studies that have assessed the toxicity of E(N)NDS aerosol mixtures. For those studies that tested aerosols both with and without nicotine, the summaries are reproduced at Annex B. These include 3 studies that investigated acute cardiovascular effects in humans (Cooke et al. 2015, Fogt et al. 2016, Moheimani et al. 2017) and a number of studies in animals: two 90-day, repeat-dose toxicity

[29] In a review of nicotine carcinogenicity, Haussman & Fariss (2016) calculated the exposure to 0.5 mg/m^3 as equivalent to 0.4 mg/kg bw/day, assuming full retention of the inhaled nicotine.

studies in rats (Werley et al. 2016, Phillips et al. 2017), 3 studies that evaluated effects of short-to-medium term exposures on the respiratory system in mice (Laube et al. 2017, Larcombe et al. 2017, Garcia-Arcos et al. 2016), and 5 studies that investigated reproductive and developmental effects in mice (Smith et al. 2015, McGrath-Morrow et al. 2015, Chen et al. 2018a, Chen et al. 2018b, Nguyen et al. 2018). The COT considered at the time that there were no unexpected findings noted and the case studies presented did not provide evidence for any cause and effect relationships above what would be expected from inhalation of vapour containing nicotine.

Summary

89. Toxicological data on nicotine were reviewed, based on literature presented in published previous toxicological reviews and an updated literature search from 2008 onwards. The aim of the review was to identify data that could be used to establish a health-based guidance value for nicotine exposure from ENDS, for which the exposure route would be expected to be mostly via inhalation. As study data on exposure to nicotine via inhalation route were limited, all routes of exposure were considered.

90. Nicotine is acutely toxic via all routes of exposure, targeting the central and peripheral nervous systems. In humans, the lethal dose is widely cited at approximately 0.6–1.0 mg/kg bw, based on historical reports of poisoning, although a recent review of the literature challenged this, suggesting that the lethal dose is in the range of 6.5–13 mg/kg bw. Cases mostly relate to accidental or deliberate ingestion or dermal exposure. A UK Rapporteur for EU peer review process for pesticides proposed a value of 0.0001 mg/kg bw/day for the ARfD, ADI, and AOEL for nicotine fumigant, using data from a report of clinical signs of toxicity in children exposed dermally to nicotine patches (UK-DAR 2007). EFSA set an ARfD and ADI of 0.0008 mg/kg bw/day for nicotine intake from ingestion of wild mushrooms, based on increased heart rate in human smoker volunteers given nicotine i.v. (EFSA 2009).

91. Hansson et al. (1994) evaluated acute effects of nicotine via inhalation exposure in human volunteers. No cardiovascular effects were noted in 5 healthy nonsmokers after inhalation of 0.64 mg nicotine in solution at 0 and 10 min. Coughing was triggered by a single inhalation at a dose of 0.04 mg. Repeated inhalation of nicotine solutions by 8 nonsmokers giving a total dose of 0, 0.4, 0.8, or 1.7 mg nicotine over 5 min caused significant increases in heart rate and systolic blood pressure at all doses compared with the vehicle controls, in a dose-related manner. Decreases in skin temperature and headache were also observed (Hansson et al. 1994).

92. LD_{50} values for nicotine in animals have been reported for oral, dermal, i.p. and i.v. routes of exposure, ranging from around 3 to 188 mg/kg bw (HCN 2005). Based on data from a study reported by Shao et al. (2013), LC_{50} values for nicotine in air of 2300 mg/m^3 (20 min), extrapolated to 190 mg/m^3 (4 h), were reported in the REACH dossier. Ahmad et al. (2019) reported that rats exposed (nose-only) to 5%

(24.47 mg/m^3) or 10% nicotine aerosol for 15 min showed decreased heart rate during the subsequent 4 h, alterations in BALF at 6 h that were considered to indicate disruption of the alveolar-capillary barrier, and some alterations in circulating white blood cell counts. Congestion in lung tissue vessels was noted at 24 h in the 10% nicotine exposure group.

93. Nicotine is reported to cause local irritation at the site of administration (e.g. dermal patch, nasal or oral sprays) in humans. The review by HCN (2005) concluded that nicotine is a skin irritant and sensitizer. The REACH dossier concluded nicotine as Category 2 (irritant), and noted that nicotine was not sensitizing in a well conducted study *in vivo* (local lymph node assay).

94. No data were identified regarding repeated or long-term inhalation exposure to nicotine *per se* in humans. Some evaluations have been made based on data from studies of NRT as an aid to quit CC smoking. The Lung Health Study reported by Murray et al. (2009) found that NRT use was not a significant predictor for lung, gastrointestinal, or all cancers over 7.5 years of follow-up. Studies relating to cardiovascular disease are generally of inadequate quality to draw clear conclusions, but have not shown evidence of serious cardiovascular events. The previous COT discussion paper, TOX/2018/45, noted that a few studies reported potential associations of NRT prescription or use during pregnancy with adverse birth outcomes but findings were difficult to evaluate due to factors including low levels of NRT use and lack of data on levels of continued CC smoking.

95. Three studies were identified that evaluated effects of repeated exposures to nicotine via inhalation in animals. Phillips et al. (2015) reported that only minor biologic effects were observed in an OECD 412 study in which rats were exposed (nose-only) for 4 weeks (6 h/day, 5 d/wk) to 50 mg/m^3 nebulised nicotine (calculated as a delivered dose of 13.6 mg/kg bw/day, or HED of 2.2 mg/kg bw/day). In the REACH dossier, data from an OECD 422 study indicated a systemic LOAEL (F_0 generation) of 10 µg/L [10 mg/m^3], and, based on no adverse effects observed at the top dose, parental reproductive NOAEL and F_1 NOAEL for growth and developmental toxicity ≥ 20 µg/L [10 mg/m^3] (Unnamed study 2018). Waldum et al. (1996) observed no treatment-related effects other than a body weight decrease of approximately 5% in rats exposed to 0.5 mg/m^3 nicotine (20 h/day, 5 d/week) for up to 103 weeks.

96. A large database of experimental studies in animals via non-inhalation routes was available, but no studies followed standard test guidelines. Previous toxicological reviews noted various adverse effects of exposure to high doses of nicotine, including locomotion and behaviour, and arteriosclerosis. In an evaluation of nicotine for use as an indoor pesticide spray, EPA (2008) considered liver effects in rats, reported from the 10-day drinking-water study of Yuen et al. (1995), calculating a LOAEL and NOAEL of 2.5 mg/kg bw/day and 1.25 mg/kg bw/day nicotine, respectively. The previous COT discussion paper, TOX/2018/45, summarised animal studies that have indicated adverse effects on the development

of several organ systems, notably the neurological and respiratory systems. Many of these studies administered nicotine during gestation via continuous s.c. osmotic minipump. Doses of 6 mg/kg bw/day were associated with reduced maternal weight gain and fetal resorption, but these effects were generally not observed at 2 mg/kg bw/day. Effects on cholinergic and serotonergic signalling in the brain were demonstrated at nicotine exposures during gestation of 0.2 mg/kg bw/day in rats, considered to represent a level equivalent to that from secondhand smoke exposure, with subsequent adverse effects on cognitive and behavioural development (Slotkin et al. 2015, Hall et al. 2016). The NIWL (2005) Group toxicological review of nicotine highlighted a study by Ankarberg et al. (2001) that showed effects on nicotine-induced motor behaviour at 4 months in offspring of mice treated by twice daily s.c. injection from PND 10–15 with 0.066 mg/kg bw/day nicotine, with no effects seen at 0.007 mg/kg bw/day.

