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# Biological Responses to the Consumption of Non-Nutritional Sweeteners

*Sage Arbor*

## Abstract

Non-nutritive sweetener (NNS) use has increased exponentially over the last 30 years as industrialized countries attempted, and failed, to battle obesity epidemics. Large studies have now shown that consumption of NNS's does not help obese individuals lose weight. A large number of scientific studies on NNS's effects have many conflicting results, methodological issues, conflicts of interest, while double blind studies represent a small minority of the studies. NNS's have often been considered as a group despite having unique in vivo absorption, distribution, metabolism, and excretion (ADME). Aspartame may be the most desirable NNS due to its rapid degradation in vivo, whereas saccharin and sucralose are worrisome due to their extended stability in vivo. This review will focus on the most ubiquitous NNS's: aspartame, saccharin, acesulfame-K, sucralose, stevia, sugar alcohols (sorbitol, xylitol, and erythritol), and discuss their different chemical structures, metabolism, effect on the gut biome and cancer.

**Keywords:** artificial sweetener, sweet receptor, gut brain axis, obesity, diet

## 1. Introduction

Non-nutritive sweeteners (NNS's) contain few to no calories or nutrients. As obesity had been rising in western countries at the end of the 20th century, NNS use increased 54% in adults and a staggering 200% in children from 2000 to 2010 [1, 2]. In 2018 over \$7 billion (USD) NNS's (also called artificial sweeteners or sugar substitutes) were sold which is projected to grow 5%/year reaching more than \$10 billion by 2025 [3]. However recent reports have shown NAS's to be ineffective at reducing obesity [4, 5], which is their primary health target. Malek et al. found that while consumers of NNS's reported lower caloric intake compared to non-NNS consumers, the NNS consumers were more overweight and obese [4]. While the "Calories in - Calories Out" (CICO) paradigm governing net weight gain/loss of has been resoundingly confirmed [6–8], the mechanism causing NAS's to be ineffective at reducing weight are still being investigated. Does NAS consumption cause increased appetite for food with caloric content (calories in)? Is metabolism and movement decreased (calories out)? Are effects mostly due to human cellular or gut microbiome response? How consistent are these effects across the NNS's, and lastly how individual are these effects? Genetic polymorphisms have been elucidated which do personalize an individual's experience to NAS's [9, 10], but this does not appear to be a primary driver since obesity has risen on a population and indeed global level.

The safety thresholds for human consumable substances are set by national or international organizations, and this review will highlight the work done by three organizations: the Joint Expert Committee on Food Additives (JECFA), European Union's Scientific Committee for Food (SCF), and the US Food and Drug Administration (FDA). Eight NNS's have been approved by the FDA: aspartame, acesulfame potassium, luo han guo (monk) fruit extract, neotame, saccharin, stevia, sucralose and advantame [11]. While this group, along with sugar alcohols, are often grouped as sugar replacements they differ in their in vivo stability, targets, and even caloric content to a minor extent. The differences between their structures, metabolism, effect on gut biome, host metabolism, obesity, cancer, and future human studies will be discussed.

## 2. Biological effects of artificial sweeteners

Non-nutritive sweeteners (NNS's) were designed to mimic the sweetness of sugar without containing any caloric content. However, over the last 40 years robust biological effects have been demonstrated beyond the desired sweetness profile. Artificial sweeteners differ in structure, in vivo half-life, molecular targets, effect on host gut-biome, and magnitude of biological effect. Consumer products increasingly have mixed NNS's to augment their flavor profile, which makes survey study results less useful since questions such as "how many diet sodas did you drink per week" can encompass biological responses from an unknown mix of NNS's. The specific attributes and effects for the major NSS's will be reviewed below.

### 2.1 Structure and metabolism of artificial sweeteners

While Non-nutritive sweeteners (NNS's) have a detectably different flavor profile [12, 13], they largely overlap in successfully replacing the sensation of sugar in foods and beverages. As a group NNS's are much more potent at stimulating the sensation of sweetness in the mouth compared to table sugar. There is concern that such swamping of one's taste receptors with sweetness through excessive NNS consumption could limit the desirability of more complex flavors, making less sweet food (e.g. fruit) less appealing, and causing healthy unsweet foods (e.g. vegetables) to be newly intolerable. NNS's differ in chemical structure by over 5-fold in molecular weight (MW) (**Table 1**). Saccharin is the lightest at 183 MW, while Rebaudioside A (a steviol glycoside) is a whopping 967 MW which causes stevias elimination (urine vs. feces) to differ between mammals based on biliary excretion MW thresholds [17, 18]. The safety of NNS's are initially determined from taxoicokinetic studies in various animal models, such as mice and rats, which absorption, distribution, metabolism, and excretion (ADME) after ingestion. The animal model with results that most closely match the ADME characteristics seen during human consumption are used to set safety limits. A wide range of NNS concentrations are used so the high doses can elucidate any adverse effects, while the lower doses titrate these potential adverse effects away until the "no observed adverse effect level" (NOAEL) is determined. Adverse effects measured to determine NOAEL may include changes in development, morphology, growth, or lifespan. The NOAEL is used to determine the acceptable daily intake (ADI), which is usually set at 100 times lower than the NOAEL. Therefore, while the ADI is often misinterpreted as a "line in the sand", a level above which a compound is instantly toxic. However, in reality the ADI is a warning level that should be much lower than toxicity while allowing up to a two fold increase before deleterious effects are seen. A significant caveat to this paradigm for NNS's is that long term human changes in

