ICBA
INTERNATIONAL COUNCIL OF BEVERAGES ASSOCIATIONS
ASIA PACIFIC REGIONAL GROUP

Australian Beverages

FIA FOOD INDUSTRY ASIA

A Spotlight on Sweeteners:

Exploring Science, Safety and Use

SYMPOSIUM

Four Seasons Hotel, Sydney
27 June 2023

Acknowledgement of Country

Geoff Parker

Chief Executive Officer, Australian Beverages Council

Executive Director, International Council of Beverages Associations Asia Pacific Regional Group

MEET THE SPEAKERS

Geoff Parker

CEO, Australian Beverages Council

Andrew Therkelsen

Managing Director, The Lab

Dr John L. Sievenpiper

Professor, University of Toronto

Dr Susan Elmore

Principal, Elmore Pathology

Christel Leehmuis

GM Science and Risk Assessment, FSANZ

EXPLORING SCIENCE, SAFETY AND USE OF SWEETENERS

AGENDA

10am	Welcome
10.10am	Non-sugar Sweetener Research Project - Key Opinion Leader Insights
10.35am	Low and No-calorie Sweeteners (LNCS) and Health: An Important Role In Sugars Reduction Strategies
11.20am	Pathologists' Perspective on Ramazzini Institute Aspartame Studies
12.05pm	Assessing Additive Risk - A Regulatory Agency's Perspective
12.40pm	Q&A Panel Discussion
1pm	Event Close and Networking Light Lunch

Andrew Therkelsen

Managing Director
The Lab

PRESENTATION

Non-sugar **sweeteners**

Explore, unpack and deliver a basis of insight from

Key Opinion Leaders

2023

thelab

In focus

The key objectives

A baseline understanding sought from key opinion leaders in Australia and New Zealand.

Key Opinion Leaders

Explore, unpack and deliver a basis of insight around their positions and sphere of influence with NSS.

40 invitations

17 participants...

- x2 Trade Associations
- x3 Health Departments
- x1 Public Health Agency
- x1 Food Regulatory Agency
- x3 Government Ministries
- x1 State Food Authority
- x5 Nutritionists/Dietitians
- x1 Member of Parliament

Methodology: indepth interviews

Baseline online survey
Consumer insight

n=1,598

AUS
1,096

NZ
502

NSW	309	28%
VIC	290	27%
QLD	225	21%
ACT	21	2%
SA	99	9%
WA	115	11%
TAS	29	3%
NT	8	1%

Auckland	183	36%
Taranaki	29	6%
Hawke's Bay	27	5%
Wellington	34	7%
Nelson	15	3%
Canterbury	148	29%
Otago	66	13%

In focus:

Key Opinion **Leaders**

thelab

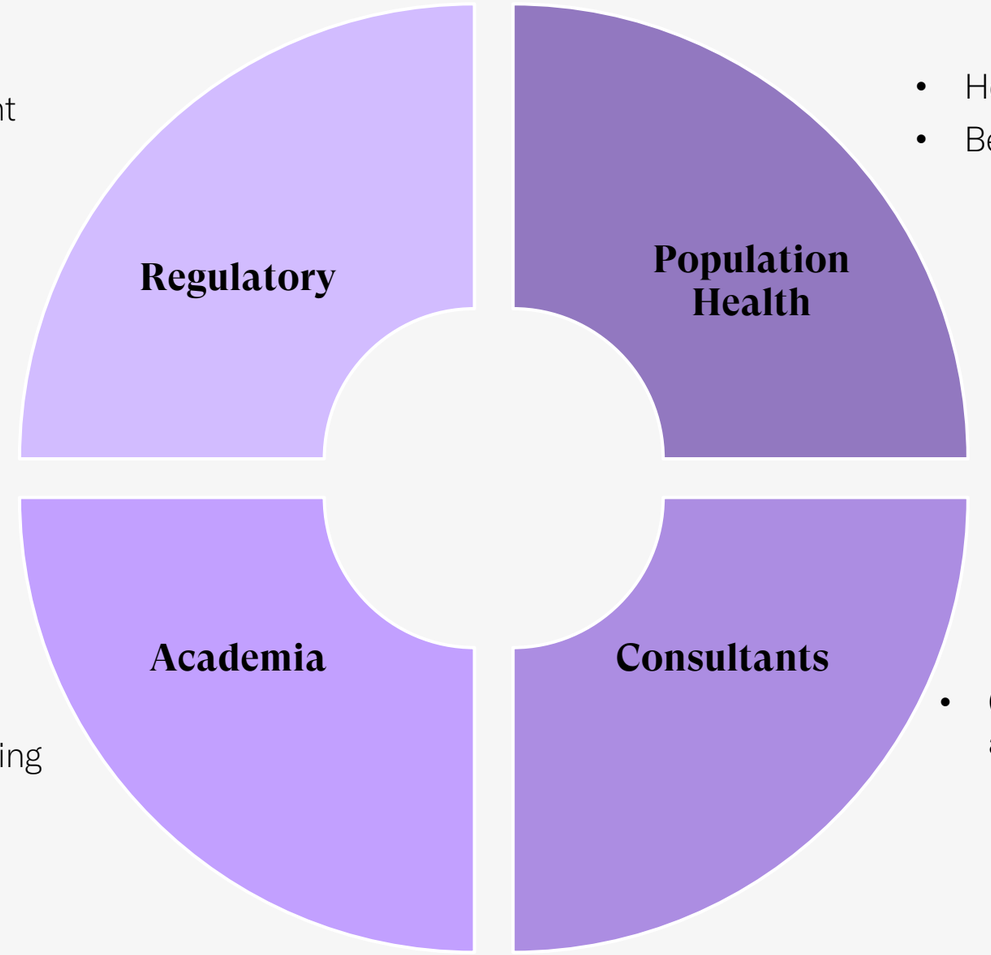
The collective position on non-sugar sweeteners is not a clear one...

It sits within a grey area – that very much depends on a range of factors that shape perspective and position.

The frame of **reference**

> “”
> Some will seek the **evidence to support their belief**, while others will **allow the evidence to drive their belief**.

Our role & organisational lens



- Independent
- Risk assessment

- Holistic focus
- Behaviour change

- The science
- Hypothesis testing

- Conduit between audiences

A question of **position**

A KOL's holistic point of view will place them on a continuum of position.

Idealist

Nutritional perfection

Water is perfection

Ideals

The ultimate position

The pinnacle of perfection

NSS = on par with sugar

Realist

Pragmatic appropriateness

Perfection is hard

Let's be practical

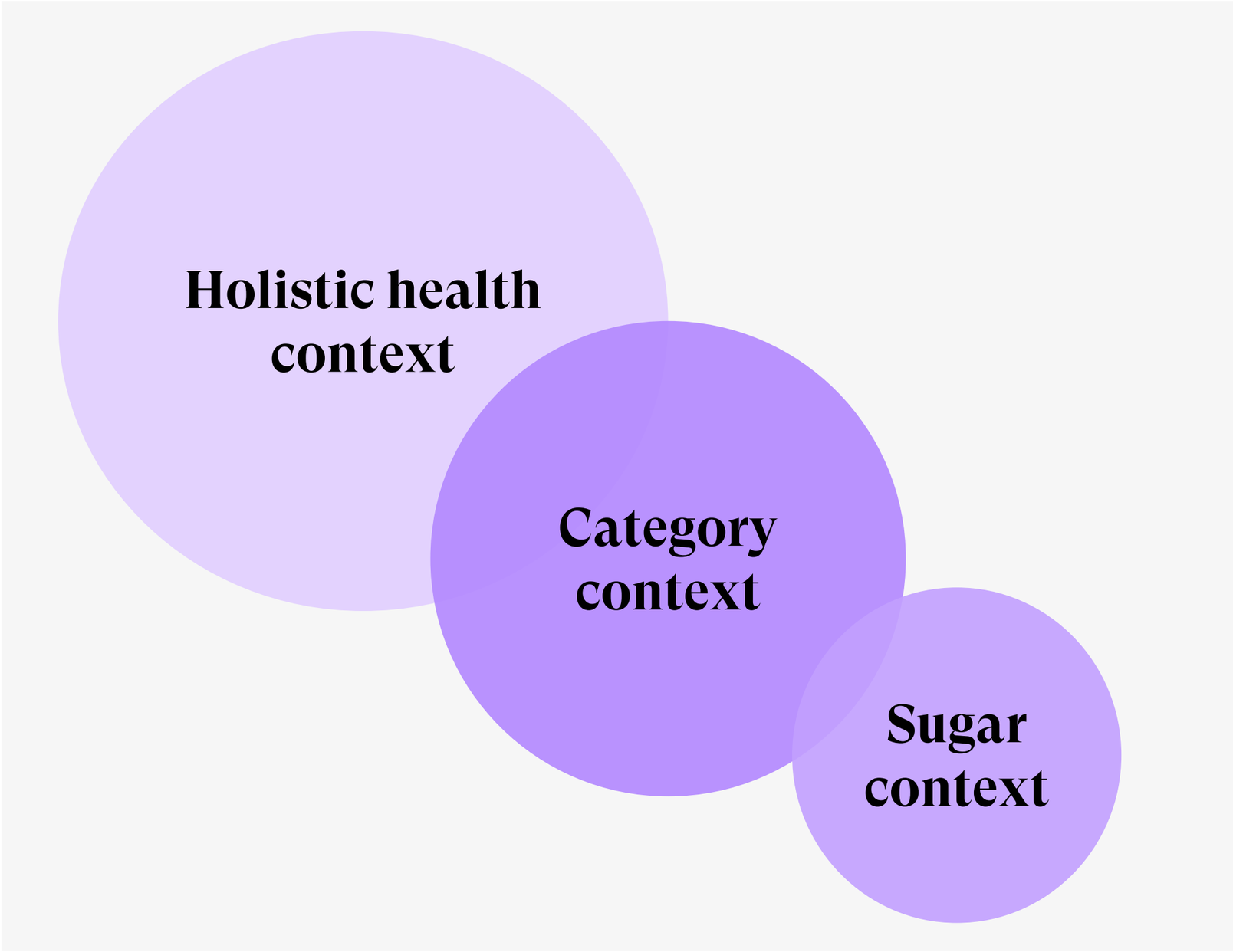
People want choice

Life is busy

Everything in moderation

Food/beverages with NSS = a 'better' choice

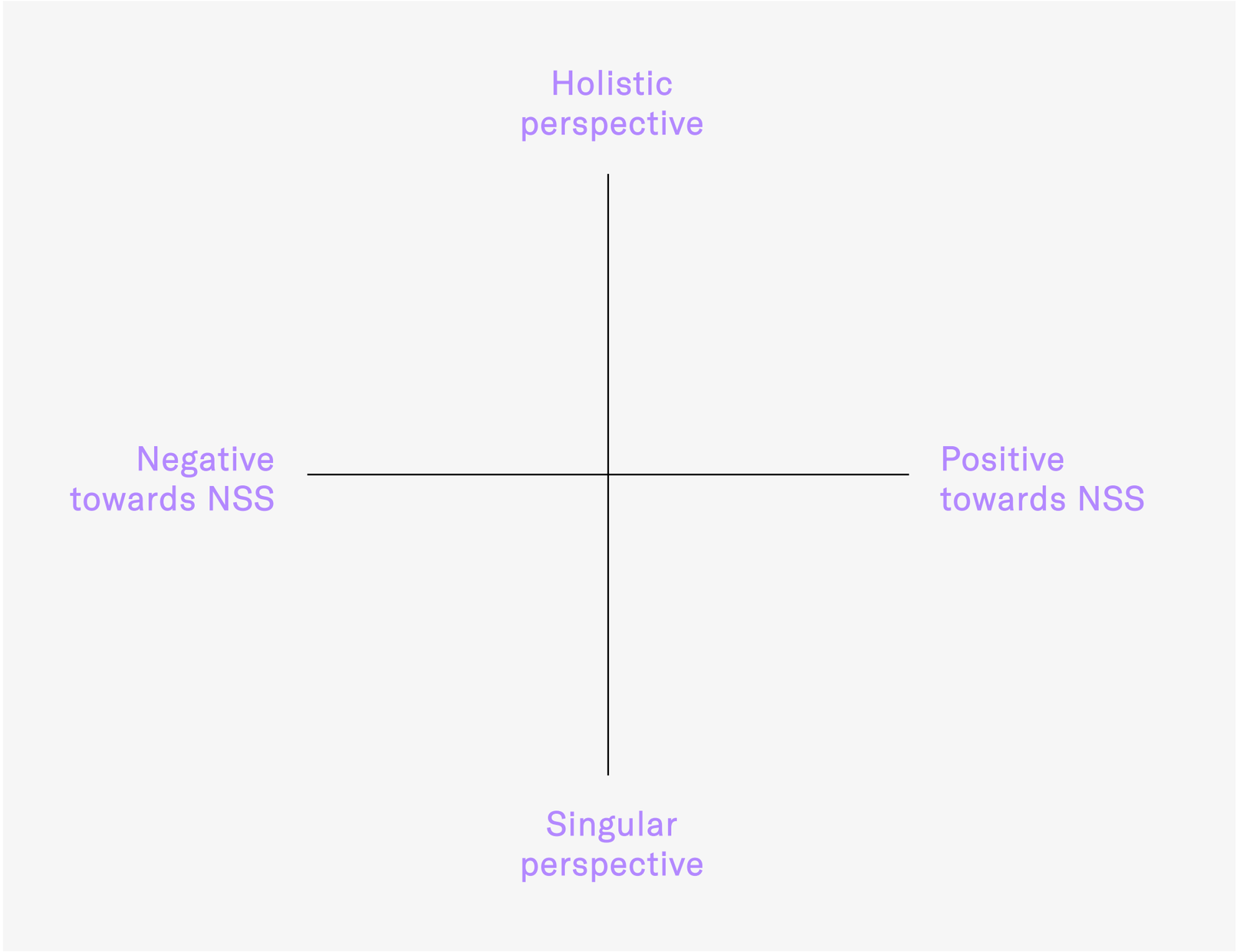
Also a question of **context**



Dimensions
Shaping perspective

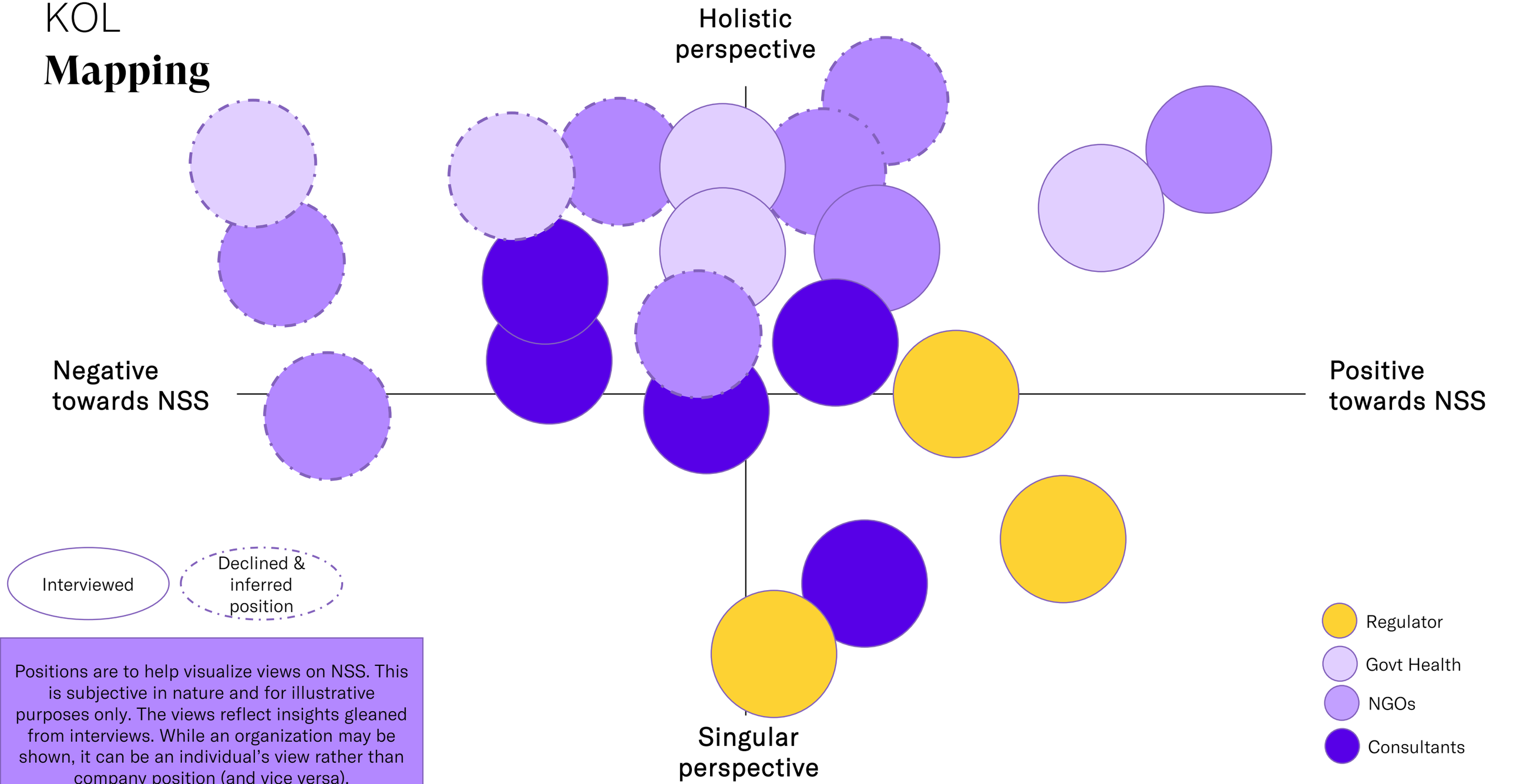
Two key variables….

1. A KOL’s position towards NSS.
2. Are they considering NSS in a vacuum or in the context of a broader ecosystem.



KOL

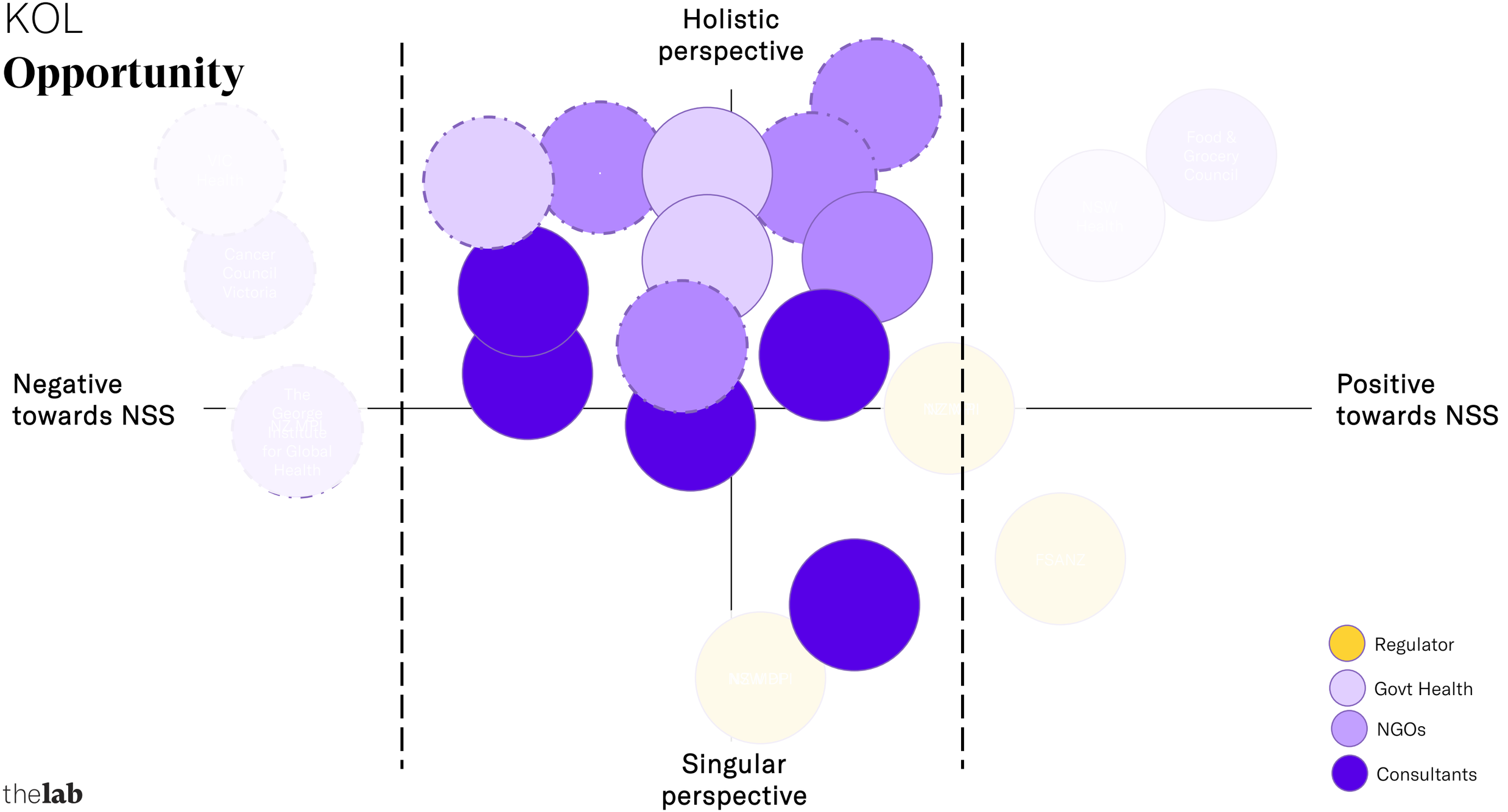
Mapping



Positions are to help visualize views on NSS. This is subjective in nature and for illustrative purposes only. The views reflect insights gleaned from interviews. While an organization may be shown, it can be an individual's view rather than company position (and vice versa).

KOL

Opportunity



The role of **non-sugar sweeteners**

Regardless of ideals, and regardless of the lens you place over your position...

Non-sugar sweeteners have a role to play in our food eco system, and within beverages.

Non-sugar sweeteners

Reflection on its role

Defensible principle

Approved use

Our region has some of the most rigid food regulatory and food additive testing regimes in the world.

Choice

Consumer options

People want choice.

To suit a need, a social scenario, a goal, or a variety of flavour, and consumption levels.

A ‘better’ choice

Alternatives

Allowing people to maintain the flavour and palette they desire, without the burden of traditional sugar based options.

Supporting health directives

A stepping stone

On a person's journey to better health and lifestyle, products with NSS work as a stepping stone to help reduce sugar intake and the associated negatives.

Sources of **influence**

Sources of **Influence**

No one source

Evolving landscape

A wide array of studies are produced on an ongoing basis.

Science evolves, positions evolve, and dietary metrics evolve – **all changing the landscape and goals over time**.

Underlying intent / influence

Devil in the detail

Care taken to review the methodology, the frame of reference, the sponsoring party, and the belief system behind the party/s

The weight of evidence

Nothing in isolation

One study from one party may be reflected upon, but most are aware of bias and **the need for a collective weight of evidence built from across sources.**

Regulation

The defensible principle

Many will fall back to **regulatory guidelines and underlying laws**.

Groups like FSANZ are seen as unbiased and strong evidence based independent bodies.

Where are the gaps in **knowledge**

Impacts of NSS on our health long term...

- Impact on gut microbiomes?
- Safe doesn't mean it is good for you!!!
- Is it a healthy(ier) option?
- Does it serve a purpose?
- A false desire / maintenance of sweetness
- How will/does overuse of NSS impact on our internal health systems?

In
Reflection

Key Opinion Leaders

Key takeaways

A complex ecosystem

Not black or white

There is no one clear position or answer to the question/s surrounding NSS.

There is a web of intertwined ways to consider its use and position.

A multitude of lens

Frame of reference

This will alter positions on NSS.

NSS has a role to play

Driven by choice

Fundamentally anchored around the essence of choice – and how our informed choices provide options to meet our goals or needs as a consumer.

Strong regulation

Defensible position

There will always be opponents to NSS.

And there will always be positions and studies released that question its role.

However, the ANZ region has some of the strictest and most comprehensive testing regimes in place – and as it stands, NSS are deemed safe for use.

The big questions

Debate

There are still universal questions remaining unanswered at this time.

What wider impacts does its use have on our systems?

What this highlights

For the industry

The category is noisy

It is not black or white

It is a very subjective topic when stating or considering the role of and position ones holds around NSS.

Frames of reference, bias, and situational perspective all influence and adjust how one looks at NSS.

There is no clear or loud voice in strong support of NSS

Subtle position

It delivers in a subtle and low key way, below the line.

While other positions and anti views tend to be louder and more prevalent.

A stronger NSS position is required

Speak up

Look to balance or shift the narrative surrounding NSS.

Audiences are open to persuasion

Opportunity is there

Consumers and KOLs (majority) appear open to creating firmer positive positions on NSS. Many are sitting on the fence.

Thank **you**

Dr John L. Sievenpiper
BASc, MD, MSc, PhD, FRCPC

Clinician Scientist

Professor, University of Toronto

Low- and no-calorie sweeteners (LNCS) and health: An important role in sugars reduction strategies

John L Sievenpiper, MD, PhD, FRCPC[1,2,3,4,5]

[1]Diabetes Canada Clinician Scientist

[2]Associate Professor, Nutritional Sciences and Medicine, University of Toronto

[3]Lifestyle Medicine Lead, MD Program, University of Toronto

[4]Consultant Physican, Division of Endocrinology & Metabolism, St. Michael's Hospital

[5]Scientist, Li Ka Shing Knowledge Institute, St. Michael's Hospital

UNIVERSITY OF TORONTO

Sydney, Australia

June 26, 2023

St. Michael's

Inspired Care.
Inspiring Science.

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FACULTY OF MEDICINE

Disclosures (past 24 months)

Board Member/Advisory Panel

–Diabetes Canada 2018 Clinical Practice Guidelines Expert Committee for Nutrition therapy

–Canadian Cardiovascular Society (CCS) 2016 Dyslipidemia Guidelines Update

–European Association for the Study of Diabetes (EASD) Clinical Practice Guidelines Expert Committee for Nutrition therapy

–Obesity Canada Clinical Practice Guidelines Expert Committee

–Institute for the Advancement of Food and Nutrition Sciences (IAFNS)

–European Fruit Juice Association Scientific Expert Panel

–SNI Scientific Advisory Committee

Research Support

–Canadian Institutes of Health Research (CIHR)

–Canadian Foundation for Innovation/Ontario Research Fund

–Diabetes Canada

–PSI Foundation

–American Society for Nutrition (ASN)

–National Honey Board (USDA “check off” program)

–INC International Nut and Dried Fruit Council Foundation

–Institute for the Advancement of Food and Nutrition Sciences (IAFNS)

–Pulse Canada

–Quaker Centre for Excellence

–Tate & Lyle Nutritional Research Fund at the University of Toronto

–Glycemic Control and Cardiovascular Disease in Type 2 Diabetes Fund at the University of Toronto (a fund established by the Alberta Pulse Growers)

–Nutrition Trialists Fund at the University of Toronto (a fund established by the Calorie Control Council)

–United Soybean Board (USDA “check off” program)

“In-kind” food product donations for trials

Almond Board of California, California Walnut Commission, Peanut Institute, Barilla/Upfield, Unico/Primo, Loblaws, Quaker, Kellogg Canada, Danone, Nutrartis, Soylent

Ad Hoc Consulting Arrangements

–Tate & Lyle

–Perkins Coie LLP

–Inquis Clinical Research

Honoraria or Speaker Fees

–Phynova

–Nestle

–IFIC

–General Mills

–Danone

–International Sweeteners Association

–International Glutamate Technical Committee

–Calorie Control Council

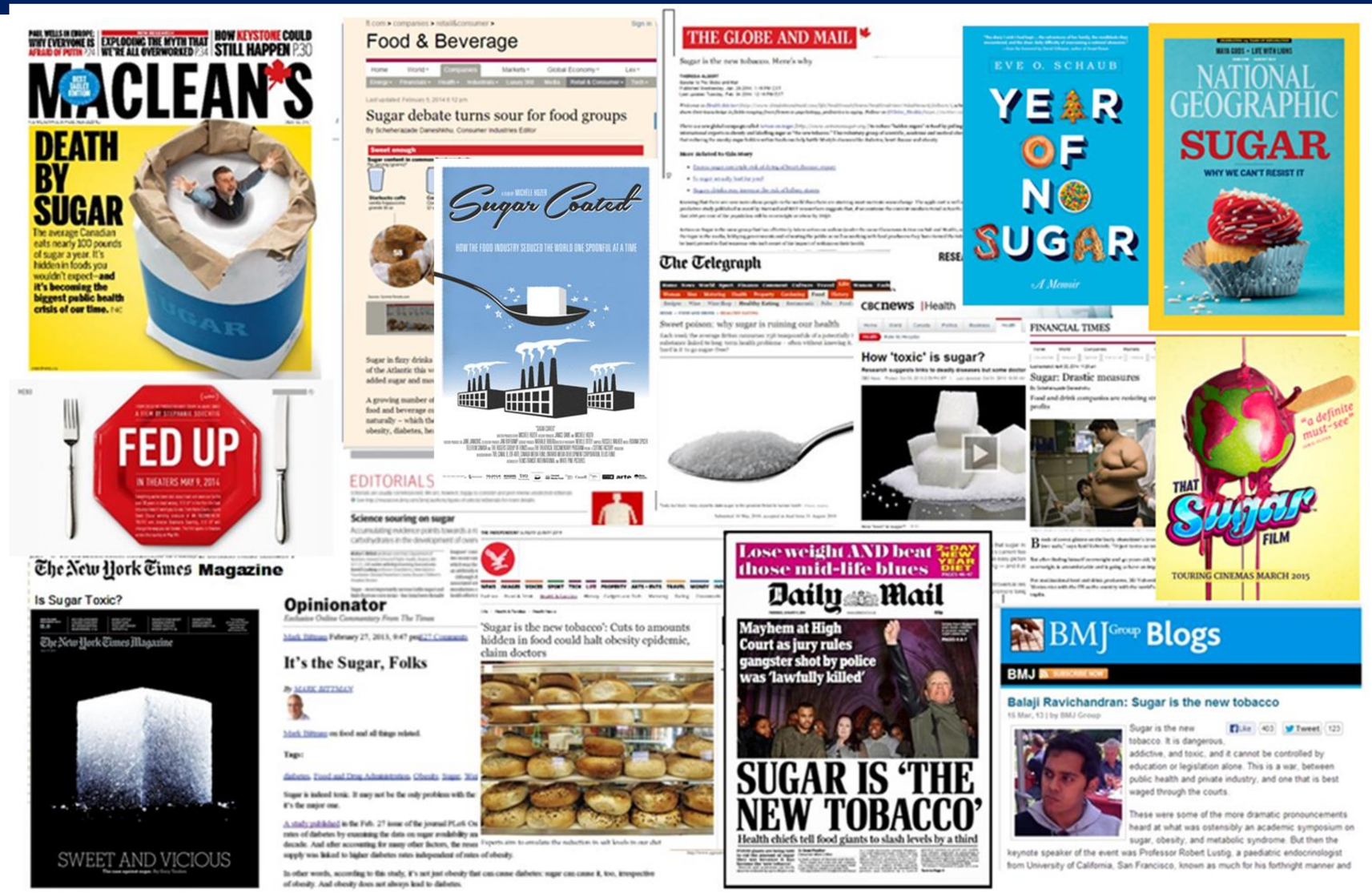
–Abbott

Other

–Spouse is an employee of AB InBev

–Director, Toronto 3D Knowledge Synthesis and Clinical Trials foundation

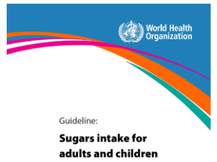
Sugars the new dominant public health concern



What do guidelines say?

Universal recommendations for sugars reduction:

Dietary guidelines and clinical practice guidelines recommend <5-10% energy from free/added sugars

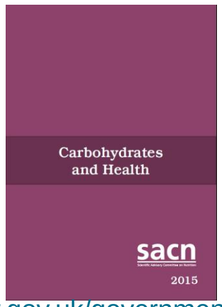


<10% energy
<5% energy (conditional)

http://www.who.int/nutrition/publications/guidelines/sugars_intake/en/

≤10% energy

https://www.dietaryguidelines.gov/sites/default/files/2020-07/ScientificReport_of_the_2020DietaryGuidelinesAdvisoryCommittee_first-print.pdf



≤5% energy

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/445503/SACN_Carbohydrates_and_Health.pdf

American Diabetes Association®

"Minimize"

ADA. Diabetes Care 2020;43(Suppl. 1):S48–S65

DIABETES CANADA

≤10% energy

Sievenpiper et al. Can J Diabetes. 2018;42 Suppl 1:S64-S79

DIABETES UK
KNOW DIABETES. FIGHT DIABETES.

≤5% energy

https://diabetes-resources-production.s3.eu-west-1.amazonaws.com/resources-s3/2018-03/1373_Nutrition%20guidelines_0.pdf

International Diabetes Federation

≤10% energy

https://alliancechronicdiseases.org/wp-content/uploads/06.-IDF_Europe_Position_on__Added_Sugar.pdf

DNSG EASD

≤10% energy

DNSG Guideline Group. Diabetologia 2023 Jun;66(6):965-98

What do Clinical Practice Guidelines for obesity and diabetes say about LNCS?

Clinical practice guidelines for obesity and diabetes recommend LNCS

American Diabetes Association

*"The use of **nonnutritive sweeteners** as a **replacement for sugar-sweetened products** may **reduce** overall **calorie and carbohydrate intake** as long as there is not a compensatory increase in energy intake from other sources. There is evidence that **low- and no-calorie sweetened beverages** are a **viable alternative to water**. B … (B)"*

American Diabetes Association. Diabetes Care 2023;46(Suppl 1):S68–S96

DIABETES CANADA

*"The evidence from systematic reviews and meta-analyses of randomized controlled trials…have shown a **weight loss benefit** when **non-nutritive sweeteners** are used to **displace excess calories** from **added sugars (especially from SSBs)** in overweight children and adults without diabetes (225), a **benefit**…**similar to** that seen with… **water** (225)."*

Sievenpiper et al. Can J Diabetes. 2018;42 Suppl 1:S64-S79

obesity canada

*"…**low-calorie sweeteners** in **substitution for sugars** or other caloric sweeteners, especially in the form of **sugar-sweetened beverages**, may have **advantages like those of water or other strategies intended to displace excess calories** from added sugars."*

Wharton S, et al. CMAJ. 2020;192:E875-E891

DIABETES UK
KNOW DIABETES. FIGHT DIABETES.

*"The use of **LNCS** for **free sugars** (**especially in sugar-sweetened beverages**) may be a useful, relatively simple strategy to help **reduce calorie intake** and **assist with weight management. Replacing free sugars with LNCS** can be helpful strategy to aid **glucose management**"*

https://www.diabetes.org.uk/professionals/position-statements-reports/food-nutrition-lifestyle/use-of-low-or-no-calorie-sweetners

diabetes australia

*"…**sweeteners** such as Equal, Stevia, Sugarine and Splenda **can be used in place of sugar**, especially if they are replacing large amounts of sugar.*

https://www.diabetesaustralia.com.au/living-with-diabetes/healthy-eating/#sugar

DNSG EASD

*"**Non-nutritive** sweeteners **may be used to replace sugars in beverages and foods. ⊕⊕⊕⊖ Moderate"***

The DNSG-EASD Guideline Development Group. Diabetologia 2023 Jun;66(6):965-98

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What do public health guidelines say about LNCS?