97. Recent evaluations in the literature have noted that there are mixed data for a genotoxic effect of nicotine. Most studies using Ames test, CA and SCE assays in Chinese hamster ovary cells, and the bacterial genotoxicity luminescence test were negative. However, some recent *in vitro* genotoxicity studies including Comet assay, CA or MN formation produced some positive findings in the concentration range of 160–650 mg/mL. The review by the US Surgeon General noted that although this range is above that of systemic levels of nicotine achieved using NRT, higher levels than this may occur at local sites of entry such as respiratory tract or oral epithelia. Genotoxic effects at lower concentrations (16 ng/mL) were noted in a small number of studies, such as cytokinesis-block micronucleus assay and CA assay. The review by US Surgeon General concluded that, overall, definitive studies to determine the genotoxic potential of nicotine in users of nicotine delivery systems are missing (HHS 2014). Experimental studies in animals have suggested that nicotine is not carcinogenic *per se*, but adequate studies of long-term exposure to assess carcinogenicity are not available.

Questions for the Committee

98. Members are asked to consider the paper and in particular:

i. Can a health-based guidance value for inhalation exposure to nicotine be calculated from the data identified?

ii. Can any conclusions be drawn about the likely risk of exposure to nicotine from ENDS:

   a. for users who are current or previous users of nicotine-containing products, including cigarettes?

   b. for users who are not current or previous users of nicotine-containing products?

   c. to bystanders?

iii. Are there any population sub-groups who may be particularly vulnerable to adverse effects of exposure to nicotine from ENDS?

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Abbreviations/Glossary

ADHD	Attention deficit hyperactivity disorder
ADI	Acceptable daily intake
AFSSA	Agence Française de Sécurité Sanitaire
ALT	Alanine aminotransferase
AOEL	Acceptable operator exposure level
AOR	Adjusted odds ratio
ARfD	Acute reference dose
AST	Aspartate aminotransferase
ATE	Acute toxicity estimate
BALF	Bronchoalveolar lavage fluid
BfR	Bundesinstitut für Risikobewertung
BUN	Blood urea nitrogen
CA	Chromosomal aberrations
CC	Conventional cigarette
CI	Confidence interval
CLH	Harmonised classification and labelling (ECHA)
DAR	Draft assessment report
DD	Delivered dose
EEG	Electroencephalogram
EFSA	European Food Safety Authority
E(N)NDS	Electronic nicotine (or non-nicotine) delivery system
ENDS	Electronic nicotine delivery system
ENNDS	Electronic non-nicotine delivery system
EU	European Union
GD	Gestational day
GLP	Good laboratory practise
GSD	Geometric standard deviation
GTS	Green tobacco sickness
HBGV	Health-based guidance value
HCN	Health Council of the Netherlands
HED	Human equivalent dose
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
LC_{50}	Lethal concentration required to kill 50% of population
LD_{50}	Lethal dose required to kill 50% of population
LDH	Lactate dehydrogenase
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOEL	Lowest observed effect level
MCA	Major congenital anomaly
MCAO	Middle cerebral artery occlusion
MMAD	Median mass aerodynamic diameter
MN	Micronucleus

MOE	Margin of exposure
NIWL	National Institute for Working Life (Sweden)
NOAEL	No observed adverse effect level
NRT	Nicotine replacement therapy
OECD	Organisation for Economic Co-operation and Development
OR	Odds ratio
PND	Post-natal day
RCT	Randomised clinical trial
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
RR	Relative risk
s.c.	Subcutaneous
SCE	Sister chromatid exchange
SNAP	Smoking, Nicotine, and Pregnancy
SOD	Superoxide dismutase
THIN	The Health Improvement Network
TSE	Tobacco smoke extract
UF	Uncertainty factor
UK	United Kingdom
US EPA	United States Environmental Protection Agency

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COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT)

Potential toxicological risks from electronic nicotine (and non-nicotine) delivery systems (E(N)NDS – e-cigarettes). Toxicological review of nicotine.

Details of literature search carried out by NCET at WRc/IEH-C

Relevant literature was obtained from reviews published by authoritative bodies, as described in paragraph 4 of the main report. In addition, searches for further literature relating to toxicity of E(N)NDS aerosol were identified as described below.

The following literature searches were performed by NCET at WRc/IEH-C under contract to PHE on 29/04/2019 in Scopus and PubMed, from 2008 to date.

Scopus

((CHEMNAME (nicotine OR "(S)-3-(1-methylpyrrolidin-2-yl)pyridine") OR CASREGNUMBER ("54-11-5") OR TITLE-ABS-KEY (nicotine)) AND PUBYEAR > 2007) AND ((TITLE-ABS-KEY (*toxic* OR carcin* OR mutagen* OR "health effect" OR "adverse effect") AND TITLE-ABS-KEY (oral OR inhal* OR derma*)) AND PUBYEAR > 2007) AND (EXCLUDE (SUBJAREA , "SOCI") OR EXCLUDE (SUBJAREA , "AGRI") OR EXCLUDE (SUBJAREA , "CENG") OR EXCLUDE (SUBJAREA , "MATE") OR EXCLUDE (SUBJAREA , "BUSI") OR EXCLUDE (SUBJAREA , "ENGI") OR EXCLUDE (SUBJAREA , "ARTS") OR EXCLUDE (SUBJAREA , "EART") OR EXCLUDE (SUBJAREA , "ECON") OR EXCLUDE (SUBJAREA , "VETE")) AND (LIMIT-TO (LANGUAGE , "English") OR EXCLUDE (LANGUAGE , "French") OR EXCLUDE (LANGUAGE , "Polish") OR EXCLUDE (LANGUAGE , "Portuguese") OR EXCLUDE (LANGUAGE , "Croatian") OR EXCLUDE (LANGUAGE , "Spanish") OR EXCLUDE (LANGUAGE , "Turkish")) AND (EXCLUDE (LANGUAGE , "German")) : 637

PubMed

((((((((((("54-11-5"[EC/RN Number]) OR nicotine[MeSH Terms]) OR nicotine[Title/Abstract]) AND ("2008/01/01"[PDat] : "2019/12/31"[PDat]))) OR ((S)-3-(1-methylpyrrolidin-2-yl)pyridine[Title/Abstract] AND ("2008/01/01"[PDat] : "2019/12/31"[PDat]))) AND ("2008/01/01"[PDat] : "2019/12/31"[PDat]))) AND ((((*toxic*[Title/Abstract] OR carcin*[Title/Abstract] OR mutagen*[Title/Abstract] OR “health effect”[Title/Abstract] OR “adverse effect”[Title/Abstract])) AND (oral[Title/Abstract] OR inhal*[Title/Abstract] OR derma*[Title/Abstract])) AND ("2008/01/01"[PDat] : "2019/12/31"[PDat]))) AND ("2008/01/01"[PDat] :

"2019/12/31"[PDat]))) AND (english[Language] AND ("2008/01/01"[PDat] : "2019/12/31"[PDat])): 289

For completeness, the reference lists of selected papers were examined for further relevant publications, and additional *ad hoc* searches were carried out as considered appropriate.