Name	Saccharin	Sucratose	Aspartame	Stevioside	Acesulfame-K	Neotame	Advantame	Cyclamate	Sugar alcohols
Structure									
Molecular weight (g/mol)	183.18	397.6	294.3	804.87	201.2	378.5	458.5	201.22	122–182
Other names	Sweet and Low, Sweet Twin, Necta Sweet	Splenda, Zerocal, Sukrana, SucraPlus, Nevella	Nutrasweet, Equal, Sugar Twin	Stevia extract, rebaudioside A	Sunett, Ace K Sweet & Safe, SweetOne	Newtame	N/A	Sucaryl, Sugar Twin	Sorbitol, Xylitol, Erythritol
Sweetness compared to 10% sucrose solution	300×	600×	200×	30-320×	200×	7000–13,000×	20,000×	30-50×	0.7×
pKa	1.6	12.5	3.1, 7.9	11.8	2.0	3.0, 8.1	2.9, 8.0	1.7	13
Year discovered	1879	1976	1965	1931	1967	FDA approved (US)	FDA approved (US) 2014	1937	1920
Nutritional calories/g	0	0	4	0	0	0	0	0	2.4
Bind receptors outside of oral cavity [10, 14, 15]	T2R8, T2R43, T2R31	t1R3	None	T2R4, T2R14	T2R9, T2R43, T2R31	No	No	T2R1, T2R31, T2R38 T2R43	T1R2, T1R3
Absorption in humans unmodified (modified)	85%	15%	0%, (100% as digested products)	0% steviol glycoside, (100% steviol)	100%	34%	6%	50%	50%

Name	Saccharin	Sucralose	Aspartame	Stevioside	Acesulfame-K	Neotame	Advantame	Cyclamate	Sugar alcohols
Main site of metabolism	None	None	GI tract	Liver	None	Entire body	None	Gut biome	Liver
ADI (mg/kg/day)	15	5	50	4	15	0.3	33	11	N/A
*ADI (# Sweetener Packets Equivalent) [16]	45	23	75	9	23	23	4920	N/A	N/A
**ADI (# 12 oz. sodas Equivalent)	10	5	16	?	20	?	?	?	?

ADI = Accepted Daily Value.  
 \*Number of Tabletop Sweetener Packets a 60 kg (132 pound) person would need to consume to reach the ADI. Calculations assume a packet of high-intensity sweetener is as sweet as two teaspoons of sugar. Adapted from: Kim Y, Keogh JB, Clifton PM. Non-nutritive Sweeteners and Glycaemic Control. *Curr Atheroscler Rep.* 2019;19(12):49. [16]  
 \*\*ADI (# 12 oz. sodas) was calculated for a 60 kg person, using the following brands and concentrations: 90 mg saccharin in Tab; 60 mg sucralose in Diet Coke with Splenda; 187 mg aspartame in Diet Coke; 45 mg acesulfame-K in Diet Coke with Splenda. Note splenda and acesulfame-K are mixed in multiple diet beverages such as Diet Coke with Splenda shown in this table.  
 ? The mg of NNS could not be found for commercially sold drinks with stevioside, neotame, advantame, cyclamate, sugar alcohols.

**Table 1.**  
 Comparison of non-nutritive sweetener characteristics.

caloric balance, such as increased caloric consumption due to unbalanced sweetness perception, would likely not be discovered in an initial NOAEL study.

There are synthetic sweeteners (acesulfame K, aspartame, cyclamate, saccharin, neotame, advantame, and sucralose), natural sweeteners (thaumatin, steviol glucosides, monellin, neohesperidin dihydrochalcone, and glycyrrhizin), and nutritive sweeteners (polyols or sugar alcohols). In 2017 the world consumed 159,000 metric tons of NNS's (valued at \$2 billion USD). China was the top region consuming NNS's (32%) followed by Asia/Oceania (23%), United States (23%), Europe (12%), and then Africa (7%) [19]. The NNS most used in food and drink was aspartame, followed by saccharin, acesulfame, and sucralose (18.5, 9.7, 6.8, and 3.3 thousand metric tons respectively) [20]. With the quantities of NNS being produced and consumed both increasing rapidly, their contamination in the environment has been of concern [21]. There has been quantitative reports finding NNS's in surface water (e.g. lakes, rivers, streams), groundwater, tap water, discharge into the sea, and even in the atmosphere [22], as well as passing through Waste Water Treatment Plants (WWTPs) [23]. NNS's have been found to interfere with photosynthesis, creating disturbances in carbon dioxide intake [24], and decreasing sugar breakdown in human body [25, 26]. The exposure to any of these environmental NNS's would likely be orders of magnitude lower than current self-selected consumption levels. Acesulfame-potassium (Ace-K) was investigated, as an environmentally persistent NNS due to low biodegradation, and found to not pose an environmental risk with very low levels in surface water and even less in groundwater (lower parts per billion [ppb] range and lower parts per trillion [ppt] respectively) [27].

The metabolism of the main NNS's can largely be put into 3 bins [28]: aspartame which is broken up when it hits the GI tract, artificial sweeteners that enter the GI tract (stevia, cyclamate, sucralose, acesulfame potassium), and sugar alcohols which can contribute calories to the diet. Cyclamate is not used in the US, and not widely used in Canada due to concerns about carcinogenesis. Advantame and neotame being newer compounds to the market also do not represent a large portion of the market.