DGAC recommendations on LNCS have changed

USDA

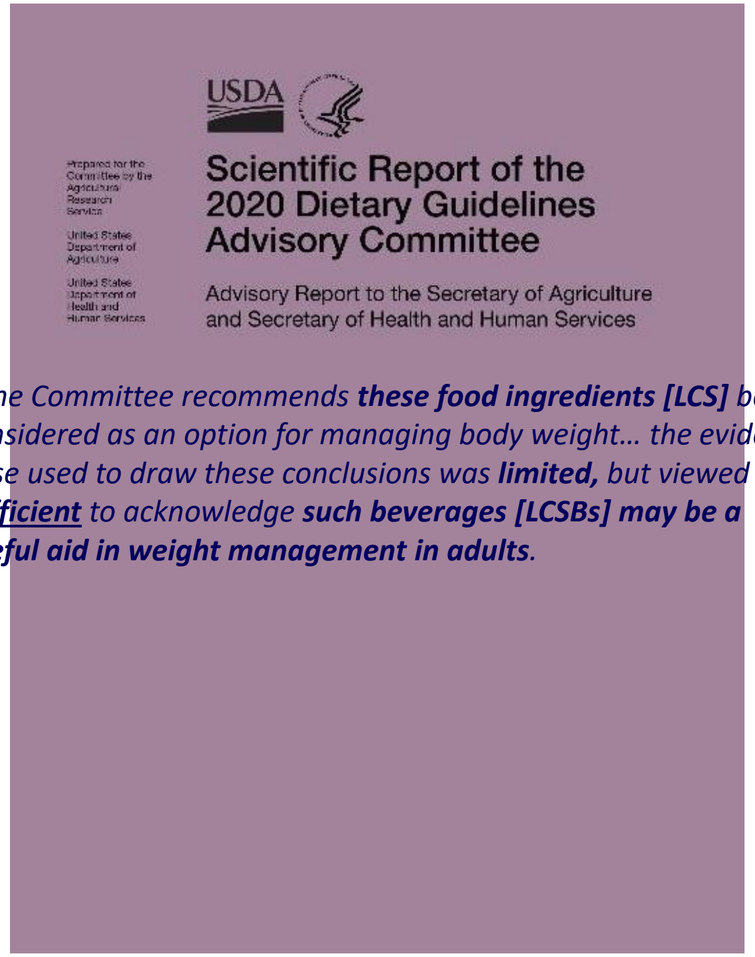
Scientific Report of the
2015 Dietary Guidelines Advisory Committee

Advisory Report to the Secretary of Health and Human Services
and the Secretary of Agriculture

*"… added sugars should be reduced in the diet and **not replaced** with **low-calorie sweeteners**, but rather with **healthy options**, such as **water** in place of **sugar-sweetened beverages**."*

First Print
February 2015

http://www.health.gov/dietaryguidelines/2015-scientific-report/



*…the Committee recommends **these food ingredients [LCS]** be considered as an option for managing body weight… the evidence base used to draw these conclusions was **limited,** but viewed as **sufficient** to acknowledge **such beverages [LCSBs] may be a useful aid in weight management in adults**.*

https://www.dietaryguidelines.gov/sites/default/files/2020-07/ScientificReport_of_the_2020DietaryGuidelinesAdvisoryCommittee_first-print.pdf

WHO guideline: Use of non-sugar sweeteners

WHO recommendation

WHO suggests that non-sugar sweeteners not be used as a means of achieving weight control or reducing the risk of noncommunicable diseases (*conditional recommendation*).

Conditional recommendations are those recommendations for which the **WHO guideline development group is uncertain** that the **desirable consequences** of implementing the recommendation **outweigh the undesirable consequences** or when the anticipated net benefits are small. Policymaking related to conditional recommendations therefore may require substantial debate and involvement of various stakeholders.

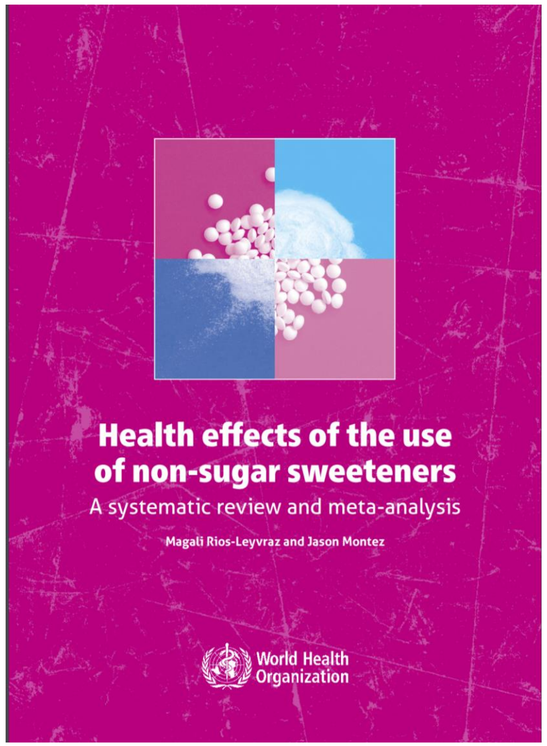
https://www.who.int/publications/i/item/9789240073616

Why the disconnect?

WHO guideline interpretation of evidence from commissioned SRMA of use of non-sugar sweeteners:

Prospective cohorts > RCTs in weighting of evidence

WHO-commissioned SRMA



WHO guideline

Randomized controlled trials

Adiposity
- ↓ Body weight −0.71 kg *(low)*
- ↓ BMI −0.14 kg/m² *(low)*
- Ø Other measures (waist-to-hip ratio, waist circumference, fat/lean mass)

Mostly in NSS→sugars

Type 2 diabetes
- Ø Intermediate markers (glucose, insulin, HOMA-IR, HbA1c)

All-cause mortality
No data

Cardiovascular diseases
- ↑ Total:HDL cholesterol +0.09 (*moderate*)
- Ø Blood pressure, cholesterol (total, LDL, HDL), triglycerides)

Cancer
No data

Total energy intake (kJ/day)
- ↓ Energy intake −569 *(low)*

Mostly in NSS→sugars

Sugars intake (g/day)
- ↓ Sugars intake −38 *(low)*

Pregnancy
No data

Low to moderate certainty

<

Cohort/case–control studies

Adiposity
- ↑ Incident obesity HR 1.76 *(low)*
- ↑ BMI +0.14 kg/m² *(very low)*
- Ø Other measures

Type 2 diabetes
- ↑ Disease (beverage) HR 1.23 *(low)*
- ↑ Disease (tabletop) HR 1.34 *(low)*
- ↑ High fasting glucose HR 1.21 *(low)*
- Ø Other measures

All-cause mortality
- ↑ Mortality HR 1.12 *(very low)*

Cardiovascular diseases
- ↑ CVD mortality HR 1.19 *(low)*
- ↑ CV events HR 1.32 *(low)*
- Ø CHD *(very low)*
- ↑ Stroke HR 1.19 *(low)*
- ↑ Hypertension HR 1.13 *(low)*

Cancer
- Ø Mortality *(very low)*
- Ø Incidence: any type *(very low)*
- ↑ Bladder cancer OR 1.31 *(very low)*

Mostly in saccharin

Total energy intake (kJ/day)
No data

Sugars intake (g/day)
No data

Pregnancy
- ↑ Preterm birth HR 1.25 *(low)*

Very low to low certainty

https://www.who.int/publications/i/item/9789240046429
https://www.who.int/publications/i/item/9789240073616

How does one reconcile the evidence from RCTs and prospective cohorts?

New EASD clinical practice guidelines:

Evidence syntheses commissioned to address the discordance in LNCS research

Diabetologia
https://doi.org/10.1007/s00125-023-05894-8

GUIDELINES

Evidence-based European recommendations for the dietary management of diabetes

The Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD)

Abstract
Diabetes management relies on effective evidence-based advice that informs and empowers individuals to manage their health. Alongside other cornerstones of diabetes management, dietary advice has the potential to improve glycaemic levels, reduce risk of diabetes complications and improve health-related quality of life. We have updated the 2004 recommendations for the nutritional management of diabetes to provide health professionals with evidence-based guidelines to inform discussions with patients on diabetes management, including type 2 diabetes prevention and remission. To provide this update we commissioned new systematic reviews and meta-analyses on key topics, and drew on the broader evidence available. We have strengthened and expanded on the previous recommendations to include advice relating to dietary patterns, environmental sustainability, food processing, patient support and remission of type 2 diabetes. We have used the Grading of Recommendations, Assessment, Development and Evaluations (GRADE) approach to determine the certainty of evidence for each recommendation based on findings from the commissioned and identified systematic reviews. Our findings indicate that a range of foods and dietary patterns are suitable for diabetes management, with key recommendations for people with diabetes being largely similar for those for the general population. Important messages are to consume minimally processed plant foods, such as whole grains, vegetables, whole fruit, legumes, nuts, seeds and non-hydrogenated non-tropical vegetable oils, while minimising the consumption of red and processed meats, sodium, sugar-sweetened beverages and refined grains. The updated recommendations reflect the current evidence base and, if adhered to, will improve patient outcomes.

Keywords Diabetes management · Dietary guidance · Eating advice · Nutrition recommendations · Type 2 diabetes prevention · Type 2 diabetes remission

Abbreviations
DASH Dietary Approaches to Stop Hypertension
DNSG Diabetes and Nutrition Study Group
IGT Impaired glucose tolerance
MUFA Monounsaturated fatty acids
NNS Non-nutritive sweetener

Introduction

Diabetes management, including the prevention and remission of type 2 diabetes, relies on effective evidence-based advice that informs and empowers individuals to manage their health. Well-designed dietary recommendations and nutrition therapy are essential to improve both life expectancy and quality. However, the flood of nutrition information available is of variable quality, creates controversy regarding the best approaches, and is likely to confuse both people with diabetes and health professionals.

These new dietary recommendations for diabetes management have been produced by the Diabetes and Nutrition Study

Members of the Guideline Development Group for the Diabetes and Nutrition Study Group (DNSG) of the EASD are listed in alphabetical order in the Appendix. Those individuals are the authors of this article.

These guidelines were reviewed for EASD by its Committee on Clinical Affairs.

The Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD)
For Guideline queries contact andrew.reynolds@otago.ac.nz

c/o Andrew Reynolds, Department of Medicine, University of Otago, Dunedin, New Zealand

Published online: 17 April 2023

Evidence-based European recommendations for the dietary management of diabetes: An EASD Clinical Practice Guideline

1. Prevention of type 2 diabetes
2. Energy-balance and weight management in diabetes
3. Carbohydrate intakes in diabetes management
4. Dietary fat intakes in diabetes management
5. Protein intakes in diabetes management
6. Food-based approaches in diabetes management
7. Traditional dietary patterns and therapeutic diets in diabetes management
8. Environmental sustainability and diabetes management
9. Food processing and diabetes management
10. Patient support and diabetes management

The DNSG-EASD Guideline Development Group. Diabetologia 2023 Jun;66(6):965-98

DNSG

EASD

European Association for the Study of Diabetes

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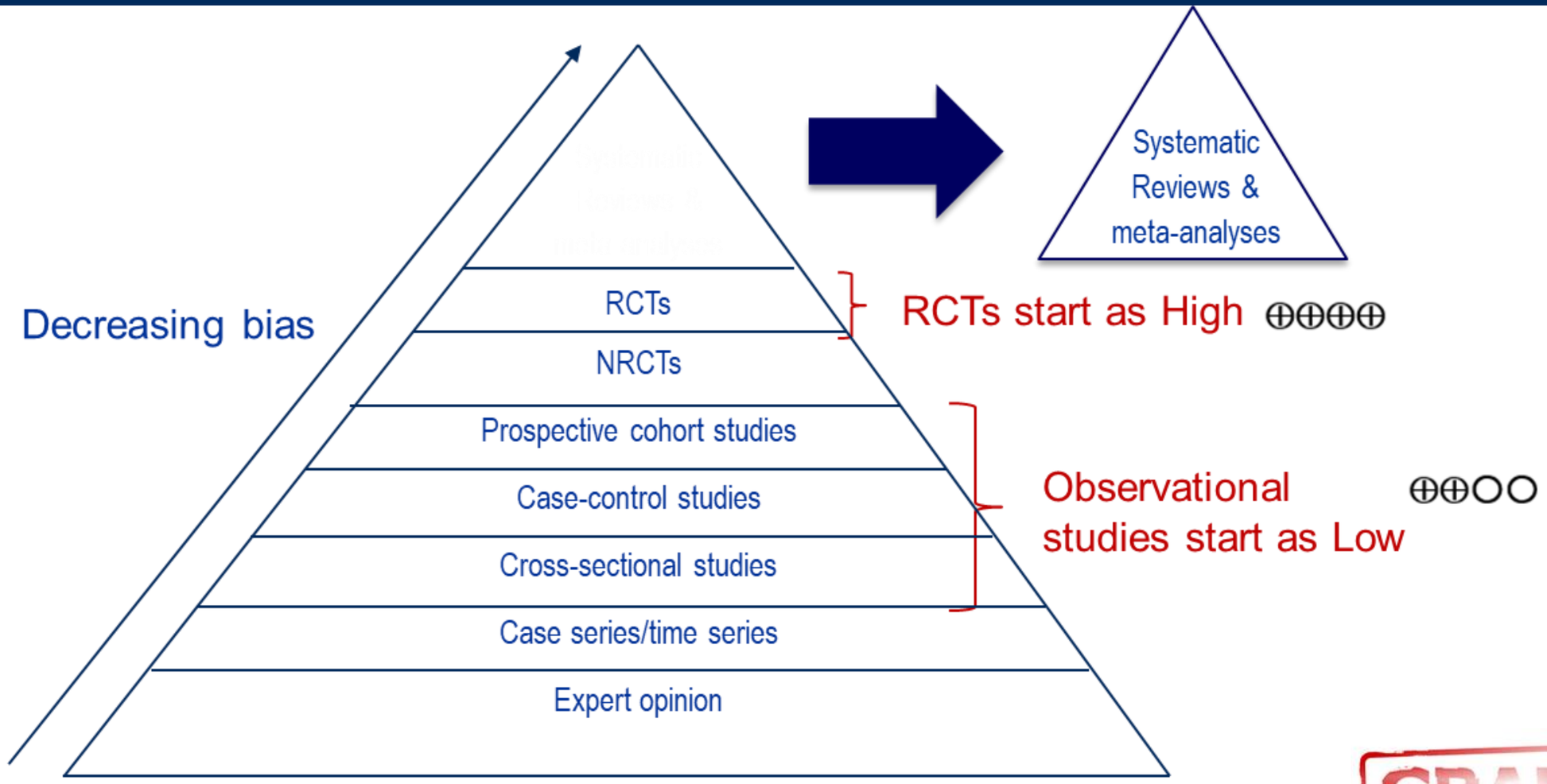
Hierarchy of evidence



GRADE

http://www.sign.ac.uk/guidelines/fulltext/50/annexb.html
http://www.cnpp.usda.gov/Publications/NutritionInsights/Insight38.pdf
http://www.nice.org.uk/niceMedia/pdf/GDM_Chapter7_0305.pdf

Letters, editorials, commentaries, expert consensus statements calling for better research design to address the nature of the comparator and reverse causality in LNCS research and evidence syntheses

Sievenpiper JL et al. CMAJ. 2017;189:E1424-E1425
Malik VS. BMJ. 2019 Jan 3;364:k5005
Mela DJ et al. Adv Nutr. 2020 Jan 10. pii

Khan TA, Malik VS, Sievenpiper JL. Stroke. 2019;50:e167-e168
Ashwell M, et al. Nutr Res Rev. 2020;33:1-10

Series of DNSG-commissioned systematic reviews and meta-analyses of RCTs and cohort studies of LNCS

Nema McGlynn, MSc, RD

**Jennifer Lee,
MPH, RD, PhD(candidate)**

**Tauseef Khan, MBBS,
PhD**

**ClinicalTrials.gov identifiers:
NCT02879500, NCT04245826**

Diabetes and **N**utrition **S**tudy **G**roup

CIHR IRSC
Canadian Institutes of Health Research
Instituts de recherche en santé du Canada

UNIVERSITY OF TORONTO

EASD
European Association for the Study of Diabetes

St. Michael's
Inspired Care.
Inspiring Science.

What is the evidence from randomized trials (RCTs)?

Approach: Network meta-analysis (NMA)

3 prespecified comparisons of clinical/public health importance

Nema McGlynn

Tauseef Khan

JAMA Network Open

Original Investigation | Nutrition, Obesity, and Exercise

Association of Low- and No-Calorie Sweetened Beverages as a Replacement for Sugar-Sweetened Beverages With Body Weight and Cardiometabolic Risk
A Systematic Review and Meta-analysis

Néma D. McGlynn, MSc, RD; Tauseef Ahmad Khan, MBBS, PhD; Lily Wang, BSc; Roselyn Zhang, MSc; Laura Chiavaroli, PhD; Fei Au-Yeung, MSc; Jennifer J. Lee, MPH, RD; Jarvis C. Noronha, MSc; Elena M. Comelli, PhD; Sonia Blanco Mejia, MSc, MD; Amna Ahmed, BSc; Vasanti S. Malik, PhD; James O. Hill, PhD; Lawrence A. Leiter, MD; Arnav Agarwal, MD; Per B. Jeppesen, PhD; Dario Rahelić, MD, PhD; Hana Kahleová, MD, PhD; Jordi Salas-Salvadó, MD, PhD; Cyril W. C. Kendall, PhD; John L. Sievenpiper, MD, PhD

Abstract

IMPORTANCE There are concerns that low- and no-calorie sweetened beverages (LNCSBs) do not have established benefits, with major dietary guidelines recommending the use of water and not LNCSBs to replace sugar-sweetened beverages (SSBs). Whether LNCSB as a substitute can yield similar improvements in cardiometabolic risk factors vs water in their intended substitution for SSBs is unclear.

OBJECTIVE To assess the association of LNCSBs (using 3 prespecified substitutions of LNCSBs for SSBs, water for SSBs, and LNCSBs for water) with body weight and cardiometabolic risk factors in adults with and without diabetes.

DATA SOURCES Medline, Embase, and the Cochrane Central Register of Controlled Trials were searched from inception through December 26, 2021.

STUDY SELECTION Randomized clinical trials (RCTs) with at least 2 weeks of interventions comparing LNCSBs, SSBs, and/or water were included.

DATA EXTRACTION AND SYNTHESIS Data were extracted and risk of bias was assessed by 2 independent reviewers. A network meta-analysis was performed with data expressed as mean difference (MD) or standardized mean difference (SMD) with 95% CIs. The GRADE (Grading of Recommendations Assessment, Development and Evaluation) system was used to assess the certainty of the evidence.

MAIN OUTCOMES AND MEASURES The primary outcome was body weight. Secondary outcomes were other measures of adiposity, glycemic control, blood lipids, blood pressure, measures of nonalcoholic fatty liver disease, and uric acid.

RESULTS A total of 17 RCTs with 24 trial comparisons were included, involving 1733 adults (mean [SD] age, 33.1 [6.6] years; 1341 women [77.4%]) with overweight or obesity who were at risk for or had diabetes. Overall, LNCSBs were a substitute for SSBs in 12 RCTs (n = 601 participants), water was a substitute for SSBs in 3 RCTs (n = 429), and LNCSBs were a substitute for water in 9 RCTs (n = 974). Substitution of LNCSBs for SSBs was associated with reduced body weight (MD, −1.06 kg; 95% CI, −1.71 to −0.41 kg), body mass index (MD, −0.32; 95% CI, −0.58 to −0.07), percentage of body fat (MD, −0.60%; 95% CI, −1.03% to −0.18%), and intrahepatocellular lipid (SMD, −0.42; 95% CI, −0.70 to −0.14). Substituting water for SSBs was not associated with any outcome. There was also no association found between substituting LNCSBs for water with any outcome except glycated

(continued)

Key Points

Question Are low- and no-calorie sweetened beverages (LNCSBs) as the intended substitute for sugar-sweetened beverages (SSBs) associated with improved body weight and cardiometabolic risk factors similar to water replacement?

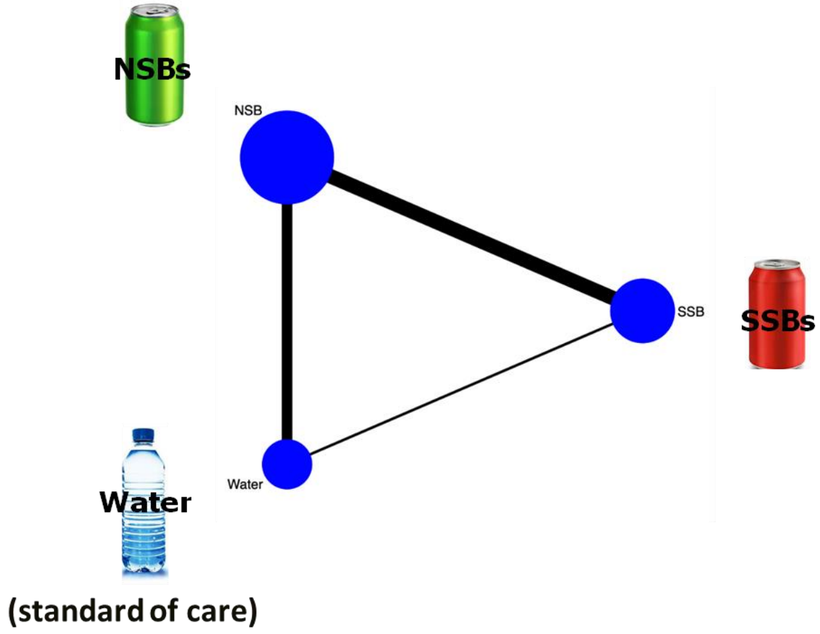
Findings In this systematic review and meta-analysis of 17 randomized clinical trials, LNCSBs as a substitute for SSBs were associated with reduced body weight, body mass index, percentage of body fat, and intrahepatocellular lipid, providing benefits that were similar to those of water, the standard-of-care substitution.

Meaning The findings of this study suggest that over the moderate term, LNCSBs are a viable alternative to water as a replacement strategy in adults with overweight or obesity who are at risk for or have diabetes.

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

1. **LNCBs for SSBs** (**“intended substitution”** with energy displacement)
2. **Water for SSBs** (**“standard of care substitution”** with energy displacement)
3. **LNCBs for water** (**“reference substitution”** with**out** energy displacement)



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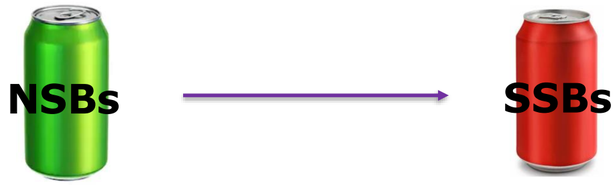
Intended substitution

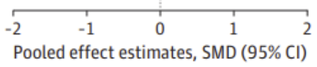
LNCSBs for SSBs (“intended substitution”)



LNCSBs for SSBs ("Intended substitution"):
Network meta-analysis 17 RCTs, N=1,733, FU=3-52 wk

Figure 2. Substitution of Low- and No-Calorie Sweetened Beverages (LNCSBs) for Sugar-Sweetened Beverages (SSBs)

Outcome	No. of trial comparisons: Direct estimate	No. of trial comparisons: Network estimate	Total No. of participants: Direct estimate	Total No. of participants: Network estimate	MD (95% CI)	Pooled effect estimates, SMD (95% CI)	GRADE certainty of the evidence
Adiposity							
Body weight, kg	12	24	467	1444	-1.06 (-1.71 to -0.41)	-0.65 (-1.05 to -0.25)	◆◆◆◇ Moderate
BMI	9	14	437	836	-0.32 (-0.58 to -0.07)	-0.67 (-1.19 to -0.14)	◆◆◇◇ Low
Body fat, %	7	14	210	559	-0.60 (-1.03 to -0.18)	-0.74 (-1.27 to -0.22)	◆◆◆◇ Moderate
WC, cm	0	6	0	868	-0.52 (-4.98 to 3.94)	-0.09 (-0.89 to 0.71)	◆◆◇◇ Low
Glycemia							
HbA_{1c}, %	4	9	154	630	0.12 (-0.08 to 0.32)	0.39 (-0.26 to 1.05)	◆◆◆◇ Moderate
FPG, mmol/L	7	19	210	1183	-0.06 (-0.16 to 0.03)	-0.32 (-0.77 to 0.13)	◆◆◆◇ Moderate
2HPP, mmol/L	4	9	154	440	0.29 (-0.45 to 1.03)	0.26 (-0.40 to 0.91)	◆◆◆◇ Moderate
FPI, pmol/L	7	16	210	512	-9.79 (-29.99 to 10.40)	-0.24 (-0.73 to 0.25)	◆◆◇◇ Low
HOMA-IR	2	7	56	265	-0.10 (-0.71 to 0.51)	-0.12 (-0.86 to 0.62)	◆◆◆◇ Moderate
Lipids, mmol/L							
LDL-C	6	16	183	894	-0.01 (-0.15 to 0.12)	-0.08 (-0.57 to 0.41)	◆◆◆◇ Moderate
Non-HDL-C	6	14	210	923	-0.08 (-0.25 to 0.09)	-0.25 (-0.77 to 0.28)	◆◆◆◇ Moderate
Triglycerides	7	17	210	923	-0.13 (-0.29 to 0.03)	-0.40 (-0.87 to 0.08)	◆◆◆◇ Moderate
HDL-C[a]	7	17	210	923	-0.05 (-0.10 to 0.01)	-0.41 (-0.88 to 0.07)	◆◆◆◇ Moderate
Total cholesterol	6	14	210	923	-0.10 (-0.35 to 0.15)	-0.21 (-0.73 to 0.31)	◆◆◇◇ Low
Blood pressure, mm Hg							
Systolic BP	3	10	56	706	-2.44 (-5.20 to 0.33)	-0.55 (-1.17 to 0.07)	◆◆◆◇ Moderate
Diastolic BP	3	9	56	483	-1.84 (-4.07 to 0.39)	-0.54 (-0.19 to 0.12)	◆◆◇◇ Low
Liver							
IHCL, SMD	2	4	49	62	-0.42 (-0.70 to -0.14)	-0.42 (-0.70 to -0.14)	◆◆◆◇ Moderate
ALT, U/L	2	6	27	143	-6.67 (-16.20 to 2.86)	-0.56 (-1.36 to 0.24)	◆◆◇◇ Low
AST, U/L	1	3	27	120	-1.50 (-7.87 to 4.87)	-0.27 (-1.40 to 0.87)	◆◆◇◇ Low
Uric acid							
Uric acid, mmol/L	3	7	49	62	-0.02 (-0.05 to 0.02)	-1.16 (-1.06 to 0.42)	◆◆◆◇ Moderate



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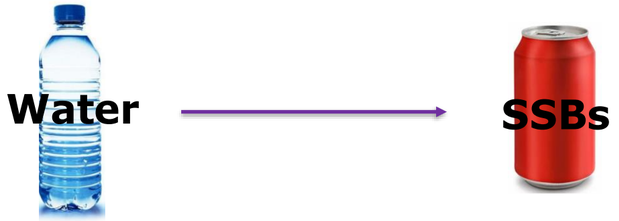
Standard of care substitution

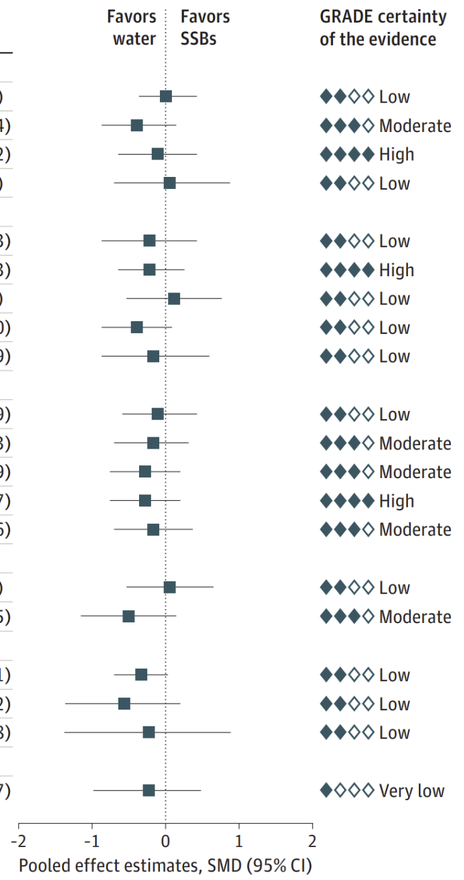
Water for SSBs (“standard of care substitution”)



Water for SSBs ("standard of care substitution"):
Network meta-analysis 17 RCTs, N=1,733, FU=3-52 wk

Figure 3. Substitution of Water for Sugar-Sweetened Beverages (SSBs)

Outcome	No. of trial comparisons: Direct estimate	No. of trial comparisons: Network estimate	Total No. of participants: Direct estimate	Total No. of participants: Network estimate	MD (95% CI)	Pooled effect estimates, SMD (95% CI)	GRADE certainty of the evidence
Adiposity							
Body weight, kg	3	24	270	1444	0.01 (-0.95 to 0.98)	0.00 (-0.40 to 0.41)	◆◆◇◇ Low
BMI	2	14	270	836	-0.35 (-0.83 to 0.13)	-0.38 (-0.90 to 0.14)	◆◆◆◇ Moderate
Body fat, %	3	14	270	559	-0.27 (-1.55 to 1.02)	-0.11 (-0.63 to 0.42)	◆◆◆◆ High
WC, cm	1	6	240	868	0.30 (-3.68 to 4.28)	0.06 (-0.74 to 0.86)	◆◆◇◇ Low
Glycemia							
HbA_{1c}, %	1	9	240	630	-0.09 (-0.33 to 0.16)	-0.24 (-0.88 to 0.43)	◆◆◇◇ Low
FPG, mmol/L	3	19	270	1183	-0.05 (-0.14 to 0.05)	-0.22 (-0.67 to 0.23)	◆◆◆◆ High
2HPP, mmol/L	0	9	0	440	0.10 (-0.67 to 0.87)	0.08 (-0.57 to 0.74)	◆◆◇◇ Low
FPI, pmol/L	2	16	30	512	-17.40 (-39.50 to 4.70)	-0.39 -(0.88 to 0.10)	◆◆◇◇ Low
HOMA-IR	1	7	30	265	-0.14 (-0.81 to 0.53)	-0.15 (-0.90 to 0.59)	◆◆◇◇ Low
Lipids, mmol/L							
LDL-C	3	16	270	894	-0.01 (-0.14 to 0.12)	-0.10 (-0.59 to 0.39)	◆◆◇◇ Low
Non-HDL-C	2	14	270	923	-0.06 (-0.22 to 0.10)	-0.20 (-0.72 to 0.33)	◆◆◆◇ Moderate
Triglycerides	3	17	270	923	-0.09 (-0.25 to 0.06)	-0.29 (-0.76 to 0.19)	◆◆◆◇ Moderate
HDL-C[a]	3	17	270	923	-0.04 (-0.09 to 0.02)	-0.31 (-0.78 to 0.17)	◆◆◆◆ High
Total cholesterol	2	14	270	923	-0.08 (-0.35 to 0.18)	-0.16 (-0.69 to 0.36)	◆◆◆◇ Moderate
Blood pressure, mm Hg							
Systolic BP	3	10	270	706	0.19 (-2.34 to 2.72)	0.05 (-0.57 to 0.67)	◆◆◇◇ Low
Diastolic BP	3	9	270	483	-1.58 (-3.61 to 0.46)	-0.51 (-1.16 to 0.15)	◆◆◆◇ Moderate
Liver							
IHCL, SMD	1	4	23	62	-0.36 (-0.74 to 0.01)	-0.36 (-0.74 to 0.01)	◆◆◇◇ Low
ALT, U/L	1	6	23	143	-7.18 (-17.01 to 2.64)	-0.58 (-1.39 to 0.22)	◆◆◇◇ Low
AST, U/L	0	3	0	120	-1.70 (-9.35 to 5.95)	-0.25 (-1.38 to 0.88)	◆◆◇◇ Low
Uric acid							
Uric acid, mmol/L	2	7	23	62	-0.01 (-0.05 to 0.03)	-0.26 (-1.01 to 0.47)	◆◇◇◇ Very low



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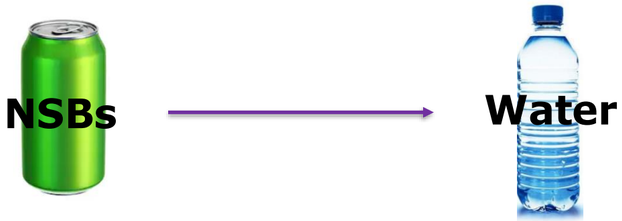
Reference substitution

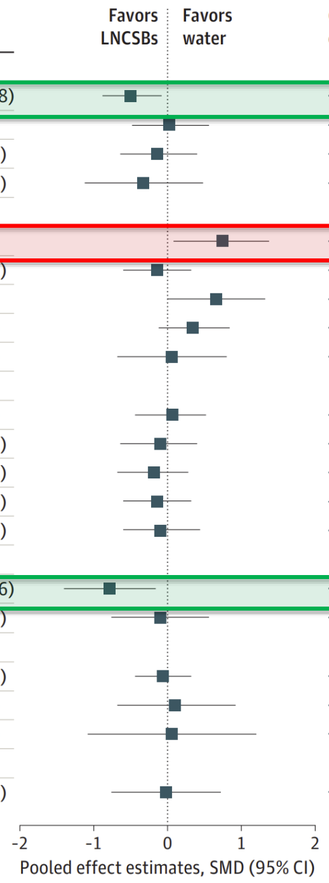
LNCSBs for Water ("reference substitution")



LNCSBs for water ("reference substitution"):
Network meta-analysis 17 RCTs, N=1,733, FU=3-52 wk

Figure 4. Substitution of Low- and No-Calorie Sweetened Beverages (LNCSBs) for Water

Outcome	No. of trial comparisons: Direct estimate	No. of trial comparisons: Network estimate	Total No. of participants: Direct estimate	Total No. of participants: Network estimate	MD (95% CI)	Pooled effect estimates, SMD (95% CI)	GRADE certainty of the evidence
Adiposity							
Body weight, kg	9	24	752	1444	-1.07 (-1.95 to -0.19)	-0.48 (-0.88 to -0.08)	◆◆◇◇ Low
BMI	3	14	174	836	0.02 (-0.46 to 0.51)	0.03 (-0.50 to 0.55)	◆◆◇◇ Low
Body fat, %	4	14	124	559	-0.34 (-1.67 to 1.00)	-0.13 (-0.66 to 0.39)	◆◆◆◇ Moderate
WC, cm	5	6	628	868	-0.82 (-2.83 to 1.19)	-0.33 (-1.13 to 0.47)	◆◆◇◇ Low
Glycemia							
HbA_{1c}, %	4	9	236	630	0.21 (0.02 to 0.40)	0.72 (0.07 to 1.38)	◆◆◇◇ Low
FPG, mmol/L	9	19	748	1183	-0.02 (-0.08 to 0.04)	-0.14 (-0.59 to 0.31)	◆◆◆◆ High
2HPP, mmol/L	5	9	286	440	0.19 (0.00 to 0.39)	0.64 (0.00 to 1.31)	◆◆◆◇ Moderate
FPI, pmol/L	7	16	317	512	7.60 (-2.95 to 18.15)	0.35 (-0.14 to 0.84)	◆◆◇◇ Low
HOMA-IR	4	7	224	265	0.03 (-0.34 to 0.40)	0.07 (-0.67 to 0.81)	◆◆◇◇ Low
Lipids, mmol/L							
LDL-C	7	16	486	894	0.00 (-0.09 to 0.08)	0.04 (-0.45 to 0.53)	◆◆◆◆ High
Non-HDL-C	6	14	488	923	-0.02 (-0.14 to 0.09)	-0.09 (-0.64 to 0.41)	◆◆◆◇ Moderate
Triglycerides	7	17	488	923	-0.04 (-0.13 to 0.06)	-0.19 (-0.66 to 0.29)	◆◇◇◇ Very low
HDL-C[a]	7	17	488	923	-0.01 (-0.05 to 0.03)	-0.14 (-0.62 to 0.33)	◆◆◇◇ Low
Total cholesterol	6	14	488	923	-0.02 (-0.14 to 0.10)	-0.09 (-0.61 to 0.44)	◆◆◇◇ Low
Blood pressure, mm Hg							
Systolic BP	4	10	425	706	-2.63 (-4.71 to -0.55)	-0.78 (-1.40 to -0.16)	◆◆◇◇ Low
Diastolic BP	3	9	202	483	-0.26 (-2.12 to 1.60)	-0.09 (-0.75 to 0.56)	◆◆◇◇ Low
Liver							
IHCL, SMD	1	4	25	62	-0.06 (-0.42 to 0.31)	-0.06 (-0.42 to 0.31)	◆◆◇◇ Low
ALT, U/L	3	6	93	143	0.51 (-2.92 to 3.95)	0.12 (-0.68 to 0.92)	◆◆◇◇ Low
AST, U/L	2	3	93	120	0.20 (-4.04 to 4.44)	0.05 (-1.08 to 1.18)	◆◆◇◇ Low
Uric acid							
Uric acid, mmol/L	2	7	25	62	0.00 (-0.04 to 0.04)	-0.02 (-0.76 to 0.72)	◆◇◇◇ Very low



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What is the evidence from Prospective cohort studies?

Approach: Substitution and change analyses

3 prespecified comparisons of clinical/public health importance

Change in intake (increase in 1 serving [330mL] per day)

Jennifer Lee

Tauseef Khan

Diabetes Care Volume 45, August 2022 1917

Relation of Change or Substitution of Low- and No-Calorie Sweetened Beverages With Cardiometabolic Outcomes: A Systematic Review and Meta-analysis of Prospective Cohort Studies

Diabetes Care 2022;45:1917–1930 | https://doi.org/10.2337/dc21-2130

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BACKGROUND

Adverse associations of low- and no-calorie sweetened beverages (LNCSB) with cardiometabolic outcomes in observational studies may be explained by reverse causality and residual confounding.

PURPOSE

To address these limitations we used change analyses of repeated measures of intake and substitution analyses to synthesize the association of LNCSB with cardiometabolic outcomes.

DATA SOURCES

MEDLINE, Embase, and the Cochrane Library were searched up to 10 June 2021 for prospective cohort studies with ≥1 year of follow-up duration in adults.

STUDY SELECTION

Outcomes included changes in clinical measures of adiposity, risk of overweight/obesity, metabolic syndrome, type 2 diabetes (T2D), cardiovascular disease, and total mortality.

DATA EXTRACTION

Two independent reviewers extracted data, assessed study quality, and assessed certainty of evidence using GRADE. Data were pooled with a random-effects model and expressed as mean difference (MD) or risk ratio (RR) and 95% CI.

