Regulatory Toxicology and Pharmacology 139 (2023) 105369

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Non-sugar sweeteners and cancer: Toxicological and epidemiological evidence

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ARTICLE INFO

Handling Editor: Dr. Martin Van den berg

Keywords:
Non-sugar sweetened beverages
Artificial sweeteners
Cancer
Toxicology
Epidemiology

ABSTRACT

Several toxicological and epidemiological studies were published during the last five decades on non-sugar sweeteners (NSS) and cancer. Despite the large amount of research, the issue still continues to be of interest. In this review, we provided a comprehensive quantitative review of the toxicological and epidemiological evidence on the possible relation between NSS and cancer. The toxicological section includes the evaluation of genotoxicity and carcinogenicity data for acesulfame K, advantame, aspartame, cyclamates, saccharin, steviol glycosides and sucralose. The epidemiological section includes the results of a systematic search of cohort and case-control studies. The majority of the 22 cohort studies and 46 case-control studies showed no associations. Some risks for bladder, pancreas and hematopoietic cancers found in a few studies were not confirmed in other studies. Based on the review of both the experimental data on genotoxicity or carcinogenicity of the specific NSS evaluated, and the epidemiological studies it can be concluded that there is no evidence of cancer risk associated to NSS consumption.

1. Introduction

Non-sugar sweeteners (NSS), also known as artificial, non-nutritive, or intense sweeteners, comprise a group of food additives that provide high sweetness intensity per gram of food and beverage products (Samaniego Vaesken et al., 2021). They are used in very small quantities and deliver no or negligible calories, replacing added sugars in a variety of food products. The use of NSS has become more common for manufacturers to develop new products and to comply with food and beverage reformulation practices with the aim to decrease energy resulting from added sugars. Furthermore, there is a general consumer interest in reducing energy intake, and food products containing NSS have become a more popular choice (Samaniego Vaesken et al., 2021).

Sweeteners, like other food additives, are subjected to strict safety control. There are currently 19 compounds authorized for use in food products by the European regulations, 7 of them being classified as polyols (low-calorie sweeteners) and the remaining 12 as non-calorie sweeteners, of which the most notable ones are acesulfame K (E950), aspartame (E951), cyclamates (E952), saccharin (E954), sucralose (E955), and steviol glycosides (E960) (Commission Regulation (EU) No 1129/2011, 2011). In addition, the new high-intensity, low-calorie sweetener, advantame, another N-substituted (aspartic acid portion) derivative of aspartame, similar in structure to neotame, is becoming popular. NSS have different chemical structures, although all of them have in common the ability to potently activate some of the multiple potential ligand-binding sites of the sweet-taste receptors in human subjects (Behrens and Ziegler, 2020).

Although, there is no consistent evidence of the association between NSS and cancer risk, the issue still continues to be of interest. Therefore, we provide a comprehensive quantitative review of the toxicological and epidemiological evidence on the possible relation between

Abbreviations: ADI, Acceptable Daily Intake; BW, Body Weight; CA, Chromosomal Aberrations; ERF, European Ramazzini Foundation; FAO, Food and Agriculture Organisation; GRAS, Generally Recognized As Safe; HR, Hazard Ratio; JECFA, Joint Expert Committee on Food Additives; NHL, Non-Hodgkin Lymphoma; NIH-AARP, National Institutes of Health - American Assiciation of Retired Persons; HPFS, Health Professionals Follow-up Study; NOAEL, No Observed Adverse Effect Level; NTP, National Toxicology Program; OR, Odd Ratio; SSB, Sugar Sweetened Beverages; SCE, Sister Chromatide Exchanges.

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https://doi.org/10.1016/j.yrtph.2023.105369
Received 9 January 2023; Received in revised form 24 February 2023; Accepted 27 February 2023
Available online 3 March 2023

Abbreviations (only firmly established)

CI	Confidence Interval
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
GLP	Good Laboratory Practice Regulations
IARC	International Agency for Research on Cancer
HR	Hazard ratio
HTS	High throughput screening
Non-SSB	Non-sugar sweetened beverages
NSS	Non-sugar sweeteners
NHL:	non-Hodgkin lymphoma
SCF	Scientific Committee on Food

consumption of NSS and cancer. A large amount of toxicological and epidemiological research was published during the last five decades on NSS and cancer and a number of warnings have been delivered to the public opinion, starting from that on saccharin and bladder cancer in the 1970s.

While the toxicological section of this review considered the most commonly used NSS separately, the epidemiological studies were unable to separate various NSS. The toxicological section includes the evaluation of genotoxicity and carcinogenicity data for acesulfame potassium (Ace K) (E950), advantame, aspartame (E951), cyclamates (E952), saccharin (E954), steviol glycosides (E960) and sucralose (E955), that are the most notable compounds. Given the recent interest in aspartame, we include a separate part in the epidemiological section (World Health OrganizationRios-Leyvraz and Montez, 2022).

2. Toxicology

2.1. Methods

The genotoxicity of acesulfame K, aspartame, saccharin, steviol glycosides and sucralose was assessed by conducting a search in PubMed and Embase databases, to identify more recent studies published since the review by Lea et al. (2021). We developed a search strategy for publications in English using search term "acesulfame K″, "aspartame", "saccharin", "steviol glycosides" and "sucralose" in combination with "genotoxicity". In addition, genotoxicity data utilized in authoritative assessments (Food and Drug Administration (FDA), Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) and European Food Safety Authority, EFSA, International agency for research on cancer (IARC)) were also reviewed. For advantame and cyclamates genotoxicity, an independent literature search was performed to identify systematic reviews and published studies. The search strategy for publications in English using search term "advantame" and "cyclamates" in combination with "genotoxicity". The carcinogenicity of acesulfame K, aspartame, steviol glycosides and sucralose was assessed by conducting a search in PubMed and Embase databases to identify more recent studies published since the reviews by Chappell et al. (2020b), Haighton et al. (2019) and Wikoff et al. (2020), Chappell et al. (2021), and Chappell et al. (2020a), respectively. In addition, carcinogenicity studies reported in authoritative assessments were also reviewed. For advantame, saccharin, and cyclamates carcinogenicity, an independent literature search was performed to identify systematic reviews and published studies.

From the articles on genotoxicity, we extracted the following data: end-point considered, test object, concentration of treatment *in vitro* and *in vivo*, results, and reference. From the included articles on carcinogenicity, we extracted the following data: species/strain/sex, dose, duration of treatment, results and reference. All selected articles were reviewed and the quality rating of the different articles was indicated as a note at the bottom of each table.

2.2. Results

Table 1 gives the number of articles retrieved by our systematic search and those not included in previous reviews.

2.2.1. Acesulfame potassium

Acesulfame potassium (Ace K) (CASRN 55589-62-3), a zero-calorie NSS, is the potassium salt of 6-methyl-1,2,3-oxathiazine-4(3*H*)-one 2,2-dioxide, a white crystalline powder having molecular formula of $C_4H_4KNO_4S$ and molecular weight 201.24 g/mol. It is approximately 120 times sweeter than sucrose and has high water solubility. Ace-K is heat stable and can be used in cooking and baking. Ace-K is often blended with other sweeteners (Sucralose or Aspartame) without adding calories upon breaking. Ace K does not undergo biotransformation *in vitro*, nor *in vivo* in both humans and experimental animals where it is excreted unchanged in urine. The breakdown product of Ace-K, acetoacetamide, is toxic if consumed in very large doses but human exposure to breakdown products is negligible (Magnuson et al., 2016).

The acceptable daily intake (ADI) for Ace K established by the Food and Drug Administration (FDA) in 1988 and by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) (JECFA, 1991) in 1991 is 0–15 mg/kg BW (FDA, 2019), in 2000, the European Scientific Committee on Food (SCF, precursor to the European Food Safety Authority, EFSA) riaffirmed an ADI of 0–9 mg/kg BW in 2000 (SCF, 2000a). The discrepancy between the FDA/JECFA and SCF/EFSA derives from the fact that SCF/EFSA consider the dog and not the rat the appropriate species; hence, although no effect of toxicological significance at dietary dose levels up to 3% in the rat (equivalent to 1500 mg/kg BW per day) or in the dog (equivalent to 900 mg/kg BW per day) has been observed, the points of departure to establish the ADI were 1500 and 900 mg/kg per day for FDA/JECFA and SCF/EFSA, respectively.

2.2.1.1. Genotoxicity. Relevant evidence available for Ace K genotoxicity can be derived from a review that includes data from the 1991 JECFA (JECFA, 1991), and the 2000 SCF reports (SCF, 2000a). Five additional studies since the SCF review (which were also evaluated in (Chappell et al., 2020b) and high-throughput screening (HTS) (from the ToxCast/Tox21 program summary files for nine assay endpoints reported by (Hsieh et al., 2019) are available and reviewed by (Lea et al., 2021); these data are shown in Table 1S. No other further data have been

Table 1
Numbers of studies on genotoxicity and carcinogenicity of non-sugar sweeteners considered in previous published reviews and new studies considered in the present review.