COMMITTEE ON TOXICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT)

Potential toxicological risks from electronic nicotine (and non-nicotine) delivery systems (E(N)NDS – e-cigarettes). Toxicological review of nicotine.

Studies reported in previous COT discussion papers on E(N)NDS that included comparison of aerosols with/without nicotine.

Paper 4: Toxicological and epidemiological evaluations of E(N)NDS aerosol exposures (TOX/2018/24).

Studies in humans

99. Cooke et al. (2015) evaluated the effect of E(N)NDS nicotine on arterial blood pressure in young, adult non-smokers (n=20, male and female). Subjects were exposed to 0 (placebo) or 18 mg/mL nicotine from a 'Green Smart Living' or 'Clean Electronic Cigarettes' device[30]: 1 puff every 30 s during 10 min. The study was a randomised, double-blind, 2-arm crossover, with a 1-week washout period. Nicotine exposure was associated with significantly increased systolic blood pressure compared with placebo, and with increased diastolic blood pressure compared with baseline and placebo values.

100. Fogt et al. (2016) assessed the effect of nicotine delivered by E(N)NDS on blood pressure, metabolic rate, metabolic responses and aerobic power in young, normotensive non-smokers (10 men and 10 women). Participants took 20 puffs of E(N)NDS ('Green Smart Living') with a 30-s puff interval. The study was carried out in a 2-arm, double-blind, crossover design, using E(N)NDS devices with cartridges containing either 0 mg nicotine or 18 mg nicotine[31]. At 40 min post exposure, resting diastolic blood pressure was significantly increased and resting systolic blood pressure was significantly reduced after 18 mg nicotine compared with 0 mg nicotine E(N)NDS exposure. There were no significant differences in other test parameters between nicotine and placebo exposures.

101. Moheimani et al. (2017) carried out an open-label, randomised, crossover study including 33 healthy volunteers who had not smoked CC or used E(N)NDS for ≥ 1 y. Participants used either a 'Greensmoke' cigalike[32] (n=15) or a second-generation 'eGo' device[33] (n=18), both with and without 1.2% nicotine, 1 puff per 30 s during either 10 min (n=6 cigalike) or 30 min (n=27), or a sham control (device without liquid[34]). Measurements (blood pressure, heart rate, electrocardiogram

[30] Constituents other than nicotine were not described
[31] The report notes that 'The 18 mg and 0 mg EC cartridges are marketed to vary only in nicotine content.'
[32] tobacco-flavoured liquid, VG/PG solvents
[33] strawberry flavour, VG/PG solvents
[34] further details not given

(ECG), blood sampling) were made before and immediately after exposures. Heart rate variability components reflecting vagal, sympathetic activity and sympathovagal balance (measured on ECG) showed a shift towards sympathetic predominance (compared with baseline) after E(N)NDS + nicotine, but not with the non-nicotine device or sham control. Plasma paraoxonase, a marker of oxidative stress, did not increase after any of the exposures. Authors concluded that the acute sympathomimetic effect of E(N)NDS is caused by the inhaled nicotine.

Studies in animals

102. Laube et al. (2017) reported that exposure to nicotine-containing, PG-based E(N)NDS aerosol via an E(N)NDS device (Joyetech 510-T) reduced mucociliary clearance (MCC) in mice in comparison with aerosol without nicotine. C57BL/6 mice were whole-body exposed to aerosol of either PG alone or PG/2.4% nicotine, 20 min/day for 1 or 3 weeks. Exposure was achieved from repeat cycles of a 6-s puff (600 µL E(N)NDS solution per puff) and 15-s puff interval into a 1 L exposure chamber during the 20 min period, although exposure concentrations were not reported. After 1 week, mean MCC was not significantly different between unexposed (8.6%), PG-exposed (7.5%), or PG/nicotine-exposed (11.2%) mice. However, after 3 weeks, MCC was significantly higher in the PG-exposed animals (8.6%, 17.2%, and 8.7% in unexposed, PG-exposed, and PG/nicotine-exposed mice, respectively). Serum cotinine levels were significantly higher in mice exposed to PG/nicotine in comparison with control and PG-only exposure groups. No differences were seen in tracheal histology. The authors concluded that chronic exposure to an aerosol containing PG and nicotine slowed MCC in comparison with the rate associated with exposure to aerosol containing PG without nicotine.

103. Exposure to E(N)NDS aerosols led to impairments in pulmonary function, without pulmonary inflammation, in a mouse model designed to simulate effects of exposure during adolescence (Larcombe et al. 2017). Groups of 12 female BALB/c mice were whole-body exposed between the ages of 4-12 weeks to control air (AIR), CC smoke (SMOKE), or 1 of 4 E(N)NDS aerosols of 'American Tobacco' flavour, as follows: PG[35] (0-PG), PG + 12 mg/mL nicotine (12-PG), VG (0-VG[36]), or VG + 12 mg/mL nicotine (12-VG[37]). Average measured chamber concentrations were reported as 0.014 g/cm^{-3} for PG and 0.018 g/cm^{-3} for VG [38]. Mice were exposed for 1 h/day, 5 days/week from weeks 4-10, then twice daily for 1 h, 5 days/week, during weeks 11 and 12. After 8 weeks of exposure, all treatment groups weighed significantly less than AIR controls, with the lowest weight gains in nicotine-exposed mice[39]. Lung mechanics and function were assessed 24 h after the final exposure.

[35] PG-based aerosols contained 100% PG as the carrier.
[36] 95.3% VG / 4.70% PG
[37] 97.53% VG / 2.47% PG
[38] The Secretariat assumes that the concentrations should be reported as g/cm^3 . Exposure concentrations of 0.014 g/cm^3 and 0.018 g/cm^3 are equivalent to 14,000,000 mg/m^3 and 18,000,000 mg/m^3, respectively, which is far higher than other studies reported in this paper so itis possible that there is a reporting error.
[39] It was noted that there were differences in starting weights and individual weight gains.