DATA SYNTHESIS

A total of 14 cohorts (416,830 participants) met the eligibility criteria. Increase in LNCSB intake was associated with lower weight (5 cohorts, 130,020 participants; MD −0.008 kg/year [95% CI −0.014, −0.002]). Substitution of LNCSB for sugar-sweetened beverages (SSB) was associated with lower weight (three cohorts, 165,579 participants; MD, −0.12 [−0.14, −0.10,] kg/y) and lower incidence of obesity (OB) (one cohort, 15,765 participants; RR 0.88 [95% CI 0.88, 0.89]), coronary heart disease (six cohorts, 233,676 participants; 0.89 [0.81, 0.98]), cardiovascular disease mortality (one cohort, 118,363 participants; 0.95 [0.90, 0.99]), and

META-ANALYSIS

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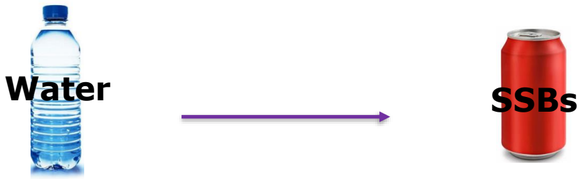
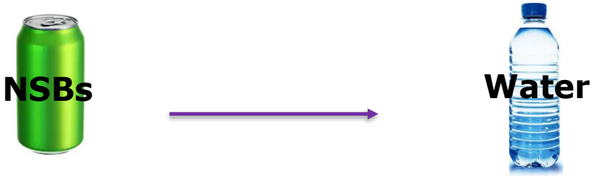
Substitution analyses

LNCSBs for SSBs ("intended substitution")

Water for SSBs ("standard of care substitution")



LNCSBs for Water ("reference substitution")



Change analyses

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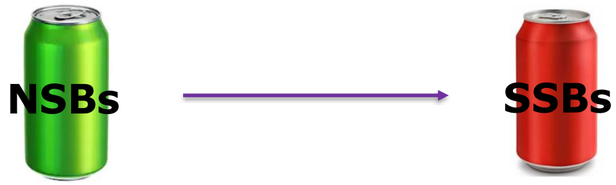
Intended substitution

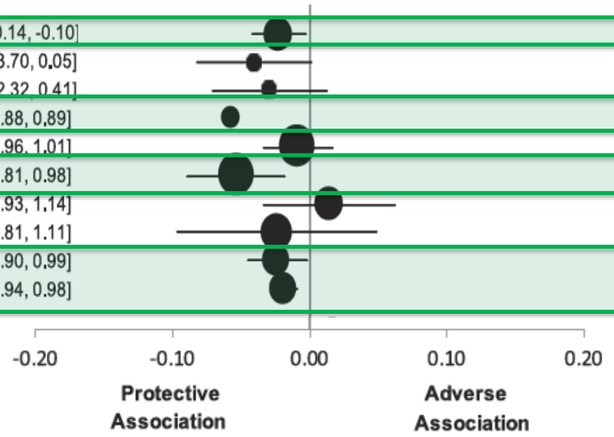
LNCSBs for SSBs (“intended substitution”)



LNCSBs for SSBs ("Intended substitution"):

SRMA of 14 unique prospective cohorts; n=416,830; FU=17.5y

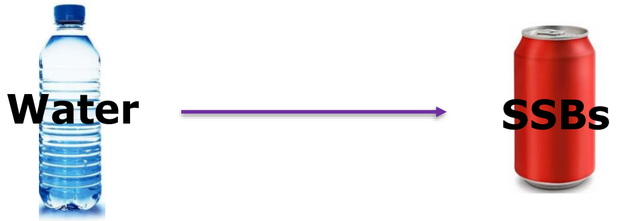
Outcome	Cohort Comparisons	N	Estimate	Pooled Estimate [95% CI]	Heterogeneity I^2	Heterogeneity P_Q	GRADE: Downgrade: Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication bias	Upgrade: Dose response	Large effect size	Attenuation	Certainty of Evidence
Substituted Beverages: LNCSBs for SSBs ('Intended Substitution')															
Body Weight (kg/y)	3	165,579	MD	-0.12 [-0.14, -0.10]	46%	0.16	□	□	□	□	□	■	□	□	⊕⊕⊕◯ Moderate
Waist Circumference* (cm/y)	1	173	MD	-1.83 [-3.70, 0.05]	N/A	N/A	□	□	■	■	□	□	□	□	⊕◯◯◯ Very Low
% Body Fat* (%/y)	1	173	MD	-0.96 [-2.32, 0.41]	N/A	N/A	□	□	■	■	□	□	□	□	⊕◯◯◯ Very Low
OB Incidence	1	15,765	RR	0.88 [0.88, 0.89]	N/A	N/A	□	□	■	□	□	■	□	□	⊕⊕◯◯ Low
T2D Incidence	5	281,855	RR	0.99 [0.96, 1.01]	69%	0.01	□	■	□	□	□	□	□	□	⊕◯◯◯ Very Low
CHD Incidence	6	233,676	RR	0.89 [0.81, 0.98]	28%	0.22	□	□	□	■	□	■	□	□	⊕⊕◯◯ Low
Stroke Incidence	1	127,456	RR	1.03 [0.93, 1.14]	N/A	N/A	□	□	■	■	□	□	□	□	⊕◯◯◯ Very Low
CHD Mortality	5	220,805	RR	0.95 [0.81, 1.11]	36%	0.18	□	□	□	■	□	□	□	□	⊕◯◯◯ Very Low
CVD Mortality	1	118,363	RR	0.95 [0.90, 0.99]	N/A	N/A	□	□	■	■	□	■	□	□	⊕⊕◯◯ Low
Total Mortality	1	118,363	RR	0.96 [0.94, 0.98]	N/A	N/A	□	□	■	■	□	■	□	□	⊕⊕◯◯ Low



Lee et al. Diabetes Care, Diabetes Care. 2022;45:1917-1930

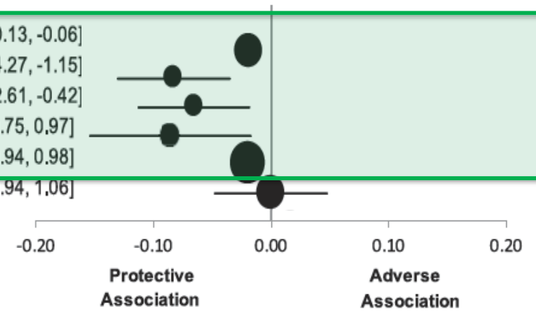
Standard of care substitution

Water for SSBs (“standard of care substitution”)



Water for SSBs (“standard of care substitution”):
SRMA of 14 unique prospective cohorts; n=416,830; FU=17.5y

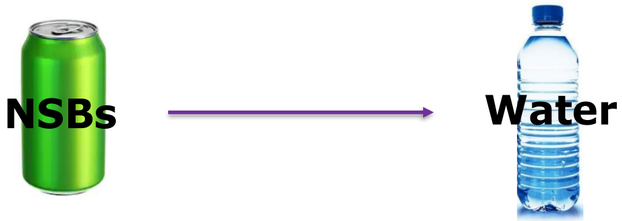
Outcome	Cohort Comparisons	N	Estimate	Pooled Estimate [95% CI]	Heterogeneity I^2	Heterogeneity P_Q	GRADE: Downgrade: Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication bias	Upgrade: Dose response	Large effect size	Attenuation	Certainty of Evidence
Substituted Beverages: Water for SSBs ('Standard of Care Substitution')															
Body Weight (kg/y)	3	165,579	MD	-0.10 [-0.13, -0.06]	82%	<0.01	□	■	□	□	□	■	□	□	⊕⊕◯◯ Low
Waist Circumference* (cm/y)	1	173	MD	-2.71 [-4.27, -1.15]	N/A	N/A	□	□	■	■	□	■	□	□	⊕⊕◯◯ Low
% Body Fat* (%/y)	1	173	MD	-1.51 [-2.61, -0.42]	N/A	N/A	□	□	■	■	□	■	□	□	⊕⊕◯◯ Low
OB Incidence	1	15,765	RR	0.85 [0.75, 0.97]	N/A	N/A	□	□	■	■	□	■	□	□	⊕⊕◯◯ Low
T2D Incidence	3	281,855	RR	0.96 [0.94, 0.98]	79%	<0.01	□	□	□	■	□	■	□	□	⊕⊕◯◯ Low
Stroke Incidence	1	127,456	RR	1.00 [0.94, 1.06]	N/A	N/A	□	□	■	■	□	□	□	□	⊕◯◯◯ Very Low



Lee et al. Diabetes Care, Diabetes Care. 2022;45:1917-1930

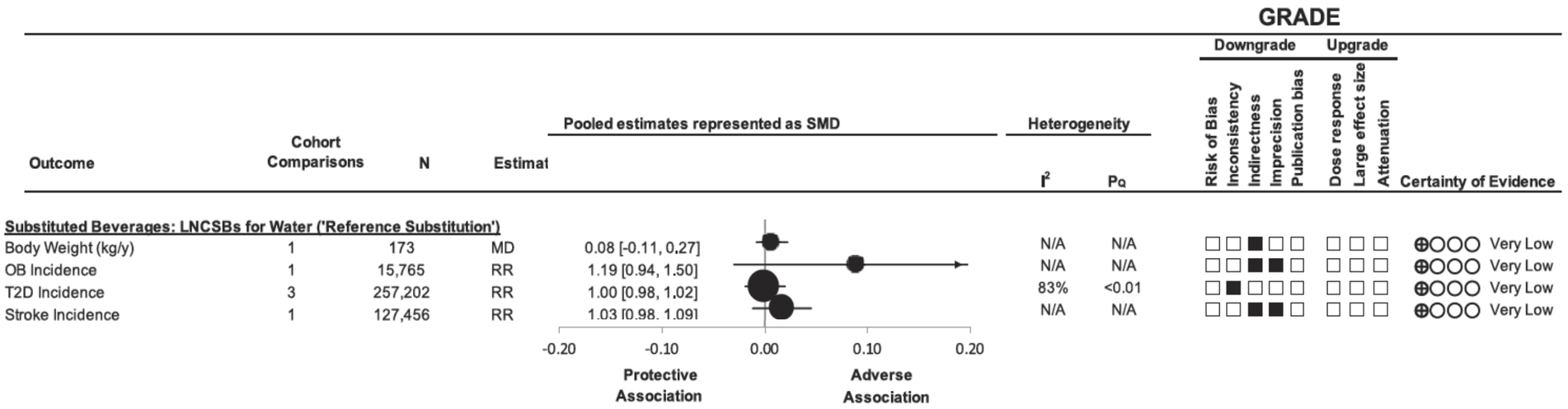
Reference substitution

LNCSBs for Water ("reference substitution")



Relation of substitution of LNCSBs for water (“reference substitution”) with cardiometabolic outcomes

SRMA of 14 unique prospective cohorts; n=416,830; FU=17.5y

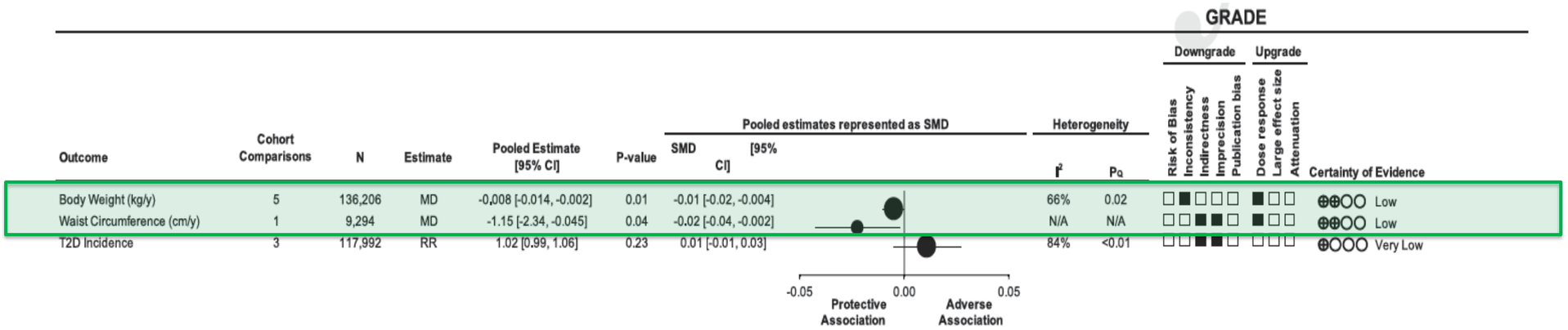


Lee et al. Diabetes Care, Diabetes Care. 2022;45:1917-1930

Change analyses

Relation of change in intake (per 330 mL serving per day) of LNCSBs with cardiometabolic outcomes

SRMA of 14 unique prospective cohorts; n=416,830; FU=17.5y



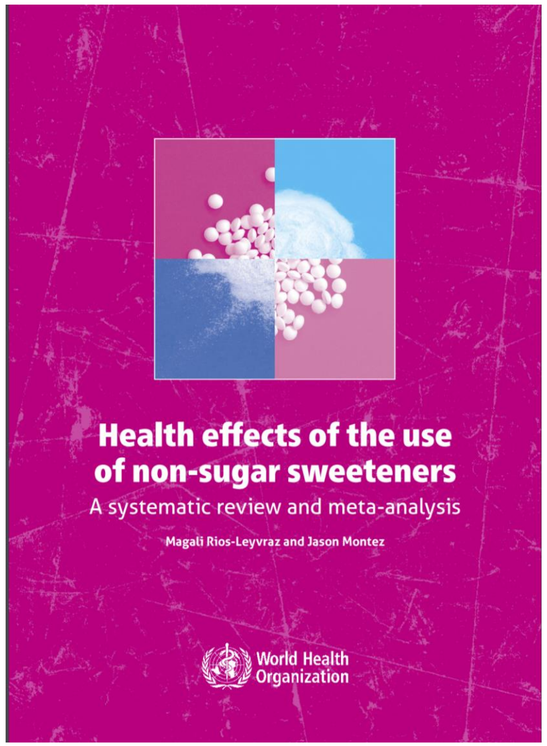
Lee et al. Diabetes Care, Diabetes Care. 2022;45:1917-1930

How do these estimates compare with prevalent exposure assessments?

WHO guideline interpretation of evidence from commissioned SRMA of use of non-sugar sweeteners:

Prospective cohorts > RCTs in weighting of evidence

WHO-commissioned SRMA



WHO guideline

Randomized controlled trials

Adiposity
- ↓ Body weight −0.71 kg *(low)*
- ↓ BMI −0.14 kg/m² *(low)*
- Ø Other measures (waist-to-hip ratio, waist circumference, fat/lean mass)

Mostly in NSS→sugars

Type 2 diabetes
- Ø Intermediate markers (glucose, insulin, HOMA-IR, HbA1c)

All-cause mortality
No data

Cardiovascular diseases
- ↑ Total:HDL cholesterol +0.09 (*moderate*)
- Ø Blood pressure, cholesterol (total, LDL, HDL), triglycerides)

Cancer
No data

Total energy intake (kJ/day)
- ↓ Energy intake −569 *(low)*

Mostly in NSS→sugars

Sugars intake (g/day)
- ↓ Sugars intake −38 *(low)*

Pregnancy
No data

Low to moderate certainty

<

Cohort/case–control studies

Adiposity
- ↑ Incident obesity HR 1.76 *(low)*
- ↑ BMI +0.14 kg/m² *(very low)*
- Ø Other measures

Type 2 diabetes
- ↑ Disease (beverage) HR 1.23 *(low)*
- ↑ Disease (tabletop) HR 1.34 *(low)*
- ↑ High fasting glucose HR 1.21 *(low)*
- Ø Other measures

All-cause mortality
- ↑ Mortality HR 1.12 *(very low)*

Cardiovascular diseases
- ↑ CVD mortality HR 1.19 *(low)*
- ↑ CV events HR 1.32 *(low)*
- Ø CHD *(very low)*
- ↑ Stroke HR 1.19 *(low)*
- ↑ Hypertension HR 1.13 *(low)*

Cancer
- Ø Mortality *(very low)*
- Ø Incidence: any type *(very low)*
- ↑ Bladder cancer OR 1.31 *(very low)*

Mostly in saccharin

Total energy intake (kJ/day)
No data

Sugars intake (g/day)
No data

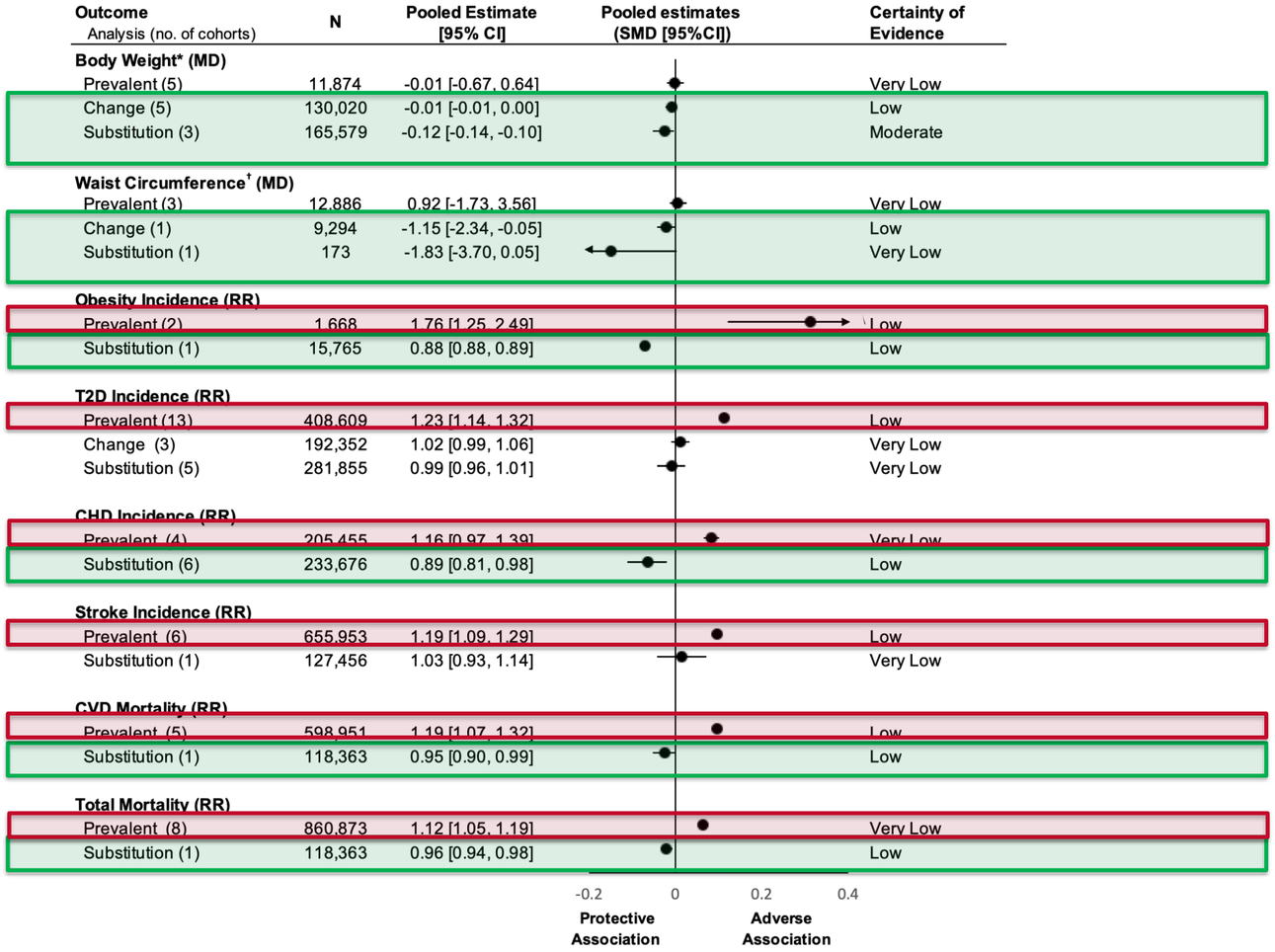
Pregnancy
- ↑ Preterm birth HR 1.25 *(low)*

Very low to low certainty

https://www.who.int/publications/i/item/9789240046429
https://www.who.int/publications/i/item/9789240073616

Comparison of prevalent, change, and substitution analyses

Prevalent exposure assessments from commissioned WHO-commissioned SRMA versus change (increase in 1 serving [330mL] per day) and substitution ("intended substitution" of LNCBs for SSBs) exposure assessments from DNSG-commissioned SMRA



- Prevalent — WHO Rios-Leyvraz M, Montez J. World Health Organization. 2022
- Change — Lee et al. Diabetes Care, Diabetes Care. 2022
- Substitution — Lee et al. Diabetes Care, Diabetes Care. 2022

Khan et al. EJCN, in press

Is there a WHO precedent for prioritizing the evidence from substitution analyses?

Lack of robust association of saturated fat and NCDs led to need for substitution analyses in updated WHO-commissioned SRMA

SRMA of 17 prospective cohort studies, N=339,090

Original 2015 WHO-commissioned SRMA

RESEARCH

OPEN ACCESS

Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies

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Accepted: 15 July 2015

ABSTRACT

OBJECTIVE
To systematically review associations between intake of saturated fat and trans unsaturated fat and all cause mortality, cardiovascular disease (CVD) and associated mortality, coronary heart disease (CHD) and associated mortality, ischemic stroke, and type 2 diabetes.

DESIGN
Systematic review and meta-analysis.

DATA SOURCES
Medline, Embase, Cochrane Central Registry of Controlled Trials, Evidence-Based Medicine Reviews, and CINAHL from inception to 1 May 2015, supplemented by bibliographies of retrieved articles and previous reviews.

ELIGIBILITY CRITERIA FOR SELECTING STUDIES
Observational studies reporting associations of saturated fat and/or trans unsaturated fat (total, industrially manufactured, or from ruminant animals) with all cause mortality, CHD/CVD mortality, total CHD, ischemic stroke, or type 2 diabetes.

DATA EXTRACTION AND SYNTHESIS
Two reviewers independently extracted data and assessed study risks of bias. Multivariable relative risks were pooled. Heterogeneity was assessed and quantified. Potential publication bias was assessed and subgroup analyses were undertaken. The GRADE approach was used to evaluate quality of evidence and certainty of conclusions.

RESULTS
For saturated fat, three to 12 prospective cohort studies for each association were pooled (five to 17 comparisons with 90 501-339 090 participants). Saturated fat intake was not associated with all cause mortality (relative risk 0.99, 95% confidence interval 0.91 to 1.09), CVD mortality (0.97, 0.84 to 1.12), total CHD (1.06, 0.95 to 1.17), ischemic stroke (1.02, 0.90 to 1.15), or type 2 diabetes (0.95, 0.88 to 1.03). There was no convincing lack of association between saturated fat and CHD mortality (1.15, 0.97 to 1.36; P=0.10). For trans fats, one to six prospective cohort studies for each association were pooled (two to seven comparisons with 12 942-230 135 participants). Total trans fat intake was associated with all cause mortality (1.34, 1.16 to 1.56), CHD mortality (1.28, 1.09 to 1.50), and total CHD (1.21, 1.10 to 1.33) but not ischemic stroke (1.07, 0.88 to 1.28) or type 2 diabetes (1.10, 0.95 to 1.27). Industrial, but not ruminant, trans fats were associated with CHD mortality (1.18 (1.04 to 1.33) *v* 1.01 (0.71 to 1.43)) and CHD (1.42 (1.05 to 1.92) *v* 0.93 (0.73 to 1.18)). Ruminant *trans*-palmitoleic acid was inversely associated with type 2 diabetes (0.58, 0.46 to 0.74). The certainty of associations between saturated fat and all outcomes was "very low." The certainty of associations of trans fat with CHD outcomes was "moderate" and "very low" to "low" for other associations.

CONCLUSIONS
Saturated fats are not associated with all cause mortality, CVD, CHD, ischemic stroke, or type 2 diabetes, but the evidence is heterogeneous with methodological limitations. Trans fats are associated with all cause mortality, total CHD, and CHD mortality, probably because of higher levels of intake of industrial trans fats than ruminant trans fats. Dietary guidelines must carefully consider the health effects of recommendations for alternative macronutrients to replace trans fats and saturated fats.

WHAT IS ALREADY KNOWN ON THIS TOPIC

Contrary to prevailing dietary advice, authors of a recent systematic review and meta-analyses claim that there is no excess cardiovascular risk associated with intake of saturated fat, and the US has recently taken policy action to remove partially hydrogenated vegetable oils from its food supply
Population health guidelines require a careful review and assessment of the evidence of harms of these nutrients, with a focus on replacement nutrients

WHAT THIS STUDY ADDS

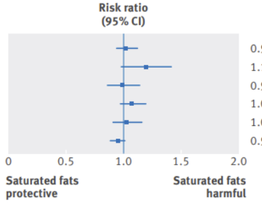
This study reviewed prospective observational studies and assessed the certainty of the associations with GRADE methods
There was no association between saturated fats and health outcomes in studies where saturated fat generally replaced refined carbohydrates, but there was a positive association between total trans fatty acids and health outcomes
Dietary guidelines for saturated and trans fatty acids must carefully consider the effect of replacement nutrients

Introduction
Recent high profile opinion pieces, informed by systematic reviews of randomized trials[1 2] and prospective cohort studies,[3] have called for a re-evaluation of dietary guidelines for intake and a re-appraisal of the effects of saturated fat on health; during this time public health efforts to remove trans fats from the food supply in several countries have intensified.

Saturated fats contribute about 10% of energy to the North American diet.[4 5] The main sources of saturated

Prevalent analyses for saturated fat and NCDs

Outcome	No of studies /comparisons	No of events /participants	Relative risk (95% CI)
All cause mortality	5/7	14 090/99 906	0.99 (0.91 to 1.09)
CHD mortality	11/15	2970/101 712	1.15 (0.97 to 1.36)
CVD mortality	3/5	3792/90 501	0.97 (0.84 to 1.12)
CHD total	12/17	6383/267 416	1.06 (0.95 to 1.17)
Ischemic stroke	12/15	6226/339 090	1.02 (0.90 to 1.15)
Type 2 diabetes	8/8	8739/237 454	0.95 (0.88 to 1.03)



"This systematic review and meta-analysis of evidence… does ***not support a robust association of saturated fats with all cause mortality, CHD, CHD mortality, ischemic stroke, or diabetes…*** *Dietary guidelines for* ***saturated and trans fatty acids*** *must carefully* ***consider*** *the effect of* ***replacement nutrients.”***

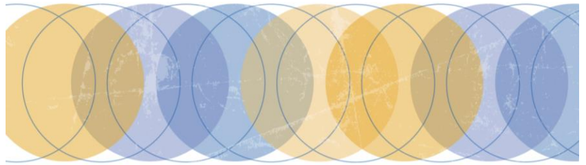
de Souza RJ et al. BMJ. 2015 Aug 11;351:h3978

Updated 2023 WHO-commissioned SRMA

Saturated fat and *trans*-fat intakes and their replacement with other macronutrients

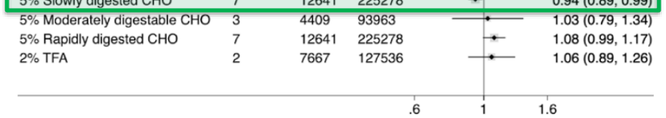
A systematic review and meta-analysis of prospective observational studies

Andrew N Reynolds, Leanne Hodson, Russell de Souza, Huyen Tran Diep Pham, Lara Vlietstra, Jim Mann



Substitution analyses for saturated fat and CHD

Replacement	Cohorts	Cases	People	ES (95% CI)
5% PUFA or Linoleic acid	17	22320	448921	0.89 (0.81, 0.98)
5% MUFA	4	10133	167855	1.00 (0.82, 1.21)
5% Plant MUFA	2	4419	93384	0.83 (0.69, 1.00)
5% Animal MUFA	2	4419	93385	1.06 (0.80, 1.41)
5% Protein	2	2466	40319	1.26 (1.06, 1.50)
5% Plant protein	2	2466	40319	0.83 (0.61, 1.12)
5% Animal protein	2	2466	40319	1.31 (1.14, 1.50)
5% Carbohydrate	6	10458	313066	0.98 (0.88, 1.09)
5% Slowly digested CHO	7	12641	225278	0.94 (0.89, 0.99)
5% Moderately digestable CHO	3	4409	93963	1.03 (0.79, 1.34)
5% Rapidly digested CHO	7	12641	225278	1.08 (0.99, 1.17)
2% TFA	2	7667	127536	1.06 (0.89, 1.26)



"Consideration of the totality of evidence available from prospective observational studies provides ***convincing evidence*** *that…* ***SFA and TFA*** *in the diet should be* ***replaced by PUFA, plant MUFA and slowly digested carbohydrates.”***

World Health Organization

Reynolds et al. Geneva: World Health Organization; 2022. Licence: CC BY-NCSA 3.0 IGO.
https://www.who.int/publications/i/item/9789240061668

UNIVERSITY OF TORONTO
FACULTY OF MEDICINE

How do we reconcile the biological mechanisms?

“Uncoupling” hypothesis

Series of systematic reviews and meta-analyses of acute RCTs

Roselyn Zhang, MSc, RD

Jarvis Noronha
MSc, MD (candidate)

Tauseef Khan, MBBS,
PhD

OSF identifier:
10.17605/OSF.IO/QSZBP

UNIVERSITY OF TORONTO

iafns

St. Michael's
Inspired Care.
Inspiring Science.

Approach:
Study Design by timing (eating occasion)
3 prespecified designs of clinical/public health importance

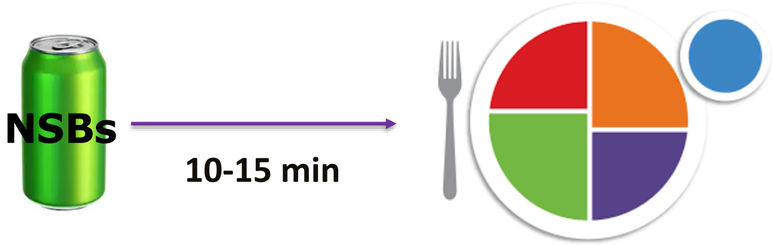
Roselyn Zhang, MSc, RD

Jarvis Noronha, MSc, MD (candidate)

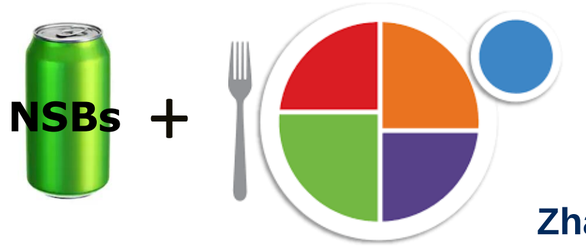
Tauseef Khan, MBBS, PhD

LNCSBs alone (uncoupling)

LNCSBs as preload (delayed coupling)



LNCSBs together with meal (coupling)



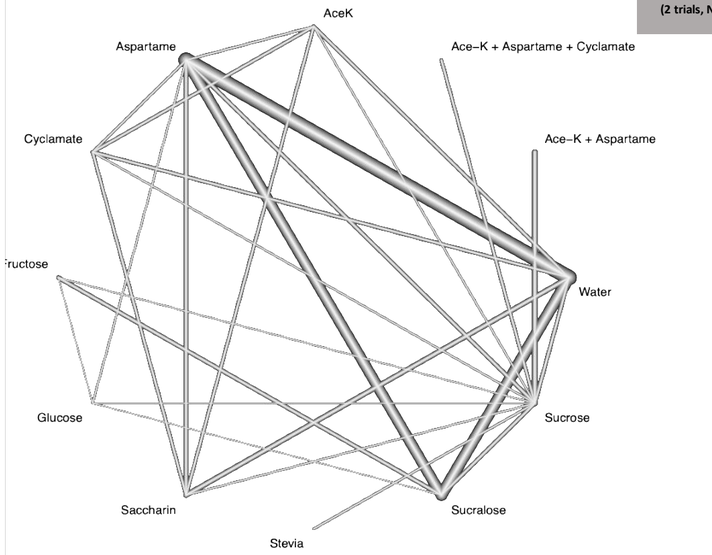
Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

Uncoupling of LNCBs

LNCSBs alone (uncoupling)

Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Uncoupling” of LNCBs and PPG in acute RCTs in NGT: Network meta-analysis 14 RCTs, N=151 (NGT)



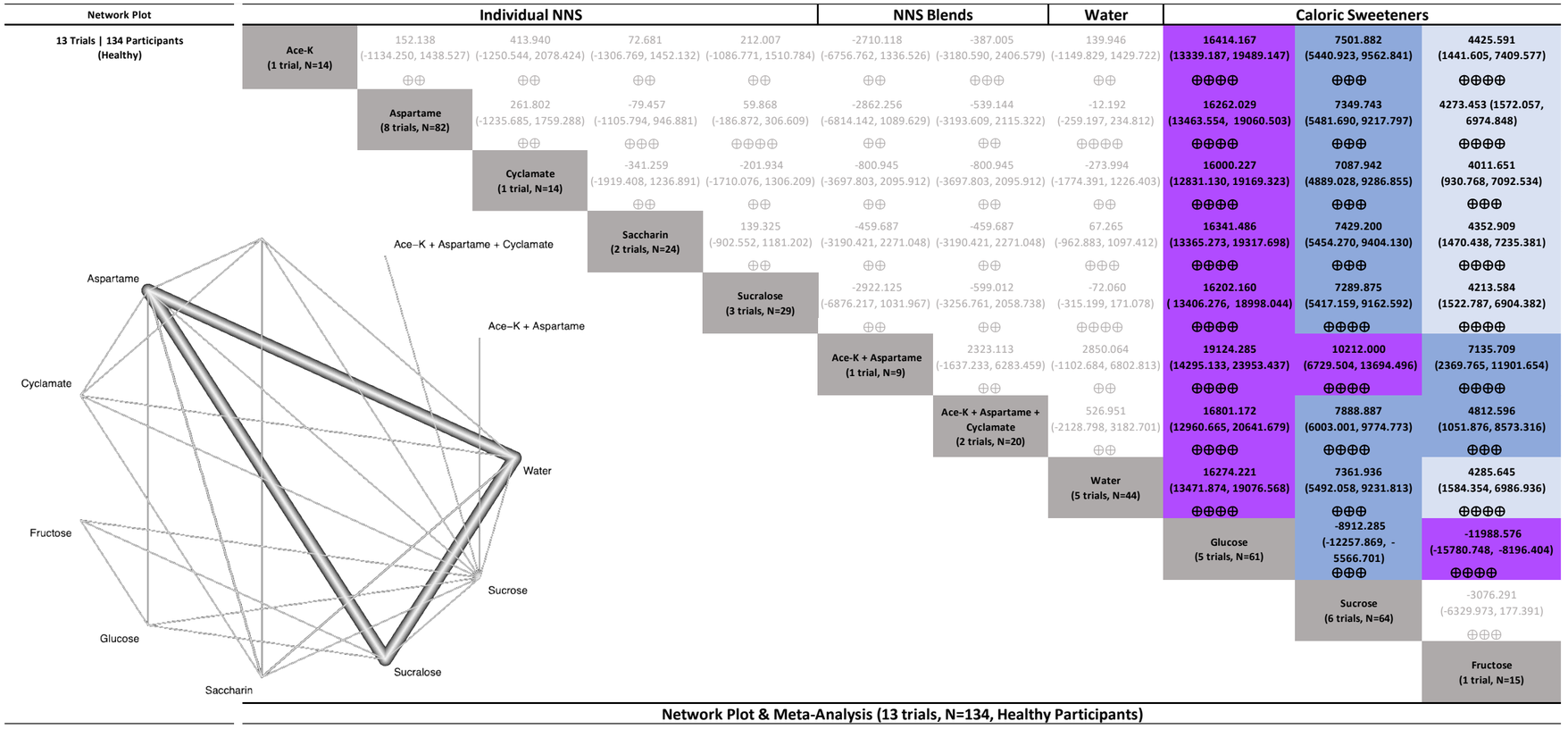
Network Plot	Individual NNS					NNS Blends		Water	Caloric Sweeteners		
14 Trials | 151 Participants (Healthy)	Ace-K (1 trial, N=14)	2.031 (-30.925, 34.988) ⊕⊕⊕	-2.280 (-39.616, 35.056) ⊕⊕⊕	9.113 (-26.058, 44.285) ⊕⊕⊕	45.809 (-14.059, 105.676) ⊕⊕	-0.649 (-35.623, 34.325) ⊕⊕⊕	**56.642 (7.942, 105.342)** ⊕⊕⊕⊕	50.982 (-8.321, 110.284) ⊕⊕⊕	-7.439 (-39.580, 24.701) ⊕⊕⊕	**239.847 (175.749, 303.945)** ⊕⊕⊕⊕	**104.809 (63.114, 146.503)** ⊕⊕⊕	40.553 (-1.824, 82.931) ⊕⊕⊕⊕
		Aspartame (8 trials, N=82)	-4.311 (-37.410, 28.787) ⊕⊕⊕	7.082 (-19.550, 33.714) ⊕⊕⊕⊕	43.777 (-9.944, 97.498) ⊕⊕⊕⊕	-2.680 (-20.680, 15.319) ⊕⊕⊕⊕	**54.611 (13.703, 95.519)** ⊕⊕⊕	48.950 (-4.141, 102.041) ⊕⊕⊕	-9.471 (-20.895, 1.953) ⊕⊕⊕⊕	**237.816 (182.307, 293.324)** ⊕⊕⊕⊕	**102.777 (70.525, 135.030)** ⊕⊕⊕⊕	**38.522 (8.565, 68.479)** ⊕⊕⊕⊕
			Cyclamate (1 trial, N=14)	11.393 (-23.912, 46.698) ⊕⊕⊕	48.089 (-11.857, 108.035) ⊕⊕	1.631 (-33.477, 36.739) ⊕⊕⊕	**58.922 (10.126, 107.718)** ⊕⊕⊕⊕	53.262 (-6.120, 112.643) ⊕⊕⊕	-5.159 (-37.446, 27.127) ⊕⊕⊕	**242.127 (177.956, 306.299)** ⊕⊕⊕⊕	**107.089 (65.282, 148.896)** ⊕⊕⊕	**42.833 (0.345, 85.321)** ⊕⊕⊕⊕
				Saccharin (2 trials, N=24)	36.696 (-20.775, 94.166) ⊕⊕	-9.762 (-39.340, 19.815) ⊕⊕⊕⊕	**47.529 (1.808, 93.249)** ⊕⊕⊕⊕	41.868 (-15.013, 98.750) ⊕⊕⊕	-16.553 (-42.664, 9.559) ⊕⊕⊕	**230.734 (169.572, 291.896)** ⊕⊕⊕⊕	**95.695 (57.523, 133.868)** ⊕⊕⊕	31.440 (-6.609, 69.490) ⊕⊕⊕⊕
					Stevia (2 trials, N=34)	-46.458 (-100.190, 7.275) ⊕⊕	10.833 (-38.956, 60.623) ⊕⊕⊕⊕	5.173 (-55.028, 65.373) ⊕⊕⊕⊕	**-53.248 (-106.064, -0.432)** ⊕⊕	**194.038 (117.465, 270.612)** ⊕⊕⊕⊕	**59.000 (16.038, 101.962)** ⊕⊕⊕	-5.255 (-64.012, 53.502) ⊕⊕⊕⊕
						Sucralose (3 trials, N=29)	**57.291 (16.368, 98.214)** ⊕⊕⊕	51.630 (-1.472, 104.733) ⊕⊕⊕	-6.790 (-21.191, 7.610) ⊕⊕⊕⊕	**240.496 (183.900, 297.092)** ⊕⊕⊕⊕	**105.458 (73.186, 137.729)** ⊕⊕⊕⊕	**41.202 (17.206, 65.199)** ⊕⊕⊕⊕
							Ace-K + Aspartame (1 trial, N=9)	-5.660 (-54.769, 43.448) ⊕⊕⊕⊕	**-64.081 (-103.794, -24.369)** ⊕⊕⊕⊕	**183.205 (115.007, 251.404)** ⊕⊕⊕⊕	**48.167 (23.002, 73.331)** ⊕⊕⊕⊕	-16.089 (-63.416, 31.239) ⊕⊕⊕⊕
								Ace-K + Aspartame + Cyclamate (2 trials, N=20)	**-58.421 (-110.596, -6.246)** ⊕⊕⊕	**188.866 (112.733, 264.998)** ⊕⊕⊕⊕	**53.827 (11.656, 95.998)** ⊕⊕⊕	-10.428 (-68.609, 47.753) ⊕⊕⊕⊕
									Water (5 trials, N=44)	**247.287 (191.300, 303.273)** ⊕⊕⊕⊕	**112.248 (81.526, 142.970)** ⊕⊕⊕⊕	**47.993 (20.043, 75.943)** ⊕⊕⊕⊕
										Glucose (5 trials, N=61)	**-135.038 (-198.425, -71.652)** ⊕⊕⊕⊕	**-199.294 (-260.661, -137.927)** ⊕⊕⊕⊕
											Sucrose (7 trials, N=81)	**-64.255 (-104.338, -24.172)** ⊕⊕⊕⊕
												Fructose (1 trial, N=15)