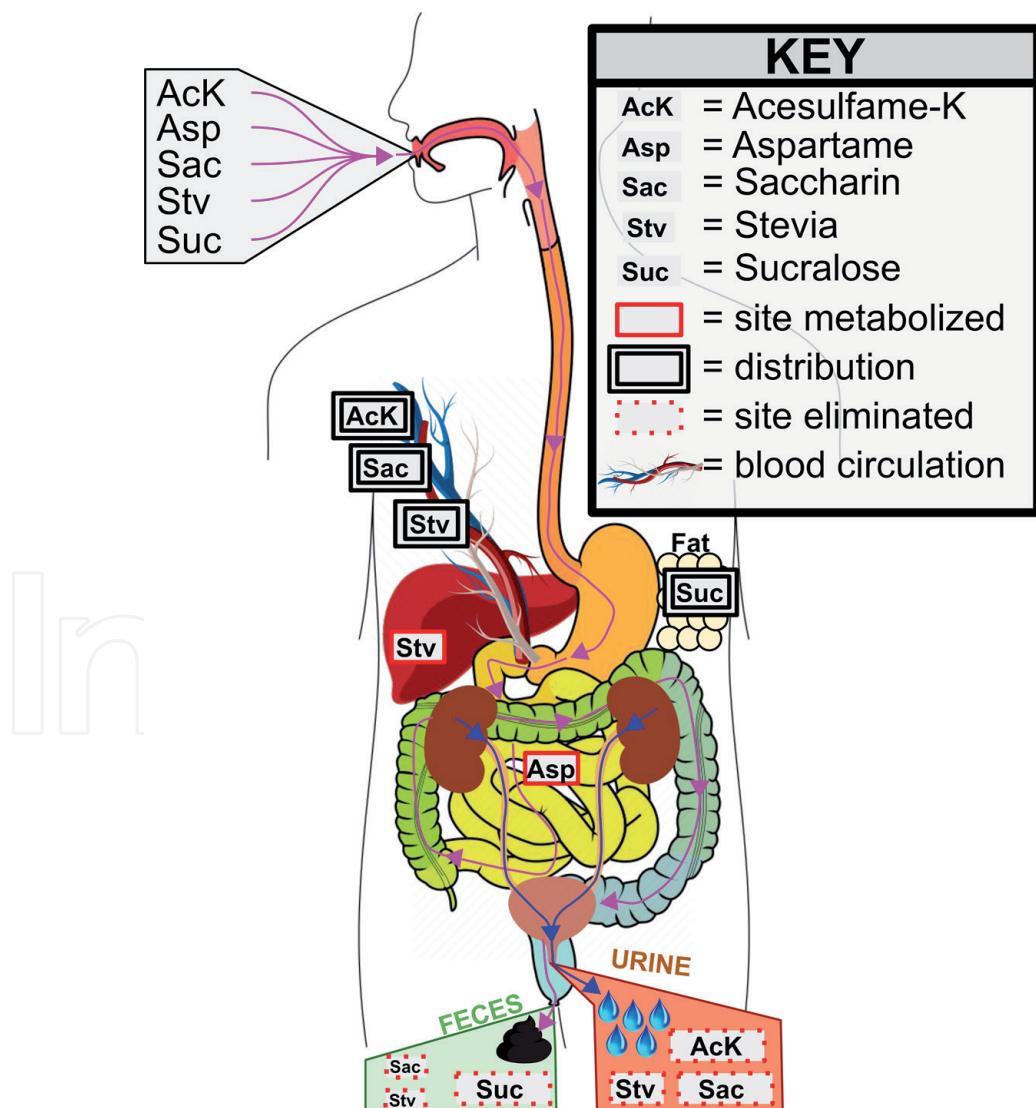
### *2.1.1 Aspartame*

Aspartame is 200 times sweeter than sugar, the most ubiquitous of the NNS's, and is quickly digested into aspartic acid, phenylalanine, and methanol. Due to its amino acid constituency aspartame is strictly a nutritive sweetener. However, because 100 g of sugar can be replaced by less than a gram of aspartame, it is consumed in such low levels that it is considered as a NNS [29]. The phenylalanine can build up to toxic levels in those with a rare genetic defect, phenylketonuria (PKU) present for 1 in 12,000 births [30], caused by mutations and lower levels in the enzyme phenylalanine hydroxylase (PAH). Infants are diagnosed with PKU shortly after birth and avoiding aspartame is achieved well by patients throughout life [31]. However, people with PKU do show increased oxidative stress [32] and the presence of comorbidities including asthma, alopecia, urticaria, gallbladder disease, rhinitis, esophageal disorders, anemia, overweight, GERD, eczema, renal insufficiency, osteoporosis, gastritis/esophagitis and kidney stones [33]. Methanol, which is converted to formaldehyde (a known carcinogen) [34], is often pointed to as a reason to avoid aspartame, but higher levels of methanol are consumed when eating fruit [35, 36]. The FDA set the ADI of aspartame at 50 mg/kg/day and, despite consumption increases since the 1980s, the US average and 95th percentile intake in 2013 was 4.9 and 13.3 mg/kg/day respectively, showing consumption levels are still 4 fold below the ADI. While some customers have complained of an aspartame sensitivity a double blind study found there to be no evidence of any acute adverse response [37]. Aspartame holds a unique position among NNS's in that its pharmacophore

structure which binds sweet receptors is obliterated by esterases and peptidases shortly after consumption when it enters the gastrointestinal (GI) tract. Many of the NNS's have been shown to bind sweet receptors outside of the oral cavity, such as in the digestive tract. Among all the NNS's consumers can be most confident that aspartame will not elicit endogenous sweet receptor cascades after swallowing due to its rapid degradation early in the GI tract (**Figure 1**).

### 2.1.2 Saccharin

Saccharin was one of the leading NNS's used during the early increase in global consumption (along with aspartame), but lost market share in the 1980s due to a 1978 paper suggesting rats had an increase in carcinomas in their urinary bladder upon exposure [38]. Since then studies have shown saccharin does not cause urinary cancer in humans and the carcinoma in mice may have been due to the large amounts of sodium administered (as sodium saccharin) [39]. Saccharin has a pKa of 1.8 and is absorbed better in species with a low stomach pH (e.g. rabbits and humans compared to the higher pH in rats). Being very water soluble there is no build up of saccharin in tissues. In humans ~90% is absorbed and excreted



**Figure 1.**

Metabolism and excretion of non-nutritive sweeteners. NNS's differ in their *in vivo* stability, distribution, and excretion. Acesulfame-K, saccharin, stevia enter the bloodstream and are mostly excreted in the urine. Sucralose exists via the feces but can accumulate in fat when in an acetylated form. Red box: Site of metabolism where NNS is degraded. Black double box: Site of distribution or accumulation. Dashed red box: Site of elimination.

in the urine while only ~10% exits in the feces. Discovered in 1878, saccharins use started to increase around 1900 when it was initially used as a sweetener by diabetic patients, but during the world wars its use in food increased because there were sugar shortages [40]. The stability of saccharin decreases above 125°C which diminishes its usefulness in cooking [40]. Saccharin is 300-fold sweeter than sugar, 1.5-fold sweeter than aspartame, and even among heavy consumers (~2 mg/kg/day) [41, 42] its usage levels are below the ADI of 5 mg/kg/day [43]. While saccharin requires less of a dose than aspartame to attain the same sweetness, it does have an undesirable metallic taste at high concentrations [44]. Saccharin can cross the placental barrier in rats, monkeys, and humans with maternal tissues having higher steady-state levels than the fetus, though the concentration in the fetal tissue does decrease slower than in maternal tissues. Therefore, mothers do not need to be as concerned with saccharin building up in their fetus, but if lowering consumption there will be a delay in lowering levels in the fetus. Of note, while saccharin buildup itself may not be an issue for the fetus, the effect of NNS's on the maternal metabolism can have knock-on effects which do seem to affect the fetus. Consistent NNS consumption is associated with glucose intolerance, interference with energy homeostasis [45–47], and if occurring during pregnancy is associated with increased chances of obesity for the child after birth [46–48].