Mice exposed to E(N)NDS aerosols showed various differences in pulmonary function compared with AIR controls, including decreased airway resistance at functional residual capacity (FRC) (0-PG and 0-VG), increased tissue damping at FRC (all 4 E(N)NDS groups), increased tissue elastance at FRC (0-PG and 0-VG), decreased lung volume and changes in volume dependence of tissue damping and elasticity (0-PG, 0-VG, 12-PG). Methacholine challenge tests showed that SMOKE- and VG-exposed mice were significantly more responsive than AIR- or PG-exposed mice, whether or not nicotine was present in the aerosol. SMOKE but not E(N)NDS exposure was associated with increased pulmonary inflammation (increased BAL cells). Overall, the authors concluded that 1] VG-based E(N)NDS aerosols induced more severe functional pulmonary impairments than PG-based aerosols, and 2] there was little effect of the presence or absence of nicotine.

104. Werley et al. (2016) conducted a 90-day OECD-guideline study of the effects of exposure to E(N)NDS aerosol mixtures in rats. Groups of Sprague-Dawley rats were nose-only exposed to aerosols of vehicle control (77% PG, 23% glycerol), Formulation 1 (75.5% PG, 22.5% glycerol, 2% nicotine), or Formulation 2 (62.3% PG, 18.1% glycerol, 2% nicotine, 17.6% 'proprietary flavour') for 16, 48, or 160 min/day to achieve daily doses of 3.2, 9.6, or 32.0 mg/kg bw/day TPM, respectively, for periods of up to 90 days, followed by a 42-day recovery period. Particle mass median aerodynamic diameter (MMAD) values were in the range 1.1-1.3 µm (GSD, 1.54-1.63). The authors reported that 'There were no treatment-related clinical observations. All clinical findings in the Formulation 1 and Formulation 2 treated groups were noted with similar incidence in the vehicle control group, or were common findings for laboratory rats of the same age and strain'. However, data were not shown. Plasma cotinine levels, measured at days 28 and 90, were reported to be 'proportional to the daily delivered dose progression', with high TPM exposures associated with approximately ten-fold higher levels than low TPM exposures. Values were not significantly different at days 28 and 90. Lower body weight gains were associated with exposure to Formulation 1 and Formulation 2, compared with vehicle, and with higher TPM exposure compared with lower TPM exposure. Food consumption was reduced in Formulation 2 males for most of the 90-day exposure period, while treatment-related effects on food consumption were generally not observed in females. At necropsy, the high TPM dose groups tended to have higher lung weights than low TPM dose groups (including significantly higher lung weight in the high compared with low TPM dose vehicle group), but these differences seemed to resolve after the recovery period. All high TPM exposures, i.e. vehicle-only or Formulation groups, were also associated with decreased serum albumin and increased serum phosphorus in males, and with increased AST (vehicle group) and serum phosphorus (Formulation 2 group) in females, compared with low TPM exposures, but these differences also resolved after recovery. Clinical chemistry and cytology analyses of BALF at days 28, 90, and 132 indicated various changes, for example increased total protein and alkaline phosphatase (ALP), and higher levels of neutrophils and reduced proportions of alveolar macrophages, associated with high compared with lower TPM exposures. A detailed breakdown of all BALF clinical chemistry and cytology findings is shown in Tables 5 (28 days), 6 (90 days), and 7

(132 days) of the publication. In general, these changes appear to have resolved after the recovery period. Histopathologic findings are summarised in Tables 9 and 10 of the publication. Histopathologic evaluation showed changes in the nose, lung, and larynx in all groups, including mild mucous cell hyperplasia in the nose, mild vacuolation of ciliated respiratory epithelium in the nose, dose-related increase in alveolar macrophages in the lungs, and non-dose-related mucin exudate in the larynx. Mucous cell hyperplasia in the nose persisted after recovery. Overall, the authors of this study determined the mid TPM dose (9.6 mg/kg bw/day) to be a no observed effect level (NOEL) for each treatment group, based on body weight decreases. These effects were greatest in high TPM Formulation groups, and authors suggested that suppressive effects of nicotine on appetite may have been involved.

105. Phillips et al. (2017) conducted a 90-day OECD TG 413 inhalation study of PG/VG mixtures at 3 test concentrations, without or with nicotine[40]. Groups of 10 male + 10 female Sprague-Dawley rats underwent nose-only exposure to nebulised test material as shown in Table A, below, for 13 weeks (6 h/day, 5 days/week). The authors calculated that the maximal exposure tested (group 'High (PG/VG)') would be a human equivalent dose (HED) for a 60 kg adult of 4.3 g/day PG and 5.3 g/day VG, corresponding to approximately 4 mL/day of a 100% solution of each compound. The maximum HED for nicotine was calculated as 66 mg, which would correspond to approximately 3.3 mL E(N)NDS liquid containing 20 mg/mL nicotine (the maximum allowed concentration in Europe).

Table A. Exposure groups in the 90-day inhalation study of Phillips et al. (2017).

Group	PG (mg/L)	VG (mg/L)	Nicotine (mg/L)	MMAD (µm)
Sham (filtered air)	0	0	0	-
Vehicle (saline)	0	0	0	1.4
Low (PG/VG)	0.174	0.210	0	1.7
Med (PG/VG)	0.520	0.630	0	2.0
High (PG/VG)	1.520	1.890	0	2.0
Nicotine + Low (PG/VG)	0.174	0.210	0.023	1.8
Nicotine +Med (PG/VG)	0.520	0.630	0.023	2.0
Nicotine + High (PG/VG)	1.520	1.890	0.023	1.9

[40] The full text of this publication is available at: https://www.sciencedirect.com/science/article/pii/S0278691517305112?via%3Dihub#mmc1 (accessed 18/06/18).