Effect size

- Trivial
- Small important
- Moderate
- Large
- Very large

Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Uncoupling” of LNCBs and PP insulin in acute RCTs in NGT: Network meta-analysis 13 RCTs, N=134 (NGT)

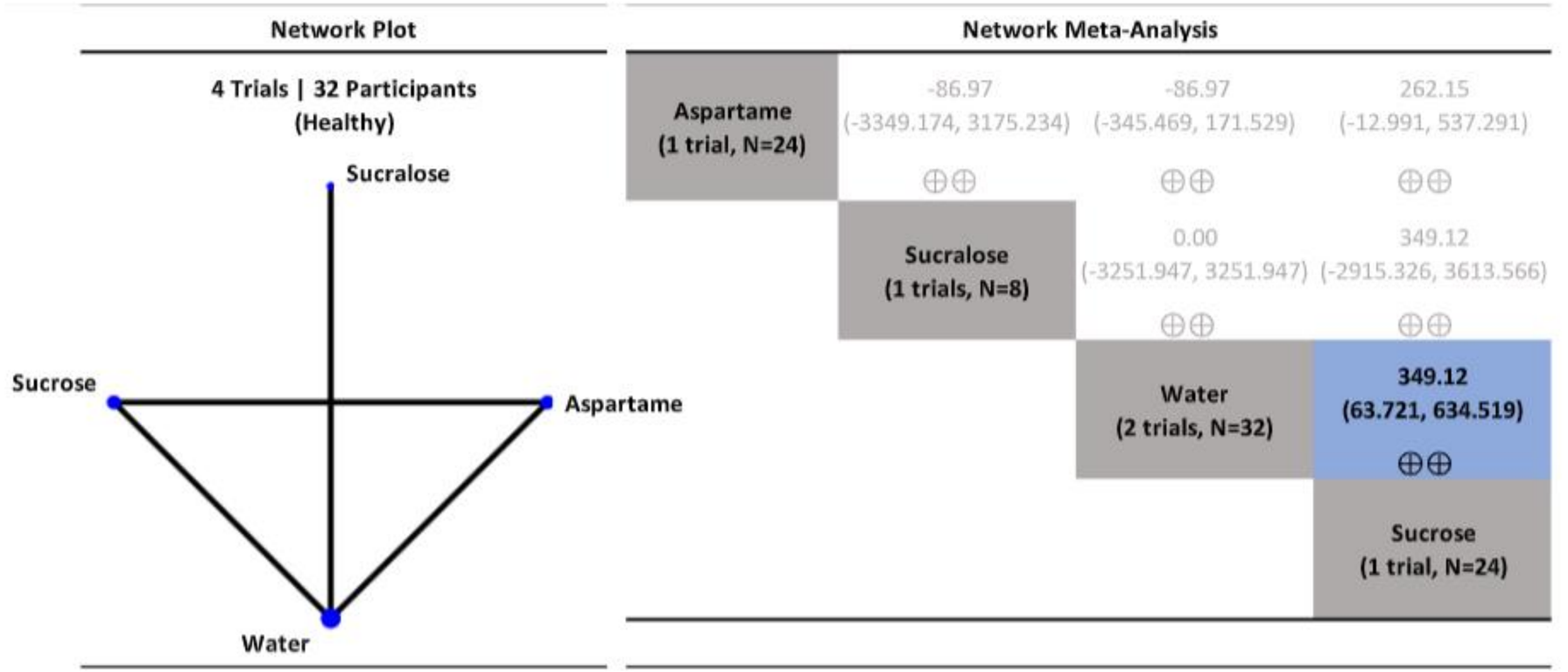


Effect size

- Trivial
- Small important
- Moderate
- Large
- Very large

Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

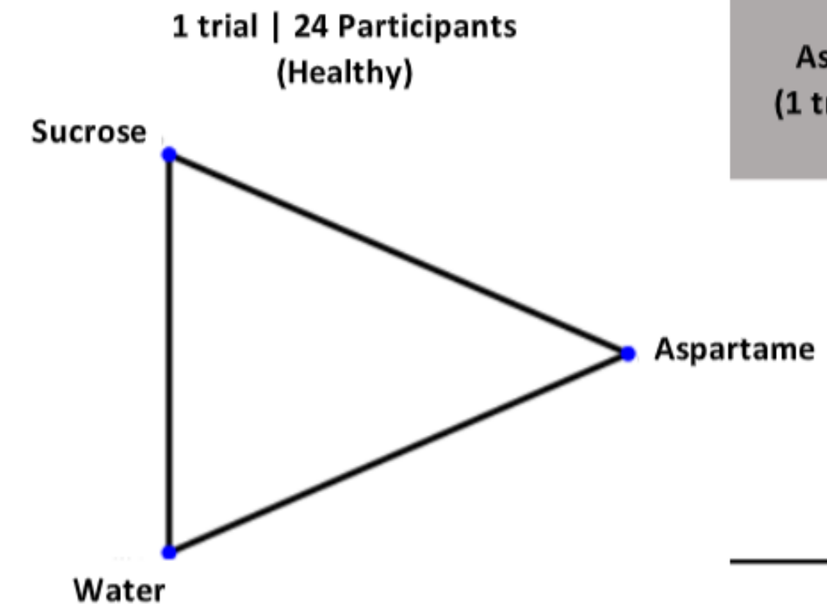
“Uncoupling” of LNCBs and PP GLP-1 in acute RCTs in NGT: Network meta-analysis 4 RCTs, N=32 (NGT)



Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Uncoupling” of LNCBs and PP GIP in acute RCTs in NGT: Network meta-analysis 1 RCT, N=24 (NGT)

Network Plot



Network Meta-Analysis

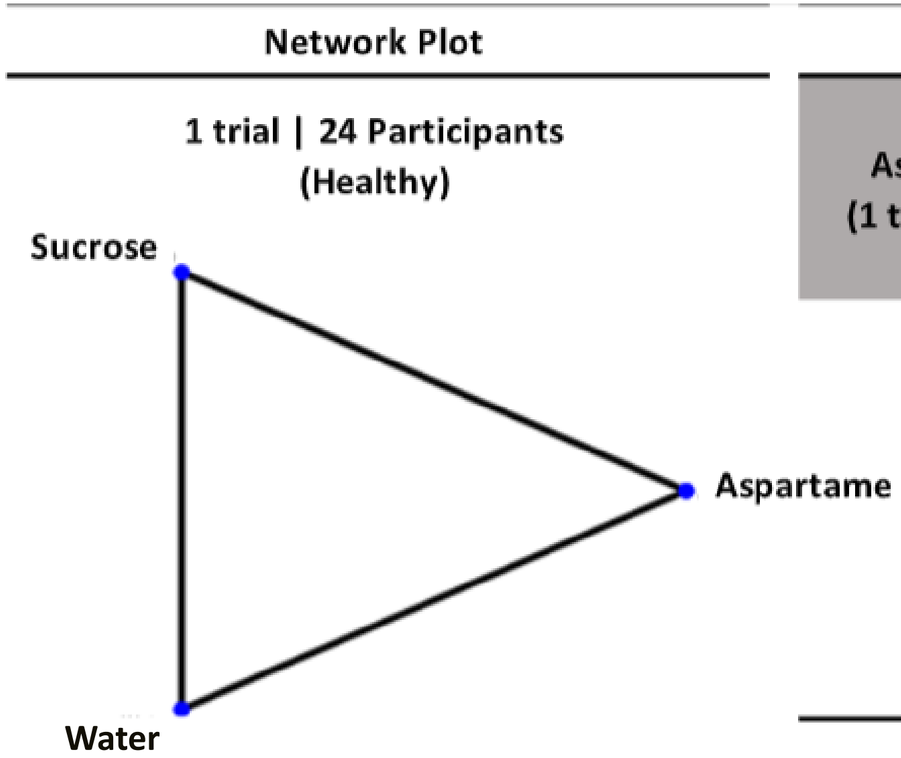
Aspartame (1 trial, N=24)	23.21 (-238.591, 285.011) ⨁⨁	1007.80 (675.588, 1340.012) ⨁⨁⨁
	Water (1 trial, N=24)	984.59 (649.367, 1319.813) ⨁⨁⨁
		Sucrose (1 trial, N=24)

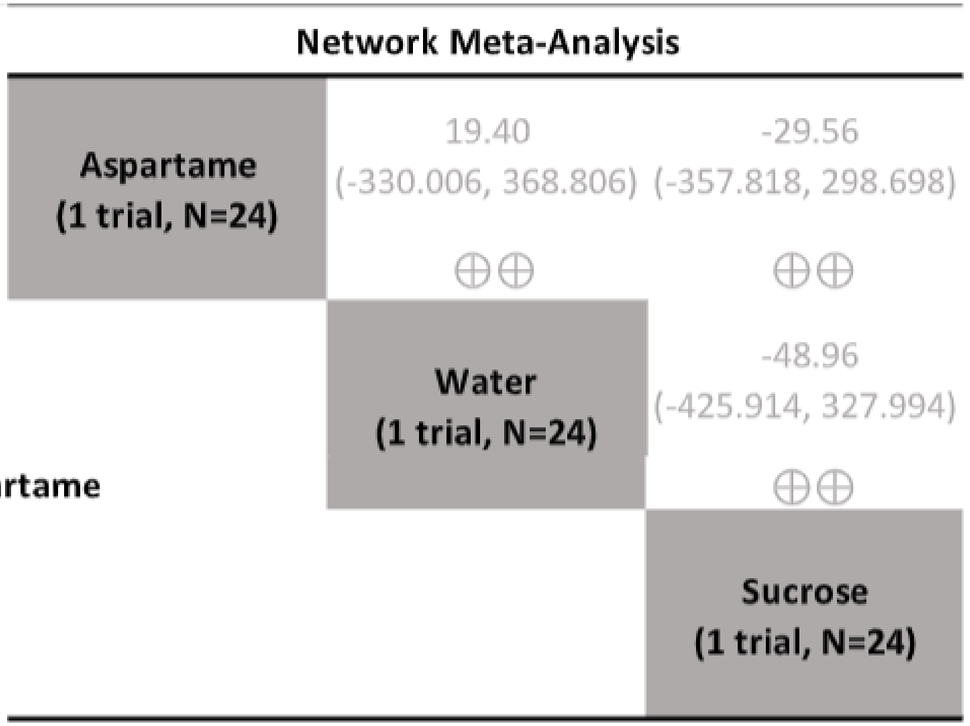
Effect size

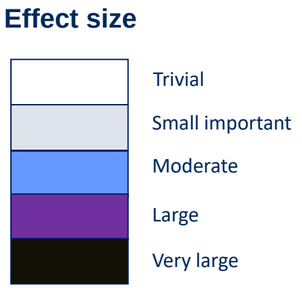
- Trivial
- Small important
- Moderate
- Large
- Very large

Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Uncoupling” of LNCBs and PP ghrelin in acute RCTs in NGT: Network meta-analysis 1 RCT, N=24 (NGT)

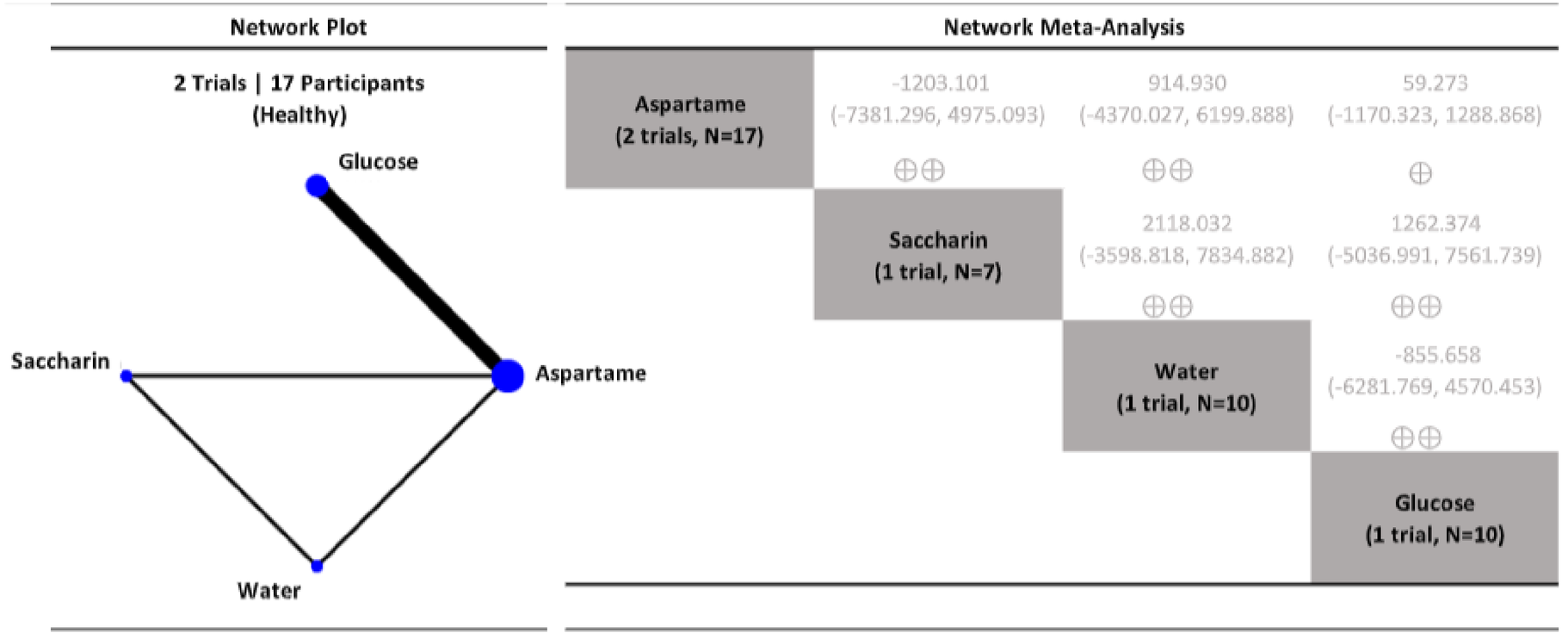






Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

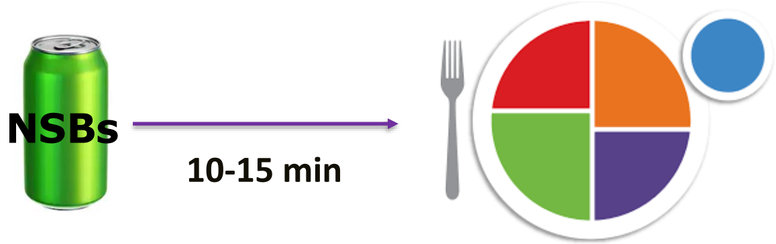
“Uncoupling” of LNCBs and PP glucagon in acute RCTs in NGT: Network meta-analysis 1 RCT, N=24 (NGT)



Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

Delayed coupling of LNCBs

LNCSBs as preload (delayed coupling)



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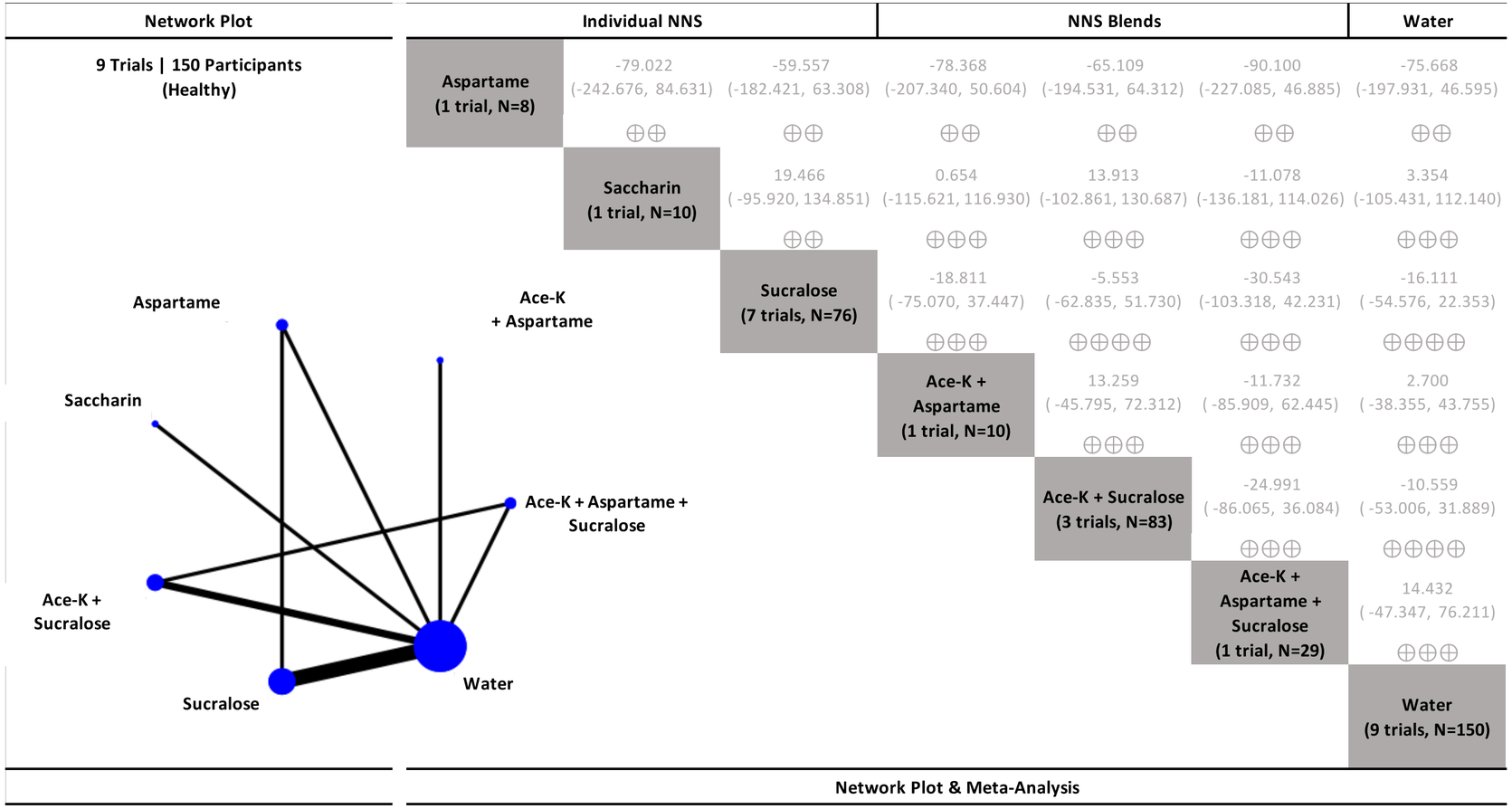
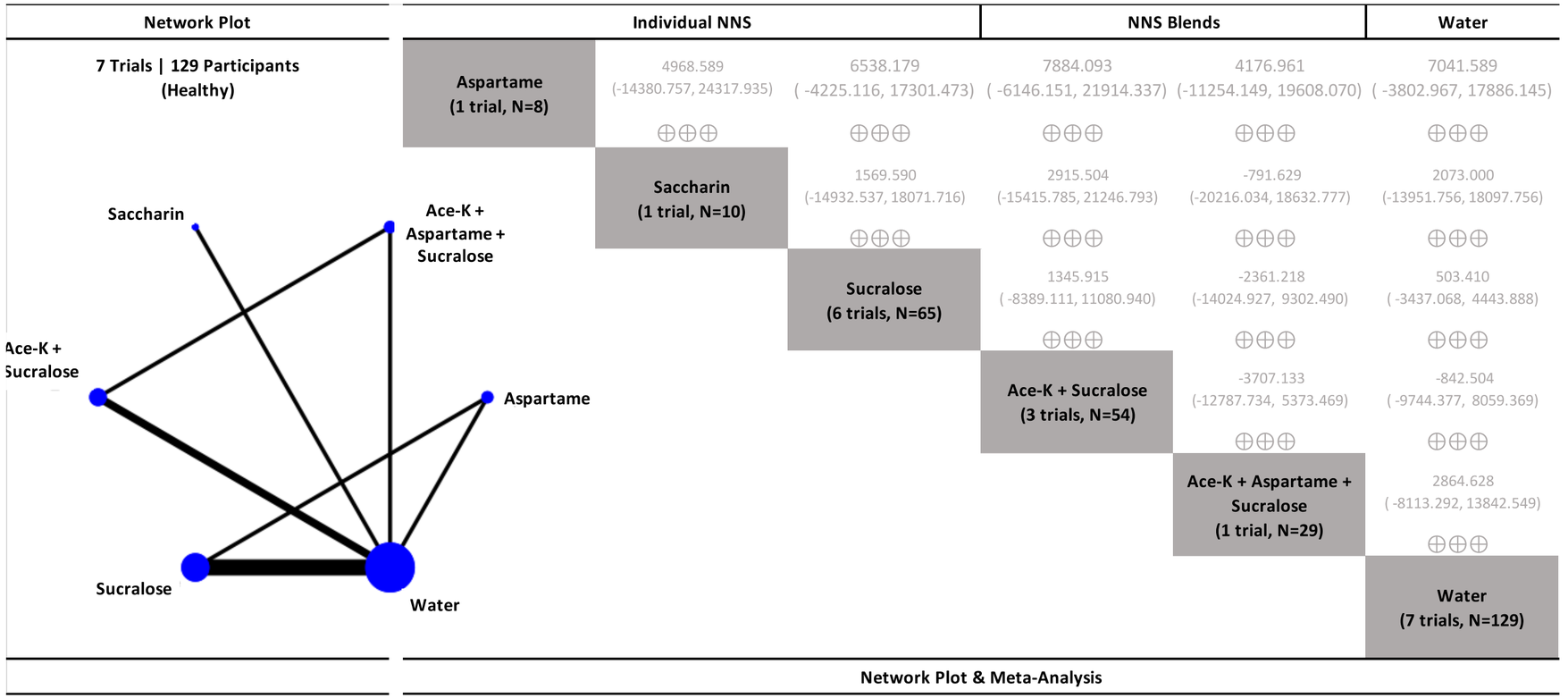
“Delayed coupling” of LNCBs and PPG in acute RCTs in NGT: Network meta-analysis 13 RCTs, N=134 (NGT)



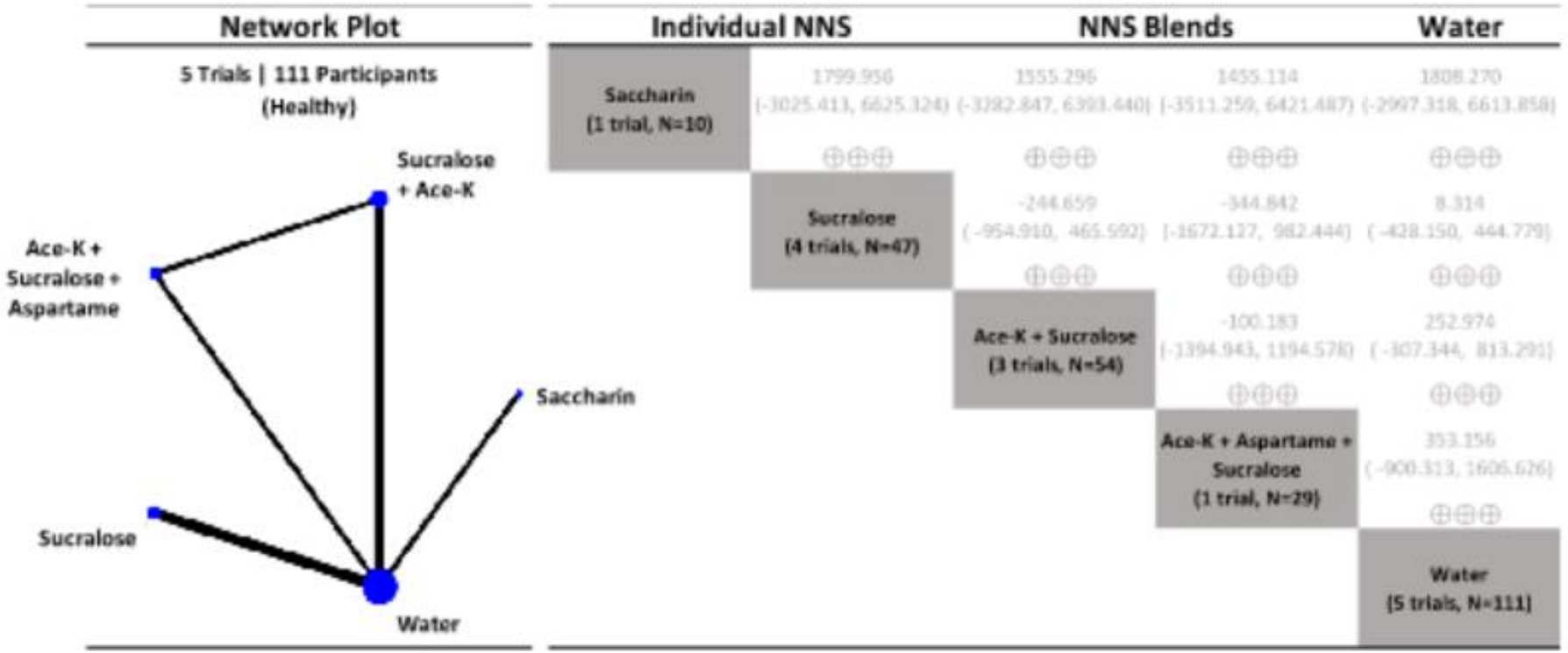
Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Delayed coupling” of LNCBs and PP insulin in acute RCTs in NGT: Network meta-analysis 7 RCTs, N=129 (NGT)



Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Delayed coupling” of LNCBs and PP GIP in acute RCTs in NGT: Network meta-analysis 5 RCTs, N=111 (NGT)



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Coupling of LNCBs

LNCSBs together with meal (coupling)

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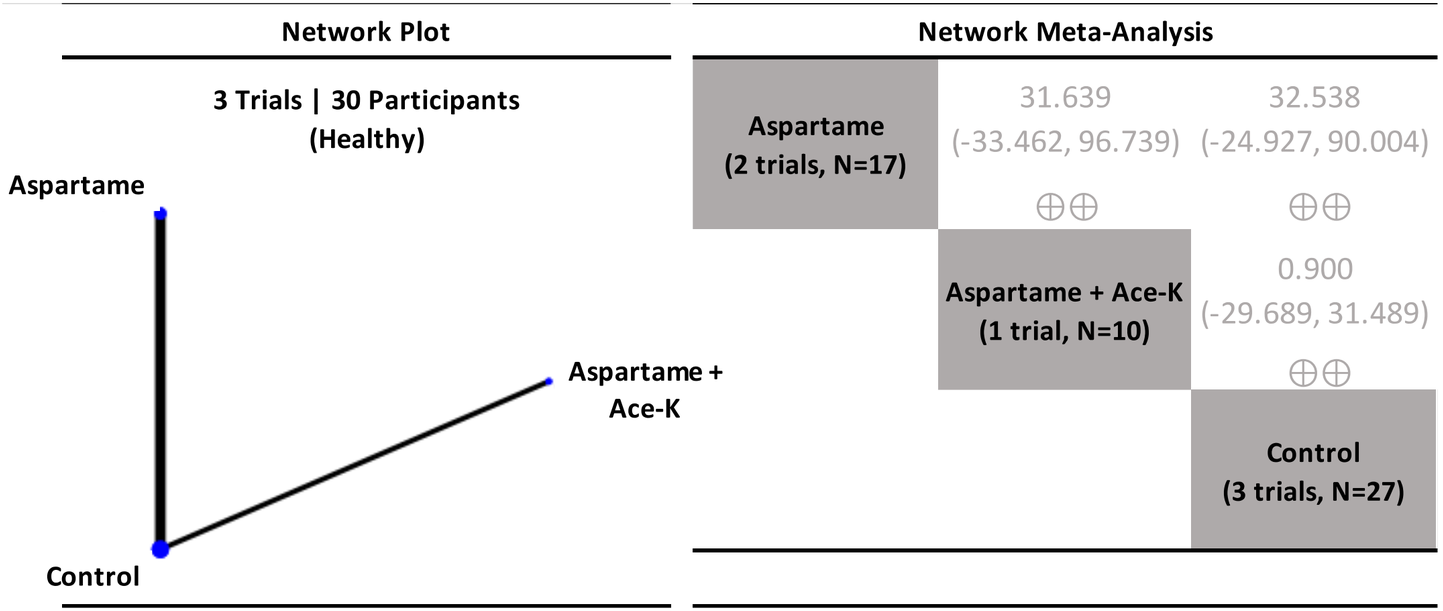
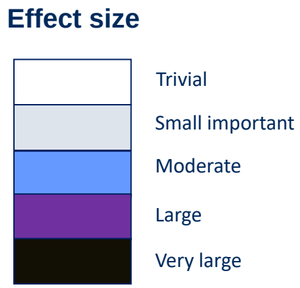
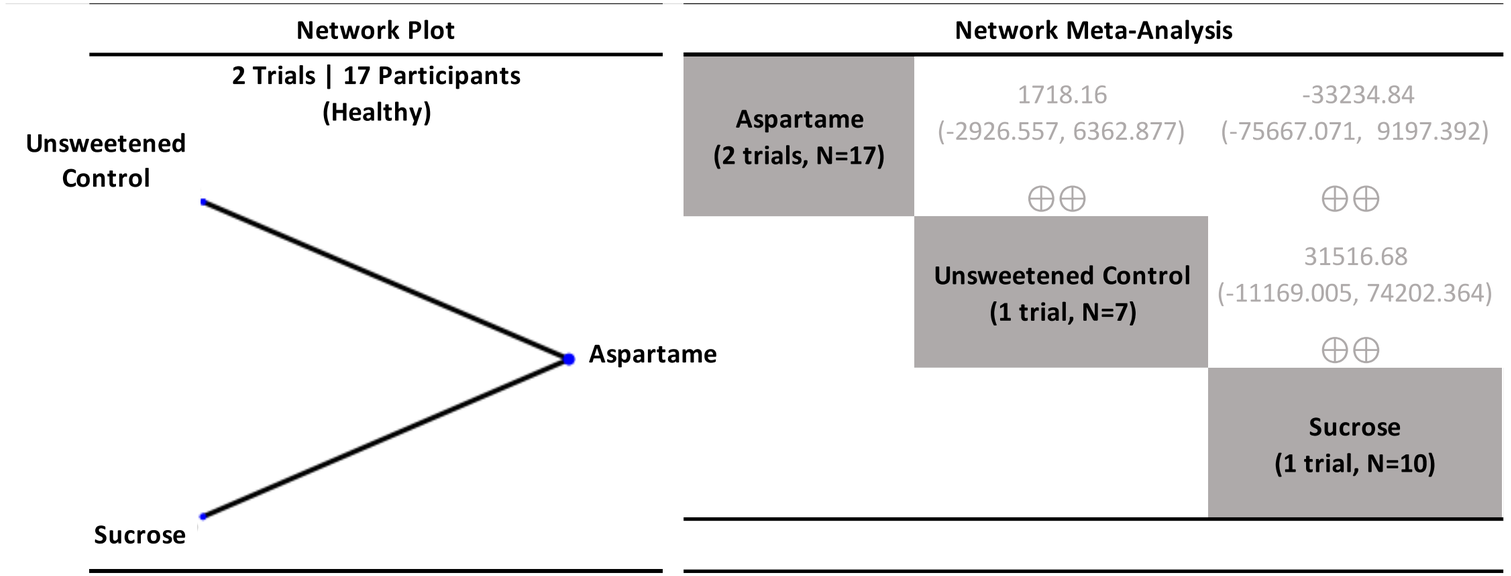
“Coupling” of LNCBs and PPG in acute RCTs in NGT: Network meta-analysis 3 RCTs, N=30 (NGT)

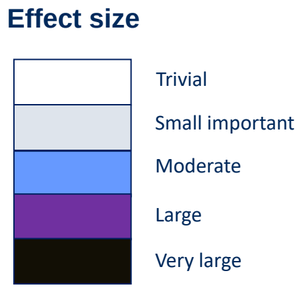




Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

“Coupling” of LNCBs and PP insulin in acute RCTs in NGT: Network meta-analysis 2 RCTs, N=17 (NGT)





Zhang R, Noronha JC, et al. 2023 Feb 20;15(4):1050

Microbiome dysbiosis hypothesis

Saccharin intake is associated with impaired glucose tolerance through changes in the gut microbiome:

Uncontrolled ("start vs. end") trial; N=7 (post hoc responders [n=4], non-responders [n=3]); dose=5 mg/kg/d saccharin (100% ADI); FU=1-wk

ARTICLE

doi:10.1038/nature13793

Artificial sweeteners induce glucose intolerance by altering the gut microbiota

Jotham Suez[1], Tal Korem[2]*, David Zeevi[2]*, Gili Zilberman-Schapira[1]*, Christoph A. Thaiss[1], Ori Maza[1], David Israeli[3], Niv Zmora[4,5,6], Shlomit Gilad[7], Adina Weinberger[2], Yael Kuperman[8], Alon Harmelin[8], Ilana Kolodkin-Gal[9], Hagit Shapiro[1], Zamir Halpern[5,6], Eran Segal[2] & Eran Elinav[1]

Non-caloric artificial sweeteners (NAS) are among the most widely used food additives worldwide, regularly consumed by lean and obese individuals alike. NAS consumption is considered safe and beneficial owing to their low caloric content, yet supporting scientific data remain sparse and controversial. Here we demonstrate that consumption of commonly used NAS formulations drives the development of glucose intolerance through induction of compositional and functional alterations to the intestinal microbiota. These NAS-mediated deleterious metabolic effects are abrogated by antibiotic treatment, and are fully transferrable to germ-free mice upon faecal transplantation of microbiota configurations from NAS-consuming mice, or of microbiota anaerobically incubated in the presence of NAS. We identify NAS-altered microbial metabolic pathways that are linked to host susceptibility to metabolic disease, and demonstrate similar NAS-induced dysbiosis and glucose intolerance in healthy human subjects. Collectively, our results link NAS consumption, dysbiosis and metabolic abnormalities, thereby calling for a reassessment of massive NAS usage.