Compound	Genotoxicity		Carcinogenicity	
	Considered in previous published reviews	New studies	Considered in previous published reviews	New studies
acesulfame potassium (Ace K) (E950)	5	0	1	0
advantame	0	1	0	1
aspartame (E951)	6	0	13	0
cyclamates (E952)	4	0	6	0
saccharin (E954)	2	0	2	0
steviol glycosides (E960)	2	0	3	0
sucralose (E955)	2	0	4	0

retrieved since that review.

Since the SCF review, three micronucleus studies for Ace K were conducted *in vivo* in mice, including a 40-week dietary study conducted by the National Toxicology Program (NTP) in transgenic mouse strains engineered to be susceptible to tumors. These studies were all negative, with the exception of an increase in micronucleated erythrocytes in p53-haploinsufficient male, but not female, mice exposed to up to approximately 4500 mg/kg BW/day for 40 weeks weeks (National Toxicology Program, 2005). However, in the NTP study, no increase in MN was observed in either sex in another transgenic mouse strain engineered to overexpress an oncogene, nor in Naval Medical Research Institute (NMRI) mice exposed to up to 4500 mg/kg BW Ace-K by oral gavage twice within 24 h (Mayer, 1991). The NTP report specifically stated that the MN response was "of uncertain biological significance" due to the weak nature of the increase and the lack of consistency across sexes.

The genotoxicity potential Using Tox21 HTS Assays for small molecules that induce genotoxicity reported by (Hsieh et al., 2019) revealed that Ace K is inactive in 17 genotoxicity-relevant HTS assays from ToxCast/Tox21. The HTS assays included those able to detect genotoxicity by increasing expression of luciferase-tagged ATAD5 and cell viability in human embryonic kidney cells, enhancing cytotoxicity in DT40 cells deficient in DNA repair proteins REV3 or KU70/RAD54, and the activation of p53. The dose tested were up to 200 μM. The FDA also evaluated these studies and reported that they did not suggest any genotoxic effects based on these findings, the overall weight of the evidence indicates that Ace K is not genotoxic.

2.2.1.2. Carcinogenicity. The carcinogenicity profile of Ace K has been recently reviewed by (Chappell et al., 2020b). An updated search did not identify additional studies.

A total of four dietary carcinogenicity studies on mice and rats were identified (Table 2). All of them did not report significant increase in tumors (Beems et al., 1991; National Toxicology Program, 2005; Reuzel and van der Heijden, 1991; Sinkeldam et al., 1991). The studies did not follow specific OECD guidance, but their protocols and reports were consistent with guideline studies. A one-year short-term study on dogs (Reuzel and van der Heijden, 1991) was not considered.

The NTP (National Toxicology Program, 2005) bioassays were conducted in transgenic mice (p53±in C57BL/6 background and Tg.AC in FVB/N) engineered to be susceptible to tumor induction by reducing the expression of genes that are important components of tumor-suppressor machinery (only a single wild type p53 allele) or over-expressing an oncogene (vHa-ras). This p53 haploinsufficient strain is sensitive and specific to mutagenic carcinogens compared to the B6C3F1 strain typically used in 2-year cancer bioassays. The Tg.AC strain develops skin papillomas and malignant tumors following exposure to genotoxic and non-genotoxic carcinogens, but not when exposed to non-carcinogens (Tennant et al., 1995). Due to the increased sensitivity and accelerated tumor development (these strains have been demonstrated to develop tumors within 40 days of treatment with a carcinogen), bioassays conducted in the transgenic strains (p53 haploinsufficient and Tg.AC) can be carried out for shorter durations than a standard 2-year bioassay.

Based on the weight of evidence on genotoxicity and the negative findings in carcinogenicity studies, there is no experimental evidence of carcinogenicity of Ace K.

Table 2
Summary report of carcinogenic studies of Ace K.

Species/strain/sex	Oral Dose	Duration	Results	Study
p53 haploinsufficient mice (B6.129-Trp53^{tm1Brd}), male and female	0%, 0.3%, 1%, or 3% (0, 3,000, 10,000, or 30,000 ppm)	40 weeks	No treatment-related increase in non-neoplastic and neoplastic lesions.	National Toxicology Program (2005)
Tg.AC hemizygous mice (FVB/N-TgN (v-Ha-ras)Led), male and female	0%, 0.3%, 1%, or 3% (0, 3,000, 10,000, or 30,000 ppm)	40 weeks	No treatment-related increase in non-neoplastic lesions.	National Toxicology Program (2005)
CPB-WK SPF rats, male and female	0%, 0.3%, 1%, or 3% (0, 3,000, 10,000, or 30,000 ppm)	Males: *in utero* through 120 weeks Females: *in utero*, through 113 weeks	No treatment-related increase in non-neoplastic lesions.	Sinkeldam et al. (1991)
Albino SPF mice, male and female	0%, 0.3%, 1%, or 3% (0, 3,000, 10,000, or 30,000 ppm)	80 weeks	No treatment-related increase in non-neoplastic lesions.	Beems et al. (1991)

2.2.2. Advantame

The new high-intensity, no-calorie sweetener, advantame, is another N-substituted aspartame. At a sweetness equivalent to 6% sucrose, advantame is approximately 116 times sweeter than aspartame and approximately 37,000 times sweeter than sucrose. The stability of advantame indicates potential functionality in a broad range of food and beverage applications, including low-pH products and products that require high-temperature processing. An ADI of 0–5 mg/kg BW for advantame was established in 2016 by WHO (World Health Organization, 2016) on the basis of a no-observed-adverse-effect level (NOAEL) of 500 mg/kg bw per day for maternal toxicity in a developmental toxicity study in rabbits and application of a 100-fold safety factor to account for interspecies and intraspecies variability. The ADI was suggested to be also applied to those individuals with phenylketonuria. Using the proposed maximum use levels and conservative assumptions, the maximum mean dietary exposure to advantame would be 1.45 mg/kg bw per day (29% of the upper bound of the ADI), and the maximum high-percentile dietary exposure would be 2.16 mg/kg bw per day (43% of the upper bound of the ADI) (World Health Organization, 2016). In 2013 EFSA (EFSA, 2013a,b) established the same ADI of 0–5 mg/kg bw/day based on the same end-point.

2.2.2.1. Genotoxicity. A series of *in vitro* and *in vivo* genotoxicity assays including bacterial mutation, mammalian cell mutation, and mouse micronucleus tests (Table 2S) has been conducted with advantame.

The genotoxicity tests were conducted in compliance with the Good Laboratory Practice (GLP) Regulations: UK GLP Regulations 1999 (Statutory Instrument No. 3106); OECD Principles of Good Laboratory Practice (as revised 1997), ENV/MC/CHEM(98)17; Japanese Ministry of Health and Welfare, Ordinance No. 21, 26 March 1997; EC Commission Directive 1999/11/EC of 8 March 1999 (Official Journal No. L 77/8); US FDA, Title 21 Code of Federal Regulations Part 58, Federal Register, 22 December 1978 and subsequent amendments. Mutagenic activity in the Ames test using standard preincubation methods in accordance with test guidelines established by the United States FDA (U.S. FDA, 2000a). Mutagenicity in the mouse lymphoma L5178Y cell mutation test in accordance with test guidelines established by (U.S. EPA, 1998), and the European Economic Community (EEC, 2000). Micronuclei formation was evaluated using male and female CD-1 mice. The procedure used was based on the test guidelines established by (U.S. FDA, 2000b). The results of these studies and of EFSA in 2013 indicate that advantame is non mutagenic and non-genotoxic.

2.2.2.2. Carcinogenicity. In short term dietary studies in Han wistar rats, advantame did not cause toxicological effects up to 50,000 ppm.

This was the highest dose used in the chronic toxicity/carcinogenicity study.

In a rat carcinogenicity study (Otabe et al., 2011), approximately 28-day-old Han Wistar rats (55 males and 55 females) were administered advantame in the diet at concentrations of 0, 2000, 10,000, or 50,000 ppm for 104 weeks. Animals were the offspring of parental animals that had been given the same dose level for 4 weeks prior to pairing, through gestation, and until weaning on the 21st day post-birth. Over the 2 years of the study, these dietary concentrations of advantame resulted in doses of 0, 97, 488, and 2621 mg/kg BW/day in males and 0, 125, 630, and 3454 mg/kg BW/day in females. Only minor reductions in BW gain were observed in high-dose males, with no changes in hematological and clinical chemistry parameters. Incidence of pancreatic islet cell carcinomas in males was 0/55, 1/55, 2/55, and 3/55 in the 0, 2000, 10,000, or 50,000 ppm groups, respectively, and mammary gland adenomas were observed in 4/41 high-dose females; these findings were not significant and were within background historical control values. The authors concluded that advantame was not carcinogenic in rats.