106. Respiratory parameters (data are provided in Supplemental Figure 1 of the publication): Male rats in the 'High (PG/VG)' exposure group had significantly lower peak inspiratory flow compared with the saline-treated group and compared with the 'Nicotine + High (PG/VG)' group. Increases in respiratory parameters (peak inspiratory flow, tidal volume, minute volume) were seen in some of the nicotine-exposure groups, most prominently in female rats. Otherwise no clear pattern of changes was seen. Food consumption and body weight (data are provided in Supplemental Figure 2 of the publication): Compared with the saline-exposed animals, food consumption was increased in association with nicotine exposure in both male and female rats (all PG/VG doses). In females, increased food consumption was seen in all nicotine exposure groups compared with non-nicotine exposure groups. On day 91 of the study, for males, body weight was slightly lower in the 'Nicotine + High (PG/VG)' compared with saline group. In females, a significantly higher body weight at day 91 was observed in all nicotine exposure groups compared with non-nicotine groups and also compared with the saline exposure group. Lung weight and histopathology (data are provided in Supplemental Figure 3 and Figure 2 of the publication): In females, absolute lung weights were increased in all nicotine exposure groups compared with saline exposure, and in 'Nicotine + High (PG/VG)' compared with 'High (PG/VG)'. There were no significant differences between groups in normalised lung weight to body weight in females. In males, absolute lung weight was lower in 'High (PG/VG)' compared with saline. Normalised lung weight was lower in the sham group compared with saline, and higher in 'Nicotine + High (PG/VG)' compared with both the saline group and the 'High (PG/VG)' group. Histopathological examination showed some changes in the respiratory tract, which the authors considered to be minimal adaptive changes (e.g. due to dehydration). The findings were mainly observed in the larynx, as follows. Compared with saline-exposed groups: basal cell hyperplasia in 'Nicotine + High (PG/VG)' females at the base of the epiglottis, and in 'Nicotine + Low (PG/VG)' females and 'Nicotine + High (PG/VG)' males at arythenoid projections; squamous metaplasia in 'Nicotine + High (PG/VG)' females and males at the base of the epiglottis and arythenoid projections, and in 'Nicotine + Med (PG/VG)' females at the base of the epiglottis. Compared with non-nicotine exposure (at the same PG/VG exposure level): increased squamous metaplasia at arythenoid projections in 'Nicotine + High (PG/VG) females and males. Compared with saline exposure, 'Low (PG/VG)' and 'Med (PG/VG)' exposures were associated with decreased infiltration of unpigmented macrophages in female rats, both with and without nicotine, but no effects were seen in male rats. BALF analysis indicated a slight increase in total cells (an indicator of inflammation) in 'Nicotine + High (PG/VG)' females compared with the equivalent non-nicotine group, but no differences were observed for any exposure groups in comparison with saline treatment. Gene and protein expression profiling (data are provided in Supplemental Figure 6 and Figure 3 of the publication): Profiling was performed on nasal epithelium and lung of female rats (6 per group). As compared with saline controls, in nasal epithelium, treated groups showed no differences in gene expression. Seven proteins were downregulated in the 'High (PG/VG)' group, but this was not observed in the 'Nicotine + High (PG/VG)' group. In the lung, some gene expression changes were observed in the 'Low

(PG/VG)' and 'Nicotine + Low (PG/VG)' groups, but these were not considered to be treatment related. Some altered gene sets were found to be associated with nicotine exposure, including upregulation of xenobiotic metabolising enzymes, Cyp1a1 and Fmo3, and downregulation of T-cell-related transcripts. Haematology and clinical chemistry (data are provided in Supplementary Figure 7 and Figure 4 of the publication): Compared with saline treatment, effects on red blood cell parameters were seen in female (but not male) rats in the 'Nicotine + High (PG/VG)' group, which the authors suggested may be related to a stress response to nicotine exposure (also indicated by changes in thymus and adrenal gland weights). The presence of nicotine was also associated with some lower total cholesterol and lower glucose concentrations (males and females) and lower creatinine and calcium (females) compared with saline and/or equivalent-dose PG/VG-only groups. Liver (data are provided in Figure 5 and Supplementary Table 4 of the publication): Exposures with nicotine were associated with effects on the liver (increased absolute and normalised liver weights, enzyme activity, hepatocyte vacuolation), with effects generally more pronounced in females than males. Effects were not seen in non-nicotine treatment groups, except for decreased alanine aminotransferase in 'High (PG/VG)' and sham-exposed males and increased alanine aminotransferase in sham-treated females, as compared with saline treatment groups. These findings were supported by findings from gene-expression studies, indicating an association of nicotine exposure with lipid oxidation, gluconeogenesis, ketone body formation, and cholesterol biosynthesis. The authors noted that although hepatocyte vacuolation might be considered as an adverse effect, studies have suggested that it is in fact an adaptive response. Overall, the authors considered that exposure to PG/VG aerosols showed minimal biological effects in comparison with exposure to nebulised saline, with no indication of toxicity. Inclusion of nicotine in the exposure led to effects that were consistent with findings from previous studies of nicotine, including upregulation of xenobiotic-metabolising enzymes in the lung and metabolic effects, such as reduced serum lipid concentrations and expression changes of hepatic metabolic enzymes. Authors concluded that 'No toxicologically relevant effects of PG/VG aerosols (up to 1.520 mg PG/L + 1.890 mg VG/L) were observed, and no adverse effects for PG/VG/nicotine were observed up to 438/544/6.6 mg/kg/day.' (Phillips et al. 2017).

107. Garcia-Arcos et al. (2016) conducted a study in which mice were whole-body exposed for 1 h/day to 0.4 mL of a 50:50 PG/VG commercial E(N)NDS liquid, with or without 18 mg/mL nicotine, or phosphate-buffered saline control, 5 days/week for 4 months. Mice exposed to nicotine-containing, but not nicotine-free, E(N)NDS liquid showed increased airway hyper-reactivity, inflammatory cell infiltration in the lung, airway enlargement, mucous production, and lung cell apoptosis. Similar findings were observed in human airways cells treated with E(N)NDS liquid with/without nicotine *in vitro*.

Paper 7: Additional information on developmental toxicity studies of E(N)NDS aerosols (TOX/2018/46).

Neurodevelopment

108. A study was carried out to assess the effects of exposure during gestation and lactation to E(N)NDS aerosol, both with and without nicotine, on gene expression markers of neurodevelopment. Pregnant C57BL/6J mice were whole-body exposed to filtered room air (control) or E(N)NDS aerosol[41] (blu, classic tobacco flavour), with or without nicotine (13–16 mg/mL)[42], for 3 hours per day, 5 days per week, throughout gestation. Average particulate concentrations in the exposure chambers were 25.6 mg/m^3 and 30.7 mg/m^3 total suspended particulates, with and without nicotine, respectively. Pups received the same exposure from PND4–PND6 through lactation to 1 month of age, at which point brain frontal cortex tissue was subjected to transcriptomic analysis. There were no effects of E(N)NDS aerosols with or without nicotine on birth weight or pup weight gain compared with controls. Results of transcriptomic analyses were stratified by gender and exposure (with/without nicotine). Alterations in gene expression in the frontal cortex were detected in both groups (with/without nicotine) compared with controls. Compared with air-exposed controls, in the groups exposed to the E(N)NDS aerosol, the numbers of genes showing significantly altered expression were: female/without nicotine (2630); female/with nicotine (1393); male/without nicotine (2615); male/with nicotine (152). Overall, 109 genes were commonly altered in all 4 groups. Looking at the top 5 predicted disease and disorder categories for each exposure group, Ingenuity Pathway Analysis (IPA) indicated predicted changes in disease and disorder outcomes including cancer, organismal injury and abnormalities, gastrointestinal disease, neurological disease, and psychiatric disorders in all groups except the male/with nicotine group, in which cancer, organismal injury and abnormalities, gastrointestinal disease, dermatological diseases and conditions, and connective tissue disorders were identified. The predominant predicted disease and biological functions included decreases in memory, cognition, learning and neurotransmission, and increases in hyperactive behaviour, emotional behaviour and organismal death (all groups except male/with nicotine); decreases in dendritic growth/branching and quantity of neurons, increases in seizure disorder and seizures in all treatment groups; and decrease in locomotion (male/with nicotine group). qPCR analysis was performed for 7 genes of interest, *Ngfr*, *Chat*, *Bdnf*, *Gdnf*, *Gal*, *Tbr1*, and *Adra1d*, and results were reported to be similar to those for RNA-Seq analysis. The authors concluded that similar gene expression changes were observed in female offspring exposed to E(N)NDS aerosol with and without nicotine as those seen in male offspring exposed to aerosol without nicotine. In contrast, male offspring exposed to aerosol with nicotine exhibited a smaller number of gene expression changes as well as different IPA-predicted diseases and disorders. The authors proposed that a possible explanation for this difference could be the inhibitory action of nicotine on aromatase enzyme (resulting in oestrogen deficiency), and they noted that the timing of euthanasia, which was performed at the beginning of puberty in this study, may be relevant to this hypothesis. The authors were

