Memorandum

Date	June 15, 2023
From	(b)(6) (HFS-255) Through (b)(6) (HFS-255) (b)(6)
Subject	An evaluation of the article "The artificial sweetener erythritol and cardiovascular event risk" by Witkowski *et al.*, Nat Med. 2023 Mar;29(3):710-718.
To	Sweetener Publication Review

In March 2023, a paper entitled "The artificial sweetener erythritol and cardiovascular event risk" by the group Witkowski et al. was published in *Nature Medicine*. The purpose of this memo is to summarize the key findings presented in the paper, placing into context the implications of this study with previously published toxicological data on erythritol. Herein, we have highlighted the strengths and weaknesses of this study and identified unanswered questions for consideration when designing future studies on erythritol.

Background on Erythritol

Erythritol is a four-carbon polyol that occurs naturally in mushrooms and fermented foods such as wine, beer, cheeses, and soy sauce. It is also present in fruits such as grapes, peaches, and watermelon. Recently, several groups have demonstrated that erythritol can be produced endogenously in mammals from glucose through the pentose-phosphate pathway (Hootman et al., 2017; Ortiz et al., 2021; Schlicker et al., 2019). Others have demonstrated that endogenous erythritol synthesis is enhanced in response to oxidative stress (Ortiz et al., 2022).

Erythritol is a relatively new food ingredient. In 2001, the FDA evaluated the first "Generally Recognized as Safe" (GRAS) notice for erythritol. It is used as a nutritive sweetener, stabilizer and thickener, flavor enhancer, formulation aid, humectant, texturizer, or sequestrant in various foods and beverages. Erythritol is produced commercially via fermentation by yeast or yeast-like fungi using substrates such as glucose and sucrose (Mazi et al., 2023). In humans, erythritol is absorbed in the small intestine, and, due to its lower molecular weight, absorption occurs more rapidly than larger sugar alcohols such as mannitol or xylitol. Blood erythritol concentrations peak 1 to 2 hours after ingestion, and erythritol is rapidly excreted unmetabolized, with 80-90% collected in the urine 24-48 hours after ingestion (Noda et al., 1994). Other human studies have confirmed that erythritol is readily absorbed and excreted unmetabolized in the urine, while further showing that unabsorbed erythritol does not undergo

colonic fermentation in the large intestine (Hiele et al., 1993; Arrigoni et al., 2005). As it is largely not metabolized by the body or gut microbiota, erythritol is considered a low-calorie sweetener and is better tolerated in comparison to other polyols. More recently, Hootman et al. (2017) has challenged the idea that erythritol is unmetabolized by showing that up to 10% of erythritol in the blood can be metabolized to erythronate.

In the United States, erythritol has been the subject of several GRAS notices. It was most recently evaluated in 2018 as GRN 000789. During the GRAS notice process, FDA has evaluated numerous published and publicly available toxicology studies, including acute; short-term (4 weeks); subchronic; chronic and carcinogenicity; reproductive and developmental studies; *in vitro* and *in vivo* mutagenicity and genotoxicity assays, and studies on the absorption, distribution, metabolism, and excretion (ADME) of erythritol (Munro et al., 1998; Til et al., 1996; Lina et al., 1996). Additionally, published human clinical studies including erythritol tolerability studies in healthy children and adults and short-term consumption studies in diabetic patients were also included as corroborative support of the safety of erythritol (Jacqz-Aigrain et al., 2015; Storey et al., 2007; Fukuda et al., 2010; Flint et al., 2014). FDA has not questioned the notifiers' GRAS conclusions for erythritol under the intended conditions of use that were identified in each GRAS notice.

Erythritol is also approved for use in food and beverages in countries throughout the world, including Japan, Australia, New Zealand, Canada, Mexico, and Brazil. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed erythritol in 1999 and established an acceptable daily intake as "not specified".[1] The European Food Safety Authority (EFSA) initially evaluated erythritol for use in food in 2003 and recently expanded its evaluation for use in beverages in 2015. The latest EFSA evaluation (2015) expanded the use of erythritol to non-alcoholic beverages at a maximum level of 1.6%. This was based on a study measuring gastrointestinal tolerance in young children (aged 4-6 years), which showed that the tolerance level for young children was similar to that observed for adults when expressed as per kg body weight (bw)/day basis (Jacqz-Aigrain et al., 2015) In this study, children who consumed a 15g beverage of erythritol (equivalent to 0.73g/kg bw for both genders) tolerated it well. In adults, the estimated laxative threshold was calculated to be 0.8g/kg bw for females and 0.66 g/kg bw for males (Oku et al., 1999). Ultimately, EFSA's recommendation was based on potential laxative effects and not based on toxicological data or any concerns regarding cardiovascular events.