In mice the carcinogenic potential of advantame was examined in 6-week-old Crl:CD-1 (ICR) BR mice who consumed diets comprising 0, 2000, 10,000, or 50,000 ppm advantame for 104 weeks (64/sex/group). The dietary concentrations of advantame resulted in achieved doses of 0, 213, 1057, and 5693 mg/kg BWt/day in male animals and 0, 272, 1343, and 7351 mg/kg BW/day in female animals. The only effect observed was reduced BW gain in the 50,000 ppm group females (76% of controls). In male mice a trend towards lower BW also was observed; however, these were not significant, likely due to the large degree of variability between animals. There were no findings of toxicity. Analysis of the tumor incidence data revealed no evidence of a carcinogenic effect of advantame (Otabe et al., 2011).

In a 1-year dog study, advantame was administered in the diet at concentrations of 0, 2000, 10,000, or 50,000 ppm (equal to 0, 83, 421, and 2058 mg/kg BW/day in males and 0, 82, 406, and 2139 mg/kg BW/day in females, respectively) to groups of 22- to 26-week-old beagle dogs (4/sex/group). No treatment-related effects were reported (Otabe et al., 2011).

In July 2013, EFSA's experts concluded that advantame and its metabolites are not carcinogenic (EFSA, 2013a,b).

2.2.3. *Aspartame*

Aspartame (CASRN 22839-47-0) is among the most used low-calorie non saccharide sweetener alternative to table sugar (Magnuson et al., 2007; Mooradian et al., 2017) of molecular formula. C14H18N2O5. After ingestion it is completely hydrolyzed in the gastrointestinal tract to methanol, aspartic acid, and phenylalanine. Since one of the metabolic products of aspartame is phenylalanine, patients with phenylketonuria should avoid excessive use of aspartame. Upon breaking Aspartame is the only NSS that produces about 4 calories of energy per gram.

With more than hundred studies conducted, aspartame is one of the most studied substances in the human food supply by regulatory bodies, including the U.S. FDA and the EFSA, that approved, and repeatedly re-evaluated it, for use in a variety of foods and beverages (EFSA, 2013a,b; FDA, 2019). The FDA (FDA, 2019) has established an ADI of 0–50 mg/kg BW (CFR 172.804), and the ADI as determined by the JECFA and SCF is 0–40 mg/kg BW, a value retained through several re-evaluations – most recently by EFSA (EFSA, 2013a,b).

2.2.3.1. Genotoxicity. The genotoxicity profile of aspartame has been assessed in a systematic review (Lea et al., 2021) that includes the studies reviewed in the 2013 EFSA opinion (EFSA, 2013a,b), and HTS data (Hsieh et al., 2019). Another review also included the majority of those studies (Magnuson et al., 2007). Our updated literature search yielded no further papers. Table 3S reports the genotoxicity data of aspartame.

EFSA (EFSA, 2006) concluded "overall the data strongly indicate that

Table 3
Summary report of carcinogenicity studies of aspartame.

Species/ Strain/Sex	Oral Dose	Study Duration	Neoplastic lesions, and dose level	Study
Charles River Albino Rats; male and female (40/sex/group; 60/sex/control)	0, 1,2, 4, or 8 g/kg/day; the very high dose group was increased from 6 to 7 g/kg/day at week 16; then from 7 to 8 g/kg/day at week 44.	104 weeks; high dose group was increased from 6 to 7 g/kg/day at week 16; then from 7 to 8 g/kg/day at week 44	None reported[a]	(Searle, 1973)
Charles River Albino Rats; male and female (40/sex/group; 60/sex/group)	0, 2, or 4 g/kg bw/d	*in utero* through 104 weeks	None reported[a]	(Searle, 1974)
ICR Swiss mice (36/sex/group; male and female (36/set/group; 72/sex/control)	0, 1,2, or 4 g/kg bw/day	104 weeks	None reported[b]	(Searle, 1974)
SLC Wistar rats, male and female (59 or 60/sex/group)	0, 1, 2, and 4 g/kg bw/d-	104 weeks	None reported[a]	Ishii (1981)
Heterozygous p53-deficient mice, male and female. (15/sex/group)	0, 3, 125, 6,250, 12,500, 25,00, or 50,000 ppm (0, 490, 970, 1,860, 3,800, or 7280 mg/kg bw in males; 0, 630, 1,210, 2,490, 5,020, or 9620 mg/kg bw in females)	40 weeks (9 months);	None reported[b]	National Toxicology Program (2005)
Cdkn2a-deficient mice, male and female (15/sex/group)	0, 3, 125, 6,250, 12,500, 25,00, or 50,000 ppm (0, 490, 960, 1,900, 3,700, or 7400 mg/kg bw in males; 0, 610, 1,200, 2,390, 4,850, or 9560 mg/kg bw in females)	40 weeks (9 months)	None reported[b]	National Toxicology Program (2005)

(*continued on next page*)

Table 3 (*continued*)

Species/ Strain/Sex	Oral Dose	Study Duration	Neoplastic lesions, and dose level	Study
Tg.AC mice, male and female (15/ sex/group)	0, 3, 125, 6,250, 12,500, 25,00, or 50,000 ppm (0, 490, 980, 1,960, 3,960, or 7660 mg/ kg bw in males; 0, 550, 1,100, 2,260, 4,420, or 8180 mg/ kg bw in females)	40 weeks (9 months)	None reported[b]	National Toxicology Program (2005)
Sprague-Dawley rats, male and female (100–150/ sex/group)	0, 80, 400, 2,000, 10,000, 50,000, or 100,000 (0, 4, 20, 100, 500, 2500, 5000 mg/ kg bw);	Daily until natural death	There was a significant increase trends in total malignant tumors, lymphomas/ leukemias, neoplastic lesions of the renal pelvis and ureter, and schwannomas of the peripheral nerves. For the mentioned cancer, there was also a significant increases of the incidence of tumors at dose level ranging from 400 to 10000 ppm.[c]	Soffritti et al. (2006)
Sprague-Dawley rats, male and female (70–95/sex/ group)	0, 400, or 2000 ppm (0, 20, or 100 mg/kg bw)	Daily, *in utero* (fetal day 12) through natural death	At 2000 ppm significant increase of total malignant tumors (male), lymphomas/ leukemia (male and females), and mammary carcinomas (females) were observed.[c]	Soffritti et al. (2007)
Swiss mice, male and female (60–122/ sex/group)	0, 2,000, 8,000, 16,000, 32,000 ppm (0, 250, 1000, 2000, and 4000 mg/ kg bw)	GD 12 to natural death	Males showed s significant increased incidence of hepatocellular carcinoma at 16000 and 32000 ppm and alveolar/ bronchiolar carcinoma at 32000 ppm.[c]	Soffritti et al. (2010)
Male C57BL/6 Ela1-Tag mice	0 or 0.035% w/v	*in utero* to 21 weeks of age	Model expresses the SV40 large T Antigen under the control of the Elastase-1 acinar cell promoter, driving spontaneous pancreatic cancer formation. All animals (control and treated) displayed tumors.[d]	Dooley et al. (2017)
Male F344/ DuCrj rats	5% in drinking water (total intake = 395.7 g/kg bw)	Administered from week 5 of experiment to week 36	Not reported[d]	Hagiwara et al. (1984)

Nine studies having complete histopathology were included: three 2-year studies by Searle; three transgenic mice studies by the NTP; three lifetime studies by the Ramazzini Institute.These studies were rated Klimisch Code 2 (reliable with restrictions). The Ramazzini Institute used a lifetime model of their own design that has been questioned due to high rates of spontaneous tumors, issues with tumor type diagnosis and concerns about the impact of chronic infections. As many of these problems could be attributed to using animals that died or were terminated near end of life, along with the other problems noted, these studies were rated Klimisch Code 3 (not reliable). As the Klimisch Code 2 studies demonstrated a lack of carcinogenic potential, and as aspartame is hydrolyzed to common components and lacks genotoxic activity, a conclusion that aspartame is not carcinogenic is supported.

[a] They were compliant with GLP and with a reliable endpoint (Klimisch scores of 2).

[b] They were compliant with GLP (Klimisch scores of 2) but no standard OECD method for conducting a study in transgenic mice.

[c] Not reliable (Klimisch scores of 3) and inappropriate study design.

[d] They were not compliant with GLP but with a reliable endpoint (Klimisch scores of 2).

aspartame is devoid of any genotoxic potential" This was reaffirmed in 2013 (EFSA, 2013a,b). Similarly (Kirkland and Gatehouse, 2015), concluded that there is no evidence for the induction of gene mutations or chromosomal damage from exposure to aspartame *in vitro* or *in vivo* (i. e. lack of micronucleus formation in bone marrow, chromosomal aberrations and comet studies, DNA damage). More recent data did not change this conclusion (Otabe et al., 2019).

2.2.3.2. Carcinogenicity. The carcinogenicity profile of aspartame has been recently reviewed (Haighton et al., 2019; Wikoff et al., 2020). An updated search did not identify additional studies.