[41] Aerosol described as 73% PG and/or VG, 15% water, 11% flavourings, 1% nicotine
[42] 35 mL puff volume, 4-s puffs, 30-s intervals, mixed with filtered air.

surprised to find that exposure to aerosols without nicotine produced the greatest number of significant gene-expression changes in offspring, and commented that this may suggest that aerosol components other than nicotine may have been involved (Lauterstein et al. 2016).

109. A follow-on paper from the set of studies described by (Lauterstein et al. 2016) reported parameters of neuroinflammation in the frontal cortex and hippocampus, neurotrophin gene expression in the hippocampus, and serum cytokine levels from the same experimental setup. A re-analysis of IPA data for the frontal cortex was also carried out. Experimental details (animals, exposures) were as reported by (Lauterstein et al. 2016) (see previous paragraph). Measured aerosol exposure concentrations were 25.6 mg/m^3 (with nicotine) and 30.7 mg/m^3 (without nicotine) total particulates. Immunostaining for markers of neuroinflammation (glial fibrillary acidic protein (GFAP) for astrocytes, ionized calcium-binding adaptor molecule 1 (Iba-1) for microglia) showed significantly increased Iba-1 in the cornis ammonis (CA) 1 region of the hippocampus in male and female mice exposed to aerosol without nicotine, but not in the groups exposed to E(N)NDS aerosol with nicotine. There was a nonsignificant tendency for the same pattern of effects in the frontal cortex. No changes in Iba-1 were observed in the hippocampus regions CA3 or dentate gyrus. There were no changes in GFAP in any exposure groups. The authors noted that exposure to aerosol without nicotine was more inflammatory than exposure to aerosol with nicotine, and suggested possible hypotheses for this including: 1. a protective effect of nicotine via promotion of neurogenesis; 2. a speculative effect of PG to increase inflammation. Expression of neurotrophin genes, *Ngfr*, *Bdnf*, and *Gdnf* was evaluated in the hippocampus. *Ngfr* and *Bdnf* were significantly downregulated in both aerosol groups, with or without nicotine (sexes pooled) compared with controls. No changes were observed for *Gdnf* expression. Serum levels were measured for the cytokines, interleukin (IL)-6, tumour necrosis factor alpha (TNFα), IL1β, interferon gamma (IFNɣ), IL-2, and monocyte chemoattractant protein-1 (MCP-1). IL1β was significantly reduced in the groups (male and female) exposed to aerosol with and without nicotine. IL-2 was significantly reduced in the aerosol with nicotine group, with a nonsignificant trend to the same finding for exposure to aerosol without nicotine. There was also a nonsignificant trend to reduction in IL-6 level in mice exposed to aerosol with and without nicotine. There were no changes in TNFα, IFNɣ, or MCP-1 levels. The authors suggested that the similar effects of aerosol with and without nicotine on neurotrophin gene expression and serum cytokine levels indicated that E(N)NDS constituents other than nicotine could be responsible for these effects. The re-evaluation of frontal cortex IPA indicated the following numbers of genes overlapping with the IPA 'affects inflammation of the CNS' molecule list: aerosol without nicotine/males (27); aerosol without nicotine/females (24); aerosol with nicotine/males (6); aerosol with nicotine/females (13). Overall, the authors concluded that exposure to E(N)NDS aerosols, both with or without nicotine, poses a risk to the developing CNS (Zelikoff et al. 2018).

110. Another study in mice indicated effects of exposure to E(N)NDS aerosol containing nicotine during late gestation and early postnatal life (correlating to the 3rd trimester of human pregnancy) on subsequent behavioural parameters (Smith et al. 2015). Pregnant C57BL/6J mice were whole-body exposed to PG or PG/2.4% nicotine aerosol from GD15 to GD19, and mothers and offspring were exposed from PND2 to PND16. Exposures were achieved as 6-s puffs every 15 s into a 13.5 x 9 x 8.7 cm chamber, from a total of 600 µL liquid, over a period of approximately 20 min, once per day. Exposure concentrations were not reported. Mean pup weight at birth and throughout postnatal development was significantly lower in the PG group without nicotine compared with the control or the group exposed to PG with nicotine. Mean pup weight in the group exposed to PG with nicotine was significantly lower than that of controls from PND7 onwards. Pups in the PG with nicotine group (postnatally) were estimated to be exposed to 2.1 mg/day, excluding exposures from additional sources such as milk and fur. Mean serum cotinine levels, measured at PND14 in female offspring, were 23.7 ng/mL in the group exposed to PG with nicotine; 2.8 ng/mL in the group exposed to PG without nicotine; and 1.0 ng/mL in controls. The authors noted that similar levels have been reported in newborns of mothers who smoked. One study reported approximately 32 ng/mL to 59 ng/mL cotinine in plasma of newborns, born to mothers who were moderate or heavy CC smokers, respectively, measured 48 hours after birth (Ivorra et al., 2014, *cited in* Smith et al. (2015)), while another study reported a mean plasma cotinine concentration of 76 ng/mL in newborns of mothers who were reported as being smokers (Ivorra et al. 2014, Chazeron et al. 2008, *cited in* Smith et al. 2015). In the study of Smith et al. (2015), behavioural tests to assess sensorimotor, affective, and cognitive domains were performed on adult male offspring at 14 weeks postnatally. Behavioural alterations were observed in offspring exposed to PG with nicotine compared to controls or offspring exposed to PG without nicotine. Authors noted this indicates increased locomotor activity (significant increase in mean number of rearing activity in open field test and head dips in zero maze test), and tendency towards increased cognitive flexibility (significantly more than 25% of time in the new location in water maze test). The authors concluded that the findings indicated that nicotine exposure from E(N)NDS may cause persistent behavioural changes when exposure occurs during a period of rapid brain growth (Smith et al. 2015).