### *2.1.3 Acesulfame-K*

Acesulfame potassium (Ace-K) is a non-nutritive sweetener discovered in 1967 when a researcher accidentally tasted a new synthesis compound. Often labeled as one of the more pleasant tasting NNS's (no metallic taste), Ace-K is commercially attractive due to its 200-fold greater sweetness than sugar, along with excellent solubility and stability in water [49, 50]. Multiple organizations (JECFA in 1983, SCF in 1985, and the FDA in 1988), agreed that there were no adverse effects in either rats or dogs over a 2-year period when fed up to 3% Ace-K [43]. Using the body weights this led to NOAELs of 900 and 1500 mg/kg/day for dogs and rats respectively. The JECFA and FDA used the rat study to set the ADI at 15 mg/kg/day while SCF used the dog study to set the ADI at 9 mg/kg/day [51]. Ace-K is not metabolized in humans, but can degrade into acetacetamide. Radiolabeling studies showing intact Ace-K (and zero breakdown products) in serum, urine, feces and/or bile [43, 50, 51]. In humans 98% of a 30 mg Ace-K dose was excreted in urine within 24 hours. In pregnant women 1.6% of radiolabeled Ace-K was excreted in milk in 24 hours, which dropped by an order of magnitude the next day (to 0.16%). The highest Ace-K concentration (0.4 mg/kg) occurred in humans 1.5 hours after consumption [52].

### *2.1.4 Sucralose*

Recently a study in rats has shown that sucralose (80 mg/kg/day for 40 days) converted to acetylated forms of sucralose, which are less polar, deposit in fat tissue due to their lipophilicity (**Figure 1**). These acetylated sucralose compounds were detected 6 days after unmodified sucralose had left the body. The original studies and regulatory decision process were unaware of these metabolites or accumulation in adipose tissue due to use of a methanol fraction from feces for analysis [27]. Sucralose is a chlorinated carbohydrate, which has been found to persistently exist in wastewater treatment plant effluents and downstream aquatic environments. Free chlorine, ozone, and ferrate did not degrade sucralose in water, but strong oxidizing agent hydroxyl radicals ( $\bullet\text{OH}$ ) was able to degrade sucralose in water [53].

In animal and human studies sucralose has been shown to not have carcinogenic activity, even when consumed at doses several orders of magnitude more than normal consumption levels [54]. Sucralose largely passes through the digestive tract and is eliminated in feces unchanged (85%) [54]. The remaining 15% of sucralose does distribute throughout the body, but because there is not active transport there are not significant levels in breast milk or across the blood–brain barrier into the central nervous system. There is 2–3% of consumed sucralose undergoing phase II metabolism (glucuronidation) and, along with the other 12% that was initially retained, is excreted in the urine [55–57].

### 2.1.5 Stevia

Stevia is not absorbed, whereas cyclamate and sucralose are partially absorbed but still not metabolized. Steviol glycosides and luo han guo fruit extracts are categorized as natural sweeteners by the FDA and therefore regulated differently [58]. Steviol glycosides are actually a group of compounds which are metabolized differently with varying biological effects, and while usually derived from the *Stevia rebaudiana* Bertoni plant, can also be produced via fermentation [59]. All steviol glycosides share a common chemical core structure, diterpene steviol (the final product of colonic bacterial metabolism). The Joint Expert Committee on Food Additives (JECFA) established an upper acceptable daily intake (ADI) of 2 mg/kg/day based on the amount of steviol after digestion which has been termed the “steviol equivalent”. For example, 3 mg of rebaudioside A converts to just 1 mg of steviol equivalent. Steviol glycosides are not metabolized in the upper gastrointestinal tract, but once reaching the colon bacteria convert them to steviol compounds which travel to the liver and are glucuronidated to steviol glucuronide [24] (**Figure 1**). Increasing levels of steviol glucuronide can be detected to peak in the plasma in humans 8 hours after administration, with a half-life of ~14 hours. In rats almost all of radiolabeled steviol glycosides were found in feces, with less than 2% showing up in urine [25]. However in humans steviol glucuronide was excreted in urine not feces, due to a difference in biliary excretion molecular weight thresholds compared to rats [17, 18]. The glucose that is removed from the glycosides in the colon is not absorbed as caloric input by humans, and is assumed to be quickly consumed by the colonic bacteria.

### 2.1.6 Sugar alcohols (sorbitol, xylitol, and erythritol)

While sugar alcohols (sorbitol, xylitol, and erythritol) are often binned with all of the sugar substitutes, they are not actually NNS's since they do contribute usable calories for humans. Sorbitol, xylitol, and erythritol are carbon chains with an alcohol attached to each carbon and differ structurally only in their carbon length with 6, 5, and 4 carbons respectively (**Table 1**). Sorbitol has low absorption, is hyperosmotic causing laxative effects, and is metabolized to fructose (via sorbitol dehydrogenase). Xylitol has low absorption in the GI tract, and is processed in the pentose phosphate pathway (PPP). Erythritol is absorbed but not metabolized and has negligible calories compared to sorbitol or xylitol. Generally the NNS's do not increase blood glucose levels with an exception being xylitol and maltitol which increase blood glucose about 40% and 80% as much as sugar respectively [24].