A total of ten cancer bioassays were identified (Table 3). Seven of these studies reported a lack of carcinogenicity following chronic, high-dose exposure to aspartame (Ishii, 1981; National Toxicology Program, 2005)(Searle, 1974a; Searle, 1974b; Searle, 1973). Three studies, that have been reported in a number of publications (Belpoggi et al., 2006; Soffritti et al., 2006, 2007, 2010), claimed increased incidences of hematopoietic, liver, lung, and peripheral nerve malignancies in Swiss mice and Sprague-Dawley rats. These studies have been conducted by the same laboratory (the European Ramazzini Foundation on Oncology and Environmental Sciences, ERF) and have been repeatedly criticized because of deficiencies in the study protocol, study conduct and reporting (EFSA, 2013a,b, 2006; FDA, 2017; Gift et al., 2013; Magnuson et al., 2007; "National Toxicology Program; Notice of Public Meeting," 2019; Schoeb et al., 2009). An EFSA(EFSA, 2006) review concluded that the studies contain "flaws which bring into question the validity of the

findings." The test animals had a high background incidence of chronic inflammatory changes that could confound the interpretations of the findings (e.g.: the observed lymphomas might have resulted from severe respiratory mycoplasmosis, caused by *mycoplasma pulmonis* disease), and the aggregation of the tumor incidences are considered not statistically justified; moreover, the life-time exposure study design may have confounded the conclusions, because older animals have a higher probability of autolytic changes when moribund animals are evaluated.

Furthermore, a recent third-party histological re-evaluation of tissues by Ishii (1981) which originally evaluated only brain neoplasms, conducted an histological evaluation of a number of organs, including those reportedly affected by the ERF studies and found no evidence of tumorigenic effects (Shibui et al., 2019).

Two additional studies, though not traditional bioassays, reported that aspartame did not promote bladder carcinogenesis in rats following a 36-week exposure at 5% in drinking water (Hagiwara et al., 1984), nor did aspartame alter pancreatic acinar carcinoma in mice exposed to 0.035% aspartame in drinking water *in utero* through 21 weeks of age water (Dooley et al., 2017).

The criticism by EFSA (EFSA, 2009) and the US FDA (U.S. FDA, https://wayback.archive-it.org/7993/20190208035817/">2007) U.S. EPA (2009) that concluded that the "increased incidence of lymphomas/leukemias reported in treated rats was unrelated to aspartame, given the high background incidence of chronic inflammatory changes in the lungs and the lack of a positive dose-response relationship" was addressed by (Tibaldi et al., 2020). These authors re-examined the lesions originally diagnosed as lymphoma or leukemia in ERF studies (Belpoggi et al., 2006; Soffritti et al., 2006), and reclassified them according to the INHAND Criteria concluding that the original diagnoses of malignancy were confirmed in 92.3% of cases. Based on this re-evaluation (Landrigan and Straif, 2021), concluded that aspartame is, in fact, carcinogenic. However, these authors (Roberts, 2021) failed to address the concerns raised by EFSA and FDA. In particular, the histological images (Tibaldi et al., 2020) proved to be inconclusive since the relationship with surrounding tissues was not provided, the absence of inflammation in other organs was not reported, and no conclusive evidence was offered to substantiate that there was no mycoplasma or other microbial infection within these animals.

Thus, available data support the conclusion, already reached by FDA and EFSA, on the lack of genotoxic and carcinogenic potential of aspartame.

2.2.4. Cyclamate

Ciclamate, another widely used artificial sweetener discovered by chance in 1937 by Audrieth and Sveda, produced from cyclohexylamine by sulfonation (Bizzari et al., 1996), was first introduced into the marketplace in 1951 (Bopp et al., 1986). Cyclamate (E952) is generally used in the form of a sodium salt because it is more soluble in water than the free acid. The calcium salt is also used as a sweetener, but, for some applications, it is not suitable as it can cause gelation and precipitation. Sodium cyclamate exhibits good stability in the solid form and is also stable in soft drink formulations within the pH range 2–10. Since it is stable following different food production processes and has a long shelf-life, cyclamate can be used in a range of food and beverage applications. It produces no calories upon breaking.

Following classification as a Generally Recognized As Safe (GRAS) article in 1958, cyclamate became the most prominently consumed NSS in food and drinks (Higginbotham et al., 1983). While only around 30 times sweeter than sucrose, it proved to have a superior taste profile when combined with saccharin in a 10:1 mixture. However, following results of a chronic study conducted in rats fed a cyclamate-saccharin mixture that indicated an increase in tumor incidence in some animals after 78 weeks of exposure (Price et al., 1970), the FDA banned the sweetener and removed cyclamate from the GRAS list. However, many countries did not follow the decision of the U.S. to ban cyclamate which continued to be used as a food additive. An ADI of 0–11 mg/kg/day was established by both the JECFA (JECFA, 1982) and the SCF (SCF, 2000b).

2.2.4.1. Genotoxicity. Cyclamate has been comprehensively reviewed several times by agencies across the world, including the JECFA (JECFA, 1982), the SCF (SCF, 2000b) and Food Standard Australia New Zealand (FSANZ, 2007). Genotoxicity data have also been extensively appraised in the 1999 by the IARC (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1999). Results on calcium and sodium cyclamate, and cyclohexylamine ("cyclamates") reveals that while cyclamates were not genotoxic in 83 studies on rodents *in vivo*, sodium cyclamate caused increased chromosomal aberrations in some mammalian cells *in vitro studies* (Collin, 1971; Kristoffersson, 1972; Nicholson and Jani, 1988; Stoltz et al., 1970; D. Stone et al., 1969; Stone et al., 1969, 1969; David Stone et al., 1969; Tokumitsu, 1971; Wolff, 1983)(Pérez Requejo (1972). The positive findings reported in some *in vitro* and *in vivo* studies on cyclamate salts are considered of insufficient reliability due to methodological shortcomings. Cyclamates however did not produce chromosomal aberrations in peripheral lymphocytes of volunteers given 70 mg/kg/day of cyclamate (Dick et al., 1974). No further data are available since (FSANZ, 2007). Therefore, the consistent conclusion is that there is no evidence of genotoxicity by cyclamate. After a reexamination, in 2022 (https://www.efsa.europa.eu/en/call/call-data-genotoxicity-data-sweeteners), EFSA concluded that additional data required would be an *in vivo* Comet assay by the oral route for the food additive cyclamates and for its metabolite cyclohexylamine (CHA). Based on the lack of data at the sites of contact, the recommended tissues to be assessed in the *in vivo* Comet assay are: stomach, colon, liver and blood cells.

2.2.4.2. Cancerogenicity. The JECFA (JECFA, 1982), the European Scientific Committee on Food (SCF, 2000b) U.S. National Cancer Institute Committee (NCIC), the Cancer Assessment Committee of the Center for Food Safety and Applied Nutrition (CAC-CFSAN) (CAC-CFSAN, 1984) at the U.S. FDA, the Food Additives and Contaminant Committee of Great Britain (MAFF) (MAFF, 1982), the National Academy of Sciences-National Research Council (NAS-NRC) (NRC, 1985) and the IARC (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1999) reviewed the carcinogenicity studies on cyclamate. No new carcinogenicity studies have been published in the literature after these evaluations. Particular attention was given to the increased incidence of bladder tumors reported in rats fed with high dietary concentrations of a mixture of sodium cyclamate and sodium saccharin (Price et al., 1970), which was the driver of the banning of cyclamate in the US (FDA, 1970). Although cyclamate was associated with bladder tumors in another chronic feeding study (Oser et al., 1976), the effect could not be reproduced in a number of other cyclamate carcinogenicity bioassays (Bopp et al., 1986). Furthemore, a long-term feeding study of sodium saccharin showed no evidence of carcinogenic effect on the urinary tract in nonhuman primates (Takayama et al., 2000). Thefore, the consistent conclusion was that there is no evidence of carcinogenicity by cyclamate.

2.2.5. Saccharin

Sodium saccharin (CASRN 128-44-9), the oldest low-calorie sweetener, 300 times sweeter than sucrose, was discovered in 1878 and has been used as a sweetener since then. Saccharin (acid form), sodium saccharin and calcium saccharin are widely used as non-caloric tabletop sweeteners, in beverages and foods, in personal care products and in a variety of nonfood applications. Saccharin is not metabolized and, thus, its caloric content is zero.

2.2.5.1. Genotoxicity. The genotoxicity profile of saccharin has been evaluated by a number of agencies and in several published studies. A systematic review was conducted by Lea et al. (Lea et al., 2021) who included and discussed the 1995 SCF report (ADI of 0–5 mg/kg BW)

(SCF, 1995), the 1993 JECFA opinion (ADI of 0–5 mg/kg BW) (FAO/WHO, Organization, W.H., Nations, F. and A.O. of the U., 1993) and also data from HTS (from the ToxCast/Tox21 program summary files for nine assay endpoints reported by (Hsieh et al., 2019). No further data have been found in the literature since the review by (Lea et al., 2021).