Non-caloric artificial sweeteners (NAS) were introduced over a century ago as means for providing sweet taste to foods without the associated high energy content of caloric sugars. NAS consumption gained much popularity owing to their reduced costs, low caloric intake and perceived health benefits for weight reduction and normalization of blood sugar levels[1]. For these reasons, NAS are increasingly introduced into commonly consumed foods such as diet sodas, cereals and sugar-free desserts, and are being recommended for weight loss and for individuals suffering from glucose intolerance and type 2 diabetes mellitus[1].

Some studies showed benefits for NAS consumption[2] and little induction of a glycaemic response[3], whereas others demonstrated associations between NAS consumption and weight gain[4], and increased type 2 diabetes risk[5]. However, interpretation is complicated by the fact that NAS are typically consumed by individuals already suffering from metabolic syndrome manifestations. Despite these controversial data, the US Food and Drug Administration (FDA) approved six NAS products for use in the United States.

Most NAS pass through the human gastrointestinal tract without being digested by the host[6,7] and thus directly encounter the intestinal microbiota, which plays central roles in regulating multiple physiological processes[8]. Microbiota composition[9] and function[10] are modulated by diet in the healthy/lean state as well as in obesity[11,12] and diabetes mellitus[13], and in turn microbiota alterations have been associated with propensity to metabolic syndrome[14]. Here, we study NAS-mediated modulation of microbiota composition and function, and the resultant effects on host glucose metabolism.

Chronic NAS consumption exacerbates glucose intolerance

To determine the effects of NAS on glucose homeostasis, we added commercial formulations of saccharin, sucralose or aspartame to the drinking water of lean 10-week-old C57Bl/6 mice (Extended Data Fig. 1a). Since all three commercial NAS comprise ~5% sweetener and ~95% glucose, we used as controls mice drinking only water or water supplemented with either glucose or sucrose. Notably, at week 11, the three mouse groups that consumed water, glucose and sucrose featured comparable glucose tolerance curves, whereas all three NAS-consuming mouse groups developed marked glucose intolerance ($P < 0.001$, Fig. 1a, b).

As saccharin exerted the most pronounced effect, we further studied its role as a prototypical artificial sweetener. To corroborate the findings in the obesity setup, we fed C57Bl/6 mice a high-fat diet (HFD, 60% kcal from fat) while consuming either commercial saccharin or pure glucose as a control (Extended Data Fig. 1b). As in the lean state, mice fed HFD and commercial saccharin developed glucose intolerance, compared to the control mouse group ($P < 0.03$, Fig. 1c and Extended Data Fig. 2a). To examine the effects of pure saccharin on glucose intolerance, we followed a cohort of 10-week-old C57Bl/6 mice fed on HFD and supplemented with 0.1 mg ml^{-1} of pure saccharin added to their drinking water (Extended Data Fig. 1c). This dose corresponds to the FDA acceptable daily intake (ADI) in humans (5 mg per kg (body weight), adjusted to mouse weights, see Methods). As with commercial saccharin, this lower dose of pure saccharin was associated with impaired glucose tolerance ($P < 0.0002$, Fig. 1d and Extended Data Fig. 2b) starting as early as 5 weeks after HFD initiation. Similarly, HFD-fed outbred Swiss Webster mice supplemented with or without 0.1 mg ml^{-1} of pure saccharin (Extended Data Fig. 1d) showed significant glucose intolerance after 5 weeks of saccharin exposure as compared to controls ($P < 0.03$, Extended Data Fig. 2c, d).

Metabolic profiling of normal-chow- or HFD-fed mice in metabolic cages, including liquids and chow consumption, oxygen consumption, walking distance and energy expenditure, showed similar measures between NAS- and control-drinking mice (Extended Data Fig. 3 and 4).

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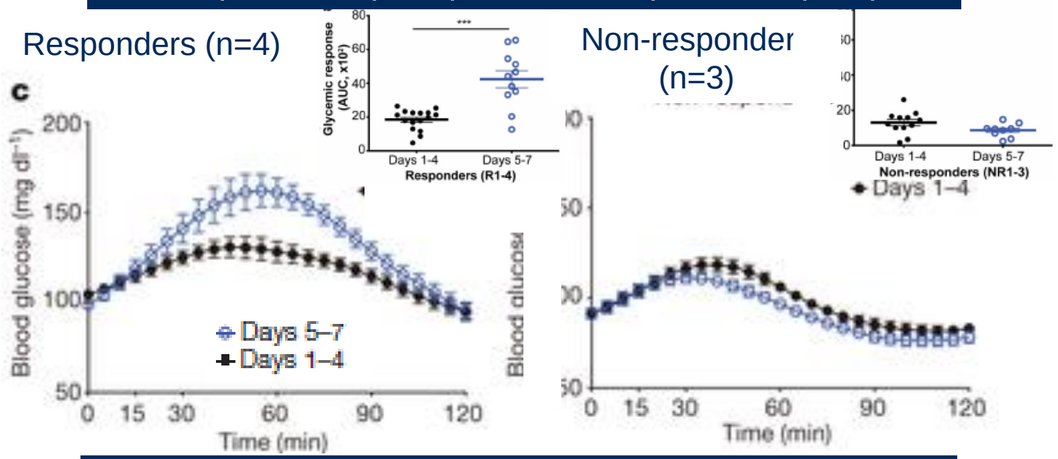
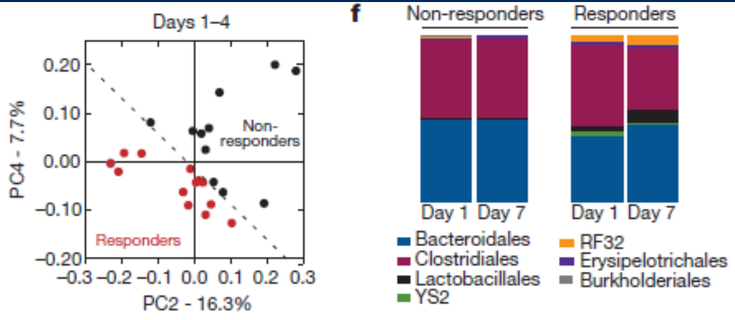
Suez J et al. Nature 2014; 514(7521):181-6

Saccharin and 75g-OGTT responses in responders (n=4) vs non-responders (n=3)



Microbiome changes (16s RNA sequencing) in responders (n=4) vs non-responders (n=3)



Saccharin intake is associated with impaired glucose tolerance through changes in the gut microbiome:

Uncontrolled ("start vs. end") trial; N=7 (post hoc responders [n=4], non-responders [n=3]); dose=5 mg/kg/d saccharin (100% ADI); FU=1-wk

ARTICLE

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Artificial sweeteners induce glucose intolerance by altering the gut microbiota

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Saccharin and 75g-OGTT responses in responders (n=4) vs non-responders (n=3)

Responders (n=4)

Non-responder (n=3)

Serious limitations

1. **ONLY** assessed **saccharin** as a "prototypical" LCS
2. **NO control group** ("before versus after" design)
3. Post-hoc separation into "**responders**" vs "**non-responders**"
4. **ONLY** assessed extreme dose at **max ADI**
5. "**Intended**" pattern of use (displacement of excess calories from sugars) **NOT** assessed

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Suez J et al. Nature 2014; 514(7521):181-6

Aspartame and sucralose intake is NOT associated with impaired glucose tolerance through changes in microbiome:

Double-blind, placebo-controlled, crossover, RCT; n=17 (healthy), Dose = 425mg (14% ADI) aspartame, 136mg (20% ADI) sucralose; FU=2-wk

NRC Research Press

ARTICLE

The effect of the artificial sweeteners on glucose metabolism in healthy adults: a randomized, double-blinded, crossover clinical trial

Samar Y. Ahmad, James K. Friel, and Dylan S. MacKay

Abstract: This study aimed to determine the effect of pure forms of sucralose and aspartame, in doses reflective of common consumption, on glucose metabolism. Healthy participants consumed pure forms of a non-nutritive sweetener (NNS) that were mixed with water and standardized to doses of 14% (0.425 g) of the acceptable daily intake (ADI) for aspartame and 20% (0.136 g) of the ADI for sucralose every day for 2 weeks. Blood samples were collected and analyzed for glucose, insulin, active glucagon-like peptide-1 (GLP-1), and leptin. Seventeen participants (10 females and 7 males; age, 24 ± 6.8 years; body mass index, 22.9 ± 2.5 kg/m^2) participated in the study. The total area under the curve values of glucose, insulin, active GLP-1 and leptin were similar for the aspartame and sucralose treatment groups compared with the baseline values in healthy participants. There was no change in insulin sensitivity after NNS treatment compared with the baseline values. These findings suggest that daily repeated consumption of pure sucralose or aspartame for 2 weeks had no effect on glucose metabolism among normoglycaemic adults. However, these results need to be tested in studies with longer durations.

Novelty

- Daily consumption of pure aspartame or sucralose for 2 weeks had no effect on glucose metabolism.
- Daily consumption of pure aspartame or sucralose for 2 weeks had no effect on insulin sensitivity among healthy adults.

Key words: non-nutritive sweetener, aspartame, sucralose, protocol, glucose metabolism, insulin, glucose, active GLP-1, leptin.

Résumé : Cette étude vise à déterminer l'effet sur le métabolisme du glucose des formes pures de sucralose et d'aspartame, à des doses reflétant la consommation courante. Les participants en bonne santé consomment des formes pures d'édulcorant non nutritif (« NNS ») mélangé à de l'eau et normalisé à des doses de 14 % (0,425 g) de la teneur journalière admissible (« ADI ») pour l'aspartame et de 20 % (0,136 g) de l'ADI pour le sucralose tous les jours durant deux semaines. Des échantillons de sang sont prélevés et analysés pour le glucose, l'insuline, le GLP-1 actif (*active glucagon-like peptide-1*) et la leptine. Dix-sept participants (10 femmes et 7 hommes; âge, 24 ± 6,8 ans; indice de masse corporelle, 22,9 ± 2,5 kg/m^2) participent à l'étude. Les valeurs de l'aire totale sous la courbe du glucose, de l'insuline, du GLP-1 actif et de la leptine sont similaires pour les groupes de traitement à l'aspartame et au sucralose comparativement aux valeurs initiales chez les participants en bonne santé. Il n'y a pas de changement de sensibilité à l'insuline après le traitement aux NNS comparativement aux valeurs initiales. Ces résultats suggèrent que la consommation quotidienne répétée de formes pures de sucralose ou d'aspartame pendant 2 semaines n'a aucun effet sur le métabolisme du glucose chez les adultes normoglycémiques. Cependant, ces résultats doivent être testés dans des études de durée plus longue. [Traduit par la Rédaction]

Les nouveautés

- La consommation quotidienne de formes pures d'aspartame ou de sucralose pendant 2 semaines n'a aucun effet sur le métabolisme du glucose.
- La consommation quotidienne de formes pures d'aspartame ou de sucralose pendant 2 semaines n'a aucun effet sur la sensibilité à l'insuline chez les adultes en bonne santé.

Mots-clés : édulcorant non nutritif, aspartame, sucralose, protocole, métabolisme du glucose, insuline, glucose, GLP-1 actif, leptine.

Introduction

Non-nutritive sweeteners (NNSs) are novel chemosensory compounds in the food additive class that have been commonly used in different foods and beverages to provide an intense sweet taste and decrease caloric content (Gardner et al. 2012). NNSs are also used and recommended for managing weight and controlling blood glucose levels in individuals with obesity and diabetes (Gardner et al. 2012; Mattes and Popkin 2009). The US Food and

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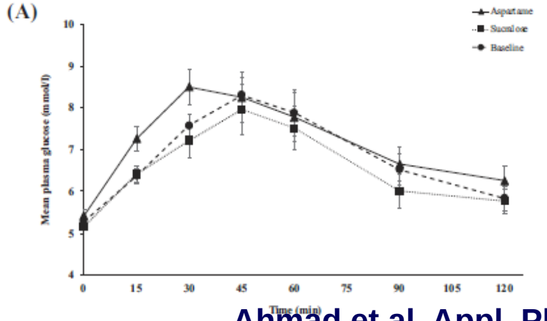
75g-OGTT



	Baseline	Sucralose	Aspartame
Glucose, mmol/L 120 min	833±143	798±145	860±205

% Change[a]	% Change[b]	$p^{c,*}$	$p^{d,*}$
−4.2	+3.1	0.54	0.65

Ahmad et al. Appl. Physiol Nutr Metab 2020; 45:606–612

Microbiome changes (16s RNA sequencing)

**	Phylum Genus	Pre-Treatment		Post-Treatment		*p*-Value	
		Sucralose	Aspartame	Sucralose	Aspartame	Sucralose	Aspartame
	Actinobacteria	0.121	0.103	0.043	0.031	0.64	0.96
	f__Clostridiaceae_ unclassified	0.000	0.000	0.000	0.000	0.55	0.31
	g__Bifidobacterium	0.102	0.095	0.026	0.028	0.54	0.88
	g__Collinsella	0.010	0.009	0.008	0.005	0.96	0.72
	g__Eggerthella	0.000	0.000	0.000	0.000	0.80	0.31
	g__Slackia	0.000	0.000	0.000	0.000	0.78	0.89
	Bacteroidetes	0.131	0.215	0.374	0.409	0.61	0.92
	g__[Prevotella]	0.000	0.000	0.000	0.000	0.21	0.48
	g__Alistipes	0.003	0.009	0.013	0.013	0.23	0.68
	g__Bacteroides	0.035	0.053	0.075	0.098	0.29	0.96
	g__Parabacteroides	0.001	0.004	0.005	0.005	0.26	0.16
	g__Prevotella	0.028	0.019	0.101	0.014	0.35	0.47
	Firmicutes	0.517	0.548	0.533	0.530	0.18	0.54
	F_Ruminococcaceae_Unclassified	0.036	0.028	0.027	0.027	0.30	0.15
	g__Blautia	0.075	0.077	0.099	0.087	0.88	0.64
	g__Coprococcus	0.036	0.043	0.033	0.024	0.96	0.76
	g__Faecalibacterium	0.026	0.024	0.066	0.033	0.41	0.10
	G_Roseburia	0.040	0.018	0.024	0.021	0.17	0.43
	Verrucomicrobia	0.000	0.000	0.000	0.000	0.92	0.44
	g__Akkermansia	0.000	0.000	0.000	0.000	0.92	0.44

Ahmad et al. Nutrients 2020 Nov 6;12(11):E3408

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Sucralose intake is NOT associated with impaired glucose tolerance through changes in microbiome:

Double-blind, placebo-controlled, parallel, RCT; n=34 (healthy), Dose=780mg (75% ADI) sucralose; FU=2-wk

British Journal of Nutrition (2019), 122, 856–862
doi:10.1017/S0007114519001570

Short-term impact of sucralose consumption on the metabolic response and gut microbiome of healthy adults

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(Submitted 19 March 2019 – Final revision received 8 June 2019 – Accepted 21 June 2019 – First published online 13 September 2019)

Abstract
Sucralose is an artificial non-nutritive sweetener used in foods aimed to reduce sugar and energy intake. While thought to be inert, the impact of sucralose on metabolic control has shown to be the opposite. The gut microbiome has emerged as a factor shaping metabolic responses after sweetener consumption. We examined the short-term effect of sucralose consumption on glucose homeostasis and gut microbiome of healthy male volunteers. We performed a randomised, double-blind study in thirty-four subjects divided into two groups, one that was administered sucralose capsules (780 mg/d for 7 d; *n* 17) and a control group receiving placebo (*n* 17). Before and after the intervention, glycaemic and insulinaemic responses were assessed with a standard oral glucose load (75 g). Insulin resistance was determined using homeostasis model assessment of insulin resistance and Matsuda indexes. The gut microbiome was evaluated before and after the intervention by 16S rRNA sequencing. During the study, body weight remained constant in both groups. Glycaemic control and insulin resistance were not affected during the 7-d period. At the phylum level, gut microbiome was not modified in any group. We classified subjects according to their change in insulinaemia after the intervention, to compare the microbiome of responders and non-responders. Independent of consuming sucralose or placebo, individuals with a higher insulinaemic response after the intervention had lower Bacteroidetes and higher Firmicutes abundances. In conclusion, consumption of high doses of sucralose for 7 d does not alter glycaemic control, insulin resistance, or gut microbiome in healthy individuals. However, it highlights the need to address individual responses to sucralose.

Key words: **Sucralose: Non-energy artificial sweeteners: Glucose control: Insulin: Gut microbiome**

Sucralose (1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside) is a non-energy artificial sweetener (NAS) synthesised by the selective halogenation of sucrose[1]. Approved by the Food and Drug Administration for use in humans, it is 600 times sweeter than sucrose. Due to its low production cost, high thermostability and solubility, sucralose has emerged as an important sugar substitute in foods and drinks. The acceptable daily intake (ADI) of sucralose has been established at 15 mg/kg body weight[2].

The concept that replacing sucrose with NAS in foods and drinks improves metabolic control has been challenged[3]. In mice, sucralose added to drinking water for 11 weeks impaired oral glucose tolerance when compared with water alone or water with sucrose or glucose[4]. Such deleterious effect was prevented when mice were treated with broad-spectrum antibiotics against Gram-negative or Gram-positive bacteria. The fact that sucralose displays bacteriostatic action on several gut microbes[5,6], and that most of the sucralose is not absorbed in the intestine[1,7,8], gives support to observations showing that sucralose can alter gut microbiome composition[6,9]. Taken together, the notion that sucralose influences glucose control through alterations in intestinal microbiota has emerged.

In humans, consumption of high doses of sucralose for 3 months has been assessed in non-diabetic[10] and type 2 diabetic[11]

Abbreviation: NAS, non-energy artificial sweetener.

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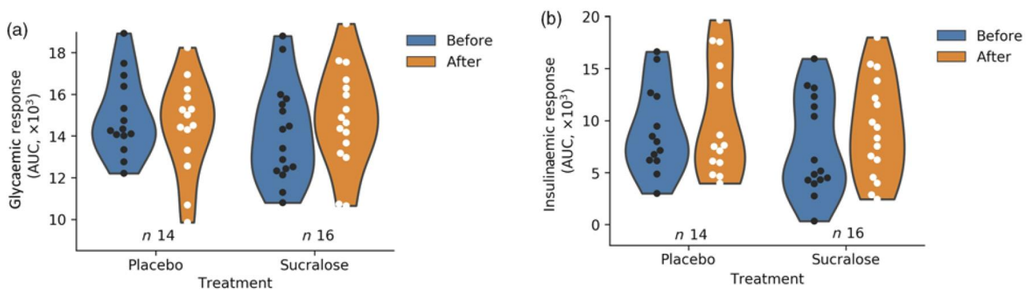
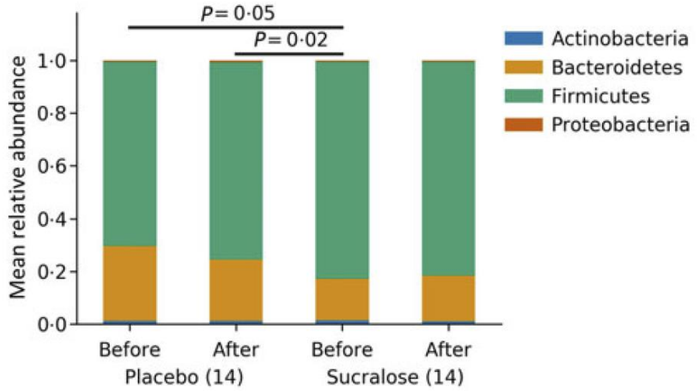
† These authors contributed equally to this work.

75g-OGTT



Microbiome changes (16s RNA sequencing)



Thomson P, et al. Br J Nutr. 2019 Oct 28;122(8):856-862.

Saccharin intake is NOT associated with impaired glucose tolerance through changes in microbiome:

Double-blind, placebo-controlled, parallel, RCT; n=46 completers of 54 (healthy), Dose = 400mg (100% ADI) saccharin; FU=2-wk

Serrano et al. Microbiome (2021) 9:11
https://doi.org/10.1186/s40168-020-00976-w

Microbiome

RESEARCH Open Access

High-dose saccharin supplementation does not induce gut microbiota changes or glucose intolerance in healthy humans and mice

Joan Serrano[1], Kathleen R. Smith[1], Audra L. Crouch[2], Vandana Sharma[3], Fanchao Yi[4], Veronika Vargova[4], Traci E. LaMoia[1], Lydia M. Dupont[1], Vanida Serna[1], Fenfen Tang[5], Laisa Gomes-Dias[6], Joshua J. Blakeslee[6], Emmanuel Hatzakis[5], Scott N. Peterson[3], Matthew Anderson[2], Richard E. Pratley[4] and George A. Kyriazis[1]*

Abstract

Background: Non-caloric artificial sweeteners (NCAS) are widely used as a substitute for dietary sugars to control body weight or glycemia. Paradoxically, some interventional studies in humans and rodents have shown unfavorable changes in glucose homeostasis in response to NCAS consumption. The causative mechanisms are largely unknown, but adverse changes in gut microbiota have been proposed to mediate these effects. These findings have raised concerns about NCAS safety and called into question their broad use, but further physiological and dietary considerations must be first addressed before these results are generalized. We also reasoned that, since NCAS are bona fide ligands for sweet taste receptors (STRs) expressed in the intestine, some metabolic effects associated with NCAS use could be attributed to a common mechanism involving the host.

Results: We conducted a double-blind, placebo-controlled, parallel arm study exploring the effects of pure saccharin compound on gut microbiota and glucose tolerance in healthy men and women. Participants were randomized to placebo, saccharin, lactisole (STR inhibitor), or saccharin with lactisole administered in capsules twice daily to achieve the maximum acceptable daily intake for 2 weeks. In parallel, we performed a 10-week study administering pure saccharin at a high dose in the drinking water of chow-fed mice with genetic ablation of STRs (T1R2-KO) and wild-type (WT) littermate controls. In humans and mice, none of the interventions affected glucose or hormonal responses to an oral glucose tolerance test (OGTT) or glucose absorption in mice. Similarly, pure saccharin supplementation did not alter microbial diversity or composition at any taxonomic level in humans and mice alike. No treatment effects were also noted in readouts of microbial activity such as fecal metabolites or short-chain fatty acids (SCFA). However, compared to WT, T1R2-KO mice were protected from age-dependent increases in fecal SCFA and the development of glucose intolerance.

Conclusions: Short-term saccharin consumption at maximum acceptable levels is not sufficient to alter gut microbiota or induce glucose intolerance in apparently healthy humans and mice.

(Continued on next page)

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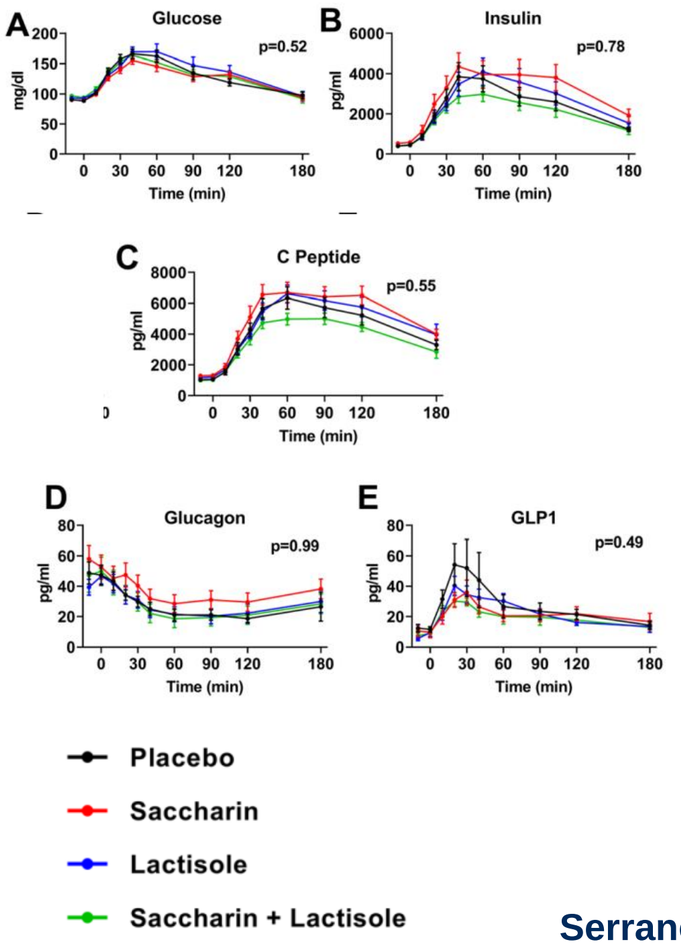

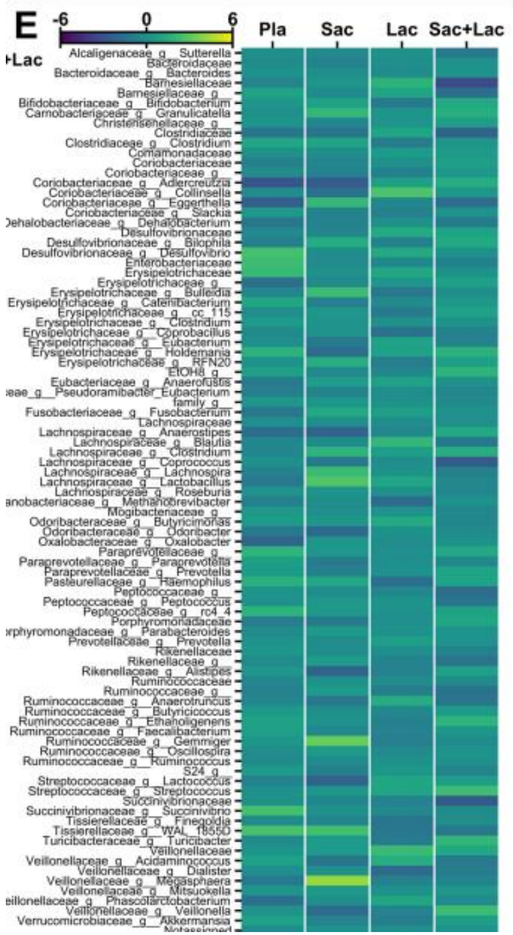

Serrano et al. Microbiome 2021;9:11

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Balancing the totality of the best available evidence, what does the data say about LNCS in sugars reduction?

New EASD clinical practice guidelines:

Evidence syntheses commissioned to address the discordance in LNCS research

Diabetologia
https://doi.org/10.1007/s00125-023-05894-8

GUIDELINES

Evidence-based European recommendations for the dietary management of diabetes

The Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD)

Abstract
Diabetes management relies on effective evidence-based advice that informs and empowers individuals to manage their health. Alongside other cornerstones of diabetes management, dietary advice has the potential to improve glycaemic levels, reduce risk of diabetes complications and improve health-related quality of life. We have updated the 2004 recommendations for the nutritional management of diabetes to provide health professionals with evidence-based guidelines to inform discussions with patients on diabetes management, including type 2 diabetes prevention and remission. To provide this update we commissioned new systematic reviews and meta-analyses on key topics, and drew on the broader evidence available. We have strengthened and expanded on the previous recommendations to include advice relating to dietary patterns, environmental sustainability, food processing, patient support and remission of type 2 diabetes. We have used the Grading of Recommendations, Assessment, Development and Evaluations (GRADE) approach to determine the certainty of evidence for each recommendation based on findings from the commissioned and identified systematic reviews. Our findings indicate that a range of foods and dietary patterns are suitable for diabetes management, with key recommendations for people with diabetes being largely similar for those for the general population. Important messages are to consume minimally processed plant foods, such as whole grains, vegetables, whole fruit, legumes, nuts, seeds and non-hydrogenated non-tropical vegetable oils, while minimising the consumption of red and processed meats, sodium, sugar-sweetened beverages and refined grains. The updated recommendations reflect the current evidence base and, if adhered to, will improve patient outcomes.

Keywords Diabetes management · Dietary guidance · Eating advice · Nutrition recommendations · Type 2 diabetes prevention · Type 2 diabetes remission

Abbreviations
DASH Dietary Approaches to Stop Hypertension
DNSG Diabetes and Nutrition Study Group
IGT Impaired glucose tolerance
MUFA Monounsaturated fatty acids
NNS Non-nutritive sweetener

Introduction

Diabetes management, including the prevention and remission of type 2 diabetes, relies on effective evidence-based advice that informs and empowers individuals to manage their health. Well-designed dietary recommendations and nutrition therapy are essential to improve both life expectancy and quality. However, the flood of nutrition information available is of variable quality, creates controversy regarding the best approaches, and is likely to confuse both people with diabetes and health professionals.

These new dietary recommendations for diabetes management have been produced by the Diabetes and Nutrition Study

Members of the Guideline Development Group for the Diabetes and Nutrition Study Group (DNSG) of the EASD are listed in alphabetical order in the Appendix. Those individuals are the authors of this article.

These guidelines were reviewed for EASD by its Committee on Clinical Affairs.

The Diabetes and Nutrition Study Group (DNSG) of the European Association for the Study of Diabetes (EASD)
For Guideline queries contact andrew.reynolds@otago.ac.nz

c/o Andrew Reynolds, Department of Medicine, University of Otago, Dunedin, New Zealand

Published online: 17 April 2023

Springer

Evidence-based European recommendations for the dietary management of diabetes: An EASD Clinical Practice Guideline

*"**Non-nutritive** sweeteners **may be used to replace sugars in beverages and foods. ⊕⊕⊕⊖ Moderate**"*

The DNSG-EASD Guideline Development Group. Diabetologia 2023 Jun;66(6):965-98

DNSG

EASD

European Association for the Study of Diabetes

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CONCLUSIONS

Conclusions

1.Although there are **concerns** that **LNCSs** may not have the intended benefits, **DNSG-commissioned network meta-analyses (NMA)** of **RCTs** with increased information size show that **LNCSBs** in displacing excess calories from SSBS (the "**intended substitution")** lead to **weight loss** and improvements in related **cardiometabolic risk factors, similar** to **water** (the **"standard of care"**).

2.These findings are supported by **network meta-analyses (NMA)** of **acute RCTs** which show no effect on metabolic and endocrine responses related to glucose and food intake regulation.

3.The improvements in intermediate risk factors appear to translate into reductions in patient and public health important cardiometabolic outcomes with **meta-analyses of prospective cohort studies** showing that the **intended substitution of LNCSBs for SSBs** is associated with **weight loss** and **reductions** in incident **obesity, CHD, and total mortality, similar** to **water** (the **"standard of care"**).

4.The certainty of the evidence is generally **moderate** for the **network meta-analyses (NMA) of RCTs** and generally **low** for the **meta-analyses of prospective cohort studies. The available evidence provides a good indication that the use of LNCSBs as an alternative replacement strategy for SSBs improves adiposity related intermediate outcomes over the moderate term in adults with overweight or obesity who are at risk for or have diabetes**

5.Ongoing RCTs (NCT01295671, NCT03259685, NCT03944616, NCT02591134) , along with our **STOP Sugars NOW trial (NCT03543644),** will contribute additional important data about the use of LNCSBs.

Acknowledgements

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Ms. Madeline Erlich(PhD student)
Ms. Diana Ghidanac (MSc student)
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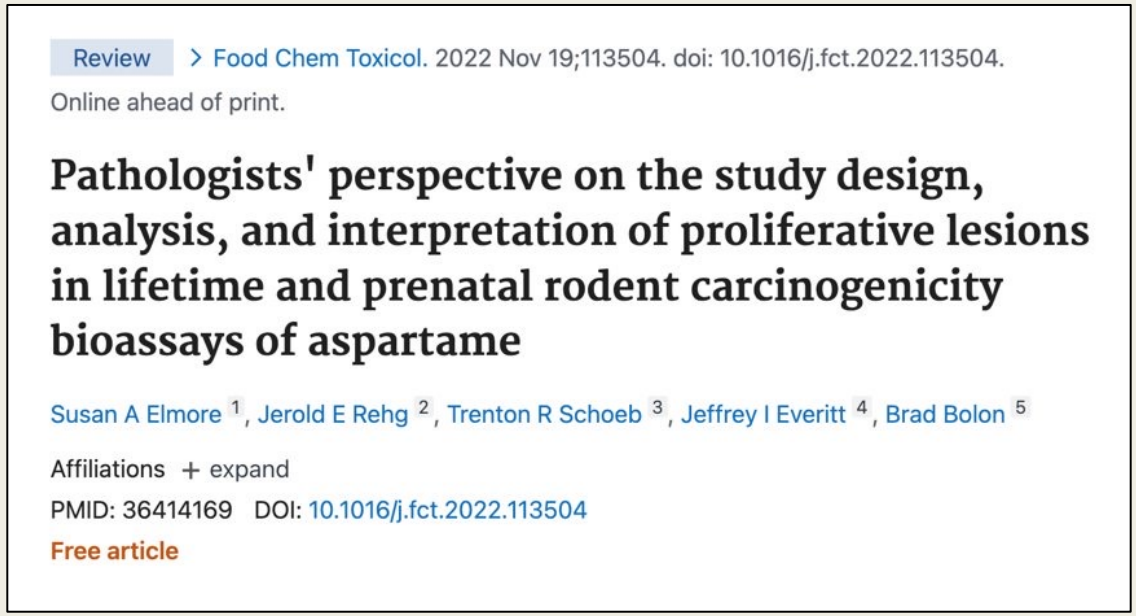
PATHOLOGISTS' PERSPECTIVE ON RAMAZZINI INSTITUTE ASPARTAME STUDIES

Susan A. Elmore, MS, DVM, DACVP, DABT, FIATP
Veterinarian Toxicologic Pathologist
ElmorePathology, LLC
On behalf of American Beverage

Aspartame has been studied extensively and evaluated for its safety in foods and beverages yet concerns for its potential carcinogenicity have persisted, driven primarily by 3 animal studies conducted at the Ramazzini Institute



Review > Food Chem Toxicol. 2022 Nov 19;113504. doi: 10.1016/j.fct.2022.113504.
Online ahead of print.