## 2.2 Effect on gut biome

The human body has been estimated to have 10-fold more bacterial symbiotic cells than human cells. The gut microbiome contains  $10^{14}$  microorganisms that play

a role in host nutrition, proliferation of intestinal cells, bone mineralization, xenobiotic metabolism, immune system regulation and protection against pathogens [60]. The effect of NAS of gut microbiome is clearly augmented by multiple NAS and could be responsible for a significant fraction of the negative population level obesity increases. In an elegant paper Suez et al. showed NAS consumption led to glucose intolerance through induction of compositional and functional alterations to the intestinal microbiota [61]. Mice fed saccharin, sucralose, or aspartame all showed increased blood glucose during an oral glucose test ( $>250$  mg/dl at 15 min. Vs 200 mg/dl for sucrose or glucose), as well as increased area under the two-hour blood glucose response curve (AUC) after 11 weeks of consuming NAS. These deleterious effects were abrogated by antibiotic treatment, but were fully transferable to germ-free mice upon fecal transplantation of microbiota configurations from NAS-consuming mice. Saccharin exhibited the greatest glucose intolerance and was further studied. Interestingly mice fed saccharin did not show differences in liquid or chow consumption, oxygen consumption, walking distance or energy expenditure. In contrast the gut microbiome of the mice fed saccharin did change significantly, with more than 40 operational taxonomic units (OTUs) significantly altered in abundance. Taxa that increased in relative abundance belonged to the *Bacteroides* genus and *Clostridiales* order, while other members of the *Clostridiales* order comprised the majority of under-represented taxa, along with *Lactobacillus reuteri*, with similar effects in germ-free recipients of stools from saccharin-consuming donors [61]. A similar NAS-induced dysbiosis and glucose intolerance was demonstrated in healthy human subjects (based on a validated food frequency questionnaire) [61]. However Ruiz-Ojeda et al. (2019) reviewed the NNS's and found only sucralose, saccharin, and stevia to have evidence of effecting the gut microbiota. Sucralose could cause dysbiosis by lowering the total number of bifidobacteria, lactobacilli, *Bacteroides*, and *Clostridiales* (both aerobic and anaerobic species) [62]. A study in 2017 found sucralose could increase *Clostridium* cluster XIVa in mice [63]. A positive correlation was found between NAS consumption and multiple metabolic-syndrome-related clinical parameters including: increased weight and waist-to-hip ratio (measures of central obesity), higher fasting blood glucose, glycosylated hemoglobin (HbA1C% indicative of glucose concentration over the previous 3 months) and glucose tolerance test (GTT, measures of impaired glucose tolerance). A small study was also done following seven healthy non-NAS consuming volunteers for 1 week (5 males and 2 females, aged 28–36) in which they consumed the FDA's maximal acceptable daily intake (ADI) of commercial saccharin (5 mg/kg body weight, as 120 mg x 3times/day) and were monitored by continuous glucose measurements and daily GTT. Even in the short 7-day exposure period, most individuals (4 out of 7) developed significantly poorer glycaemic responses [61].

Consuming larger amounts of non-caloric artificial sweeteners (NAS) have been found to increase in hemoglobin (HbA1C %), as well as increased levels of Enterobacteriaceae family, Deltaproteobacteria class, and Actinobacteria phylum found in fecal microbiota [61]. There have been literature reviews that found such effects to be inconsistent with methodological or reporting problems concluding that NAS's (e.g., acesulfame K, aspartame, cyclamate, neotame, saccharin, sucralose, steviol glycosides) have no evidence of an actual adverse effect on human health [64]. Such reviews often point out that studies showing changes in the diet unrelated to NAS consumption may be confounding the data. It is worth noting that such studies can be funded by corporate entities with conflicts of interest, such as the Calorie Control Council (established in 1966 as an international association representing the low- and reduced-calorie food and beverage industry) [64]. A 2016 systematic study on risk of bias screened 493 papers publishing about NNS and, after analyzing the 31 papers that met the eligibility requirements, found extremely

statistically significant bias [65]. Reviews that were sponsored by the artificial sweetener industry were 1700% more likely to have favorable results (Relative Risk (RR) = 17.25). Not only do industry reported papers supporting the use of NNS's seem unreliable, but all 4 papers analyzed that were funded by NNS competitor companies (sugar and water industry) found unfavorable conclusions. A disturbing 42% of reviews did not have authors' financial conflicts of interest disclosed. Lastly reviews performed by authors with financial conflicts of interest almost always had favorable conclusions (18/22, RR = 7.36) [65].

Aspartame and acesulfame-K have been found to not interact with the human colonic microbiota, whereas saccharin and sucralose change the gut microbiome populations (though those effects are not clearly positive or detrimental) [66]. Sugar alcohols can also reach the colonic microbiota and act as prebiotics, with accompanying negative laxative effects [67], particularly in patients with inflammatory bowel syndrome (IBS). Since sucralose can deposit in fat tissue in an acelatved version (see metabolism section), studies that have looked at the effect of sucralose with less than a week washout period could have to be revisited [68].

While most studies have just measured disturbances or lack thereof from NNS's there have been some advancements in the physiological and mechanistic effects gut microbiome dysbiosis could cause. One leading concern would be if NNS's can lead to Non-alcoholic fatty liver disease (NAFLD). NAFLD is denoted by an excess fat in the liver in people who do not drink significant alcohol. Gut dysbiosis has a continually increasing number of known effects including altering caloric absorption [69], development of a "leaky gut", increase in bile acid receptors and transporters, as well as choline deficiency. A leaky gut allows proinflammatory molecules and bacterial endotoxins (e.g. LPS and ethanol) to enter the bloodstream, travel through the portal vein to the liver and increase hepatic inflammation which leads to NAFLD. Increases in bile acid lead to insulin resistance and NAFLD [60]. Dysbiosis in the gut increases conversion of choline to dimethylamine (DMA) and trimethylamine (TMA) [70, 71], potentially leading to choline deficiency which decreases the transportation of VLDL which is used to mobilize fat from the liver [60].