The genotoxicity Potential Using Tox21 HTS assays for small molecules that induce genotoxicity reported by (Hsieh et al., 2019) revealed that saccharin is inactive in 17 HTS assays. The HTS assays included those able to detect genotoxicity by increasing expression of luciferase-tagged ATAD5 and cell viability in human embryonic kidney cells (TOX21_ELG1_LUC_Agonist and TOX21_ELG1_LUC_Agonist_viability), enhancing cytotoxicity in DT40 cells deficient in DNA repair proteins REV3 or KU70/RAD54 (TOX21_DT40), TOX21_H2AX_HTRF_CHO_Agonist_ratio and a and the activation of p53 (TOX21_p53_BLA_p1_ratio and TOX21_p53_BLA_p1_viability). The dose tested were up to 200 μM. Table 4S reports available genotoxicity studies.

Initial genotoxicity studies on sodium saccharin that produced some positive results for *in vitro* chromosomal aberrations (CA) and sister chromatide exchanges (SCE) tests are now considered non-specific effects likely resulting from the very high doses used (SCF, 1995). In fact, these high concentrations increased the osmolality in *in vitro* systems, producing spurious results. Furthermore, other *in vitro* positive results (van Eyk, 2015) have been attributed to reduction in viability in all cell lines. *In vivo* studies have similarly been prone to methodological flaws with positive results being attributed to impurities or contaminants in the test article. *In vivo*, comet assays showed inconsistent results; DNA fragmentation was observed in ddY mouse stomach and colon at 1000 and 2000 mg/kg (after a single oral dose) but not in liver, kidney, bladder, lung, brain and bone marrow (Sasaki et al., 2002) while in a study designed to establish an OECD test guideline for the *in vivo* rodent alkaline comet assay by the Japanese Center for the Validation of Alternative Methods (JaCVAM), DNA fragmentation was not observed in rat stomach and liver (Uno et al., 2015).

In summary, the conclusion by the SCF and JECFA that sodium saccharin is not genotoxic is supported by new data. In 2016 sodium saccharin was identified as a reference chemical for negative *in vitro* mammalian cell genotoxicity tests (European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)) (Kirkland et al., 2016).

2.2.5.2. Carcinogenicity. Sodium saccharin was tested by oral administration in numerous experiments in rats and mice, and in a few studies in hamsters, guinea-pigs and monkeys (Table 5S). Sodium saccharin caused urinary bladder tumors in male rats in two-generation studies (Schoenig and Anderson, 1985) (Taylor et al., 1980; Tisdel et al., 1974), but negative in (Schmähl and Habs, 1980), in one study in male rats in which administration began at birth (Schoenig and Anderson, 1985) and in one study (Arnold et al. 1980) at 30 days of age. Sodium saccharin was not carcinogenic for the urinary bladder in several one-generation studies in male and female rats or in mice. Furthermore, saccharin (acid form) did not produce tumors in one study (Cohen et al., 1995) in male and female mice, in one study in male rats or in one study in female rats (Schmähl, 1973; Lessel, 1971). Calcium saccharin did not produce tumors in one study in male rats (Cohen et al., 1995). A few studies with sodium saccharin in hamsters and guinea-pigs also showed no induction of bladder tumors but were considered inadequate (Althoff et al., 1975), (Hagiwara et al., 1983),(Hagiwara et al., 1983). In one long-term (up to 283 months) study (Takayama et al., 1998) in monkeys in which oral administration of sodium saccharin begun shortly after birth, no bladder tumors were observed, but a relatively low dose (25 mg/kg BW) and relatively few animals were used.

In order to understand the specific effect of sodium saccharin, numerous experiments were performed in adult rats involving administration concurrently or, more frequently, sequentially with other chemicals or treatments. Enhanced bladder tumorigenesis has been observed after prior treatment with known urinary bladder genotoxic carcinogens (Cohen, 1985). Thus, the only organ affected by sodium saccharin is the urinary bladder only in rats exposed for periods including pre- and/or postnatal periods and/or when exposure was begun by 30 days of age.

These potential toxic and carcinogenic effects have been at the center of several controversies (Baran and Yilmaz, 2006; Ellwein and Cohen, 1990; Kroger et al., 2006). The mechanism for saccharin-induced bladder cancer involves the binding of saccharin to urinary proteins, initiating the subsequent formation of silicate-containing precipitate and crystals; the urinary crystals act as an abrasive to the bladder epithelium, causing cytotoxicity with resultant regenerative hyperplasia (Cohen et al., 1995). The increase in cell proliferation of the rat urothelium by saccharin is modified by the salt form in which it is administered, despite equivalent concentrations of saccharin in the urine. The chemical form of saccharin in the urine is unaffected, and there is no evidence for a specific cell receptor for the saccharin molecule. The effect in rat urinary bladder is species-specific and not relevant to human

Table 4
Summary report of carcinogenicity studies of steviol glycosides.

Species/ strain/sex	Dose with diet	Duration	Neoplastic lesions and dose level	Study
Fisher 344 rats, male and female	0.1, 0.3, or 1% steviol glycosides	22 (males) or 24 (females) months	No treatment-related increase in tumors.	Yamada et al. (1985)
Wistar rats, male and female	0.2, 0.6, or 1.2% steviol glycosides	6, 12, or 24 months	No treatment-related increase in tumors.	Xili et al. (1992)
Fisher 344 rats, male and female	2.5 or 5% steviol glycosides	104 weeks	No treatment-related increase in tumors.	Toyoda et al. (1997)
C57BL/6 Ela1-Tag mice, male	0.02% w/v steviol glycosides Oral (drinking water)	*in utero*+ 21 weeks	No treatment-related increase in tumors.	Dooley et al. (2017)

Table 5
Summary report of carcinogenicity studies of sucralose.

Species/ strain/sex	Dose Oral (diet)	Duration	Neoplastic lesions and dose level	Study
Sprague-Dawley rats, male and female	0, 0.3, 1, or 3% (0, 3000, 10000, or 30000 ppm)	*in utero*+ 52, 78 or 104 weeks	No treatment-related increase in tumors.[a]	Mann et al. (2000b)
CD-1 mice, male and female	0, 0.3, 1, or 3% (0, 3000, 10000, or 30000 ppm)	104 weeks	No treatment-related increase in tumors.[a]	Mann et al. (2000b)
Swiss mice, male and female	0, 500, 2,000, 8,000, and 16,000 ppm	Gestational day 12 through the lifespan (i.e. until natural death)	No tumor response in female mice. In Males, significant dose-related increase in number of tumor-bearing mice, and a significant dose-related increase in incidence of hematopoietic neoplasias.[b]	Soffritti M et al. (2016)

[a] They were compliant with GLP and with a reliable endpoint with Klimisch scores of 2.
[b] The study was not reliable and issues with reporting, study design, and use of appropriate historical controls.

expected exposures (Ellwein and Cohen, 1990). On these bases, both IARC and the US NTP modified their previous classification of saccharin that is currently classified in Group 3 by IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1999) and not anticipated to be a human carcinogen by the NTP (NTP, 2001).

2.2.6. Steviol glycosides

Steviol glycosides (stevioside and rebaudioside A) exist naturally in the leaves of the *Stevia rebaudiana* (Bertoni) plant (i.e. stevia). They are estimated to be 150–400 times sweeter than saccharose. Used for centuries in parts of South America, stevia has been adopted in recent years by much of the calorie-conscious modern world (Planas and Kucacute, 1968). When consumed, steviol glycosides are hydrolyzed in the gut to the common metabolite steviol which is absorbed and metabolized into steviol glucuronide, and excreted primarily in urine. It produces no calories upon breaking.

2.2.6.1. Genotoxicity. The genotoxicity profile for stevia has been the subject of the systematic review by Lea et al. (2021) that includes the 2010 EFSA opinion (EFSA, 2010) (ADI, expressed as steviol equivalents, of 4 mg/kg BW/day) (Misra et al., 2011), four additional studies published (Chappell et al., 2021) since the EFSA (2010), and HTS data (from the ToxCast/Tox21 program summary files for nine assay endpoints reported by Hsieh et al. (2019). No other further data have been found in the literature published after the review by (Lea et al., 2021).

The genotoxicity Potential Using Tox21 HTS assays for small molecules that induce genotoxicity reported by (Hsieh et al., 2019) revealed that steviol glycosides is inactive in 17 HTS assays. The HTS assays included those able to detect genotoxicity by increasing expression of luciferase-tagged ATAD5 and cell viability in human embryonic kidney cells (TOX21_ELG1_LUC_Agonist and TOX21_ELG1_LUC_Agonist_viability), enhancing cytotoxicity in DT40 cells deficient in DNA repair proteins REV3 or KU70/RAD54 (TOX21_DT40), TOX21_H2AX_HTRF_CHO_Agonist_ratioand a and the activation of p53 (TOX21_p53_BLA_p1_ratio and TOX21_p53_BLA_p1_viability). The dose tested were up to 200 μM.