Pathologists' perspective on the study design, analysis, and interpretation of proliferative lesions in lifetime and prenatal rodent carcinogenicity bioassays of aspartame

Susan A Elmore [1], Jerold E Rehg [2], Trenton R Schoeb [3], Jeffrey I Everitt [4], Brad Bolon [5]

Affiliations + expand

PMID: 36414169 DOI: 10.1016/j.fct.2022.113504

Free article

Objective: To provide the perspective of experienced pathologists on publicly available pathology data regarding purported aspartame-related proliferative lesions in liver, lung, lymphoid organs, and mammary gland as well as their implications for carcinogenic hazard as reported for three lifetime rodent carcinogenicity bioassays of aspartame conducted at the Ramazzini Institute (RI) in Bologna, Italy

Funding: American Beverage provided funding to Dr. Susan Elmore alone in this review

Background
Ramazzini Aspartame Rodent Studies

- The RI has conducted 3 rodent bioassays to investigate the carcinogenic potential of aspartame:
  - A lifetime study in male and female Sprague Dawley rats (2005, 2006*, 2006*)
  - A prenatal lifetime study in male and female Sprague Dawley rats (2007, 2008*, 2011*)
  - A prenatal lifetime study in male Swiss mice (2010)
- Later publications* are just re-analyses of the original studies
- An additional study that attempted to characterize hematopoietic and lymphoid tumors using immunohistochemistry (IHC) was published in 2020

Outline: Summary of Pathologists' Specific Concerns

1. Method of combining lymphomas and leukemias with other neoplastic lesions
2. Method of combining proliferative lesions
3. Weight-of-evidence approach
4. Immunohistochemical evaluation of lymphomas and leukemias
5. Rodent health monitoring program
6. Concurrent infections
7. Historical control data
8. Methods of tissue fixation
9. Limitations of lifetime rodent carcinogenicity studies
10. High-quality images at suitable magnifications
11. Diagnostic criteria for immunoblastic lymphoma
12. Pathology peer review and public scientific review procedures
13. Prior comprehensive evaluation by United States government agencies
14. Determination of human relevancy for animal data

References for Standards of Practice

1. OECD Guideline for GLP Studies. https://www.oecd.org/chemicalsafety/testing/good-laboratory-practiceglp.htm#:~:text=The%20OECD%20Principles%20of%20Good,Acceptance%20of%20Data%20(MAD).
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1. Method of Combining Lymphomas and Leukemias with Other Neoplastic Lesions

- The RI interpretations of the two aspartame lifetime Sprague Dawley rat bioassays diverged from current practice in combining different types of pathological entities for carcinogenic hazard identification
  - *Example: Combined lymphoma and lymphoblastic/lymphocytic leukemias with histiocytic sarcomas, monocytic leukemias, and myeloid leukemias*
- Industry standard for chronic studies is to consider lymphoblastic / lymphocytic (i.e., “lymphoid”) leukemias and lymphomas under the single term “lymphoma”
- Other types of leukemias (e.g., myeloid, monocytic, erythroid) and histiocytic sarcomas are diagnosed separately as they arise from non-lymphoid cell lineages

2. Method of Combining Proliferative Lesions

By Type	By Location	By Sex
The RI diverged from current practice by combining tumor incidences with varying morphologies (i.e., combining non-neoplastic and neoplastic lesions)	The RI also diverged from current practice by combining tumor incidences from multiple topographies	The RI also diverged from current practice by combining incidences from males and females
Example: Dysplastic hyperplasias (non-neoplastic lesions), dysplastic papillomas (benign tumors), and carcinomas (malignant tumors) were combined (Table 4, 2006)	*Example: Combined tumor incidences from renal pelvis and ureter (Table 4, 2006)*	*Example: Reported incidences for males and females separately AND in combination (Tables 2, 3, 4, and 5, 2006)*

Long term carcinogenicity bioassay on ASPARTAME, administered with feed, supplied *ad libitum,* to male (M) and female (F) Sprague-Dawley rats

PRENEOPLASTIC AND NEOPLASTIC LESIONS OF THE TRANSITIONAL CELL EPITHELIUM OF THE RENAL PELVIS AND URETER

Group No.	Concentration (ppm)	Animals Sex	Animals No.	Animals with preneoplastic or neoplastic lesions [a,b,c,d] Dysplastic hyperplasias No.	%	Dysplastic papillomas No.	%	Carcinomas No.	%	Total No.	%
I	100,000	M	100	3	3.0	0	–	1	1.0	4	4.0
		F	100	8	8.0	3	3.0	4	**4.0**[#]	15	**15.0**[##]
		M+F	200	11	5.5	3	1.5	5	2.5	19	9.5
II	50,000	M	100	2	2.0	0	–	1	1.0	3	3.0
		F	100	6	6.1	1	1.0	3	**3.0**	10	**10.1**[##]
		M+F	200	8	4.0	1	0.5	4	2.0	13	6.5
III	10,000	M	100	2	2.0	0	–	1	1.0	3	3.0
		F	100	6	6.0	1	1.0	3[d]	**3.0**	10	**10.0**[##]
		M+F	200	8	4.0	1	0.5	4	2.0	13	6.5
IV	2,000	M	150	4	2.7	0	–	1	0.7	5	3.3
		F	150	6	4.0	1	0.7	3[d]	**2.0**	10	**6.7**[#]
		M+F	300	10	3.3	1	0.3	4	1.3	15	5.0
V	400	M	150	4	2.7	1	0.7	0	–	5	3.4
		F	150	5	3.3	1	0.7	3	**2.0**	9	**6.0**[#]
		M+F	300	9	3.0	2	0.7	3	1.0	14	4.7
VI	80	M	150	3	2.0	0	–	0	–	3	2.0
		F	150	4	2.7	1	0.7	1	**0.7**	6	**4.0**
		M+F	300	7	2.3	1	0.3	1	0.3	9	3.0
VII	0 (control)	M	150	1	0.7	0	–	0	–	1	0.7
		F	150	2	1.3	0	–	0	–[*#]	2	**1.3**[**##]
		M+F	300	3	1.0	0	–	0	–	3	1.0

Hyperplasias, papillomas and carcinomas should not be combined

Male and female incidences should not be combined

Lesions in the renal pelvis and ureter should be reported separately

3. Consideration of Tumor Interpretation Based on Weight-of-Evidence Approach

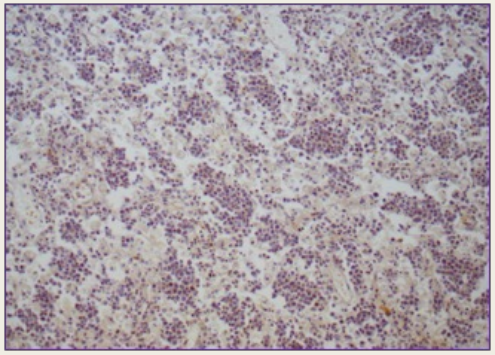
- Decisions regarding a carcinogenic effect should be based on a weight-of-evidence approach that considers the totality of the pathology data derived from one or more long-term carcinogenicity studies (generally in rodents) along with other appropriate experimental investigations
- Factors used to determine the weight-of-evidence approach are generally outlined and discussed when presenting data for hazard assessment and this was not done for the RI aspartame studies
- A mechanism of carcinogenesis should be supported by the finding *in vivo* of any dose-related tumors coupled with data showing that the test article induces major molecular-initiating events and cellular key events needed for tumor formation

3. Consideration of Tumor Interpretation Based on Weight-of-Evidence Approach

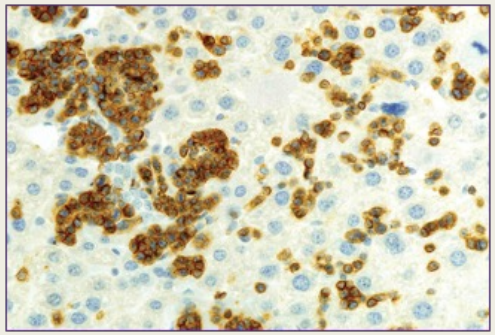
- Factors to be considered include, but are not limited to:
  - the presence of hyperplastic (non-neoplastic but potentially pre-neoplastic) lesions as well as benign and malignant neoplasms of the same cell lineage
  - similar lesions in other organs
  - tumor latency (especially accelerated time of tumor onset)
  - early mortality
  - tumor frequency (how common or rare a tumor is)
  - known sensitivity/resistance of the animal species and stock/strain to tumor induction
  - dose response (increasing incidence with increasing dose [whether the response curve is linear or non-linear])
  - concurrent control and historical control data
  - human relevance (based on cross-species or species-specific modes of action)
  - dose-related weight changes (loss or gain)
  - pharmacokinetic-pharmacodynamic (PK/PD) relationships
  - testing in more than one species and sex; etc.

4. Immunohistochemical Evaluation of Lymphomas and Leukemias

- To address the issue of distinguishing lymphoid tumors involving the lung from large lymphocyte-rich inflammatory cell aggregates that result from chronic respiratory tract infections, the RI published IHC data for the aspartame rodent bioassays in Sprague Dawley rats and discussed their relevance to the re-evaluation of lymphoid tumors
- Unfortunately, neoplastic and florid non-neoplastic lymphoid lesions in rodents cannot be definitively discriminated from one another in all circumstances using conventional IHC techniques (to assess cell type-specific but not disease-specific cell markers) and routine histopathologic evaluation
- Instead, IHC may be used to determine the lineage (B or T) of cells within a lymphoid tumor or inflammatory lesion
- TdT was the only useful marker to distinguish a neoplasm from a non-neoplastic lesion and essential data was lacking for this marker
- Methods such as polymerase chain reaction (PCR), flow cytometry, or Southern blot analysis are necessary to definitively determine clonality of the cells in question in order to differentiate a neoplasm (monoclonal or oligoclonal) from an inflammatory lesion (typically polyclonal)



Tibaldi 2020, Figure D



INHAND 2019, Figure 131

5. Rodent Health Monitoring Programs

- It is standard practice in North America and many European countries to use specific pathogen-free (SPF) animals at the start of toxicity studies and to employ rodent health surveillance programs to assure appropriate ongoing health status of the research animal test system
  - Conventional rodent health monitoring systems employ diagnostic examinations including molecular tests for nucleic acid sequences or antigens, bacterial cultures, serologic tests for existing immune responses to prior infections, parasite examinations, and microscopic evaluation of tissues
  - Animal colonies may be monitored directly or via use of sentinel animals within the study rooms to survey the collective health of the colony

5. Rodent Health Monitoring Programs

- The Internal Cancer Research Center colony was used for source animals
  - There was no indication of the health status of the colony at the time the animals were bred for the aspartame studies
  - There was no indication that the animals were monitored for genetic drift
  - This information is lacking in the published papers
- From 2009 onward, the RI has implemented practices in accordance with OECD guidelines, but this quality control procedure was implemented after the RI aspartame studies were either completed or underway

6. Concurrent Infections

- In the three RI lifetime rodent carcinogenicity bioassays for aspartame, the presence of lung lymphomas, liver carcinomas, and mammary adenocarcinomas cannot be reliably attributed to the effects from aspartame exposure since the same findings may be the result of chronic pathogen infections
- There was known mycoplasma positive serology and therefore infection within the rat colony https://downloads.regulations.gov/EPA-HQ-ORD-2009-0398-0021/attachment_4.pdf
- In both species, potential pathogen confounders render the tumor data questionable as a basis for making decisions regarding carcinogenic hazard

6. Concurrent Infections (Mycoplasma pulmonis)

- Data from aspartame and other rodent carcinogenicity bioassays (e.g., MTBE) conducted by the RI and reported in 1995 and 2002, especially those using rats, have been heavily criticized
- The most prominent concern was the presence of pronounced, coalescing lymphocyte-rich nodules associated with major pulmonary airways, which experienced laboratory animal pathologists believe were misinterpreted to be definitive evidence of test article-related lymphoma without acknowledging their substantial resemblance to classic bacteria-induced inflammatory lesions, such as *Mycoplasma pulmonis* (~90% were reported to have bronchitis, the signature lesion of *M pulmonis* disease)
- Pulmonary lymphoma and lymphocytic leukemia are difficult to distinguish from the excessive hyperplastic bronchus-associated lymphoid tissue that develops secondary to chronic subclinical infection with *M. pulmonis*
- Zella and coworkers have recently linked *Mycoplasma* infections in rodents with induction of lymphoma in *Prkdscid* (scid) mice of two genetic backgrounds (C57BL/6 and NOD)
- This confirmation that lymphoma may arise secondary to chronic bacteria-associated inflammation is an additional confounder for the interpretation of any putative chemically-induced lymphomas in animals infected with *Mycoplasma*
- Therefore, the RI should have assessed and reported at the time, using PCR or IHC, whether *M. pulmonis* DNA was detectable in the putative neoplastic tissues; therefore, these studies are not usable or interpretable

6. Concurrent Infections (Mycoplasma pulmonis)

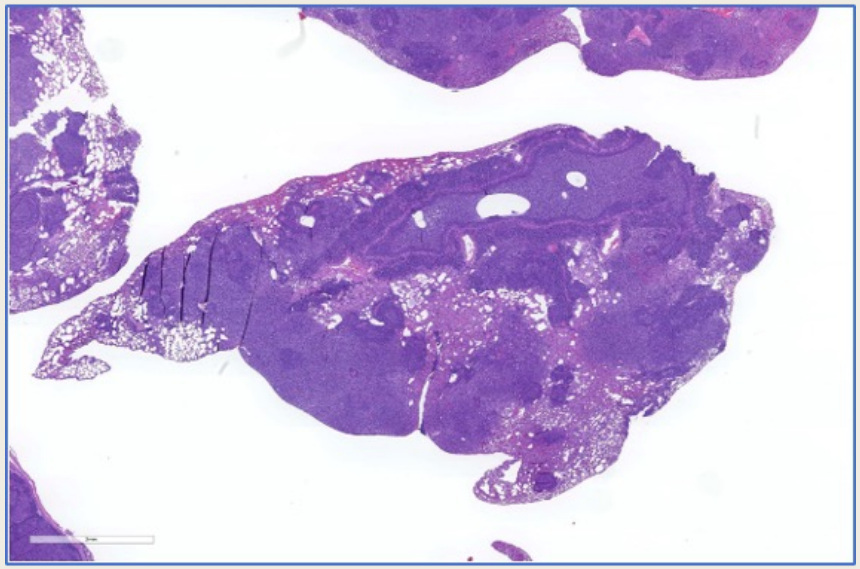
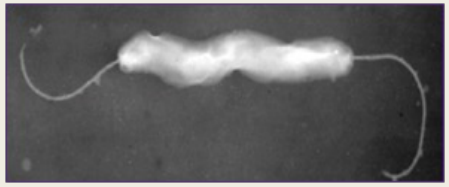


Image from: Joint Pathology Center
Veterinary Pathology Services
Wednesday Slide Conference
2019-2020
Conference 15
22 January 2020

- PCR for Mycoplasma spp. on fresh frozen lung was positive
- Illustrates the proliferative nature of this infection

6. Concurrent Infections (Helicobacter hepaticus)



- Prenatal infection with *Helicobacter hepaticus* induces liver tumorigenesis in mice via a cytolethal distending toxin
- *H. hepaticus* has also been shown to induce mammary adenocarcinomas in mice via a TNF*α*-dependent mechanism
- Another study found that host neutrophil-associated immune responses to intestinal tract microbes, such as *H. hepaticus*, have the potential to significantly impact cancer progression in mammary glands
- Given the reported hepatocellular carcinomas in male mice and mammary adenocarcinomas in female rats in the RI prenatal lifetime studies, evidence demonstrating SPF rodent colonies, and an appropriate health monitoring program, should have been published to confirm the absence of confounding tumorigenic mechanisms

6. Concurrent Infections - Example

- When lymphomas or leukemias related to test article exposure involve multiple tissues during 2-year rodent bioassays, there are generally clinical signs and/or an increased incidence of early deaths in treated groups that occur in a dose-related manner
- A search in the U.S. National Toxicology Program (NTP) archives for lung lymphomas and leukemias in rats indicates that most animals in a 2-year study with these tumors will be found moribund or dead before the designated terminal necropsy date, with these conditions serving as the sole or a major contributing cause of death
- In the three RI aspartame rodent studies, there is no dose-related increase in mortality due to the reported hematolymphoid tumors (HLTs), even with the incorrect inclusion of histiocytic sarcomas, monocytic leukemias and myeloid leukemias to this tumor category
- This stated pattern is consistent with the pathologists' perspective, bolstered by the NTP archival data, that the lymphoproliferative lesions in the RI rodent bioassays are indicative of a confounding inflammatory process in response to a chronic microbial infection rather than evidence of aspartame-related induction of lymphoid neoplasia

7. Historical Control Data

- The RI does not provide the historical control (HC) data in enough detail to determine whether one can be confident in the significance of the lesions noted for the three aspartame bioassays, even in the concurrent control group
- Although the concurrent control group is critical to assist in the interpretation of tumor incidence, reliable HC data are an important component of a holistic evaluation of tumor data
  - HC should also be divided into categories depending on the species, sex, route of administration, vehicle, study type, and breeder
  - The nomenclature conventions and diagnostic criteria should remain constant between studies
  - Criteria should be established to help determine if a study should be excluded from the HC database
  - HC tables should be updated periodically (~5 years) because animal colonies can vary over time due to extrinsic and intrinsic factors
- Providing HC information as supplementary data for published carcinogenicity studies is good practice

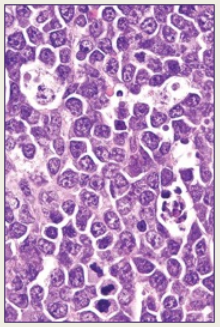
8. Methods of Tissue Fixation

- For the aspartame studies, organs and tissues (except for bone) were preserved in 70% ethyl alcohol (ethanol)
- This fixative has an advantage for immunohistochemistry and for laser capture microdissection studies of clonality but is a poor fixative for routine microscopic analysis of H&E-stained tissue sections
- The vast majority of toxicologic pathology studies use neutral buffered 10% formalin because it is far superior to 70% ethanol for preservation of fine structural detail in cells and tissues
- Fixation with 70% ethanol results in significant morphological artifacts that distort or obscure cell and tissue features
  - Artifacts include marked cell and tissue shrinkage, fragmentation of sections, and poor staining quality
  - These artifacts often compromise the pathologist's ability to provide accurate, reliable, and specific diagnoses
- Importantly, chronic inflammation may be difficult to distinguish from lymphoma unless the tissues are adequately preserved with no artefactual changes that would hinder diagnosis
- Such artifacts will also hinder the ability to distinguish hepatocellular tumors from pre-neoplastic foci

9. Limitations of Lifetime Rodent Carcinogenicity Bioassays

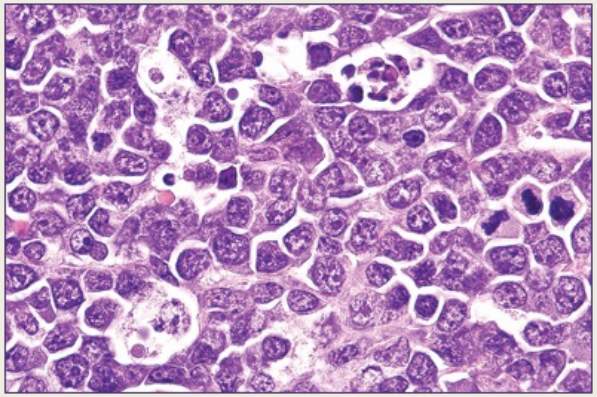
- OECD guidance for the testing of chemicals in carcinogenicity studies indicates that "[t]he duration of the study will normally be 24 months for rodents, representing the majority of the normal life span of the animals to be used."
- Issues with lifetime studies:
  - Increased neoplasms and non-neoplastic lesions
  - Comparison of lesion incidences becomes more complicated as the study progresses because older animals will tend to have more neoplastic and non-neoplastic lesions
  - Increased infections w/o effective health monitoring and infectious disease control measures
  - Presence of pathogenic organisms results in clinical and subclinical infections that often produce structural changes that accompany or obscure any test article-related effects, confounding interpretation
  - Death of animals at different days/weeks/months
  - Death from natural causes rises gradually, beginning at about 1 year of age
  - Allowing animals to die naturally increases the potential for animals to die overnight, increases the time between death and necropsy, increases the potential for autolysis of tissues
- These factors, taken together, may make it difficult to ascertain the biological relevance of any statistically significant differences in tumor incidences among control and treated animals past 2 years of age

10/11. Figures and Diagnostic Criteria that Illustrate Neoplasms of Concern



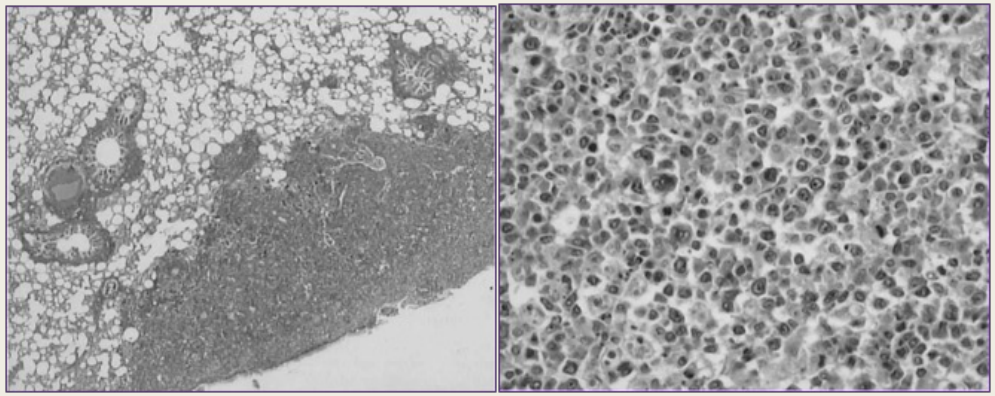
- Authors tend to publish their best (presumably “representative”) image examples as confirmation of diagnoses
- Importantly, images used for this purpose should be high-resolution, correctly formatted depictions (i.e., appropriate brightness, contrast, and colors) at a magnification that accurately displays key cell and tissue diagnostic features; more images at several different magnifications with a better narrative description would be warranted when documenting new or rare lesions
- The RI failed to provide such images to confirm diagnoses
- Example: Figures 9 and 10 in Soffritti et al., 2005 are reported to support a diagnosis of lymphoimmunoblastic [immunoblastic] lymphoma, but the histological criteria are not well described, the cellular features are not clear in the provided images and are not consistent with an immunoblastic lymphoma

10/11. Comparison of Published Figures and Diagnostic Criteria



2019 INHAND publication illustrating the cytomorphology of immunoblastic lymphoma

- Large, fairly monotypic cells
- Abundant amphophilic cytoplasm
- Large vesicular nuclei with prominent nucleoli
- Frequent mitotic figures



Figures 9 and 10 from Soffritti et al., 2005 illustrating a purported immunoblastic lymphoma

- Fixation artifact/autolysis
- Cellular features not described
- Variable cell appearance so not consistent with a lymphoid (especially immunoblastic) neoplasm
- Variable cell features are more consistent with severe infection

12. Pathology Peer Review and Public Scientific Review Procedures

- Independent scientific review is an essential element of modern scientific inquiry, a means by which data generated during experiments are cross-checked by other scientists as a quality control procedure to maximize data accuracy
- Pathology peer review is generally performed by one or more pathologists who will often have different subject matter expertise compared to that of the study pathologist
  - When animal studies are involved, qualified and experienced veterinary toxicologic pathologists are an essential part of the pathology peer review process
- In contrast, public scientific review is performed by scientists with variable degrees of pathology expertise and experience
- For animal studies slated for regulatory review, a post-publication peer review (PPPR) takes place when an article is published and is a valuable additional means of verifying diagnostic accuracy and interpretation to maximize the quality of the pathology raw data
- The RI did not invite a full independent PPPR for the aspartame carcinogenicity bioassays specifically, including a peer review of all tissue sections from all relevant studies as selected by the reviewing pathologists
- Allowing relevant stakeholders, including regulatory agencies and industries, to review the raw data, analyses and corresponding interpretations provides transparency in the testing program and increases confidence in situations where such data are used to make decisions regarding human health risk

13. Prior Comprehensive Evaluation by U.S. Government Agencies

- Two divisions of the U.S. Environmental Protection Agency (EPA), performed a comprehensive evaluation of the RI study designs, protocol differences, and accuracy of tumor diagnoses for their impact on carcinogenic hazard characterization
- Also considered an NTP report for a focused quality assurance (QA) and pathology working group (PWG) review of a subset of tissues from RI carcinogenicity studies of methanol, methyl tertiary-butyl ether (MTBE), ethyl tertiary-butyl ether (ETBE), vinyl chloride, and acrylonitrile (not a complete QA review or PWG evaluation)
- For the methanol and MTBE studies, the reviewing pathologists from the NTP pathology contractor diagnosed fewer lymphoid neoplasms, mainly of the respiratory tract, and indicated that there was chronic inflammation of the nasal cavity, ear canal, trachea, and lungs indicative of long-standing infection by one or more respiratory pathogens

13. Prior Comprehensive Evaluation by U.S. Government Agencies

- The NTP concluded that the findings suggest that the male and female rats in these lifetime drinking water studies had a persistent respiratory infection that confounded carcinogenic hazard identification in terms of lung-centered lymphoproliferative lesions
- The NTP also concluded that it is not unusual in the setting of pronounced chronic inflammation that inflammatory or regenerative lymphoid proliferations take on some neoplastic-like features
- This limited review by qualified pathologists underscores the difficulty that the RI had in diagnosing hematolymphoid tumors, most likely given the background inflammatory lesions, method of tissue fixation, autolysis, and lack of appropriate pathology peer review

14. Human Relevance

- The utility of animal data in identifying a hazard, and thus in assessing risk, is undercut if other factors confound diagnostic terminology, analyses and interpretation, which remains the case for the RI lifetime rodent bioassays for aspartame
- Understanding mechanistic pathways or mode of action for any chemical with significant human exposure is important
- Biological plausibility: Assuming RI-noted lesions are neoplastic in origin, the relevance of these lesions to human carcinogenesis can be assessed and ruled out if there is no mechanistic justification from aspartame exposure, particularly since aspartame has been present in the environment and consumed by humans for years without significant epidemiological results linking aspartame to increased incidences of hematolymphoid tumors
- These lesions (and others) might then be explained by the prevailing infection(s) in the study animals

Updated Systematic Assessment of Human, Animal and Mechanistic Evidence

- An updated systematic review of available human, animal, and mechanistic data was conducted leveraging critical assessment tools to consider the quality and reliability of data. The evidence base includes 12 animal studies and >40 epidemiological studies reviewed by the World Health Organization which collectively demonstrate a lack of carcinogenic effect
- Assessment of >1360 mechanistic endpoints, including many guideline-based genotoxicity studies, demonstrate a lack of activity associated with endpoints grouped to key characteristics of carcinogens
- Other non-specific mechanistic data (e.g., mixed findings of oxidative stress across study models, tissues, and species) do not provide evidence of a biologically plausible carcinogenic pathway associated with aspartame

Summary

- Taken together, available evidence supports that aspartame consumption is not carcinogenic in humans and that the inconsistent findings of the RI studies may be explained by flaws in study design and conduct (despite additional analyses to address study limitations), as acknowledged by authoritative bodies

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Review

Pathologists' perspective on the study design, analysis, and interpretation of proliferative lesions in lifetime and prenatal rodent carcinogenicity bioassays of aspartame

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ABSTRACT

Aspartame, an artificial sweetener commonly used as a sugar substitute, is currently authorized for use in more than 100 countries. Hundreds of studies, conducted in various countries dating back to the 1970s, have shown that aspartame is safe at real-world exposure levels. Furthermore, multiple human epidemiology studies have provided no indication that consumption of aspartame induces cancer. Given the continued controversy surrounding the Ramazzini Institute's (RI) studies suggesting that aspartame is a carcinogenic hazard in rodents and evaluation by the International Agency for Research on Cancer, this report aims to provide the perspective of experienced pathologists on publicly available pathology data regarding purported proliferative lesions in liver, lung, lymphoid organs, and mammary gland as well as their implications for human risk assessment as reported for three lifetime rodent carcinogenicity bioassays of aspartame conducted at the RI. In the authors' view, flaws in the design, methodology and reporting of the RI aspartame studies limit the utility of the data sets as evidence that this agent represents a carcinogenic hazard. Therefore, all three RI studies, and particularly the accuracy of their pathology diagnoses and interpretations, should be rigorously reviewed by qualified and experienced veterinary toxicologic pathologists in assessing aspartame's carcinogenic risk.

1. Introduction

Aspartame, an artificial sweetener that is 200 times sweeter than sucrose, is composed of the two naturally occurring amino acids phenylalanine and aspartic acid, modified by the addition of a methyl group to the phenylalanine (which yields the sweet taste). It is commonly found in prepared foods, low-calorie beverages, and as a table-top sweetener under trade names such as Equal®, NutraSweet®, and Canderel®. In the intestine, aspartame is metabolized rapidly and completely to the two parent amino acids while the methyl group is released as methanol. Over a hundred animal studies, including additional toxicological and mechanistic studies, conducted in various countries dating back to the 1970s, have shown that aspartame is safe at real-world exposure levels (see Table 1 for select examples) (Molinary et al., 1984; EFSA, 2013a; FDA, 1983; FDA, 2007). Furthermore, the vast majority of human epidemiology studies have provided no indication that consumption of aspartame induces cancer (WHO, 2022; Toews et al., 2019; Borghoff et al., 2022). As a result, aspartame has been deemed safe for human consumption by many regulatory agencies in their respective countries, including the United States (FDA, 2018), United Kingdom (Food Standards Agency, 2019), European Union (EFSA, 2013b), Canada (Health Canada, 2005), Japan (Japan External Trade Organization, 2011), China (USDA Global Agricultural Information Network, 2015), and India (Food Safety and Standards Authority of India, 2009) as well as the health authorities of Australia (Food Standards Australia New Zealand, 2021) and New Zealand (Food Standards Australia New Zealand, 2021).

The Ramazinni Institute (RI) in Bologna, Italy has conducted three rodent bioassays to determine the carcinogenic potential of aspartame. These include a lifetime study in male and female Sprague Dawley rats

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(Soffritti et al., 2005, 2006; Belpoggi et al., 2006), a prenatal lifetime study in male and female Sprague Dawley rats (Soffritti et al., 2007), and a prenatal lifetime study in male Swiss mice (Soffritti et al., 2010) (Tables 2–4). An additional study that attempted to identify hematopoietic and lymphoid tumors using immunohistochemistry (IHC) was published in 2020 (Tibaldi et al., 2020).

Given the expressed concerns regarding the interpretation of these studies within the scientific community, including the European Food Safety Authority (EFSA, 2013a; EFSA, 2006a; EFSA, 2013b), this critical review provides an expert pathology perspective on multiple biological factors identified as potential complicating factors in these RI bioassays. Particular issues that will be considered are listed below and included in part in Table 5:

- practices of combining proliferative lesions for interpretation (e.g., lymphomas with leukemias, hyperplastic [non-neoplastic] and neoplastic lesions),
- methods for reporting tumor incidences,
- weight-of-evidence approach to enhance the assessment of carcinogenic risk to humans,
- immunohistochemical (IHC) evaluation of hematolymphoid proliferation to determine whether lymphoid proliferative findings represent lymphoma, leukemia, or an inflammatory reaction,
- relevance of rodent health monitoring programs in rodent carcinogenicity testing,
- the potential for concurrent infections (e.g., *Mycoplasma pulmonis* and *Helicobacter hepaticus*) to complicate data interpretation in animal research,
- methods of tissue fixation for histopathological evaluation,
- utility and limitations of lifetime rodent carcinogenicity studies,
- importance of high-quality images at suitable magnifications to accurately illustrate the diagnostic features in tumors,
- diagnostic criteria for immunoblastic lymphoma,
- pathology peer review and public scientific review procedures,
- prior comprehensive evaluation by United States (U.S.) government agencies, and
- determination of human relevancy for animal data.

Table 1
Outcomes of select aspartame rodent studies.

Finding	Laboratory	Species and Stock/Strain	Route	Duration	Source
Positive	RI	Sprague Dawley rats	Diet	Lifetime	Soffritti et al., 2005, 2006; Belpoggi et al., 2006
Positive	RI	Sprague Dawley rats	Diet	Prenatal/Lifetime	Soffritti et al., 2007; Chiozzotto et al., 2011
Positive	RI	Swiss mice	Diet	Prenatal/Lifetime	Soffritti et al., 2010
Null	Non-RI	C57BL/6 Ela-Tg mice	Drinking water	GD 0–104 weeks	Dooley et al., 2017
Null	Non-RI	FVB Tg.AC hemizygous mice	Diet	9 months	NTP 2005
Null	Non-RI	B6.129-Cdkn2a deficient mice	Diet	9 months	NTP 2005
Null	Non-RI	P53 haploinsufficient mice	Diet	9 months	NTP 2005
Null	Non-RI	F344 rats	Diet	36 weeks	Hagiwara et al., 1984
Null	Non-RI	Wistar rats	Diet	2 years	Ishii 1981; Ishii et al., 1981
Null	Non-RI	CR Albino rats	Diet	104 weeks	E33-34, Searle, 1973
Null	Non-RI	CR Albino rats	Diet	GD 0–104 weeks	E70, Searle, 1974a
Null	Non-RI	ICR Swiss mice	Diet	104 weeks	E75, Searle, 1974b

GD = gestational day
NTP = U.S. National Toxicology Program
RI = Ramazinni Institute

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Table 2
Aspartame administered to male and female Sprague Dawley rats in feed, supplied *ad libitum*, from 8 weeks of age until spontaneous death.[a]

Tumors	Lesion Incidence (%)						
Dose (ppm)[b]	0	80	400	2,000	10,000	50,000	100,000
Lymphoma and Leukemia in Females	8.7	14.7	20.0##	18.7#	19.0#	25.0##	25.0##
Renal Pelvis Carcinomas in Females	0	0.7	2.0	2.0	3.0	3.0	4.0#

#Statistically significant ($p < 0.05$) using Poly-k test ($k = 3$).
##Statistically significant ($p < 0.01$) using Poly-k test ($k = 3$).
[a] Source: Belpoggi F et al. Results of long-term carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame administered in feed. *Ann N Y Acad Sci.* Sep 2006; 1076:559–77.
[b] Doses equivalent to 0, 0.004, 0.02, 0.1, 0.5, 2.5 and 5g/kg body weight/day (as defined in: European Food Safety Authority (EFSA). Scientific opinion on the re-evaluation of aspartame (E 951) as a food additive. *EFSA Journal.* 2013; 11(12):3496).

Table 3
Aspartame administered to male and female Sprague Dawley rats prenatally and postnatally (in milk and later feed, supplied *ad libitum*) until spontaneous death[a,b].

Tumors	Lesion Incidence (%)		
Dose (ppm)[c]	0	400	2000
Lymphoma and Leukemia in Males[d]	9.5	15.7	17.1*
Lymphoma and Leukemia in Females[d]	12.6	17.1	31.4**
Mammary Gland Adenocarcinoma in Females[e]	5.3	7.1	15.7*

*Statistically significant ($p < 0.01$) using Cox regression model.
**Statistically significant ($p < 0.05$) using Cox regression model.
[a] Source of original study: Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M. Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. *Environ Health Perspect.* Sep 2007; 115(9):1293–7.
[b] Source of updated information with historical control data: Chiozzotto D SM et al. Results of life span carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame since foetal life. *Eur J Oncol.* 2011; 16(2):81–97. Tables 1 and 3.
[c] Doses equivalent to 0, 0.02, and 0.1 g/kg body weight/day (as defined in: European Food Safety Authority (EFSA). Scientific opinion on the re-evaluation of aspartame (E 951) as a food additive. *EFSA Journal.* 2013; 11(12):3496.
[d] Historical control data not reported for lymphoma and leukemia.
[e] In the past 20 years the overall incidence of mammary adenocarcarcinomas in the females of the RI colony was 9.0% (range 4.0–14.2%) among 2,424 females; however, it was not reported that the historical control database was specifically based on prenatal studies or divided into other appropriate categories such as route of administration, vehicle, etc.

Table 4
Aspartame administered to male Swiss mice prenatally and postnatally (in milk and later feed, supplied *ad libitum*) until spontaneous death.[a]

Tumors	Lesion Incidence (%)				
[b]Dose (ppm)	0	2,000	8,000	16,000	32,000
Lung Alveolar Carcinoma	6.0	5.8	11.3	12.5	13.3**
Hepatocellular Carcinoma	5.1	11.7	14.5	15.6*	18.1**

*Statistically significant ($P < 0.05$) using Cox proportional hazard model.
**Statistically significant ($P < 0.01$) using Cox proportional hazard model.
[a] Source: Soffritti M et al. Aspartame administered in feed, beginning prenatally through life span, induces cancers of the liver and lung in male Swiss mice. *Am J Ind Med.* Dec 2010; 53(12):1197–206.
[b] Doses equivalent to 0, 0.25, 1, 2, and 4 g/kg body weight (as defined in: European Food Safety Authority (EFSA). Scientific opinion on the re-evaluation of aspartame (E 951) as a food additive. *EFSA Journal.* 2013; 11(12):3496.

Table 5
Comparison of methods used in Ramazzini Institute aspartame studies.

Method	Lifetime study in rats[a,b,c,d]	Lifetime and prenatal study in rats[d,e,f]	Lifetime and prenatal study in mice[g]
Appropriate combination of proliferative lesions for interpretation	No	No	Yes
Weight-of-evidence approach used to determine relevance of tumor incidences	No	No	No
Relevant IHC evaluation of hematolymphoid proliferation	No	No	N/A
Appropriate rodent health monitoring program	No	No	No
Potential for concurrent infections to complicate data interpretation	Yes	Yes	Yes
Appropriate method of tissue fixation for histopathological evaluation	No	No	No
Public access to high-quality images	No	No	No
Presentation of detailed diagnostic criteria and descriptions for proliferative lesions	No	No	No
Appropriate and timely pathology and public review procedures	No	No	No
Appropriate determination of human relevance	No	No	No

IHC = immunohistochemical; N/A = not applicable.
[a] Soffritti MBF, Esposti DD, Lambertini L. Aspartame induces lymphomas and leukaemias in rats. *Eur J Oncol.* 2005; 10(2):107–116.
[b] Belpoggi F et al. Results of long-term carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame administered in feed. *Ann N Y Acad Sci.* Sep 2006; 1076:559–77.
[c] Soffritti M, Belpoggi F, Degli Esposti D, Lambertini L, Tibaldi E, Rigano A. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. *Environ Health Perspect.* Mar 2006; 114(3):379–85.
[d] Tibaldi E, Gnudi F, Panzacchi S et al. Identification of aspartame-induced hematopoietic and lymphoid tumors in rats after lifetime treatment. *Acta Histochem.* Jul 2020; 122(5):151548.
[e] Soffritti M, Belpoggi F, Tibaldi E, Esposti DD, Lauriola M. Life-span exposure to low doses of aspartame beginning during prenatal life increases cancer effects in rats. *Environ Health Perspect.* Sep 2007; 115(9):1293–7.
[f] Chiozzotto D SM, Falcioni L, Tibaldi E, Manservisi F, Manservigi M, Bua L, Belpoggi F. Results of life span carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame since foetal life. *Eur J Oncol.* 2011; 16(2):81–97.
[g] Soffritti M, Belpoggi F, Manservigi M et al. Aspartame administered in feed, beginning prenatally through life span, induces cancers of the liver and lung in male Swiss mice. *Am J Ind Med.* Dec 2010; 53(12):1197–206.