## **2.3 Effect on host metabolism and obesity**

Glucose (which NSS's mimic) has long been known as the *in vivo* "nutrient sensor", not only for carbohydrates consumed but as a "fed state" signal in general since muscular protein can be broken down to produce glucose (via gluconeogenesis). However it appears that the dose dependent glucose detection of sweetness, which matches the caloric load of that glucose, is also an important input for our *in vivo* nutrient sensor. Recent evidence suggests NNS consumption disrupts our normal association if sweetness levels detected differ from caloric intake, causing us to crave even more sweets and gain weight. Indeed, the San Antonio Heart Study found those who drank more than 21 diet drinks per week were found to be twice as likely to become overweight or obese as people who did not drink diet soda [72].

### *2.3.1 Sweetness and caloric load alignment for maximal metabolic response*

A ground breaking study by Veldhuizen et al. found that for carbohydrate reward to maximize its biological response the level of sweet taste must match the caloric load that natural carbohydrate food would provide [73]. The level of caloric load was manipulated using maltodextrin, a tasteless carbohydrate, while the sweetness levels were manipulated with the NNS sucralose. Beverages with differing amounts of maltodextrin + sucralose showed a non-linear response between caloric load, metabolic response and reinforcement potency. When sweetness

is proportional to caloric load greater metabolic responses are observed. Lower calorie beverages were able to produce greater metabolic response than a beverage with more calories but the same level of sweetness, and that data was integrated in the Central Nervous System (CNS). fMRI studies showed the nucleus accumbens (NAcc) response was also greatest when sweetness and caloric load were balanced [73]. It understandably appears humans have evolved to elicit a maximum metabolic response to carbohydrate when the caloric load matches the sweetness that natural sugars such as glucose induce. The use of NNS's are almost always used to replace the sweetness lost when calories are removed from processed foods or beverages, which is now known to elicit a submaximal metabolic response.

### 2.3.2 Nutrient sensing

Approximately 1% of the mucosal epithelial layer of the gastrointestinal (GI) tract are made up of hormone-secreting enteroendocrine (EE) cells, which by mass are the largest endocrine tissue in the body. It is now understood that whole-body metabolism integrates signals from many metabolically active tissues (GI tract, pancreas, adipose tissue, liver, and the CNS). These organs can release both nutrient storage hormones in the fed state postprandially (e.g. glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide (GIP, also known as: glucose-dependent insulinotropic polypeptide), peptide tyrosine tyrosine (PYY), Cholecystokinin (CCK), Oxyntomodulin (OXM)) or after a period of fasting (e.g. ghrelin, 5-hydroxytryptamine (5-HT, also known as gut-derived serotonin when released by the intestine)). Agonists of the sweet taste receptors in the GI tract modify secretion of GLP-1, PYY, ghrelin, and GIP which appears to increase the storage of blood glucose [5] (to glycogen or triacylglycerides and preferential differentiation into adipocytes) [58]. Glucose causes GLP-1 secretion via glucose-dependent Na<sup>+</sup> uptake via SGLT-1 and intracellular glucose metabolism, closing the KATP channels, causing further depolarisation and exocytosis [74]. The distal portion of the small intestine in particular regulates glucose uptake via release of incretins (GLP-1, GIP) [74]. While many of these hormonal links to metabolic syndrome have been elucidated, such as increased 5-HT gut-derived serotonin levels in type II diabetics, most of the knowledge covers the correlation of hormone levels with disease and the downstream effects (such as increased lipogenesis) but not the breadth and magnitude of molecular mechanisms causing this. For example, while serotonin is most often thought of for its neurotransmitter effects in the CNS, more than 90% of whole body 5-HT levels are produced in enterochromaffin (EC) cells in the intestinal epithelium lining and platelets. These EC cells in the gut lumen detect multiple nutritional moieties such as glucose and fructose [75, 76], medium chain fatty acids, as well as various tastants and olfactants [77]. Studies of such EC cell stimulation are confounded by the fact that they are also regulated by mechanical stimulation, neural and endocrine inputs (e.g. adrenergic stimulation and GABA and somatostatin inhibition) [78]. The increased gut-derived 5-HT during a fasted state, along with glucagon, increases hepatic gluconeogenesis and glycogenolysis and therefore glucose output from the liver, while also inhibiting glucose uptake or glycogen synthesis at the liver. At the same time 5-HT increases lipolysis in white fat cells.

Under fasting conditions, gut-derived 5-HT, together with glucagon, markedly increases hepatic glucose output, a main driver of fasting euglycaemia, by increasing hepatic gluconeogenesis and glycogenolysis (29), while inhibiting glucose uptake and glycogen synthesis in the liver (30). 5-HT promotes not only lipolysis, but also energy conservation and weight gain via reduced thermogenesis in brown adipose tissue (and hence reduced energy expenditure) [79]. Antagonists and agonists of these caloric receptors, such as NNS's, have to be considered in the

hormone secretion per cell. However as an example, HT-5 also increases the density or glucose-sensitivity of duodenal EC cells, which can be measured in obese human duodenal EC cells [80], but the molecular mechanisms producing this increased density have not been elucidated.

Because adipocytes (both undifferentiated and mature) express sweet and bitter taste receptors [69], a direct role of NNS's in adipocyte differentiation and function is likely. Both acesulfame-K and saccharin bind the bitter taste receptors at higher concentrations adding the complexity of a second pathway they stimulate compared to many other NNS's [70]. Sucralose was found to promote fat accumulation and upregulate adipogenesis, inflammation, and antioxidant pathways [71]. While rodents lacking sweet taste receptor subunits were shown to have less adiposity and smaller adipocytes on a Western diet [32], showing the importance of sweet taste receptor signaling for normal adipose tissue development. One study suggested NNS's were not dependent on sweet taste receptors to stimulate adipogenesis or suppress lipolysis [36]. However in contrast, Masubuchi et al. reported that adipogenesis was inhibited by sucralose and saccharin in preadipocytes [72], and suggested NNS exposure leads to microtubule disassembly and alteration of the PI3K–Akt signaling pathway [72]. The rodent and *in vitro* studies do not provide conclusive results [58].