The summary report of genotoxicity studies of steviol glycosides are showed in Table 6S. Most genotoxicity study were negative; positive studies suffered from a number of drawbacks highlighted in EFSA opinion that included non-adherence of accepted OECD protocols or effects observed as secondary to high doses. Moreover, rebaudioside A (the steviol glycoside tested in the ToxCast/Tox21 program) was inactive in all HTS assays. This supports the EFSA conclusion (EFSA, 2010) that stevioside and rebaudioside lack genotoxic potential.

2.2.6.2. Carcinogenicity. The carcinogenicity profile of steviol glycosides has been recently reviewed (Chappell et al., 2021). An updated search did not identify additional studies.

A summary of experimental animal studies evaluating the carcinogenic potential of steviol glycosides is given in Table 4. There were three standard cancer bioassays in rats, as well as a study conducted in transgenic mice sensitized to pancreatic cancer development. All studies are compliant with GLP. There was no treatment effect on the incidence of tumors and non-neoplastic lesions. The single exception was a decreased incidence of mammary adenomas in female Fisher 344 rats treated with 2.5 or 5% stevioside relative to controls (Toyoda et al., 1997). Furthemore, the study in transgenic mice (C57BL/6 Ela1-Tag), which are genetically engineered to be particularly susceptible to the spontaneous formation of pancreatic cancer of acinar origin, showed no increased incidence of tumors following exposure to stevia leaf extract (Dooley et al., 2017). All studies are considered reliable with Klimisch scores of 2. The EFSA Panel on Food Additives and Flavourings (FAF) (EFSA Panel on Food Additives and Flavourings, 2022) considered that all steviol glycosides share the same metabolic fate, and therefore, the safety of the 60 identified steviol glycosides can be based on read-across.

In conclusion, there is no experimental evidence that steviol glycosides are genotoxic or carcinogenic.

Table 6
Numbers of epidemiologic studies on non-sugar sweeteners and cancer risk considered in the WHO review and new studies considered in the present review, by study type.

Study type	Considered in the previous published WHO review	New studies
Cohort studies on cancer incidence	10	8
Cohort studies on cancer mortality	3	3
Case-control studies	37	9

2.2.7. Sucralose

Sucralose is a non-nutritive sweetener, approximately 600 times sweeter than table sugar. Sucralose (CASRN 56038-13-2) is a widely used sweetener currently approved for use in over 80 countries. It does not produce any calories upon breaking.

2.2.7.1. Genotoxicity. The genotoxicity profile for sucralose has been evaluated by the SCF in 2000 (ADI of 0–15 mg/kg BW) (SCF, 2000c), by the U.S. FDA in 1998 (ADI of 0–5 mg/kg BW) (FDA, 1998) and recently reviewed by Lea et al. (2021). No other further data have been found in the literature since the Lea et al., review (Lea et al., 2021).

The SCF (SCF, 2000a) and FDA (FDA, 1998) concluded that the data were generally negative or inconclusive for genotoxicity and mutagenicity. According to Lea et al. (2021) the genotoxicity Potential Using Tox21 HTS assays for small molecules that induce genotoxicity reported by (Hsieh et al., 2019) revealed that sucralose is inactive in 17 HTS assays. The HTS assays included those able to detect genotoxicity by increasing expression of luciferase-tagged ATAD5 and cell viability in human embryonic kidney cells (TOX21_ELG1_LUC_Agonist and TOX21_ELG1_LUC_Agonist_viability), enhancing cytotoxicity in DT40 cells deficient in DNA repair proteins REV3 or KU70/RAD54 (TOX21_DT40), TOX21_H2AX_HTRF_CHO_Agonist_ratioand a and the activation of p53 (TOX21_p53_BLA_p1_ratio and TOX21_p53_BLA_p1_viability). The dose tested were up to 200 μM.

The summary report of studies of genotoxicity of sucralose are showed in Table 7S. Sucralose was negative in all mutagenesis assays (Brusick et al., 2010). While inconclusive results have been obtained in chromosomal aberration test in cultured human lymphocytes (Pasqualli et al., 2020), two *in vivo* studies were negative for CA in the bone marrow of both rats and mice (Brusick et al., 2010; Heredia-García et al., 2019). A recent study showed increased incidence of micronuclei in the blood of carp (*Cyprinus carpio*) (Heredia-García et al., 2019). However, the non-standard nature of this assay raises questions as to the applicability of these results to humans (Heredia-García et al., 2019).

In summary, there is no evidence to modify the SCF and US FDA conclusions (FDA, 1998; SCF, 2000c) that sucralose lacks genotoxic potential.

2.2.7.2. Carcinogenicity. The carcinogenicity profile of sucralose has been recently reviewed (Chappell et al., 2020a). An updated search did not identify additional studies.

Three cancer bioassays conducted with sucralose were identified (Table 5). Two of them were conducted by the same investigators, and reported no increased incidence of carcinogenicity in male and female Sprague-Dawley rats and CD-1 mice (Mann et al., 2000b; Mann et al., 2000a). These studies were compliant with GLP and with a reliable endpoint with Klimisch scores of 2.

In the third study, conducted by the ERF (Soffritti et al., 2016), sucralose was administered in feed to male and female Swiss mice at concentrations of 0, 500, 2000, 8000, or 16,000 ppm throughout life,

beginning *in utero* on gestational day 12 and continuing to natural death. The authors reported a significant increase in the incidence of malignant tumour-bearing male mice exposed to sucralose at 16,000 ppm with a significant dose-related trend. Hematopoietic neoplasias represented the neoplasms that contributed most to the increased incidence of tumour-bearing mice. There was no significant increase in tumour-bearing female mice associated with treatment. The study has been reviewed by the EFSA (EFSA, 2013) that identified several limitations and issues with its protocol and reporting. The included dosing duration (dosing until death), the fact that the data for historical controls were not collected within five years of the contemporary study, a high rate of bronchiolar/alveolar and peribronchiolar inflammation in the control groups, the lack of a dose-response relationship, and the lack of a mode of action with general defined criteria for establishing a reported cause-and-effect relationship. The same critical issues have been raised by others (Berry et al., 2016; Magnuson et al., 2017).

In conclusion, there is no evidence of genotoxicity and carcinogenicity of sucralose.

3. Epidemiology

3.1. Methods

We conducted a systematic review by searching PubMed and Embase databases for original articles reporting the results of cohort and case-control studies evaluating the potential association between NSS and cancer incidence or mortality. Articles evaluating recurrence/survival after a diagnosis of cancer were not considered. The search strategy is outlined in Table 8S. Citations were exported from the databases and then imported to Rayyan for duplicates removal and title and abstract screening (Ouzzani et al., 2016).

All original articles published in English up to July 29, 2022 were included, whereas reviews, case reports and conference proceedings were excluded. The full text of the eligible articles and systematic reviews on the topic were hand-searched for studies that could have been missed. When results are reported in more than one articles, only the most informative one (i.e. the one including more cases) was considered.

From the included articles, we extracted the following data: year of publication, country where the study was conducted, study design, number of participants, number of cancer cases or deaths, measure of exposure, categories of comparison, study outcome (incidence/mortality), cancer site and an estimate of the association in terms of relative risk (RR), hazard ratio (HR) or odds ratio (OR) with their 95% confidence intervals (CI). When more than one estimate was provided, we selected the one obtained from the model including the highest number of adjustments. Estimates with CI not including unity were considered statistically significant.

Results were summarized according to type of study (cohort or case-control studies) and epidemiological measure evaluated (incidence or mortality). Results on aspartame are presented in a specific section.

When the evaluations of the exposure and the outcome were comparable and when at least three estimates were available from different studies, the results of the comparison between the highest and the lowest level of NSS consumption obtained from cohort studies were pooled using a meta-analytic approach based on a random effect model. Briefly, each study-specific measure of association was weighted by the inverse of its variance plus the between studies variance component τ^2 computed through the moment estimator (DerSimonian and Laird, 1986). Between-study heterogeneity was assessed by Q statistics based on a Chi-squared test, and inconsistency was measured through the I^2 statistic, representing the proportion of total variation due to between-study variance (Higgins and Thompson, 2002).