111. A group of researchers based at the University of Sydney have carried out studies of the effects of exposure to E(N)NDS aerosol, with and without nicotine, during gestation and lactation in mice (Chen et al. 2018a, Chen et al. 2018b, Nguyen et al. 2018).

112. One report from this set of studies described assessment of effects on brain development and behaviour (Nguyen et al. 2018). Adult female Balb/c mice (n = 8 per group) were exposed to E(N)NDS aerosol (50/50 PG/VG, tobacco flavour), either with [Ecig(+nic)] or without [Ecig(–nic)] 18 mg/mL nicotine, or ambient air (sham), in a 9 L exposure chamber, for 15 min twice per day with a 5-min interval, from 6 weeks before mating until pups were weaned at PND20. Measured aerosol exposure concentrations were not reported, nor mouse cotinine levels. Behavioural

assessments were carried out on adult male offspring at 12 weeks postnatally. Compared with the sham group, the [Ecig(+nic)] but not [Ecig(–nic)] offspring showed indicators of short-term memory deficit (assessed by novel object recognition test). Both [Ecig(–nic)] and [Ecig(+nic)] aerosol exposures were associated with indicators of increased anxiety in some aspects of the elevated plus maze (EPM) test (significantly increased time in open arms of maze by [Ecig(–nic)] and [Ecig(+nic)] groups; significantly increased number of head dips in the [Ecig(+nic)] group; significantly increased unprotected stretches in the [Ecig(–nic)] group). Both [Ecig(–nic)] and [Ecig(+nic)] showed significantly increased locomotor activity (number of centre crosses in the EPM test) compared with the sham control group. Tissues were collected from male offspring euthanized at PND1, PND20, or 13 weeks postnatally. Global DNA methylation was significantly increased in whole brain of [Ecig(–nic)] but not [Ecig(+nic)] offspring compared with the sham group, evaluated at PND1 and PND20. Evaluation of global DNA methylation was performed specifically for the hippocampus at 13 weeks postnatally, with no significant differences between the 3 groups. Detailed gene expression analyses were performed, showing various changes in genes associated with the modulation of neurological activity. The authors suggested that the changes in global methylation at PND1 and PND20 (in the Ecig(-nic) group only) might be related to substances transferred via the breastmilk. The authors concluded that the memory deficits seen in this study were due to intrauterine nicotine exposure, and that changes in other behaviours seen in EPM tests may be due to other constituents within E(N)NDS aerosols (Nguyen et al. 2018).

Respiratory system development

113. McGrath-Morrow et al. (2015) found that nicotine exposure from E(N)NDS aerosol affected alveolar growth and lung cell proliferation. Neonatal C57BL/6J mice were whole-body exposed to aerosol produced from 400 µL E(N)NDS liquid (PG or PG/1.8% nicotine; no flavouring) from PND1 to PND10, once per day on PND1 and PND2, then twice per day. Exposure was achieved from 6-s puffs every 15 s over approximately 20 min. Exposure concentrations were not reported. Control mice were kept in room air. Results were reported for ‘Trial 1’ and ‘Trial 2’, although the report does not describe what Trial 1 and Trial 2 were. Plasma and urinary cotinine levels in offspring were measured at PND10, reported for Trial 1. Mean levels in plasma and urine, respectively, were: < 5 ng/mL and < 10 ng/mL for both control and PG/no-nicotine groups; 63.3 ng/mL and 892.5 ng/mL for the PG/nicotine group. As compared with controls, total body weight at PND10 was significantly decreased (Trial1 and Trial 2) in both the PG/no-nicotine (11.5% decrease) and PG/nicotine (13.3% decrease) groups. Body weight reduction in PG/nicotine offspring was significantly correlated with plasma cotinine level. Mean linear intercept (MLI), a measure of mean free distance into airspaces, taken as a marker of postnatal alveolar growth, was measured in offspring at PND10. The MLIs were reported as higher in offspring exposed to PG/nicotine compared with controls in Trial 1 ($p < 0.054$) and Trial 2 ($p < 0.006$), but were not associated with cotinine levels. The MLI was not significantly different between PG/no-nicotine and control offspring (Trial 1, p

= 0.27; Trial 2, p = 0.79). The MLI was significantly higher in PG/nicotine offspring than PG/no-nicotine in Trial 2 ($p < 0.014$) but not in Trial 1 ($p < 0.34$). Alveolar cell proliferation was evaluated by immunohistochemical staining for the marker, KI67. Staining was significantly decreased in offspring exposed to PG/nicotine compared with controls and compared with those exposed to PG/no-nicotine. There were no differences in levels of markers of apoptosis or oxidative stress. The authors concluded that the findings from this study indicated that exposure to nicotine-containing E(N)NDS can cause systemic absorption of nicotine and modest impairment of lung growth during early postnatal life. The authors also commented that exposure to aerosol without nicotine caused decreased body weight gain compared with room air-exposed controls, suggesting that PG exposure alone may disrupt postnatal growth, although it was noted that others factors may also need to be taken into consideration, such as stress/altered feeding behaviours related to the different treatment protocol of controls compared with aerosol exposure groups (McGrath-Morrow et al. 2015).

114. Chen et al. (2018a) reported that maternal exposure to E(N)NDS aerosol, with or without nicotine, altered cytokine levels in both maternal and offspring lungs. Adult female Balb/c mice were exposed to E(N)NDS aerosol (50/50 PG/VG, tobacco flavour), either with ('E-cig18') or without ('E-cig0') 18 mg/mL nicotine[43], for 15 min, twice per day with a 5-min interval, from 6 weeks before mating until pups were weaned at PND20. To achieve this, the adult females were removed from the home cage and placed in a 9 L exposure chamber. Adult male mice and offspring did not undergo exposures, but remained in the home cage. Measured exposure concentrations were not reported. Control animals underwent the same procedure, but were exposed to room air instead of E(N)NDS aerosol. Male offspring were studied at PND1, PND20, and at 13 weeks postnatally. Mothers were studied when pups were weaned. Mean blood cotinine levels in offspring at weaning were 9.12 ng/mL (E-cig18), 3.31 ng/mL (E-cig0), and 2.83 ng/mL (control). After the first 6 weeks of exposure, measurement of maternal lung proinflammatory cytokine levels showed increased IL1-β in E-cig18, increased IL-6 in E-cig0, and increased TNF-α in both E-cig0 and E-cig18, compared with controls. Proinflammatory cytokines were measured in adult offspring lung at 13 weeks postnatally. Compared with controls, IL1-β was significantly decreased in both E-cig0 and E-cig18, TNF-α was significantly increased in E-cig18 only, and IL-6 was increased non-significantly ($p = 0.05$) in E-cig0 only. These changes were correlated with changes in signalling pathway protein expression levels. Gene expression markers of alveolar development were studied in PND1 and PND20 offspring lungs. Increased expression of *PDGF* mRNA was noted at PND20, but not PND1, in E-cig18 and E-cig0 offspring, compared with controls. There were no differences between groups in ephrine B2 or surfactant protein C (*Sftpc*) mRNA levels at either PND1 or PND20. Global DNA methylation in the lungs was increased 3-fold in E-cig0 and 2-fold in E-cig18 offspring at PND1, compared with controls. The authors concluded that both nicotine and non-nicotine constituents induce inflammatory responses in the lungs of

[43] Aerosol generated by KangerTech NEBOX, 4 x 5-s puffs at 30 W, 20-s interval

both mothers and offspring, that different signalling pathways may be altered in mothers and offspring, and that epigenetic modifications are likely to contribute in the offspring (Chen et al. 2018a).