A pattern has emerged in which observational studies make NNS's appear harmful causing increased weight, while intervention studies find NNS's reduce weight. A natural hypothesis was that in the observational studies the people consuming NNS's must have compensated by eating more solid food calories at subsequent meals, which indeed was found in some studies, but others reported a reduction in total energy expenditure after NNS consumption [81]. When sweet receptors in the oral cavity are activated not only does the brain perceive sweetness but the body prepares to digest calories through the cephalic phase responses, most notably the cephalic phase insulin response (CPIR) which is a neurally-mediated release of insulin prior to nutrient absorption [82]. Not only do glucose [83] and sucrose [82] stimulate CPIR, but saccharin [82] and possibly sucralose [84] also to activate CPIR. Of note, all these molecules which activate CPIR seem to bind the same VFD region on the T1R3 receptor [84]. In contrast aspartame [84, 85], stevioside and cyclamate [86] all bind a different site on the receptor and fail to elicit the CPIR response. If this CPIR were the main driver of obesity and driven by binding the VFD region on T1R3 then those consuming saccharin and sucralose would be expected to have increased Total Body Weight (TBW) compared to those consume aspartate, stevioside, or cyclamate. However, that has been neither proven nor disproven. In addition while CPIR may be activated through the sweet receptor recent studies have suggested that CPIR may actually be activated through the adenosine triphosphate (ATP)-sensitive potassium channel ( $K_{ATP}$  channels) [87] which are also found on T1R3-containing taste cells [88].

The magnitude with which the combination of circadian rhythm and eating controls metabolism at a cellular level is currently a productive field of research. The use of NSS's during fasting would be useful to not only elucidate the pathways augmented by NNS's while removing confounding variables, but would also shed some needed information on to what degree, if any, NNS's inhibit the positive molecular effects of fasting. It is now well documented that extended fasts ( $> = 1$  day) increase autophagy (recycling of cellular content) [89–93] which has been shown to be anti-cancer [94], anti-aging [93, 95–100], beneficial to sleep [101–103], protective of muscle loss [89, 104], and clearly helpful to reduce weight [105–109]. Many people that undergo intermittent fasting still consume diet beverages because they are viewed as low or zero calorie. Quality-Adjusted Life Years (QALYs) analyses for NNS's, incorporating data across multiple disease states, would be useful in determining the utility of NSS's (e.g. positive tastant stimulant vs. negative microbiome modulator). The main QALY studies

found in pubmed by the author concerned the efficacy of sugar taxes to increase QALYs [110–113]. Sirtuin 1 (SIRT1) is a deacetylase involved in normal circadian rhythm modifications, has been linked to increasing longevity, and increases with fasting. Recently NNS's (aspartame and saccharin) were used to suggest the metabolic effects of sugars (not perception of sweetness) resulted in the SIRT1 regulation of a simple sugar preference [114]. It is unknown if NNS consumption during fasting effects such calorie specific pathways as quality of sleep, autophagy, mTOR inhibition, muscle sparing during exercise, or increased efficacy of cancer therapy.

## 2.4 Effect on cancer

Non-nutritive sweeteners (NNS's) have had a rollercoaster history in being viewed at either increasing or decreasing lifespan, with quality of life being less often measured. An early study by Reuber in 1978, which received tremendous attention in the popular press, appeared to show very high levels of saccharin causing bladder cancer in rodents [38]. However, earlier and later studies did not support this conclusion and mechanistic data [115] showed different saccharin metabolism in rodents and humans [39, 116, 117]. Yet as a result of the Reuber study, saccharin was listed from 1981 to 2000 as a substance reasonably anticipated to be a human carcinogen in the U.S. National Toxicology Program's Report on Carcinogens [118, 119]. Since 2000 the view of NNS's has increasingly become Generalized Recognized as Safe (GRAS). This continued for maybe 15 years, until recent data showing a failure to reduce obesity has again ramped up questions regarding their utility. However large cohort studies of NNS consumption showed all-cause mortality to increase in a range from 4% [120] - 24% [121]. The correlation with increased risk from cardiovascular (13% [120]) and circulatory disease (52% [121]) was higher.

Recent data suggests that NNS's may actually be beneficial in the treatment of cancer patients [122, 123], in contrast to the earlier attention to carcinogenicity or current concern over obesity in the general public. Cancer cells are well known to be energy hungry, activating molecular pathways of growth to multiply quickly. It has been found that fasting can improve outcomes for cancer patients [123]. The specific food that a cancer patient consumes, or lack thereof, can cause tumor starvation, have cancer-specific toxicity, activate anticancer immune response, or enhance drug-based therapy. Reducing calories have been shown to attack, stress, and kill cancer cells. Low calorie or ketogenic diets increase tumor-infiltrating CD8+ T cells [94, 124] and reduce immune inhibitory ligand expression such as PDL-1/CD137 [125]. If NNS's were used to decrease calories consumed in cancer patients the type and time of remaining calories consumed is still important. Modifying consumption of specific amino acids can be useful when treating cancer patients. For example, both glucose (sugar) and glutamine (protein) are central to cell growth (e.g. TCA cycle, Nucleotide synthesis, Cellular redox balance, Lipid synthesis) [123]. Glucose contributes pyruvate and acetyl-CoA to the TCA cycle while glutamine adds  $\alpha$ -ketoglutarate, producing ATP and NADH which is used in oxidative phosphorylation to create even more ATP generation. Both glucose and glutamine are needed for different steps in nucleotide synthesis so lowering consumption of both will synergistically inhibit cancer cells more than inhibition of either alone. Likewise glucose and glutamine work orthogonally to maintain cellular redox potential balance. Glucose is needed for NADPH production via the pentose-phosphate pathway, and glutamine in conjunction with NADPH forms the antioxidant GSH. Lipid synthesis needs both glucose for cytoplasmic acetyl-CoA and glutamine as a carbon source, particularly under hypoxic conditions which are common in tumors. Methionine restriction reduces one-carbon metabolism and nucleotide synthesis, thereby improving cancer inhibition by chemotherapy and radiation. Histidine catabolism

reduces cells levels of tetrahydrofolate, which is a cofactor in nucleotide synthesis. The cancer drug Methotrexate inhibits dihydrofolate reductase which generates tetrahydrofolate, therefore histidine supplementation could act synergistically with methotrexate in killing cancer cells [123]. Increasing arginine in mice was found to increase T-cell activation and T-cell mediated antitumor response [126].