3.2. Results

3.2.1. Retrieved articles

The electronic search yielded 1590 unique articles; after conducting the electronic search, an additional article was published online in September 2022 (McCullough et al., 2022), whose results were also included in this review. The selection procedure showed in Fig. 1 yielded 68 articles which were included in this systematic review (22 cohort studies (Bao et al., 2008; Bassett et al., 2020; Chazelas et al., 2019; Debras et al., 2022; Heath et al., 2021; Hodge et al., 2018; Hur et al., 2021; Inoue-Choi et al., 2013; Jones et al., 2022; Lee et al., 2006; Lim et al., 2006; Liu et al., 2022; Malik et al., 2019; McCullough et al., 2022, 2014; Mullee et al., 2019; Navarrete-Muñoz et al., 2016; Romanos-Nanclares et al., 2021; Schernhammer et al., 2012, 2005; Stepien et al., 2016; Zamora-Ros et al., 2022; Zhang et al., 2021) and 46 case-control studies (Akdaş et al., 1990; Andreatta et al., 2008; Asal et al., 1988; Bosetti et al., 2009; Bravo et al., 1987; Bruemmer et al., 1997; Cabaniols et al., 2011; Cartwright et al., 1981; Chan et al., 2009; Chang et al., 2021; Connolly et al., 1978; Ewertz and Gill, 1990; Gallus et al., 2007; Gold et al., 1985; Goodman et al., 1986; Gurney et al., 1997; Hardell et al., 2001; Hoover and Strasser, 1980; Howe et al., 1977, 1980; Kessler and Clark, 1978; Kobeissi et al., 2013; Li et al., 2006; Maclure and Willett, 1990; Mahfouz et al., 2014; Mettlin, 1989; Møller-Jensen et al., 1983; Momas et al., 1994; Mommsen et al., 1983; Morgan and Jain, 1974; Morrison et al., 1982; Morrison and Buring, 1980; Murtaugh et al., 2004; Najem et al., 1982; Nomura et al., 1991; Norell et al., 1986; Ohno et al., 1985; Piper et al., 1986; Radosavljević et al., 2001; Risch et al., 1988; Silverman et al., 1983; Simon et al., 1975; Wang et al., 2013; Wu et al., 1997; Wynder and Goldsmith, 1977; Yu et al., 1997)).

Table 6 gives the number of studies considered in our systematic review and those which were not included in the previous published WHO review (World Health OrganizationRios-Leyvraz and Montez, 2022).

Most studies were focused on bladder cancer. A minority of them considered other cancer sites, including stomach, pancreas, colorectum, prostate, kidney, breast, endometrium, brain and hematopoietic cancers; one study considered lung cancer and another thyroid cancer. Eighteen cohort studies (Bao et al., 2008; Bassett et al., 2020; Chazelas et al., 2019; Debras et al., 2022; Heath et al., 2021; Hodge et al., 2018; Hur et al., 2021; Inoue-Choi et al., 2013; Jones et al., 2022; Lee et al., 2006; Lim et al., 2006; McCullough et al., 2014; Navarrete-Muñoz et al., 2016; Romanos-Nanclares et al., 2021; Schernhammer et al., 2005, 2012; Stepien et al., 2016; Zamora-Ros et al., 2022) measured the association between NSS and cancer incidence and six evaluated the association with cancer mortality (Heath et al., 2021; Liu et al., 2022; Malik et al., 2019; McCullough et al., 2022; Mullee et al., 2019; Zhang et al., 2021).

3.2.2. Cancer incidence from cohort studies

The characteristics and the main results of the 18 cohort studies (Bao et al., 2008; Bassett et al., 2020; Chazelas et al., 2019; Debras et al., 2022; Heath et al., 2021; Hodge et al., 2018; Hur et al., 2021; Inoue-Choi et al., 2013; Jones et al., 2022; Lee et al., 2006; Lim et al., 2006; McCullough et al., 2014; Navarrete-Muñoz et al., 2016; Romanos-Nanclares et al., 2021; Schernhammer et al., 2005, 2012; Stepien et al., 2016; Zamora-Ros et al., 2022) evaluating the association between NSS and cancer are summarized in Table 9S. They were based on large cohorts from Europe, the USA and Australia. The main measure of exposure was the consumption of all non-sugar sweetened beverages (non-SSB) with only four studies measuring the intake of aspartame (Debras et al., 2022; Lim et al., 2006; McCullough et al., 2014; Schernhammer et al., 2012) and one also the intakes of acesulfame K and sucralose (Debras et al., 2022). Two studies evaluated the association with all cancers (Chazelas et al., 2019; Debras et al., 2022) and three studies with pancreatic cancers (Bao et al., 2008; Navarrete-Muñoz et al., 2016; Schernhammer et al., 2005). Other cancers considered

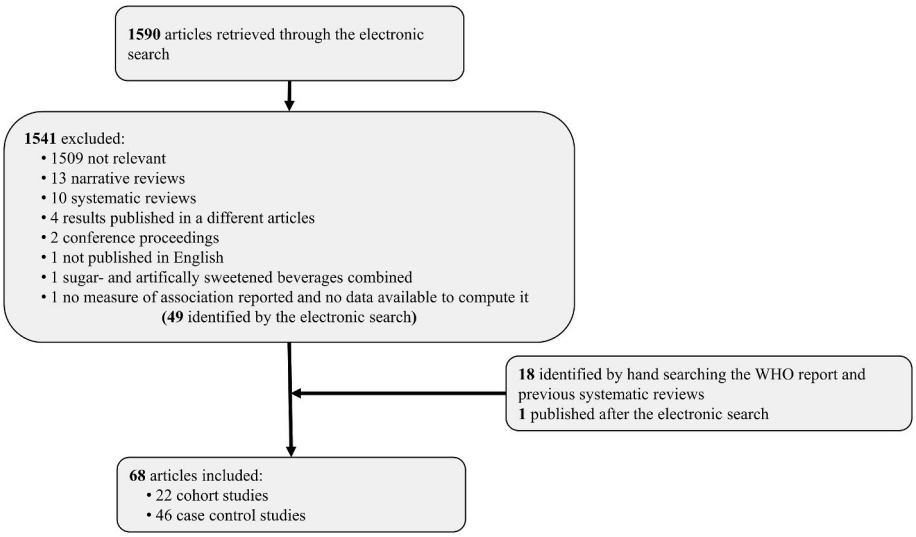

Fig. 1. Flow diagram of epidemiologic study selection.

were: colorectum, pancreas, liver and biliary tract, breast, prostate, kidney, endometrium, brain, thyroid, hematopoietic cancers and obesity-related cancers.

Eleven out of the 18 studies did not find significant associations between NSS and cancer (Bao et al., 2008; Heath et al., 2021; Hodge et al., 2018; Hur et al., 2021; Inoue-Choi et al., 2013; Lee et al., 2006; Lim et al., 2006; Navarrete-Muñoz et al., 2016; Romanos-Nanclares et al., 2021; Schernhammer et al., 2005; Zamora-Ros et al., 2022), while six reported HR ranging between 1.12 and 3.36 (Bassett et al., 2020; Chazelas et al., 2019; Debras et al., 2022; McCullough et al., 2014; Schernhammer et al., 2012; Stepien et al., 2016) and one found a significant association only in a subgroup of subjects (Jones et al., 2022).

A study, published in 2012 (Schernhammer et al., 2012) and based on the Nurses' Health Survey (NHS) and the Health Professionals Follow-Up Study (HPFS) cohorts, found an increased risk of leukemia (HR: 1.42), non-Hodgkin lymphoma (NHL) (HR: 1.31, only in men) and multiple myeloma (HR: 2.02, only in men) among subjects consuming 1 or more serving of diet soda as compared to subjects having less than 1 serving per week, and excess risks for NHL (HR: 1.64, only in men) and multiple myeloma (HR: 3.36, only in men) among aspartame consumers. Those risks were not confirmed in a study published in 2014 (McCullough et al., 2014) and based on the Cancer Prevention Study II Nutrition Cohort. That study did not find any excess risk of NHL associated with the consumption of NSS carbonated beverages, and an increased risk only for the intermediate, but nor for the highest levels of aspartame intake.

An excess risk of hepatocellular carcinomas was observed in a study (Stepien et al., 2016) based on the EPIC cohort with a HR of 1.06 for consumption of 100g/day of non-SSB. In a subsequent study, based on two large US cohorts (Jones et al., 2022) (NIH-AARP Diet and Health Study and PLCO Cancer Screening Trial), the HR of liver cancer was 1.13 among subjects consuming diet beverages as compared to non-drinkers. The excess risk was, however, limited to subjects with diabetes and to the first follow-up time (<12 years).

Excess risks for cancers not related to obesity (i.e. excluding oesophageal, pancreatic, colorectal, breast, endometrial, renal, ovarian, gallbladder, liver, cardia, thyroid cancers, multiple myeloma and meningiomas) were observed among subjects enrolled in an Australian study (Bassett et al., 2020) consuming 1 or more servings of non-SSB as compared to those consuming less than one serving per month, with a HR of 1.23.

A study, published in 2019 and based on a French cohort (the NutriNet-Santé cohort) (Chazelas et al., 2019), reported an increased risk of prostate cancer for the highest level of non-SSB as compared to the lowest level (HR: 1.33). In an updated analysis of the same cohort (Debras et al., 2022), the authors evaluated the consumption of specific sweeteners and found increased risks of all cancers among lower and higher intakes of aspartame and acesulfame K (HR of 1.13–1.15), but not for sucralose intake. Higher intakes of aspartame were also associated with excess risks of breast (HR: 1.22) and obesity related cancers (HR: 1.15), but not for prostate cancer.