Summary and Discussion of the Witkowski et al. (2023) Epidemiological Studies

In their paper in *Nature Medicine*, Witkowski et al. (2023) investigated the relationship between erythritol and cardiovascular event risk. In their observational epidemiologic study, the authors performed untargeted metabolomics studies on a discovery cohort of 1,157 U.S. patients that underwent a cardiac risk assessment between 2001 and 2007. The authors found that circulating levels of several polyols, including erythritol, were associated with incident risk for major adverse cardiovascular events

[1] JECFA states that 'ADI not specified' means that "on the basis of the available data (chemical, biochemical, toxicological, and other), the total daily intake of the substance, arising from its use or uses at the levels necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health."

(MACE).[2] As noted by the authors, there were several limitations with this initial study. Untargeted metabolomics studies offer a comprehensive and qualitative analysis of metabolites in the plasma. Moreover, this approach does not readily differentiate erythritol from its structural isomers, such as threitol.

To address these caveats, the authors carried out two targeted metabolomics studies: one from the U.S. involving a distinct cohort of 2,149 subjects and another from Europe involving a cohort of 833 subjects. Like the discovery cohort, subjects in these validation cohorts were all undergoing elective cardiac risk assessments, and longitudinal outcome data were collected for 3 years after enrollment in the studies. Subjects in each cohort were grouped into four quartiles based on plasma levels of erythritol in each cohort. Kaplan–Meier estimates and Cox proportional hazards regression models were appropriately used to compare the risk of MACE over three years of follow up among those four groups. Only subjects in the highest quartile in both the U.S. and European cohorts had a significantly increased incident MACE risk when compared to subjects in the lowest quartile. To confirm these results were not confounded by factors other than plasma levels of erythritol, the investigators reconsidered their findings in light of traditional risk factors of MACE such as age, gender, diabetes status, blood pressure, cholesterol and triglyceride levels, and smoking status by adjusting the Cox proportional hazards regression models for those risk factors. As was observed in the discovery cohort, the association between the highest erythritol levels and MACE risk was still statistically significant following adjustment for these various cardiovascular risk factors in both the U.S. and European cohorts (adjusted HR (95% CI) 1.80 (1.18-2.77) and 2.21 (1.20-4.07), $P = 0.007$ and $P = 0.010$, respectively). They also performed subgroup analyses of these risk factors and other factors and confirmed that the association observed between plasma levels of erythritol, and the incidence of MACE are homogeneous across the study population. From a statistical standpoint, we believe the authors have used the appropriate statistical methods to make sound conclusions about their epidemiological studies and have acknowledged and discussed the limitations of these studies.[3]

There are several important limitations to bear in mind when interpreting the findings of these epidemiological studies. First, there is an inherent limitation of observational studies to establish causation, and in fact, the authors have appropriately acknowledged that they have provided evidence of an association, and not necessarily causation, between circulating erythritol levels and MACE. Second, with clinical observational studies, we need to consider what confounding variables may be influencing the described association. The authors have correctly adjusted for many variables, including age, gender, and known cardiovascular risk factors such as diabetes and hypertension status. However, there were critical unmeasured variables[4] that could be resulting in bias that leads to an over or underestimation of

[2] The authors defined MACE as death, nonfatal myocardial infarction, or a nonfatal stroke that occurred after enrollment in the study.

[3] From (b)(6) at the Office of Analytics and Outreach (OAO), Biostatistics and Bioinformatics Staff (BBS), email correspondence dated March 13, 2023.

[4] In their analysis of the epidemiological studies, the authors did not adjust for kidney function in their discovery or validation cohorts. As the kidneys are involved in the excretion of erythritol, impaired kidney function may result in delayed clearance and elevated levels of erythritol. The authors did assess risk of MACE in various subgroups, including in patients with an estimated glomerular filtration rate (eGFR) greater than and less than 60. Risk of MACE was elevated in patients with the highest erythritol levels and impaired kidney function (eGFR <60) in both the U.S. and European validation cohorts. The subgroup difference did not reach statistical significance; however, the sample sizes for patients with impaired kidney function were small in both cohorts.

the association. For example, the authors did not collect data on overall diet or food consumption in any of their subjects. Others have demonstrated that glucose can be metabolized to erythritol via the pentose-phosphate pathway, and therefore, the patients in this study with the highest levels of erythritol in their plasma may reflect those who consume higher levels of glucose in their diet. Additionally, in the European cohort, body mass index (BMI) data was not available, and therefore was not adjusted for. Another important factor to consider pertains to the patient populations utilized for these observational studies. Patients in all three cohorts were already at much higher risk for incidents of MACE given the high prevalence of multiple risk factors such as diabetes and hypertension in these cohorts. Given that the association of erythritol to cardiovascular events was interrogated in an at-risk population rather than in a healthy population, there is limited applicability of these findings to the general population.