All of these example diets with glucose and specific amino acids suggest a cancer patient on a high fat/low carb/low protein diet with NNS's could have better outcomes than if on a normal diet.. While NNS's could increase adherence to such diets it is not currently known if such a diet would be any better than intermittent fasting for cancer patients [95, 98, 105, 127–130], and in fact intermittent fasting has been shown in analysis to have the best adherence of common diets.

NNS's have been shown to increase death, though studies have varied largely in the magnitude of the effect. In 2018 Mulle et al. found NNS's to cause more deaths than natural sugar. The study was done with a 451,743 cohort from across 10 European countries. Participants were fairly healthy and excluded if they had preexisting conditions (cancer, heart disease, stroke, or diabetes at baseline), with a mean (SD) age of 50.8 (9.8) years. The participants were mostly women (71.1%). Participants that consumed 2 or more glasses per day of total soft drinks (with NNS's or sugar beverages, compared to <1 glass per month) died earlier (hazard ratio [HR], 1.17; 95% CI, 1.11–1.22;  $P < .001$ ). Sugar-sweetened soft drinks only had a HR of 1.08 (95% CI, 1.01–1.16;  $P = .004$ ), compared to NNS's with a HR of 1.26 (95% CI, 1.16–1.35;  $P < .001$ ). While NNS's seemed to increase participants risk of deaths from circulatory diseases (HR, 1.52; 95% CI, 1.30–1.78;  $P < .001$ ), sugar-sweetened soft drinks increased deaths from digestive diseases (HR, 1.59; 95% CI, 1.24–2.05;  $P < .001$ ) [121].

Some studies have found aspartame to be carcinogenic in large animal studies with rats [131, 132] and mice [131, 133] (many animals per sex and group of exposure, numerous dose levels tested, and with observation extending to death) [134]. In 2012 a prospective epidemiological study showed diet soda with aspartame to increase risk of non-Hodgkin's lymphoma and multiple myeloma in men [135]. Some have theorized that increased aspartame consumption could be linked to an increase in brain tumors [136], but a later study looking at almost half a million people between 50 and 71 years of age found there to be no link between aspartame and either hematopoietic or brain cancer [137]. However, a metaanalysis of 22 studies found aspartame to not cause cancer while looking at low, medium, and high doses in rodents, however it is worth noting that the odds ratio had an extraordinarily large range from 0.115 to 3.500 [138]. While case control studies have found an increased risk of cancer, such as a relative risk (RR) 1.3 for bladder cancer from heavy sweetener consumption, the same study found heavy coffee consumption (50 cups/week) had a RR of 1.4 for bladder cancer [34, 139]. While cancer can result in death, and therefore has large negative effects on Quality of Life Year calculations (QALYs), the data does not suggest NNS's increased cancer risk as much as other diseases, such as circulatory disease and metabolic syndromes.

### **3. Conclusions**

While the continually growing list of NNS's still have many unanswered questions regarding their biological effect in humans, the knowledge we are gaining about each NNS continues to grow as well. Sucralose is now known to become acylated and accumulate in fat tissue. Future studies with sucralose could require long wash out periods, and consumers could expect a delay in biological changes if they removed sucralose from their diet. Aspartame holds a preferential spot among the major NSS's in that it is quickly broken down when consumed so less

likely to have some of the deleterious effects by binding sweet receptors outside the oral cavity. Many NNS's alter the gut biome, with sucralose and saccharin seeming particularly deleterious. Unlike aspartame, saccharin is very stable in vivo and binds sweet receptors in the GI tract. Sucralose on the other hand becomes acetylated and accumulates in fat tissue, thereby potentially posing side effects for days long than other NSS's. The altered gut biome reduces satiety, causing consumption of more calories later. Obesity in mice has been alleviated through a fecal transplant which restores the gut biome [140]. This NNS induced dysbiosis also increases digestion of choline, needed for synthesis of VLDL which mobilizes fat from the liver. The choline deficiency and decreased VLDL leads to metabolic issues such as non-alcoholic fatty liver disease (NAFLD) [60].

Future double-blind human studies that would benefit the field would test individual NNS's to determine the causative effect of NNS consumption on:

1. Sleep quality and quantity (when consumed close to sleep)
2. Resting metabolic rate (RMR) during fasting
3. Energy expenditure (EI) during fasting
4. Autophagy during fasting

While the use of NNS's primary goal (reduced obesity) appears discreet, the multiple interacting pathways involved in effecting that change make the cause and effect complex to disentangle (e.g. changing gut biome, leaky gut, NAFLD, increased appetite, decreased RMR).

## Conflict of interest

The authors declare no conflict of interest.

## Appendices and nomenclature

5-HT	5-hydroxytryptamine, (also) serotonin
Ace-K	Acesulfame-potassium
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism, and Excretion
AUC	Area Under the Curve
CCK	Cholecystokinin
CICO	Calories-In-Calories-Out
CPIR	Cephalic Phase Insulin Response
DMA	Dimethylamine
EC	Enterochromaffin cells
EE	Enteroendocrine cells
FDA	US Food and Drug Administration
GIP	gastric inhibitory peptide, (also) glucose-dependent insulinotropic polypeptide
GERD	Gastroesophageal Reflux Disease
GI	Gastrointestinal
GLP-1	Glucagon-Like Peptide-1
GTT	Glucose Tolerance Test

HbA1C	Glycosylated Hemoglobin
JECFA	Joint Expert Committee on Food Additives
OTUs	Operational Taxonomic Units
OXM	Oxyntomodulin
PAH	Phenylalanine Hydroxylase
PKU	Phenylketonuria
PPP	Pentose Phosphate Pathway
PYY	Peptide Tyrosine Tyrosine
MW	Molecular Weight
NAFLD	Nonalcoholic Fatty Liver Disease
NNS	Non-Nutritional Sweetener
NOAEL	No Observed Adverse Effect Level
QALY	Quality Adjusted Life Year
RR	Relative Risk
SCF	European Union's Scientific Committee for Food
TBW	Total Body Weight
TMA	Trimethylamine
USD	United States of America Dollars
WWTP	Waste Water Treatment Plant

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