Effects on body weight, body fat, and energy homeostasis in offspring

115. As described above, in the study reported by Chen et al. (2018a), adult female Balb/c mice were exposed to E(N)NDS aerosol (50/50 PG/VG, tobacco flavour), either with ('E-cig18') or without ('E-cig0') 18 mg/mL nicotine[44], for 15 min, twice per day with a 5-min interval, from 6 weeks before mating until pups were weaned at PND20. Control animals underwent the same procedure, but were exposed to room air instead of E(N)NDS aerosol. After the first 6 weeks of exposure, E-cig0 mothers had only gained one-third of the weight of control mice, while weight gain in E-cig18 mice was not affected. There were no differences in weight gain or liver weight between maternal groups at the end of the experiment, but retroperitoneal fat mass was significantly reduced in both groups of aerosol-exposed mothers compared with controls. In offspring, body weight did not differ between the 3 groups at PND1. At PND20, E-cig0 offspring were significantly heavier and E-cig18 were significantly lighter than controls. Liver weight, as a percentage of body weight, was significantly increased in E-cig18 offspring but not E-cig0 offspring compared with controls. E-cig0 and E-cig18 offspring had significantly increased retroperitoneal fat mass compared with controls (E-cig0 > E-cig18). Epididymal fat mass was significantly increased in E-cig0 compared with both E-cig18 and controls. At 13 weeks postnatally, body weights did not differ between groups. Liver weight was reduced in E-cig0 compared with the other 2 groups. Retroperitoneal fat mass was increased in both E-cig0 and E-cig18 compared with controls. Mean blood cotinine levels in offspring at weaning were 9.12 ng/mL (E-cig18), 3.31 ng/mL (E-cig0), and 2.83 ng/mL (control) (Chen et al. 2018a).

116. A follow-on study from those of Chen et al. (2018a) and Nguyen et al. (2018) compared effects on energy homeostasis of maternal exposures during pregnancy and lactation to cigarette smoke extract and E(N)NDS aerosol, with or without nicotine, on offspring in mice (Chen et al. 2018b). Adult female Balb/c mice (n = 8 per groups) were exposed to E(N)NDS aerosol (50/50 PG/VG, tobacco flavour, Vaper Empire, Australia) either with [E-cig18] or without [E-cig0] 18 mg/mL nicotine[45], smoke extract [SE][46], or ambient air [Sham]. Exposures were carried out in a chamber, for 30 min, twice per day, from 6 weeks before mating until pups were weaned at PND20. The adult male mice and offspring were not exposed. Measured aerosol exposure concentrations were not reported, however nicotine dosage per treatment was described as "equivalent to 2 cigarettes (2.4 mg)". Two additional subgroups were created from the [SE] group: replacement of SE with E-cig18 vapour

[44] Aerosol generated by KangerTech NEBOX, 4 x 5-s puffs at 30 W, 20-s interval
[45] Aerosol generated by KangerTech NEBOX, 4 x 5-s puffs at 30 W, 20-s interval
[46] 2 cigarettes (Winfield Red, VIC, Australia)

from mating until weaning [Replacement]; supplementation of SE with L-carnitine[47] during gestation and lactation [SE + LC]. Male offspring (1 per litter) were euthanized at weaning (PND20). Body weight and epididymal fat mass were recorded, and total mRNA extracted from whole brain was analysed for expression of the genes encoding the regulators of appetite and food intake, neuropeptide Y (NPY), NPY Y1 receptor (Y1R), pro-opiomelanocortin (POMC), melanocortin receptor (MC4R), single-minded gene (Sim) 1, and leptin receptor (Ob-Rb), and also the oxidative stress marker, inducible nitric oxide synthase (iNOS). Analysis of expression levels of proteins was not undertaken due to insufficient sample availability. For all measured parameters, 2 sets of comparisons were made: 1] [Sham], [e-cig0], and [e-cig18]; 2] [Sham], [SE], [Replacement], and [SE + LC]. At PND20, [E-cig0] offspring had significantly higher body weight, fat mass, and % fat compared with [E-cig18] and with [Sham], while values for [E-cig18] were not significantly different from [Sham]. There were no significant differences in body weight, fat mass, or % fat between [Sham] and either [SE] or [SE + LC]. However, in [Replacement] offspring, fat mass and % fat were significantly reduced compared with [SE]. For gene expression analyses, NPY and iNOS were significantly higher in [E-cig0] compared with [Sham] and with [E-cig18], and MC4R and Ob-Rb were significantly higher in [E-cig0] compared with [E-cig18]. There were no significant differences between [E-cig18] and [Sham]. Exposure to [SE] was associated with significantly increased expression of NPY, NPY Y1R, and iNOS compared with [Sham], [Replacement], and [SE + LC], and also with increased expression of MC4R and Ob-Rb compared with [Sham]. In [Replacement] offspring, NPY, NPY Y1R, MC4R, Sim, Ob-Rb, and iNOX were significantly lower compared with [SE], and NPY, MC4R, and Ob-Rb were significantly lower compared with [SE + LC], with the authors noting that “the effect of Replacement to normalise brain markers was more potent than maternal L-carnitine supplementation.”. The authors commented that this study showed that nicotine-free E(N)NDS aerosol induces significant adiposity, dysregulation of brain metabolic regulatory pathways and increase iNOS expression, and concluded that nicotine-free may be more harmful than nicotine-containing E(N)NDS aerosol if the same amount is inhaled. They also commented on the fact that replacement of CC smoke with nicotine-containing aerosol normalised the effects of CC smoke on the regulatory pathways evaluated, supporting an assumption that E(N)NDS vapour may be safer than smoking CC, and highlighting the potential role of CC components other than nicotine in maternal programming of metabolic regulation in offspring (Chen et al. 2018b).

[47] An antioxidant that has shown benefits on ‘offspring brain health’ when supplemented to pregnant mice exposed to SE.