Another caveat with this study is that it did not address to what extent dietary exposure to erythritol is contributing to circulating erythritol levels in patients. Sources of erythritol include dietary exposure to foods that naturally contain it, such as fruits and fermented foods; dietary exposure to foods or beverages where it is added; as well as endogenous production from glucose through the pentose-phosphate pathway (Hootman et al., 2017; Schlicker et al., 2019). The authors state that the U.S. patient cohort samples, which account for approximately 80% of the total samples analyzed, were collected between 2001 and 2007, prior to the widespread addition of erythritol to foods and beverages in the U.S. Additionally, patients were fasting prior to blood collection. Therefore, at least in these samples, detected erythritol levels are likely a reflection of endogenous production of erythritol rather than from significant exposure to erythritol as a food ingredient. The authors do note that plasma from the European cohort was collected between 2016-2018, and therefore erythritol levels in this subset of patients are more likely to reflect consumption of erythritol as well as endogenous production. The European subjects were also fasting prior to sample collection. Pharmacokinetic data from this study as well as others indicate that erythritol concentrations in blood peak within the first two hours following consumption but circulating levels can remain elevated for two days (Ishikawa et al., 1996; Munro et al., 1998). Unfortunately, no data on dietary exposure to erythritol was collected in any of the patient cohorts in these studies, and thus, it remains unknown to what extent consumption of erythritol from food sources is contributing to elevated circulating levels.

As discussed above, one limitation of the initial untargeted metabolomics approach was that the data generated is not quantitative, and the method is not able to distinguish a compound from its structural isomers. To address this, the authors designed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay with isotope dilution quantification to measure erythritol content in human and mouse plasma and serum. The internal standard spike for erythritol was D_6-erythritol. This technique is suitable for the quantification of erythritol in human and mouse plasma as the deuterated erythritol used as an internal standard spike can compensate for losses during sample preparation while having identical retention time as erythritol. However, the study lacks details on the validation of the method and thus the contribution of the uncertainty from the serum and plasma erythritol analyses cannot be ascertained. More information is necessary on: (1) the source and purity of the analytes and their internal standards; (2) calibration range; (3) how were the LOD and LOQ determined; (4) source and/or preparation, and concentrations of the erythritol QC samples; and (5) the evaluation of recovery and matrix effect may be

needed. Without this information, we are not able to evaluate the accuracy and precision of their method to quantify erythritol in human plasma.[5]

Summary and Discussion of the Witkowski et al. (2023) Preclinical Platelet Studies

In addition to the epidemiological studies, the authors also performed a series of *in vitro*, *in vivo*, and *ex vivo* preclinical studies aimed at offering potential mechanistic insight into the association between erythritol levels and incidents of MACE in at-risk individuals. To interrogate the effects of erythritol on platelet aggregation, the authors isolated platelet-rich plasma from healthy volunteers and incubated it with a range of erythritol concentrations (0, 4.5, 18, 45, 90, and 270μM) which largely corresponded to levels seen in the highest quartile in their observational study cohorts. They observed a dose-dependent increase in platelet responsiveness in platelet-rich plasma exposed to erythritol but not to glucose or another polyol, 1-5-anhydroglucitol. Additionally, the authors showed that when human platelets from healthy controls were incubated with erythritol, markers of platelet activation (intracellular cytosolic Ca^{2+} levels, P-selectin surface expression and glycoprotein α2β3 activation) were observed. As was seen in the platelet aggregation assay, higher levels of erythritol (generally, greater than 18μM) were needed to see a significant increase in platelet reactivity markers. Using whole blood from healthy patients, the authors performed a thrombosis assay to assess the impact of erythritol exposure on platelet adhesion. They incubated whole blood with 45μM of erythritol, a dose corresponding to levels at the high end of the 4th quartile in the cohorts of their observational studies. Under these conditions, erythritol did significantly increase platelet adhesion. Finally, they used a $FeCl_3$-induced carotid artery injury model in mice to show that erythritol, given at a dose of 25mg/kg body weight, significantly decreased the time to clot formation. The dose of erythritol given to mice in this experiment resulted in circulating levels of erythritol around 290μM, which was much higher than values seen in patients in the observational studies. Overall, the authors used appropriate assays to evaluate platelet reactivity and these mechanistic studies suggest that erythritol can augment platelet activation and enhance thrombosis potential.[6] However it is important to consider that platelet activation and blood clotting are influenced by many factors, all of which cannot be accounted for in these types of preclinical studies.