We performed a meta-analysis only for the relationship between non-SSB and pancreatic cancer incidence as it was the only exposure-cancer site combination with at least three estimates available. All estimates were close to unity, thus indicating no association, with low-to-moderate heterogeneity. The pooled estimate based on the results of three studies using different cohorts (Bao et al., 2008; Navarrete-Muñoz et al., 2016; Schernhammer et al., 2005) gave no excess risk for the highest compared to the lowest level of non-SSB consumption (pooled HR: 1.05, 95% CI: 0.89, 1.25) (Fig. 1S).

3.2.3. *Cancer mortality from cohort studies*

Table 10S summarizes the characteristics and the main results of the six cohort studies (Heath et al., 2021; Liu et al., 2022; Malik et al., 2019; McCullough et al., 2022; Mullee et al., 2019; Zhang et al., 2021) evaluating the relationship between NSS intake and cancer mortality. All studies used the consumption of non-SSB as a measure of exposure, and all except one (McCullough et al., 2014) did not find any significant excess mortality among consumers of non-SSB.

That study (McCullough et al., 2022) was based on a large cohort of more than 900 thousand individuals enrolled in 1982 and found a significant excess risk for pancreatic and gall bladder cancers for the highest (≥2 drinks/day) compared to the lowest level of intake (no consumption), with HR between 1.09 and 1.26, while the association with obesity-related cancers was weak (HR ~ 1.05).

We performed a meta-analysis using the four studies including information on non-SSB and mortality from all cancer since it was the only exposure-outcome combination having at least three estimates available. All estimates were close to unity with no between-study heterogeneity. Our pooled estimate was 1.01 (95% CI: 0.96, 1.06), thus indicating no excess risk for the highest level of consumption (Fig. 2S).

3.2.4. *Evidence from case-control studies*

Table 11S summarizes the characteristics and the main results of the 46 case-control studies (Akdaş et al., 1990; Andreatta et al., 2008; Asal et al., 1988; Bosetti et al., 2009; Bravo et al., 1987; Bruemmer et al., 1997; Cabaniols et al., 2011; Cartwright et al., 1981; Chan et al., 2009; Chang et al., 2021; Connolly et al., 1978; Ewertz and Gill, 1990; Gallus

et al., 2007; Gold et al., 1985; Goodman et al., 1986; Gurney et al., 1997; Hoover and Strasser, 1980; Howe et al., 1977, 1980; Kessler and Clark, 1978; Kobeissi et al., 2013; Li et al., 2006; Maclure and Willett, 1990; Mahfouz et al., 2014; Mettlin, 1989; Møller-Jensen et al., 1983; Momas et al., 1994; Mommsen et al., 1983; Morgan and Jain, 1974; Morrison et al., 1982; Morrison and Buring, 1980; Murtaugh et al., 2004; Najem et al., 1982; Nomura et al., 1991; Norell et al., 1986; Ohno et al., 1985; Piper et al., 1986; Radosavljević et al., 2001; Risch et al., 1988; Silverman et al., 1983; Simon et al., 1975; Wang et al., 2013; Wu et al., 1997; Wynder and Goldsmith, 1977; Yu et al., 1997); two of them based on the same population but using different exposures (Howe et al., 1977, 1980) and other two using partially overlapping populations with different exposures (Morrison et al., 1982; Morrison and Buring, 1980).

Most case-control studies evaluated the association of NSS with bladder cancer, four with renal cancer and three with pancreatic cancer. A few studies evaluated also the association with cancers of oral cavity and pharynx, oesophagus, stomach, small intestine, colorectum, larynx, lung, breast, brain, ovary and prostate, acute myeloid leukemia and childhood brain tumors. Fourteen studies found significant associations (Akdaş et al., 1990; Andreatta et al., 2008; Asal et al., 1988; Bravo et al., 1987; Cartwright et al., 1981; Chan et al., 2009; Howe et al., 1977; Kobeissi et al., 2013; Maclure and Willett, 1990; Mahfouz et al., 2014; Mommsen et al., 1983; Silverman et al., 1983; Yu et al., 1997): most of them with bladder/urinary tract cancers (Akdaş et al., 1990; Andreatta et al., 2008; Asal et al., 1988; Cartwright et al., 1981; Gallus et al., 2007; Howe et al., 1977; Kobeissi et al., 2013; Maclure and Willett, 1990; Mommsen et al., 1983; Yu et al., 1997) (with ORs up to 6.7 (Mommsen et al., 1983)), one study with larynx (Gallus et al., 2007), one with pancreas (Chan et al., 2009) and another study with colorectal cancer (Mahfouz et al., 2014).

3.2.5. *Aspartame and cancer*

We reviewed seven studies evaluating the association between aspartame and cancer (Cabaniols et al., 2011; Debras et al., 2022; Gurney et al., 1997; Hardell et al., 2001; Lim et al., 2006; McCullough et al., 2014; Schernhammer et al., 2012).

A French study (Debras et al., 2022), based on 102,865 individuals enrolled in the NutriNet Santé cohort, found a slight increased risk (HR ~1.15) for all cancers also at low levels of aspartame consumption (i.e. <5.06 mg/day in men and <15.39 mg/day in women). The same study also found an increased risk for breast cancer (HR: 1.22) and obesity-related cancer (HR: 1.15), but only for levels of consumption above those values. An excess risk of similar magnitude (HR ~ 1.30) was reported for NHL in a study from the US (McCullough et al., 2014) based on 100,442 individuals of the Cancer Prevention Study II Nutrition cohort, in which individuals in the lowest three quintiles of intake (corresponding to intakes up to 27 mg/day among men and up to 19.6 mg/day among women) were associated with a 30% increased risk compared to the lowest level of intake. However, results were not consistent since no excess risk was observed for higher levels of consumption. Higher excess risks for NHL (HR: 1.64) and multiple myeloma (HR: 3.36) were reported at level of consumption ≥149 mg/day and only among men, in a different American study, based on two cohorts (NHS and HPFS) and including 125,028 subjects. However, their results were not consistent with those of a larger study (Lim et al., 2006), including 437,984 individuals from the NIH-AARP Diet and Health Study which did not find any excess risk for NHL and other hematopoietic malignancies even for very high levels of consumption (i.e. 400-599 and ≥ 600 mg/day).

In a case-control study (Gurney et al., 1997) including 56 children with brain tumors, maternal consumption and use of aspartame in childhood were not significantly associated with an increased risk of brain tumors. Null associations were also found for adult brain tumors in two larger case-control studies in adults (Cabaniols et al., 2011; Hardell et al., 2001) and in the NIH-AARP Diet and Health Study cohort, where intakes ≥600 mg/day were not associated with an increased risk of glioma (Lim et al., 2006).

4. Conclusions

NSS have been extensively studied for genotoxicity and carcinogenicity effects but no consistent evidence was found. A number of previous studies suffer from experimental drawbacks that results in unreliable outcomes. Most recent studies provide general and consistent reassurance on the lack of evidence of either genotoxic or carcinogenic effects by NSS. This is also consistent with the conclusions of several regulatory bodies that evaluated NSS over the years.

Based on the epidemiological evidence, a consistent association between consumption of NSS and cancer risk can now be excluded. Several studies have been conducted and the majority showed no significant relationship. Some risks for bladder, pancreas and hematopoietic cancers found in a few studies were not confirmed in others, suggesting a role of chance, multiple testing and selected reporting of positive results.

The heterogeneity in the results relies on several factors including differences in the pattern of NSS consumption across populations from different countries and differences in the study design, but on top of that the assessment of exposure is likely an important source of heterogeneity. Indeed, some studies evaluated the consumption of any NSS, others evaluated specific substances, some assessed the consumption of non-SSB. Moreover, some cohort studies evaluated the consumption of NSS over the follow-up, whereas others limited the assessment to a questionnaire administered at start of follow-up even if the outcome was registered decades later. Groups using in the comparisons also differed, including current users *vs* non-users, ever use *vs* never use, categories according to level of consumption *vs* no consumption or duration of use *vs* never use. Finally, some studies provided sex-specific estimates of associations in the absence of an overall association, while in others males and females were considered together. All these factors contributed to the apparent heterogeneity of the results. On the basis of the toxicological and epidemiological data summarized in this review there is no consistent evidence to advice against the use of NSS on the basis of cancer risk.

Funding

The study was supported by Melete s.r.l., Como, Italy through an unconditional grant provided by the International Sweeteners Association (ISA), Bruxelles, Belgium. The sponsors did not review the manuscript prior to submission.

CRediT authorship contribution statement

Sofia Pavanello: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Angelo Moretto:** Conceptualization, Supervision, Validation, Writing – review & editing. **Carlo La Vecchia:** Conceptualization, Supervision, Validation, Writing – review & editing. **Gianfranco Alicandro:** Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Melete srl provided financial support through a grant form ISA (International Sweeteners Association). The conclusions are those of the authors; the sponsors did not have any role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Data availability

No original data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2023.105369.

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