Current industry standards that represent acceptable methods used in toxicologic pathology and recommended by the U.S. National Toxicology Program (NTP) and national and international regulatory agencies are also discussed.

2. Considerations for diagnosing and combining proliferative lesions for interpretation

Proliferative findings with different presumed biological behaviors (e.g., localized vs. systemic neoplasms) arising from the same cell of origin may be combined for analysis under some circumstances (McConnell et al., 1986; Brix et al., 2010). In such situations, great care must be exercised to only combine similar entities and not comingle unrelated lesions. The RI interpretations of the two aspartame lifetime Sprague Dawley rat bioassays (Soffritti et al., 2005, 2006, 2007; Belpoggi et al., 2006) diverged from current practice in combining different types of pathological entities for carcinogenic hazard identification and risk assessment.

2.1. Combining lymphocytic leukemias and lymphomas

The hematolymphoid tumors (HLTs) that were combined for analysis and carcinogenic hazard assessment in the RI aspartame studies were "lymphoblastic lymphoma and leukemia, lymphocytic lymphoma, lymphoimmunoblastic lymphoma, histiocytic sarcoma, monocytic leukemia and myeloid leukemia" (Soffritti et al., 2005). Industry standard for chronic studies is to consider lymphoblastic/lymphocytic (i.e., "lymphoid") leukemias and lymphomas under the single term "lymphoma" (McConnell et al., 1986; Brix et al., 2010). *Other types of leukemias (e.g., myeloid, erythroid) and histiocytic sarcomas are diagnosed separately as they arise from non-lymphoid cell lineages* (McConnell et al., 1986; Brix et al., 2010; Willard-Mack et al., 2019). Therefore, histiocytic sarcomas, monocytic leukemias and myeloid leukemias should not be combined with lymphoid tumors, as was done by the RI, for determining group incidences for analyses of potential test article effects. Without knowledge of the incidences of these different tumors, any potential relationship to the test article cannot be determined if this degree of combination were to be employed.

Regarding the diagnosis and combination of leukemia vs. lymphoma, both can occur spontaneously in aged rodents, although the tissue of origin may differ. Lymphoma originates from lymphocytes of lymphoid tissues such as the spleen, thymus, or lymph nodes; typically presents as single or multicentric solid tumors; and may ultimately disseminate to many tissues/organs through the vasculature. Primary lung lymphomas are exceedingly rare in all species. Leukemia is a neoplasm that originates from blood cells of the bone marrow or spleen (especially in the mouse/rat) which then spreads in the blood to infiltrate multiple organs including thymus, spleen, liver, and lymph nodes. Leukemia develops from precursors of any hematopoietic cell class: erythrocytes (red blood cells); leukocytes (white blood cells including lymphocytes, granulocytes, monocytes, mast cells, etc.); and megakaryocytes (platelet precursors). Thus, one may diagnose lymphocytic (or lymphoblastic) leukemia, granulocytic leukemia, monocytic leukemia, erythroid leukemia, megakaryocytic leukemia, etc.—all of which are classified as leukemias even though they arise from distinct marrow cell lineages. The cell morphology and biomarkers of lymphoid leukemia (and also lymphoma) are generally different from those of leukemias that originate from other blood cell lineages (Willard-Mack et al., 2019). Therefore, combining lymphoid tumors with leukemias of other cell origins is inappropriate.

As indicated previously, it is industry standard to consider lymphoblastic/lymphocytic (i.e., "lymphoid") leukemias and lymphomas under the single term "lymphoma" for chronic studies. In the context of a 2-year rodent carcinogenicity bioassay, and especially a lifetime study without interim time points, the longer lifespan provides increased time to allow hematopoietic neoplasia to arise and spread to multiple organs, often to such an extent that the initial site of origin cannot be determined. For this reason, combining all lymphoid neoplasms as a single finding for interpretation, whether they are lymphomas or lymphoid leukemias, is warranted in mice and rats from chronic bioassays regardless of the presence of neoplastic lymphocytes in various organs (spleen, liver, lymph node, etc.), peripheral blood, and/or bone marrow. Understandably, combining the lymphomas and lymphocytic leukemias may give a falsely high risk ratio because lymphomas are not leukemias and both categories are composed of various lymphoid subtypes. If lymphomas and lymphoid leukemias could be reliably separated out as individual entities, the incidence of the lymphoma or leukemia may not be statistically increased and thus might not be relevant for assessing human risk. However, since a precise distinction is not always possible in chronic rodent studies, the conservative approach to human risk assessment is to combine lymphoid-derived neoplasms (leukemia and lymphoma) for data interpretation. Since the RI aspartame studies combined all hematolymphoid tumors, rather than just the lymphomas and lymphoid leukemias, for statistical analysis, this data should not be considered for determining risk assessment.

2.2. Combining other tumor types in assessing carcinogenic risk

The RI analysis and interpretation of the aspartame lifetime rodent bioassays did not follow industry standard practices in combining proliferative lesions. First, combining the incidences of rodent tumors with varying morphologies and topographies is of questionable relevance for predicting carcinogenic potential in humans (Haseman et al., 1986). Second, combining non-neoplastic and neoplastic lesions for body systems is contrary to recognized industry standards for data analysis and interpretation. For example, the 2006 RI study (Belpoggi et al., 2006) approach to combine atypical hyperplasias and dysplasias (non-neoplastic precursor changes) with benign (papilloma) and malignant (carcinoma) tumors of the renal pelvis and ureter is not standard scientific practice. Moreover, epithelial tumors present in distinct organs (e.g., renal pelvis [kidney] vs. ureter) should be reported separately. In following the standard practice for carcinogenic study evaluation, renal pelvis tumors are diagnosed separately from ureteral tumors; if the same tumor (based on histological features) appears to occur in both sites, an attempt would be made to determine the organ (site) of tumor origin or confirm the induction of separate tumors arising independently from the same cell lineage at two sites. If the origin could not be determined, the pathology report would indicate which organ had the largest mass of neoplasm and discuss the likelihood of local extension to the adjoining organ/tissue.

In limited instances, some benign and malignant tumors may be combined as long as they occur in the same organ (e.g., hepatocellular adenoma [benign] and carcinoma [malignant] in liver), are of the same cellular origin (e.g., hepatocyte), and it has been documented that there is a spectrum of progression in proliferative lesions leading to carcinoma formation over time. For such situations, incidences of the benign and malignant lesions would be recorded separately, and also considered in combination (for each sex separately), for interpretation. Although tumor combinations were done inappropriately for the two RI rat aspartame studies (Soffritti et al., 2005, 2006, 2007; Belpoggi et al., 2006), they were combined appropriately for liver and lung tumors in the male Swiss mice prenatal lifetime study (Soffritti et al., 2010). When benign and malignant tumors are not of the same cellular origin and/or are associated with a known spectrum of tumor progression, incidences of benign and malignant lesions represent distinct entities and should be recorded, analyzed, and interpreted separately.

3. Consideration of tumor interpretation based on weight-of-evidence approach

Decisions regarding a carcinogenic effect should be based on a weight-of-evidence approach that considers the totality of the pathology

data derived from one or more long-term carcinogenicity studies (generally in rodents) along with other appropriate experimental investigations. Factors used to determine the weight-of-evidence approach are generally outlined and discussed when presenting data for hazard assessment or risk analysis and this was not done for the RI aspartame studies. The variety of factors to be considered include the presence of hyperplastic (non-neoplastic but potentially pre-neoplastic) lesions as well as benign and malignant neoplasms of the same cell lineage, similar lesions in other organs, tumor latency (especially accelerated time of tumor onset), early mortality, tumor frequency (how common or rare a tumor is), known sensitivity/resistance of the animal species and stock/strain to tumor induction, dose response (increasing incidence with increasing dose [whether the response curve is linear or non-linear]), concurrent control and historical control data, human relevance (based on cross-species or species-specific modes of action), dose-related weight changes (loss or gain), pharmacokinetic-pharmacodynamic (PK/PD) relationships, testing in more than one species and sex, etc. In addition, tumor types should be reported separately for male and female animals as the combination of tumors in both sexes is inappropriate (Tumors in males and females were inappropriately reported separately and in combination [for each sex, benign and malignant tumors considered together] for the RI lifetime and prenatal carcinogenicity studies in Sprague Dawley rats (Soffritti et al., 2005; Belpoggi et al., 2006; Chiozzotto et al., 2011)). A mechanism of carcinogenesis should be supported by the finding *in vivo* of any dose-related tumors coupled with data showing that the test article induces major molecular-initiating events and cellular key events needed for tumor formation (Jacobs et al., 2020; Downes and Foster, 2015; Perkins et al., 2019). Examples of additional experimental investigations include, but are not limited to, shorter *in vivo* toxicity studies and selected *in vitro* studies that provide evidence of particular modes of action (e.g., genotoxicity leading to mutagenicity, hormonal disruption, immunosuppression, and long-term toxicity causing repeated cycles of cell death and cell proliferation) that have relevance for human carcinogens. Such an approach enhances the assessment of carcinogenic risk to humans.

4. Immunohistochemical evaluation of lymphomas and leukemias

To address the issue of distinguishing HLTs involving the lung from large, lymphocyte-rich inflammatory cell aggregates that result from chronic respiratory tract infections, the RI published IHC data for the aspartame rodent bioassays in Sprague Dawley rats and discussed their relevance to the re-evaluation of HLTs (Tibaldi et al., 2020). The RI authors indicated that their aim was to reaffirm the previous HLT diagnoses using updated morphological terminology and criteria (Willard-Mack et al., 2019), attempting to exploit IHC analysis to further characterize the lymphoid tumors and their association with aspartame exposure. The results of this study purported to reinforce the hypothesis that aspartame has a "leukaemogenic and lymphomatogenic effect" (Tibaldi et al., 2020). Unfortunately, neoplastic and florid non-neoplastic lymphoid lesions in rodents cannot be definitively discriminated from one another in all circumstances using conventional IHC techniques and routine histopathologic evaluation. Instead, IHC may be used to determine the lineage (B or T cell) of a lymphoid tumor while methods such as polymerase chain reaction (PCR), flow cytometry, or Southern blot analysis are more capable of determining clonality of the cells in question, if needed in order to differentiate a neoplasm from an inflammatory lesion.

Chronic inflammation in the lung may have different characteristics depending on the duration of the lesion and initiating cause (Renne et al., 2009). The distribution may involve the bronchi, terminal bronchioles, alveoli, and/or pleura. When chronic, the cellular infiltrate in rodents may comprise predominantly lymphocytes with moderately fewer plasma cells and variable numbers of macrophages. Chronic inflammation may be difficult to distinguish from lymphoma unless the tissues are adequately preserved with no artefactual changes that would hinder diagnosis. Airway insufflation using a fixative solution that adequately preserves cellular architecture, such as neutral buffered 10% formalin, is imperative. Continuous exposure to toxic or infectious agents – or inability to clear these from lung parenchyma – leads to more severe and widespread airway and interstitial inflammation over time in an attempt to eliminate or sequester the causative agent (Renne et al., 2009).

Table 6
International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) diagnostic features for immunoblastic lymphoma in rodents.

- Cells are large, non-cohesive, and monotypic
- Cytoplasm is conspicuously amphophilic
- Nuclei are large and vesicular with one large, sometimes bar-shaped, central, or peripheral nucleolus
- Mitotic figures may be numerous
- May be of B cell (more commonly) or T cell origin
- Plasmacytoid cells and plasma cells may be present
- Rare in most non-genetically engineered strains
- Pattern of organ involvement shows diffuse infiltration of lymph nodes, spleen, liver, kidneys, and ovaries and along the vascular tree in the lung (similar to lymphoblastic lymphoma); not primarily leukemic in distribution
- When of B cell origin, cells produce immunoglobulin (Ig) heavy chains or kappa light chains but rarely express lambda light chains

Reprinted in part from Willard-Mack CL et al. Nonproliferative and proliferative lesions of the rat and mouse hematolymphoid system. *Toxicol Pathol.* Aug 2019; 47(6):665–783, with permission of Sage.

In rodent toxicologic pathology, the industry standard practice is that a hematopoietic neoplasm is generally diagnosed with a combination of cellular and tissue morphological features (as seen in routine hematoxylin and eosin [H&E]-stained sections) using harmonized terminology (e.g., INHAND [International Harmonization of Nomenclature and Diagnostic Criteria] (Table 6; Willard-Mack et al., 2019)). Thereafter, IHC may be used to determine the lineage of neoplastic cells and, if warranted, the lineages of leukocyte populations within inflammatory foci. Based on a detailed morphologic examination, the overall nature of the lesion would be characterized at low magnifications using H&E to define cell location, density, arrangement (sheets, aggregates), cohesion (solid, papillary, etc.), and invasion of surrounding tissues. Higher magnifications would then be used to evaluate cytoarchitectural traits such as cell size as well as specific features of the cytoplasm, nuclei, nucleoli, nuclear to cytoplasmic ratio, number and appearance of mitotic figures, presence of apoptotic cells, etc. (Table 6). This information is evaluated in a holistic manner to determine if the lesion is inflammatory or neoplastic in character. If neoplastic, IHC could then be used at the discretion of the pathologist to define the cell lineage (B cell, T cell, etc.).

The diagnosis and classification of hematolymphoid malignancies in rodents may be undertaken in several ways. Flow cytometry for immunophenotyping has several advantages over IHC in accomplishing this purpose, including more accurate cell sub-type identification and clonality determination, but flow cytometry is best accomplished using fresh tissue specimens to maintain the intact conformation of leukocyte antigens. Routine IHC is performed commonly on tissues fixed in neutral buffered 10% formalin and then embedded in paraffin (i.e., FFPE); in such FFPE preparations, robust leukocyte antigens may be preserved (e.g., B cell and T cell markers) while more labile antigens needed for molecular clonality analysis are often disrupted. *Under the best circumstances, it is often not possible to determine whether a lymphocyte-rich proliferative lesion represents a neoplasm, reactive hyperplasia, or inflammation using IHC markers.* In such cases, specific clonality analyses should be performed. PCR and Southern blot analyses are commonly used to detect either clonal immunoglobulin (Ig) heavy chain (IgH) or clonal T cell receptor (TCR) gene rearrangements. These analyses are dependent on the test specimen having DNA of good quality (i.e., minimal strand

disruption associated with tissue preservation and processing conditions).

In some circumstances, when dealing with B cell proliferations, IHC can be used in some species to test for immunoglobulin light chain (IgL) restriction. This assay may be used as a surrogate marker for clonality in some species. Unfortunately, detection of kappa and lambda light chain clonality is not useful in rodents because the majority (~95%) of neoplastic and inflammatory lymphocytes in rodents express kappa light chains (Woloschak and Krco, 1987). Detecting clonality is in itself inadequate for rendering a diagnosis of lymphoma because benign lymphoid infiltrates can harbor clonal gene rearrangements (Schafernak et al., 2014). Except for TdT (terminal deoxynucleotidyl transferase), the limited panel of standard antibodies used in the RI study (Tibaldi et al., 2020) was not capable of differentiating a hematopoietic lymphoid tumor from a lymphocyte-rich reactive (i.e., non-neoplastic inflammatory) process. Additionally, the RI investigators did not indicate how many neoplasms and what percentage of their presumed neoplastic cells expressed the TdT antigen. Moreover, the criteria for classifying a neoplasm as positive for any of the biomarkers in the antibody panel were not provided. Therefore, it is not possible to assess the degree of homogeneity for a specific IHC marker within a presumed neoplasm for the RI rodent bioassays for aspartame. The specificity of CD33 (a marker for multi-lineage hematopoietic progenitors) varies by species (Brinkman-Van der Linden et al., 2003), and the RI authors have not documented the utility of this biomarker in rats.

Lymphoblastic lymphoma is a rapidly progressive disease that can occur at any age in rodents, but it is more common in animals younger than 18 months of age. Therefore, in lifetime rodent bioassays it is very unusual to have an increased incidence of lymphoblastic lymphoma in aged animals as this highly lethal tumor typically precludes animals from surviving to 2 years of age. On the other hand, lymphoblasts and immunoblasts are often present in reactive inflammatory processes of rodents at all stages of life. Unfortunately, the RI investigators do not clearly describe the morphological criteria they used for discriminating various lymphoid lesions in the aspartame lifetime rodent bioassays. In particular, lack of reporting of key features prevented the differentiation of the generic term “lymphoma” as either lymphoblastic lymphoma, immunoblastic lymphoma, or any other recognized variant of hematolymphoid neoplasm (Table 6). Furthermore, the RI investigators do not identify any significant correlation between cytomorphology and the immunoreactivity of the lymphomas other than whether the tumor expressed CD3 (a T cell marker) or CD20 (a B cell marker). Similarly, no biomarker expression patterns are noted that distinguish presumed lymphocytic neoplasms from lymphocyte-rich reactive (non-neoplastic) lesions. Consequently, except for the use of TdT, it is not clear how the use of IHC as described in the publication by Tibaldi and coworkers (Tibaldi et al., 2020) publication was interpreted to provide definitive evidence that any lymphoid proliferation represented a clonal neoplastic lesion (i.e., test article-related lymphoma) and not a polyclonal immune response (i.e., confounding pathogen-induced chronic inflammatory reaction).

Two members of the Scientific Advisory Board to the RI published a commentary that claims the increases in various types of lymphomas and lymphocytic leukemias reported in earlier rodent carcinogenicity studies of aspartame at the RI have been “validated” (Landrigan and Straif, 2021). They stated that the RI subjected all HLTs from aspartame-exposed animals to IHC analyses and appropriate morphological reclassification. As described above, routine IHC analyses the RI performed could not confirm that the lesions were neoplastic in nature, and the morphology of the lesions were neither described in detail nor presented as suitable figures (i.e., of high resolution and multiple magnifications with large image sizes in print) to allow readers the chance to confirm for themselves the diagnoses and interpretations reported by the RI team in the paper. Importantly, IHC of non-neoplastic lesions in proliferative *M. pulmonis*-infected lung tissue of rodents would be expected to exhibit similar results as those obtained from IHC of HLTs.

In conclusion, the use of IHC to characterize lymphoid lesions in rodent tissues may determine whether the lymphocytes are B cells or T cells, but IHC cannot discern conclusively whether the lymphocyte population is neoplastic or inflammatory in nature. As stated by Gift and coworkers (Gift et al., 2013), IHC may be used as a part of clonality assays, but should not serve as the sole clonality assay *per se*. Although Gift and colleagues also indicate that ethanol-fixed tissue should be adequate for molecular clonality (PCR) assays, the RI did not report any PCR findings for the three aspartame rodent bioassays in question. Given the controversy around potential *M. pulmonis* infection in the two Sprague Dawley RI lifetime rodent bioassays of aspartame, PCR tests for *M. pulmonis* and other rodent pathogens that cause marked lymphoid hyperplasia would have been useful, even essential parameters to determine if chronic inflammation due to a bacterial or viral infection was present in the suspect lymphoproliferative lesions (Loens and Ieven, 2016).

5. Rodent health monitoring programs

Rodent health monitoring programs, including indirect health surveillance and sentinel monitoring programs, are designed to detect subclinical infections that may detrimentally impact biological research (NRC, 1991). Such programs employ batteries of diagnostic tests in order to define the pathogen load and health status of a research animal population. It is standard practice in North America and many European countries to use specific pathogen-free (SPF) animals at the start of toxicity studies and to employ rodent health surveillance programs to assure appropriate ongoing health status of the research animal test system (Nicklas et al., 2002). Conventional rodent health monitoring systems employ diagnostic examinations including molecular tests for nucleic acid sequences or antigens, bacterial cultures, serologic tests for existing immune responses to prior infections, parasite examinations, and microscopic evaluation of tissues. Animal colonies may be monitored directly or via use of sentinel animals to survey the collective health of the colony. In addition to monitoring the health status throughout the life span of a rodent bioassay, it is considered imperative to understand the health status of the starting population and for the study director and study pathologist to know the pathogen exclusion list employed. Not all potential pathogens are considered to be of equal importance, depending on the type/objective of the study and the type of research animal population. *Mycoplasma pulmonis* and other major respiratory pathogens of rodents are on all pathogen exclusion lists of major rodent producers due to the potential clinical and subclinical effects, which if sufficiently severe may invalidate entire studies.

The RI aspartame publications indicate that the internal Cancer Research Center (CRC) colony was utilized for source animals with no indication of the health status of the colony at the time animals were bred for the aspartame studies (Belpoggi et al., 2006; Soffritti et al., 2007; Chiozzotto et al., 2011). While some pathologists may feel that the use of institutional colonies makes for a more consistent historical database of lesions, specific scientific justification is still needed as these colonies must be managed for a variety of factors including genetic drift, which can impact any aspect of an animal’s phenotype (Elliott et al., 2018). Unless a unique genotype is required for experimental cohorts, the benefits of institutional colonies generally do not outweigh their risks. The benefits of sourcing animals from major commercial suppliers include a more uniform health status and genetic background. The latter enables easier detection of a pathogen outbreak (and resultant complications) by virtue of having a wider swath of user groups of the source colonies. Commercial vendors also regularly employ well-accepted methods to ensure the health status of both the colony and individual animals, and they periodically publish pathogen exclusion lists and health monitoring data for production rooms. Even when SPF animals are sourced from a reputable facility, as was the case for the RI aspartame prenatal lifetime study in Swiss mice (Soffritti et al., 2010), the

implementation of a health monitoring system at the laboratory performing the carcinogenicity study remains critical as animals continue to be susceptible to infections, particularly in a facility that historically lacked such health monitoring systems.

Beyond the lack of reporting the health status of rodents at the start of the study, the RI aspartame publications do not indicate that a health surveillance program was in place for these studies yet indicate instead that "creatures that die naturally are subject to infectious pathologies, whether they be rodents or humans" (Chiozzotto et al., 2011). The RI recognized this laissez faire approach (i.e., a choice to accept rather than control confounding influences of natural origin) is not common practice for animal research facilities and decided to implement practices in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines (GLP Life Test) from 2009 onward, which was after all the RI aspartame studies were completed or well underway (Gift et al., 2013). The presumption is that the RI maintained its Good Laboratory Practice (GLP) certification and adherence to OECD guidelines regarding the monitoring and control of infectious agents, including regular serological testing, and diligent sacrifices of moribund test animals since 2009. Many complications may arise in lifetime rodent carcinogenicity bioassays when pathogen infections occur during rodent bioassays, including difficulty in distinguishing lesions secondary to chronic infection from those neoplastic lesions that may be morphologically similar (Everitt and Richter, 1990). Without a clear understanding of their inciting agent (test article and/or concurrent pathogen infection) and time course, proper interpretation of the biological relevance of such lesions may be impossible.

5.1. *Mycoplasma pulmonis* infections may mimic carcinogen-induced tumor responses in lung

As indicated previously, the potential for infectious agents to complicate biomedical research and bioassays for toxicity and carcinogenicity has been well recognized for decades (Everitt and Richter, 1990; Baker, 1998, 2003; Bhatt et al., 1986; Lindsey et al., 1991; Hamm, 1986). Most animal research facilities today experience no or minimal

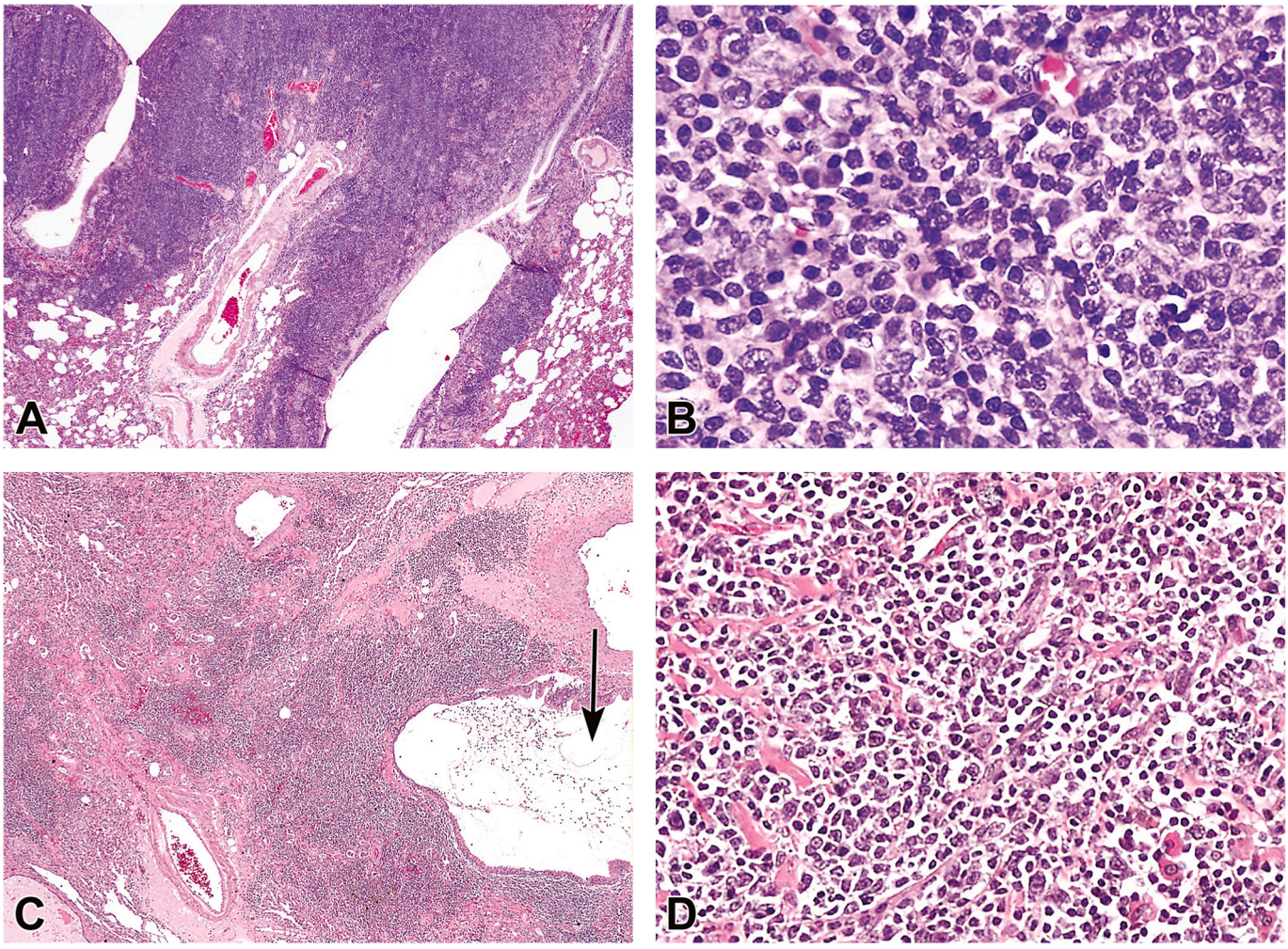

Fig. 1. Examples of *Mycoplasma pulmonis*-induced pulmonary inflammation. A) Low magnification (10×) of infected lung with peribronchial and peribronchiolar accumulations of coalescing nodules of inflammatory cells in a male F344 rat. In some areas, the cells infiltrate through the entire thickness of the airway wall and extend through the underlying basement membrane. B) High magnification (40×) of the inflammatory cells demonstrating myriad lymphocytes with fewer macrophages and plasma cells. Low (C) 10x and mid (D) 20× magnifications of *M. pulmonis* and Sendai virus infection in a male aged Long Evans rat. This rat most likely had a coinfection with *Filobacterium rodentium* (formerly cilia-associated respiratory [CAR] bacillus). The lungs were reported to have variable peribronchiolar and bronchiolar infiltration of lymphocytes and plasma cells, intrabronchiolar accumulation of neutrophils and mucin (arrow), segmental necrosis and loss of bronchiolar epithelial cells, and suppurative (neutrophil-rich) bronchiectasis. The mid magnification (D) illustrates alveolar spaces filled primarily with mononuclear inflammatory cells (lymphocytes, with fewer plasma cells and macrophages).
A and B: 1980-Armed Forces Institute of Pathology (AFIP) case MS 12. H&E.
C and D: Conference 6 Case 1 of the 1990–1991 AFIP 2287127 Wednesday Slide Conference, slides 23 and 24. http://www.askjpc.org/wsco/wsc/proceedings/WSCPB1990-1991.pdf. H&E.

complications with study conduct from infectious diseases as modern standards for research animal care and health monitoring have been widely accepted and rigorously implemented.

These RI aspartame studies of concern include very prominent examples of problems that can arise when health surveillance programs are not used regularly and rigorously, particularly with the difficulty in distinguishing lesions resulting from inadequately controlled infectious diseases from bona fide neoplastic lesions. In addition to the aspartame studies, data from other contemporaneous rodent carcinogenicity bioassays conducted by the RI and reported in 1995 and 2002, especially those using rats, have been heavily criticized; these include investigations of methanol, methyl-tertiary-butyl ether (MTBE), and other chemicals (Soffritti et al., 2002; Belpoggi et al., 1995, 2002). Those criticisms arose for several reasons, but the most prominent concern was the presence of pronounced lymphocyte-rich nodules associated with major pulmonary airways, which were misinterpreted to be definitive evidence of test article-related lymphoma (NTP, 2011; Schoeb and McConnell, 2011a, 2011b; Ward and Alden, 2009; Schoeb et al., 2009) without acknowledging their substantial resemblance to classic bacteria-induced inflammatory lesions. In particular, pulmonary lymphoma and lymphocytic leukemia are difficult to distinguish from the exuberant hyperplastic bronchus-associated lymphoid tissue (BALT) that develops secondary to chronic *M. pulmonis* infection (Fig. 1A–B). Moreover, the lesions seen with *M. pulmonis* can vary substantially in severity and spectrum depending on other factors such as level of ammonia in cages, co-infections with other agents, etc. Fig. 1C–D provides an example of severe lymphoid-predominant pulmonary inflammation associated with coinfection of *M. pulmonis,* Sendai virus and possibly *Filobacterium rodentium* (cilia-associated respiratory [CAR] bacillus). Importantly, *M. pulmonis* is an important, highly contagious respiratory pathogen of rodents that may go undiagnosed and be perpetuated in a diseased colony due to its sometimes subclinical (asymptomatic) protracted nature in the absence of a well-designed health surveillance program. *M. pulmonis* can be transmitted vertically through the placenta and horizontally through direct contact or aerosol, and is therefore difficult to eliminate without following a strict infection control policy (i.e., test and remove) (Schoeb et al., 1996).

In contrast to the RI aspartame studies, another lifetime carcinogenicity bioassay of aspartame, conducted at another institution that used SPF Slc:Wistar rats, reported no increased incidence of pre-neoplastic lymphoid lesions or lymphomas and no neoplasms in the lungs after 104 weeks of dietary exposure (Ishii et al., 1981; Shibui et al., 2019). In the test animals, the incidence of leukemia/lymphoma and related hematopoietic system tumors was unaffected by aspartame treatment even at doses of 4 g/kg body weight/day. This exposure level can be compared to the RI lifetime and prenatal lifetime carcinogenicity studies in rats where statistically significant incidences of lymphoma and leukemia in females were reported at doses from 0.02 g/kg body weight/day (Tables 2 and 3) with no additional increase in tumor incidence up to 5 g/kg (Table 2). Moreover, there were no incidences of lymphomas in either concurrent control or aspartame-treated animals in this other study (Shibui et al., 2019). The NTP also conducted studies of aspartame in 3 genetically altered male and female mouse models in 9-month feed studies with average daily high doses ranging from 7.3 to 9.6 g/kg body weight and found no evidence of carcinogenic activity (NTP, 2005). Because the genetically altered mouse line was a new model, there was uncertainty whether the study possessed sufficient sensitivity to detect a carcinogenic effect. Nevertheless, we consider the contrasting evidence in these publications as well as many other negative studies (select examples in Table 1) to be highly convincing that aspartame does not pose a carcinogenic hazard (Molinary et al., 1984).

Furthermore, Zella and coworkers have recently linked *Mycoplasma* infections in rodents with induction of lymphoma in *Prkd*^{scid} (scid) mice of two genetic backgrounds (C57BL/6 and NOD) (Zella et al., 2018). This confirmation that lymphoma may arise secondary to chronic bacteria-associated inflammation is an additional confounder for the interpretation of any putative chemically-induced lymphomas in animals infected with *Mycoplasma*. Considering the Zella report and Gift et al.'s assertion of the adequacy of ethanol-fixed tissues for molecular assays, the RI should assess whether *M. pulmonis* DNA is detectable in the tissues from their three rat aspartame bioassays using, for example, PCR (Loganbill et al., 2005; Sanchez et al., 1994). The sensitivity and reliability of such a study would depend on degradation of nucleic acid and this would be highly dependent on, among other factors, storage conditions. However, the ethanol fixation used by the RI should be superior to formalin for PCR on archived materials. Lack of positivity is not absolute for a negative study; however, if positive then the animals were likely infected with *M. pulmonis*. The lymphoma-negative Slc:Wistar rat study of aspartame (Ishii et al., 1981; Shibui et al., 2019) and the lymphoma-positive study in *M. pulmonis*-infected scid mouse study (Zella et al., 2018) underscore the importance of an active and rigorous health monitoring program in research facilities performing animal studies, especially when findings from these studies are intended to inform human health risk assessment.

5.2. *Helicobacter hepaticus* infections may induce carcinogenic responses in liver and mammary gland

Some rodent pathogens actually contribute to induction of neoplasia. For example, prenatal infection with *H. hepaticus* induces liver tumorigenesis in mice (Diwan et al., 2008). *H. hepaticus* expresses a cytolethal distending toxin, which is essential for *H. hepaticus* colonization of the intestines in normal mice (Chien et al., 2000). This toxin has been linked to tumor development via immune system imbalance, apparently by fostering a pro-inflammatory environment that promotes hepatocyte proliferation (Chien et al., 2000). This toxin also enhances DNase activity, causing DNA damage that could potentially contribute directly to tumor initiation. *H. hepaticus* has also been shown to induce mammary adenocarcinomas in mice via a tumor necrosis factor alpha (TNFα)-dependent mechanism (Rao et al., 2006). Another study in mice found that host neutrophil-associated immune responses to intestinal tract microbes, such as *H. hepaticus,* have the potential to significantly impact cancer progression in mammary glands (Lakritz et al., 2015). Given the reported hepatocellular carcinomas in male mice in the RI prenatal lifetime studies of aspartame (Soffritti et al., 2010), evidence demonstrating an SPF mouse colony is warranted to confirm the absence of confounding tumorigenic mechanisms. These confounding factors further reinforce the importance of implementing an active and rigorous health monitoring program that is able to adequately detect subclinical infections in rodent colonies that may otherwise significantly affect the study quality, accuracy of results, and measured interpretation of the findings.

5.3. Interpretation of putative tumor responses reported in RI lifetime rodent carcinogenicity bioassays with concurrent chronic infections

One of the most basic and important elements of robust study design is to eliminate confounding factors so that an observed effect of an experimental treatment can be confidently ascribed to the treatment itself. This design objective is applicable not only to bioassays carried out for regulatory purposes to assess product safety and potential human health risk but also for basic biomedical research. In the three RI lifetime rodent carcinogenicity bioassays for aspartame, the presence of lung lymphomas, liver carcinomas, and mammary adenocarcinomas cannot be reliably attributed to the effects from aspartame exposure, particularly since the same findings may instead be the result of chronic pathogen infections. In view of the absence of a suitable health monitoring system during the conduct of the RI aspartame studies, we interpret the findings reported in the rodents used in these three studies to be highly confounded by infections such as *M. pulmonis* (Schoeb and McConnell, 2011b; Schoeb et al., 2009); therefore the tumor results in question could be neoplasia, or inflammation misdiagnosed as

neoplasia, due to the pathogen. Thus, whether the proliferative lesions are inflammatory, neoplastic, or a mixture of the two processes, the studies are irrevocably confounded by the potential presence of chronic infection in the rodents. In such a research environment, it is highly likely that the existence of unmonitored pathogenic microbes compromised findings in both rats and mice, although here the data regarding what pathogens may have been involved are less certain. In both species, potential pathogen confounders render the tumor data questionable as a basis for making decisions regarding carcinogenic hazard even if the diagnoses for lymphoproliferative lesions were 100% accurate.

6. Historical controls

Although the concurrent control is the most appropriate control group to assist in the interpretation of tumor incidence, reliable historical control (HC) data is an important component of the holistic evaluation of tumor incidences from carcinogenicity studies in rodents (Haseman et al., 1984; Greim et al., 2003). For example, HC data may help one determine if a tumor is rare or not, if the study under question has tumor incidences that exceed HCs, or if a concurrent control has an unusually high or low tumor incidence. HC data should also be divided into categories depending on the species, sex, route of administration, vehicle, study type, and breeder. Importantly, the nomenclature conventions and diagnostic criteria should remain constant between studies. Moreover, criteria should be established to help determine if a study should be excluded from the HC database. HC animals can vary substantially over time, even with institutional sourcing, as changes in intrinsic factors (e.g., unavoidable genetic drift) and extrinsic factors (e.g., pathogen status, food components) do occur over time in animal colonies. Therefore, HC data tables should be updated periodically (e.g., every 5 years). Providing this information as supplementary data for published carcinogenicity studies is needed to provide confidence in the interpretation of actual tumor incidences. The RI does not provide the HC data in enough detail to determine whether one can be confident in the significance of the lesions noted for the three aspartame bioassays, even for findings in the concurrent control group.