Summary and Discussion of the Witkowski et al. (2023) Erythritol Pharmacokinetics Study

In addition to these preclinical studies, the authors conducted a small pharmacokinetics study with eight healthy volunteers to determine how long circulating erythritol levels remain elevated after dietary exposure. Subjects fasted overnight, and baseline blood samples were collected before the volunteers were given a beverage containing 30g of erythritol. In agreement with prior pharmacokinetics studies, levels of erythritol in plasma peaked rapidly in the first hour at a concentration nearly 1,000-fold higher than baseline levels. The authors note that the observed erythritol levels in all participants in the first two days after ingestion (millimolar concentrations) greatly exceeded the levels that were needed to elicit the platelet activation phenotypes in their *in vivo* studies (4.5-45μM). Interestingly, they repeated the platelet aggregation assay using a 6mM dose of erythritol to model the concentrations they saw following their pharmacokinetics studies. Platelet aggregation was only slightly higher at this millimolar

[5] From (b)(6) and (b)(6) at the Office of Food Additive Safety (OFAS), Division of Food Ingredients (DFI), email correspondence dated March 8, 2023.

[6] From (b)(6) at the National Center for Toxicological Research (NCTR), Division of Systems Biology, email correspondence dated March 9, 2023.

dose compared to what they saw at micromolar doses of erythritol. Overall, this was a very small pharmacokinetics study, done in a healthy population, but it does recapitulate prior findings on the absorption, distribution, and excretion of erythritol in humans.

The authors stated that they chose a dose of 30g of erythritol based on data available from prior GRAS notices. It is important to point out that the estimated daily intake of erythritol from the intended uses proposed in the most recent GRAS notice on erythritol for the total population was 32.1 and 63.0 g/person/day for the mean and 90th percentile, respectively. These are considered highly conservative estimates based on assumptions that erythritol is being used at the maximum use levels in all intended foods. The actual dietary exposure to erythritol is likely to be considerably lower. Also, these are exposure estimates for consumption over the course of an entire day, whereas the beverage administered to the volunteers in this study containing 30g of erythritol was consumed over a two-minute period. Moreover, in a study by Bornet et al. (1996), plasma concentrations of erythritol were found to be lower when consumed in food as compared to an aqueous solution, which could be due to slower gastric emptying. Thus, it is not clear whether a typical dietary exposure scenario from the intended uses would result in clinically relevant adverse events. Considering these factors, future studies should be designed with more typical dietary exposure patterns in mind.[7]

Conclusions and Unanswered Questions

In their study, Witkowski et al. (2023) report an association between circulating erythritol levels and the incidence of MACE. They also provide preliminary evidence that erythritol can affect platelet activation and thrombosis potential in preclinical models. While the data reported warrants further investigations into the potential link between erythritol and MACE, there are several caveats with this study that would require further research to address unanswered questions relevant to food safety assessment. Most notably, the observational studies in this paper are incapable of establishing causation, and there were critical confounding variables, such as diet, that were not accounted for in their analysis. Moreover, the choice to interrogate this relationship in a patient population with known cardiovascular risk factors limits our ability to extrapolate their findings to the general, healthy population.

Until a causative role of dietary erythritol on MACE is reasonably established, we need to consider this paper in the context of other literature available on erythritol. Over the past thirty years, numerous toxicological, metabolic, biochemical, and clinical studies have been published using animal models and human subjects, and erythritol has consistently been shown to be safe (EFSA ANS Panel, 2015; JECFA, 1999a). As dietary exposure to erythritol has increased in recent years, more studies investigating the health benefits of the sweetener have been carried out, often with conflicting results. For example, there are studies in healthy and diabetic patients that demonstrate that dietary erythritol consumption does not influence glucose and insulin levels (Noda et al., 1994; Ishikawa et al., 1996). However, there are also papers that suggest elevated circulating erythritol levels are associated with type II diabetes and coronary heart disease (Rebholz et al., 2018; Wang et al., 2019). Like the study by Witkowski et al. (2023), the samples for these metabolomics studies were collected prior to the introduction of erythritol into foods, and so these observations also likely reflect endogenous production of erythritol. In fact, several studies have suggested that endogenous erythritol may be a marker or a result of metabolic stressors (Ortiz et al., 2020). Together, these findings highlight the need for a better understanding of

[7] It would also be beneficial to obtain additional clinical data from future studies, such as coagulation testing in these volunteers at various time points after ingestion, to validate the preclinical findings presented in the paper.

how diet, especially diets that are high in refined sugars, influences blood erythritol levels. Carefully designed and controlled studies should help to clarify whether circulating erythritol levels are a biomarker of cardiovascular and metabolic disease or actively contributing to the pathogenesis of these diseases.

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