7. Methods of tissue fixation

For the aspartame studies performed by the RI, organs and tissues (except for bone) were preserved in 70% ethyl alcohol (ethanol) (Soffritti et al., 2005, 2006, 2007, 2010; Belpoggi et al., 2006; Tibaldi et al., 2020; Chiozzotto et al., 2011). Although Gift and coworkers (Gift et al., 2013) indicate that the use of ethanol fixation by the RI has an advantage for immunohistochemistry and laser capture microdissection studies of clonality, the vast majority of toxicologic pathology communities use neutral buffered 10% formalin (NBF) because tissue preservation using cross-linking fixatives (like NBF) for the histopathology evaluation of tissues is far superior to that provided by coagulating agents (like 70% ethanol) in preserving fine structural detail (Survana et al., 2019). Fixation with ethanol results in significant morphological artifacts that distort or obscure cell and tissue features, and such artifacts often compromise the pathologist's ability to provide accurate and specific diagnoses. Notable artifacts commonly seen with 70% ethanol fixation include marked cell and tissue shrinkage (due to water extraction), fragmentation of sections ("chattering" due to the microtome blade snagging on brittle tissues), and poor staining quality. Clear spaces may appear around and inside cells as well as between tissues, and cytoplasmic and nuclear components often appear condensed and lack subtle cytoarchitectural details. Such artifacts make diagnosis more difficult for the pathologist, especially limiting the ability to definitively (1) discriminate lymphocyte-rich nodular inflammatory lesions from lymphoid leukemias and lymphomas in the lung and (2) distinguish hepatocellular foci (pre-neoplastic lesions) in the liver. For these reasons, ethanol is not recommended as a substitute for NBF or other cross-linking fixatives (Chatterjee, 2014).

8. Limitations of lifetime rodent carcinogenicity bioassays

Extending the length of studies to encompass the entire lifespan of study animals is generally not desirable. OECD guidance for the testing of chemicals in carcinogenicity studies indicates that "[t]he duration of the study will normally be 24 months for rodents, representing the majority of the normal life span of the animals to be used." (OECD, 2018) Longer study durations may be used but should be justified. Senescent animals (i.e., near the end of life) are replete with disease, often including multiple neoplasms per individual even in controls. In the absence of effective health monitoring and infectious disease control measures, infections from various pathogenic organisms often produce subclinical but profound structural changes that accompany or obscure any test article-related effects, thus confounding interpretation. Aged animals die at different days/weeks/months over the course of a lifetime study, with the pace of death from natural causes in rodents rising gradually beginning at about 1 year (52 weeks) of age. Accordingly, comparison of lesion incidences becomes complicated as the study progresses because older animals will tend to have more neoplastic and non-neoplastic lesions. Conventional chronic rodent carcinogenicity bioassays have all surviving animals terminated at 78 (for some mouse studies) or 104 weeks (some mouse and all rat studies) of exposure, where exposure is usually initiated at 6 to 8 weeks of age (OECD, 2018). The 2-year termination not only maximizes the number of control and treated animals available at the same age for pathological comparisons but also minimizes incidental late-developing background tumors that may limit the ability to detect chemically induced effects (Melnick et al., 2008). These factors, taken together, may make it difficult to ascertain the biological relevance of any statistically significant differences in tumor incidences among control and treated animals past 2 years of age.

The length of time between death and the postmortem examination (necropsy [i.e, an animal autopsy]) substantially impacts diagnostic accuracy. In well-designed toxicity studies, the surviving animals are all processed on a specific termination date, which minimizes the amount of time between induced death and placement of tissue specimens in appropriate preservatives. In rodents, necropsy times range from 15 to 45 min per animal depending on the objectives of the study, so fixation begins no more than an hour after their demise. In contrast, animals that expire from natural causes typically are not found until the morning after; in rodent colonies, the presence of rigor mortis (body stiffening due to post-mortem muscle contraction) indicates that the animal has been dead for at least 3 h at room temperature (Krompecher, 1981), a time span in which autolysis (i.e., digestion of cells and tissues by their own enzymes and microenvironmental conditions) is well-advanced. Unscheduled deaths in rodent studies typically are processed by refrigerating the carcass at 4°C until an abbreviated necropsy (often limited to macroscopic and microscopic evaluations) can be completed; refrigeration slows but does not stop autolysis. The autolytic process is more severe and more widespread the longer the interval between death and necropsy, making diagnoses difficult at best and impossible for some lesions. The RI indicates that a patrol to perform cage-side observations of animals was done three times daily during weekdays and twice on weekends and holidays (Soffritti et al., 2010; Chiozzotto et al., 2011). However, this would not address the problem in animals that die overnight and not identified for necropsy/tissue fixation until the following morning. The RI indicates that deceased animals were refrigerated for a maximum of 16–19 h (Chiozzotto et al., 2011). What was not reported was the percentage of animals found dead and refrigerated prior to necropsy. Given the uncertainty surrounding data interpretation for the three RI lifetime rodent carcinogenicity bioassays for aspartame, data from each study should be compiled and evaluated fully to help determine whether any of these studies provide enough reliable data to inform the hazard identification and carcinogenic risk assessment for this chemical.

When lymphomas or leukemias related to test article exposure involve multiple tissues during 2-year rodent bioassays, there are

generally clinical signs and/or an increased incidence of early deaths in treated groups that occur in a dose-related manner. A search in the NTP archives for lung lymphomas and leukemias in rats indicates that most animals in a 2-year study with these tumors will be found moribund or dead before the designated terminal necropsy date, with these conditions serving as the sole or a major contributing cause of death (NTP, 2022). In the three RI aspartame rodent studies, there is no dose-related increase in mortality due to the HLTs, even with the incorrect addition of histiocytic sarcomas, monocytic leukemias and myeloid leukemias to this tumor category. This stated pattern is consistent with the pathologists' perspective, bolstered by the NTP archival data, that the lymphoproliferative lesions in the RI rodent bioassays are indicative of a confounding inflammatory process in response to a chronic microbial infection rather than evidence of aspartame-related induction of lymphoid neoplasia.

9. Figures that illustrate neoplasms of concern

As a general practice, authors tend to publish their best image examples as confirmation of their diagnoses. Importantly, images used for this purpose are high-resolution, correctly formatted depictions (i.e., appropriate brightness, contrast, and colors) at a magnification that accurately displays key cell and tissue diagnostic features; more images at several different magnifications with a better narrative description would be warranted when documenting new or rare lesions. To illustrate, while Figure 9 in the 2005 RI study (Soffritti et al., 2005) – reported to be a "lymphoimmunoblastic" lymphoma – is not well described nor are the cellular features clear in the image provided, Fig. 1 in the publication by Ott and colleagues (Ott et al., 2010) demonstrates the importance of well-illustrated, high-resolution images for properly demonstrating the morphology of immunoblastic lymphoma. As described in the INHAND publication on lesion nomenclature entitled "Nonproliferative and Proliferative Lesions of the Rat and Mouse Hematolymphoid System", an immunoblastic lymphoma without a concurrent microbial co-infection should show a pattern of involvement in the lung tracking along the bronchovascular tree rather than occurring as a flat, non-bulging plaque in the subpleural parenchyma (Fig. 1A–B) (Willard-Mack et al., 2019). In addition, the cellular features shown at higher magnification in Figure 10 from the Soffritti et al. (2005) article are not consistent with a neoplasm (due to the variable cell appearance and paucity of mitotic figures) but instead are more consistent with a severe *M. pulmonis* infection (see Fig. 1C–D). The morphological criteria for immunoblastic lymphoma, as described by INHAND, are provided in Table 6 (Willard-Mack et al., 2019). These include large, fairly monotypic cells with abundant amphophilic cytoplasm, large vesicular nuclei with prominent nucleoli, and frequent mitotic figures (Fig. 2). The reported tumor in Figures 9 and 10 of the 2005 RI study (Soffritti et al., 2005) is also very unlikely to be an immunoblastic lymphoma because this is an extremely uncommon tumor in rodents and does not originate in the lung. After a search of all NTP rodent bioassays performed since the late 1970s and a search of the NTP image archives (NTP, 2022), this type of tumor was never reported or depicted, respectively.

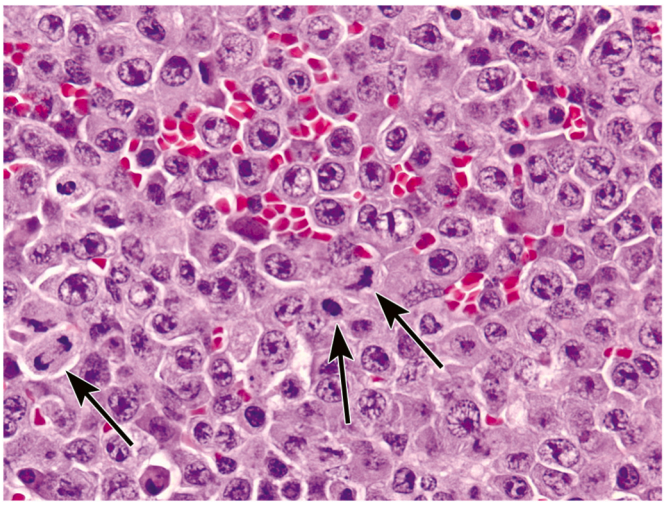

Fig. 2. Immunoblastic lymphoma in an 11-month-old female *Arf* null mutant mouse. This tumor is characterized by large monomorphic (cytologically identical) cells with abundant amphophilic cytoplasm, large vesicular nuclei, and prominent nucleoli. Mitotic figures are frequent (arrows). This rare tumor has similar morphological features in both rats and mice. 60x, H&E. Image reprinted with permission from Willard-Mack CL et al. Nonproliferative and proliferative lesions of the rat and mouse hematolymphoid system. *Toxicol Pathol.* Aug 2019; 47(6):665–783, with permission of Sage.

Lymphomas that originate in the hematolymphoid organs (spleen, lymph nodes, thymus, bone marrow, mucosa-associated lymphoid tissue) and then spread hematogenously to the lung typically have a perivascular and intra-alveolar pattern (Fig. 3) (Willard-Mack et al., 2019). If a lymphoma first originates within the lung, which is exceedingly rare, then one would expect a predominantly well-defined, nodular lesion within the pulmonary parenchyma in association with hyperplastic BALT and not a subpleural plaque-like lesion with irregular edges. In contrast, in rodents a focal or multifocal accumulation of various (i.e. non-monomorphic) leukocyte cell types within the alveolar spaces and/or along airways (comprised predominantly of lymphocytes) almost always is a response to a chronic microbial infection in which the pathogen localizes to lung tissue.

Neoplastic lesions can usually be differentiated from inflammatory/proliferative lesions based on cellular morphology, tissue distribution, clinical history, and related lesions in other tissues. Fig. 4 illustrates an intrapulmonary metastatic histiocytic sarcoma in a rat. The cellular morphology of the malignant histiocytes (a myeloid lineage) and the presence of multinucleated giant cells (MNGCs) makes histiocytic sarcoma easy to distinguish from lymphomas, which are composed of malignant lymphocytes and do not contain MNGCs. Because the cell of origin for a histiocytic sarcoma is the macrophage, it should not be combined with lymphoma or lymphoid leukemias when analyzing HLT incidence. Fig. 5 illustrates a myeloid leukemia. The cells are granulocytic, composed in this instance of neoplastic neutrophils. Myeloid leukemias may be of neutrophilic, eosinophilic, basophilic, myelomonocytic or monocytic origin and therefore should not be combined with lymphomas, which are of lymphoid origin when analyzing tumor incidences.

10. Pathology peer review and public scientific review procedures

Independent scientific review is an essential element of modern scientific inquiry, a means by which data generated during experiments are cross-checked by other scientists as a quality control procedure to maximize data accuracy. In terms of toxicologic pathology data sets, pathology peer review is generally performed by one or more pathologists who will often have different subject matter expertise compared to that of the study pathologist. In contrast, public scientific review is performed by scientists with variable degrees of pathology expertise and experience.

Pathology peer review procedures for studies conducted under GLP guidelines should always be described in study protocols in some detail. The pathology peer review process improves the accuracy and quality of the pathology raw data that is ultimately used in risk assessments and thus may impact human health (Boorman et al., 2002; Mann and Hardisty, 2014). Most GLP studies submitted to regulatory agencies and other global health authorities are subjected to conventional quality assurance (QA) as well as pathology peer review and sometimes pathology working group (PWG) procedures (Boorman et al., 2002; Mann

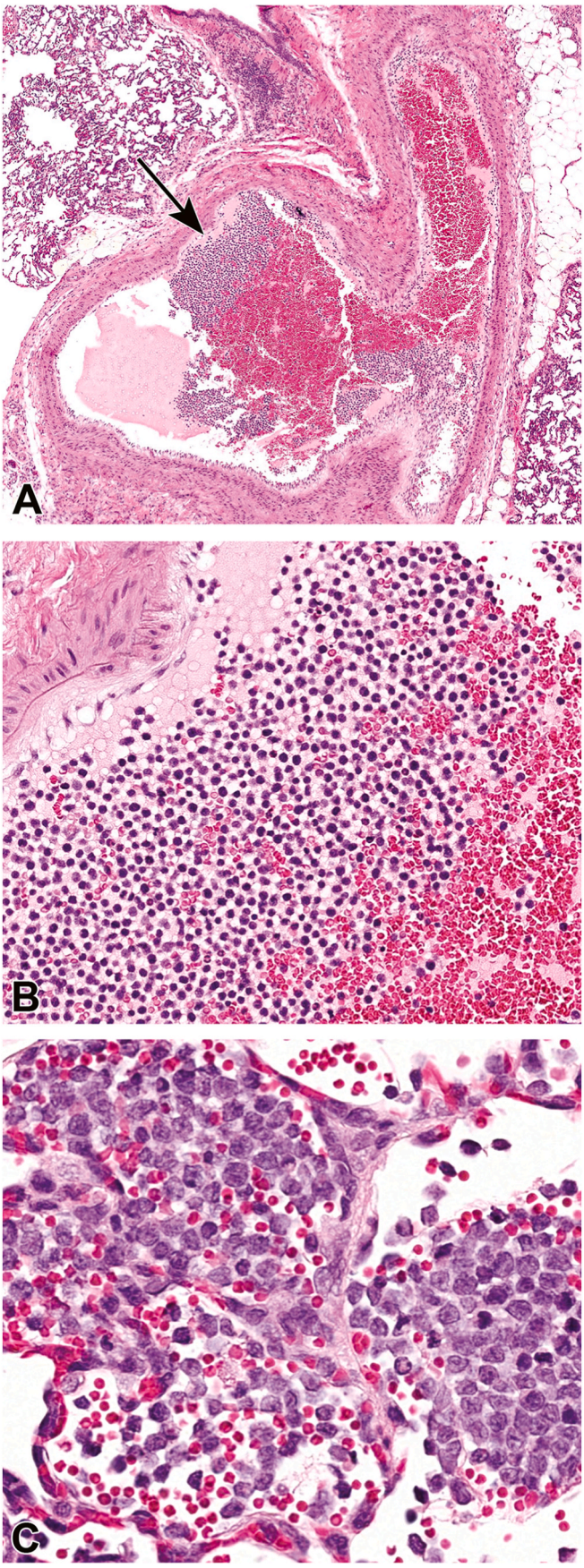

Fig. 3. Hematogenous spread of lymphoma in the lungs of a 2-year-old male Harlan Sprague Dawley rat. A) Low magnification (10×) illustrates neoplastic lymphocytes (arrow) admixed with red blood cells within the pulmonary artery. B) Higher magnification (20×) of (A) showing sheets of uniform small, round, neoplastic lymphocytes. C) High magnification (40×) of neoplastic lymphocytes within the alveolar spaces. All magnifications indicated at original objectives. H&E.

and Hardisty, 2013, 2014; Elmore and Boorman, 2013; Morton et al., 2010). When animal studies are involved, qualified and experienced veterinary toxicologic pathologists are an essential part of the pathology peer review process (Ettlin et al., 2008). Inclusion of veterinary pathologists with substantial experience in toxicologic pathology is critical because they have obtained comprehensive training in laboratory animal biology, medicine, and pathology as well as toxicology, extended by regular, lifelong continuing education in these disciplines that makes them uniquely qualified, compared to pathologists with other educational backgrounds, to review regulatory-type nonclinical toxicology studies (Bolon et al., 2010).

A public scientific review of the data prior to publication is also critical to the quality of the study presentation. In the experience of the authors, and pathologists generally (Cardiff et al., 2008), public reviews of pathology findings (both descriptions and images) are substantially improved if this task is performed by pathologists with subject matter expertise in detecting and describing cell and tissue morphological changes. For animal studies slated for regulatory review, a post-publication peer review (PPPR) takes place when an article is published before a pathology peer review has been done, and such a PPPR of pathology findings is a valuable additional means of verifying diagnostic accuracy and interpretation to maximize the quality of the pathology raw data. The RI has not invited a full independent PPPR of their studies generally, and for the aspartame carcinogenicity bioassays specifically, including a peer review of all tissue sections from all relevant studies as selected by the reviewing pathologists. For clarification, a PWG (e.g., as reported by Gift et al. (2013) for the RI rodent carcinogenicity bioassays of aspartame) would review a subset of tissues, usually selected by the study pathologist or PWG organizer rather than the reviewing pathologists, so a PWG constitutes a directed reevaluation to address a specific question and is not a full independent peer review. Allowing relevant stakeholders, including regulatory agencies and industries, to review the raw data, analyses and corresponding interpretations would provide transparency in the testing program and increase confidence in situations where such data are used to make decisions regarding human health risk.

11. Prior comprehensive evaluation by U.S. government agencies

Investigators from the U.S. National Center for Environmental Assessment (NCEA), the U.S. National Health and Environmental Effects Research Laboratory (NHEERL), and the U.S. Environmental Protection Agency (EPA) performed a comprehensive evaluation of the RI study designs, protocol differences, and accuracy of tumor diagnoses for their impact on carcinogenic hazard characterization (Gift et al., 2013). This joint effort also considered an NTP report for a focused QA and PWG review of a subset of tissues from RI carcinogenicity studies of methanol, MTBE, ethyl tertiary-butyl ether (ETBE), vinyl chloride, and acrylonitrile (NTP, 2011). According to the NTP report, the review entailed an audit of pathology specimens (APS) and the QA review of selected rat tissues in the RI methanol study but did not constitute a complete NTP pathology QA review or NTP PWG evaluation. The findings of this focused NTP review were submitted as a preliminary report, intended as a basis for recommendations for further evaluations around pathology data quality concerns, rather than as final conclusions about any possible or reported effects of the chemical under study. For the methanol and MTBE studies, the reviewing pathologists from the NTP

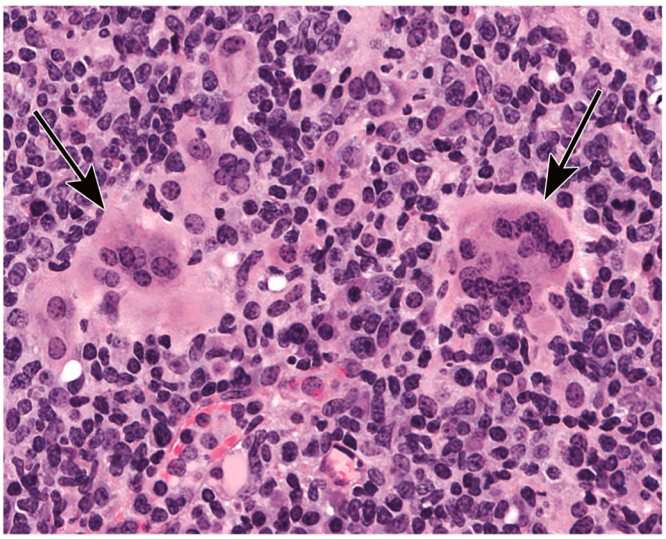

Fig. 4. Pulmonary histiocytic sarcoma in a 2-year-old female F344/N rat. The origins for this tumor are cells of the mononuclear phagocyte system (i.e., a myeloid lineage), and it should therefore not be combined with tumors composed of neoplastic lymphocytes. The neoplastic cells have nuclei that are round, irregular, elongated, folded, or indented with abundant eosinophilic cytoplasm. Multinucleated giant cells (MNGCs, arrows) are often scattered throughout the tumor. Original objective magnification 40×. H&E.

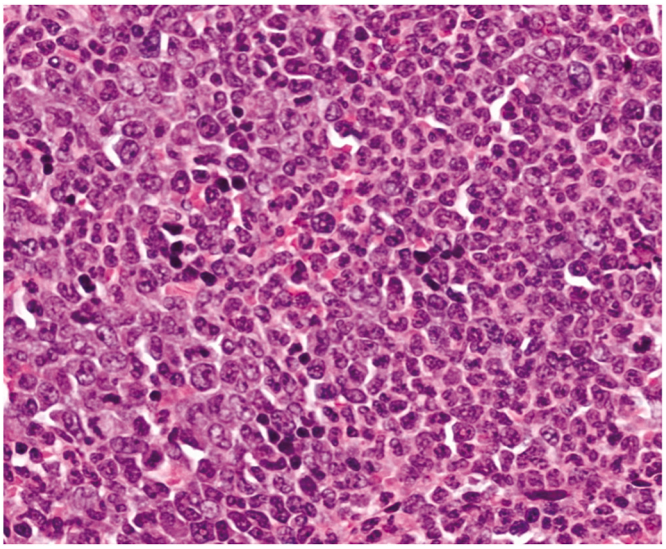

Fig. 5. Pulmonary myeloid (granulocytic) leukemia in a 2-year-old female F344/N rat. The most common form of myeloid leukemia arises from the neutrophilic lineage of granulocytes, as depicted in this image, and therefore should not be combined with tumors composed of neoplastic lymphocytes. Cell differentiation can vary from poorly differentiated to mature and well differentiated, with variable proportions of blastic to segmented forms. In this example, the neoplastic cells are well differentiated with nuclei that are segmented, indented, and donut shaped. Original objective magnification 40×. H&E.

pathology contractor diagnosed fewer lymphoid neoplasms, mainly of the respiratory tract, and indicated that there was chronic inflammation of the nasal cavity, ear canal, trachea, and lungs indicative of infection by one or more respiratory pathogens. The NTP concluded that the findings suggest that the male and female rats in these lifetime drinking water studies had a respiratory infection that confounded carcinogenic hazard identification in terms of lymphoproliferative lesions. The NTP also concluded that it is not unusual in the setting of pronounced chronic inflammation that inflammatory or regenerative lymphoid proliferations take on some neoplastic features (NTP, 2011).

This NTP review underscores the difficulty the RI had in diagnosing HLTs, most likely given the background inflammatory lesions as discussed by Schoeb and McConnell as a secondary review of the results of the RI methanol bioassay review undertaken via a Freedom of Information Act request (Schoeb and McConnell, 2011a, 2011b; Ward and Alden, 2009; Schoeb et al., 2009). The NTP pathologists only agreed with the diagnoses of the RI study pathologist for 54% of hematopoietic neoplasms and 36% of lymphomas while identifying about twice as many inflammatory lesions in the airways (nose, trachea, and lung) and ear. The inflammatory lesions that were described were consistent with *M. pulmonis* (Schoeb and McConnell, 2011a). Importantly, this pathogen does colonize solid surfaces and can form biofilms and survive for extended periods of time when dried onto surfaces in facilities with insufficient health monitoring programs (Eterpi et al., 2011). The NTP also concluded that the RI's practice of combining myeloid leukemias and histiocytic sarcomas with lymphomas for statistical analyses and data interpretation was not acceptable because these neoplasms are of different cellular origins. Most companies and institutions agree that these malignancies should be treated as separate entities and not combined with the lymphomas or lymphoid leukemias.

The RI has reported dose-related increases in incidences of lymphomas/leukemias for aspartame, chlorinated drinking water, diisopropyl-ether (DIPE), formaldehyde, mancozeb, methanol, MTBE, tert-amyl methyl-ether (TAME), toluene, and vinylidene chloride. A similar induction of lymphomas/leukemias has not been associated with these chemicals for lifetime rodent bioassays performed by other institutions and, as noted above, evidence of a chronic respiratory infection with a *Mycoplasma* organism known to produce marked lymphoid proliferation has been brought forth for agents tested by the RI. By the law of parsimony ("Occam's razor," which holds that the most straightforward explanation for a set of facts likely represents the truth), the simplest interpretation of these data is that the marked, airway-localized lymphoproliferative lesions in this group of outlier RI studies are inflammatory (non-neoplastic) responses to chronic microbial colonization and not chemically induced neoplasms.

Regarding the putative immunoblastic lymphomas of the lung (typically reported as "lymphoimmunoblastic" in RI publications), the published descriptions reported "diffusion" of neoplastic tissue sometimes involving the lung but concurrently impacting other organs (most often liver, spleen, and/or mediastinal and peripheral lymph nodes). In the RI lifetime study of Sprague Dawley rats exposed to aspartame since early fetal life, the most frequent subtypes observed were immunoblastic lymphomas mainly involving lung and mediastinal/peripheral lymph nodes in males (Soffritti et al., 2007; Chiozzotto et al., 2011; Schoeb and McConnell, 2011a; Ward and Alden, 2009). In almost half of the rats with this diagnosis from the RI lifetime rodent bioassay for methanol, Schoeb and McConnell reviewed lesion data and determined that the lung was the only affected organ (Schoeb and McConnell, 2011a); in the Tibaldi report, 69% of the immunoblastic lymphomas are listed as occurring only in the lung (Tibaldi et al., 2020). Primary lung lymphomas, and this tumor type in particular, are exceedingly rare in rats generally and Sprague Dawley rats specifically. The review of the RI lifetime rodent bioassay for methanol by Schoeb and McConnell also demonstrated that the distribution of the lymphoproliferative lesions mainly followed the major airways (Schoeb and McConnell, 2011a). It is not uncommon that inflammatory cells from a chronic, marked respiratory infection in rodents are misdiagnosed as lymphoma and also cause marked expansion in the mediastinal (bronchial) lymph nodes (as they are involved in lung drainage and sometimes may spread to other organs that sequester leukocytes, especially hematopoietic and lymphoid tissues) (Tilney, 1971). Taken together, these data indicate that (1) the lymphoproliferative lesions observed in the RI rodent cancer bioassays for aspartame (and the other chemicals) arose in the lung as an inflammatory response to a chronic, severe but nonetheless subclinical

infection of airway mucosa and that (2) the lung foci exhibiting this pattern are not neoplastic in nature.

12. Human relevance

Not all agents that cause cancer in experimental animals will also cause cancer in humans, although it is considered biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals may also present a carcinogenic hazard to humans (Melnick et al., 2008). Accordingly, in the absence of additional scientific information, these agents would be considered to pose a carcinogenic hazard to humans. Examples of additional scientific information are data that demonstrate that a given agent causes cancer in animals through a species-specific mechanism that does not operate in humans, or data that demonstrate that the mechanism in experimental animals is a mode of action that is not relevant in humans (EPA, 2005). The use of experimental animal responses that are highly suspect can have adverse public health implications; therefore, rodent cancer studies with questionable data should be periodically reexamined for consistency and coherence with data from emerging studies. An additional point is that hazard identification ("Is an agent capable of causing cancer under any conditions?") is not identical to carcinogenic risk ("Is an agent capable of causing cancer under real-world conditions?"); hazard identification represents the first of four steps in carcinogenic risk assessment (EPA, 2005; OECD, 2014; NRC, 1983). The utility of animal data in identifying a hazard, and thus in assessing risk, is undercut if other factors confound diagnostic terminology, analyses and interpretation, which remains the case for the RI lifetime rodent bioassays for aspartame.

For aspartame, the RI reported statistically significant increases in the incidences of mammary adenocarcinomas in female Sprague Dawley rats treated prenatally ($p < 0.05$) (Soffritti et al., 2007), hepatocellular carcinomas in male Swiss mice treated prenatally ($p < 0.01$) (Soffritti et al., 2010), and alveolar/bronchiolar carcinomas in male Swiss mice treated prenatally ($p < 0.05$) (Soffritti et al., 2010). For all these tumors, a weight-of-evidence approach should consider and report the types of lesions (non-neoplastic precursors vs. benign vs. malignant neoplasms of the same cell lineage), accelerated tumor onset, early mortality, tumor frequency (how common or rare), dose response, and presence of other factors such as data from subchronic studies, findings in concurrent and historical control animals, infectious agents, and known sensitivity/resistance of the animal stock/strain.

Importantly, induction of lymphomas in rodents by true carcinogens would be expected to yield significant dose-related increases in lesion incidence and early mortality. Interestingly, the RI studies of aspartame at 400 ppm (0.02 g/kg) and at 100,000 (5 g/kg) ppm resulted in lymphomas/leukemias affecting 20% and 25% of rats, respectively (Belpoggi et al., 2006). One would expect to see a more pronounced increase in tumors at the high dose given the very large difference in exposure if aspartame were a true carcinogen. By comparison, a similar lymphoproliferative lesion incidence at widely divergent dose levels is consistent with colony-wide microbial flora leading to chronic infection and a progressive rise in inflammatory lesions.

During the course of analyzing and reporting on more than 500 2-year rodent carcinogenicity bioassays at the NTP, some chemicals appeared to have a sex- and/or species-specific effect, underscoring the importance of elucidating the chemical's mode(s) and mechanism(s) of action. In 2005, the EPA provided a framework for the critical analysis of mode of action information to address the extent to which the available information supports the hypothesized mode of action, whether alternative modes of action are also plausible, and whether there is confidence that the same inferences can be extended to populations and life stages (including humans) that are not represented among the experimental data (EPA, 2005). This "mode of action" analysis is based on physical, chemical, and biological information and includes a variety of data such as tumor types, whether tumors are responsive to endocrine influences, similarity of metabolic activation and detoxification between humans and the test species, influence of routes of exposure, development of tumors that invade locally or systemically, or lead to death, tumor latency, etc. A few examples of possible modes of carcinogenic action include mutagenicity, mitogenesis, inhibited cell death, chronic cytotoxicity with reparative cell proliferation, and immune suppression. In contrast, "mechanism of action" implies a more detailed understanding and description of events, often at the molecular level. Therefore, understanding the mode of action and mechanism of action can aid in identifying processes that may help explain how chemical exposures may differentially affect a particular population segment or life stage. Understanding mechanistic pathways or mode of action for aspartame will help assess the relevance of these lesions to human carcinogenesis, especially since the chemical has been present in the environment and consumed by humans for years without significant epidemiological results linking aspartame to increased incidences of hematolymphoid tumors.

13. Conclusions

The 2013 EFSA scientific opinion on carcinogenic risk of aspartame as a food additive concluded that aspartame and its breakdown products were safe for human consumption at current levels of exposure (EFSA, 2013a). It is our opinion that the existing RI bioassay data, analyses and interpretation provide neither compelling nor conclusive evidence that aspartame represents a carcinogenic hazard in rodents. By extension, this agent is unlikely to pose a carcinogenic risk to humans.

Given the myriad flaws in study design, methodology and reporting for the totality of the RI studies, and particularly the lack of accuracy of pathology diagnoses and interpretations, these studies should be rigorously reviewed by regulatory authorities and their qualified and experienced veterinary toxicologic pathologists. It is industry standard to conduct lifetime rodent studies for 1.5–2 years with at least 50 animals per group, to fix tissues in NBF or a similar cross-linking fixative, and to maintain animals in a pathogen-free facility. An active health monitoring program is an essential element of well-run research facilities to be able to adequately detect subclinical infections in rodent colonies that may interfere with the accurate diagnosis, analysis and interpretation of animal carcinogenicity bioassays. It is also industry standard to combine lymphoid leukemias and lymphomas under the common diagnosis "lymphoma" while maintaining leukemias of other hematopoietic cell lineages and histiocytic sarcomas as separate diagnoses since they arise from different lineages. In general, pre-neoplastic lesions (e.g., hyperplasias) should not be combined with neoplasias, and the same tumor evolving in different organs or tissues should not be combined. It is also industry standard to have qualified and experienced veterinary toxicologic pathologists provide histopathological review of all tissues including QA, peer, and PWG reviews and for the slides from which pathology raw data were generated to be made available freely for peer review upon request by regulatory agencies or their designees. Detailed descriptions (ideally narrative and visual) of the various tumors and access to individual animal tables that list types of neoplasms, non-neoplastic lesions, locations of lesions, and animal ages at death are needed to adequately evaluate the true incidence and biological significance of reported proliferative lesions. Rodent bioassays that use accepted industry standards reliably provide high-quality samples, promote more consistent microscopic diagnoses, and thus simplify the interpretation of statistical significance and biological relevance. The authors believe that an independent review of the pathology findings in the three RI rodent carcinogenicity bioassays for aspartame would go far to addressing the nature of the lymphoproliferative lesions (to wit, neoplastic vs. non-neoplastic) and thus would be useful in better defining the actual carcinogenic hazard (if any) posed by aspartame.

The attempt to provide reassurance regarding the diagnosis of HLTs in the RI aspartame bioassays using IHC alone is not valid. The limited antibody panel employed by RI is not capable of either definitively differentiating HLTs (especially lymphomas) from non-neoplastic

lesions (lymphoid hyperplasia, inflammation) or determining the clonality of rodent lymphoid tumors. Molecular evidence of clonality (e.g., PCR) might be gained if unfixed frozen samples of lung are still available in the RI archive, and this would provide the most straightforward demonstration that the findings (or some of them) represent lymphoid neoplasms.

Demonstration of a pathogen-free rodent facility at the time the RI aspartame lifetime carcinogenicity bioassays were conducted, along with mode and mechanism of action studies, would offer perspective for the liver, lung, and mammary proliferative lesions observed in the prenatal studies. As stated previously, without an active health monitoring system in place, the animals in the RI studies most likely had underlying infections that would have compromised the ability to determine, with confidence, if a lesion were due to the subclinical but chronic infection or the test article. This opinion is based on historical data in the scientific literature regarding rodent colony health prior to the institution of rigorous health surveillance systems in accredited laboratory animal facilities worldwide.

In summary, the issues discussed here as well as a reported lack of carcinogenicity and lack of relevant modes of action (e.g., genotoxicity leading to mutagenicity) for lifetime rodent carcinogenicity bioassays of aspartame (performed by other institutions who have documented their use of industry standard practices in study design, methodology and reporting (Ishii et al., 1981; Shibui et al., 2019; NTP, 2005; Otabe et al., 2019; Magnuson et al., 2007; Ishii, 1981)) necessitate a full audit and comprehensive pathology peer review of all slides from these RI studies. This full audit and pathology peer review should be conducted by a group of independent, qualified and experienced veterinary toxicologic pathologists. A rigorous public peer review of all data would provide assurance to the scientific and lay communities as to the accuracy, relevance, and biological plausibility of the pathology data and conclusions reported by the RI.

CRediT authorship contribution statement

Susan A. Elmore: Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing. **Jerold E. Rehg:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing. **Trenton R. Schoeb:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing. **Jeffrey I. Everitt:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing. **Brad Bolon:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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Data availability

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

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Elmore is a veterinary anatomic pathologist, board-certified by the American College of Veterinary Pathologists (ACVP) and the American Board of Toxicology (ABT) and credentialed as a Fellow of the International Academy of Toxicologic Pathologists (IATP). She has enjoyed a 35-year career as a molecular biologist and a 19-year career as a veterinary toxicologic pathologist and was previously employed by the U.S. National Toxicology Program (NTP) and U.S. National Institute of Environmental Health Sciences (NIEHS).

Rehg is a veterinarian board-certified by both the ACVP and the American College of Laboratory Animal Medicine (ACLAM). He specializes in hematopathology and oncogenesis. He has been a comparative/veterinary pathologist and laboratory animal specialist for 51 and 49 years, respectively. Currently, he is a full Member (Professor) in the Department of Pathology at St. Jude Children's Research Hospital.

Schoeb is an ACVP board-certified veterinary anatomic pathologist and Professor Emeritus of the University of Alabama at Birmingham. His professional career in laboratory animal pathology spanned 41 years, and he continues to consult part-time.

Everitt is ACVP and ACLAM board-certified and is an IATP Fellow. He is currently Professor of Pathology at the Duke University School of Medicine and formerly served as laboratory animal veterinarian for the Chemical Industry Institute of Toxicology (CIIT)/CIIT Centers for Health Research during a 17-year career at that institution.

Bolon is a veterinary anatomic pathologist with board certifications by the ACVP and ABT and also Fellow status by the IATP, Academy of Toxicological Sciences (ATS), and Royal College of Pathologists (RCPath). He has been a comparative and toxicologic pathologist for 30 years in various settings, most recently as a self-employed consultant at GEMpath, Inc.

Christel Leehmuis

General Manager Science and Risk Assessment
Food Standards Australia New Zealand (FSANZ)

Q&A Panel Discussion

Panelists:

Dr Susan Elmore

Veterinary Anatomic Pathologist; Principal

Elmore Pathology

Christel Leehmuis

General Manager Science and Risk Assessment

Food Standards Australia New Zealand

Moderated by Geoff Parker

Let's continue the dialogue on the science of sweeteners

Thank you for being part of the conversation

Scan here to share
your thoughts!

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A Spotlight on Sweeteners

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Thank you.

Please join us for a light networking lunch from 1pm - 2pm.

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