Xylitol is prothrombotic and associated with cardiovascular risk

Marco Witkowski 1,3, Ina Nemet 1, Xinmin S. Li 1, Jennifer Wilcox 1, Marc Ferrell 1, Hassan Alamri 1,4, Nilaksh Gupta 1, Zeneng Wang 1, Wai Hong Wilson Tang 1,2, and Stanley L. Hazen 1,2*

1Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA; and 2Department of Cardiovascular Medicine, Heart, Vascular and Thoracic Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA

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Abstract

Background and Aims The pathways and metabolites that contribute to residual cardiovascular disease risks are unclear. Low-calorie sweeteners are widely used sugar substitutes in processed foods with presumed health benefits. Many low-calorie sweeteners are sugar alcohols that also are produced endogenously, albeit at levels over 1000-fold lower than observed following consumption as a sugar substitute.

Methods Untargeted metabolomics studies were performed on overnight fasting plasma samples in a discovery cohort (n = 1157) of sequential stable subjects undergoing elective diagnostic cardiac evaluations; subsequent stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were performed on an independent, non-overlapping validation cohort (n = 2149). Complementary isolated human platelet, platelet-rich plasma, whole blood, and animal model studies examined the effect of xylitol on platelet responsiveness and thrombus formation in vivo. Finally, an intervention study was performed to assess the effects of xylitol consumption on platelet function in healthy volunteers (n = 10).

Results In initial untargeted metabolomics studies (discovery cohort), circulating levels of a polyol tentatively assigned as xylitol were associated with incident (3-year) major adverse cardiovascular event (MACE) risk. Subsequent stable isotope dilution LC-MS/MS analyses (validation cohort) specific for xylitol (and not its structural isomers) confirmed its association with incident MACE risk [third vs. first tertile adjusted hazard ratio (95% confidence interval), 1.57 (1.12–2.21), P < .01]. Complementary mechanistic studies showed xylitol-enhanced multiple indices of platelet reactivity and in vivo thrombosis formation at levels observed in fasting plasma. In interventional studies, consumption of a xylitol-sweetened drink markedly raised plasma levels and enhanced multiple functional measures of platelet responsiveness in all subjects.

Conclusions Xylitol is associated with incident MACE risk. Moreover, xylitol both enhanced platelet reactivity and thrombosis potential in vivo. Further studies examining the cardiovascular safety of xylitol are warranted.
Role of the artificial sweetener xylitol in cardiovascular event risk. In initial untargeted metabolomics studies (discovery cohort) and subsequent stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) studies (validation cohort), fasting levels of xylitol are associated with incident major adverse cardiovascular events (MACE). Using human whole blood, platelet-rich plasma, and washed platelets, xylitol enhances multiple indices of platelet reactivity in vitro. Xylitol also was shown to enhance thrombosis formation in a murine arterial injury model in vivo. In human intervention studies, when subjects ingested a typical dietary amount of xylitol in an artificially sweetened food, multiple functional measures of platelet responsiveness were significantly increased. Xylitol is both clinically associated with and mechanistically linked to heightened atherothrombotic and cardiovascular disease risks. Long-term safety studies and reappraisal of labelling mandates for the sugar alcohol xylitol are warranted.

Keywords
Artificial sweetener • Low-calorie sweetener • Cardiovascular disease • Platelet • Thrombosis • Heart attack • Stroke • Sugar alcohol • Polyol • Nutrition
Introduction

In light of the obesity pandemic worldwide, measures to reduce sugar intake have become a public health priority. One such measure is the proliferation of artificial sweeteners including non-nutritive and low-calorie sweeteners (which collectively are also often called ‘artificial sweeteners’) in processed foods that are promoted as healthy sugar alternatives. While artificial sweeteners have generally recognized as safe (GRAS) status and are approved by public health authorities [e.g. US Food and Drug Administration (FDA) and European Union (EU)], the World Health Organization recently advised against the use of non-sugar sweeteners for weight control in newly released guidelines. Over the past decade, the use of artificial sweeteners has markedly increased in near-universal fashion across all subject categories including gender, age, race/ethnicity, weight status, and geographic and socio-economic subgroups. Of note, the use of artificial sweeteners is recommended for subjects that suffer from cardiometabolic diseases including obesity, diabetes, and cardiovascular disease (CVD) by multiple guideline organizations (e.g. American Heart Association and diabetes associations in the USA, UK, Canada, and Australia), while their potential long-term cardiovascular adverse effects are rarely studied. Contrary to their intended benefits, a history of artificial sweeteners in a number of epidemiological studies has been associated with cardiometabolic adverse effects including insulin resistance, type 2 diabetes, and CVD, including atherothrombotic complications and death. Moreover, in recent studies, the endogenous sugar alcohol erythritol was both clinically and mechanistically linked to CVD. Only a limited number of randomized controlled trials have examined the short-term effects of sweeteners on metabolic indices with results that generally fail to support the promoted benefits, though some studies have suggested potential metabolic improvements. The limited randomized controlled trials available on artificial sweeteners have been criticized for not reflecting real-life exposure and for typically being conducted over a short period of time, making them unable to recapitulate long-term exposures appropriately for CVD endpoints.

Xylitol is a 5-carbon sugar alcohol (polyol) that is commonly used as a low-calorie sweetener. It can be found in small quantities in fruits and vegetables but is highly enriched (often over 1000-fold higher than found in nature) in numerous artificially sweetened foods and beverages. Because of its anti-cariogenic properties, xylitol is also commonly used in candy, gum, and oral care products. Xylitol is also produced endogenously as a low abundance intermediate side product of human glucose metabolism (part of the glucuronate pathway) and has negligible impact on blood sugar or insulin secretion. In contrast to traditional high-intensity sweeteners, xylitol has comparable sweetness to sucrose. Thus, when used as a sugar substitute, it is added in much larger amounts to processed food (i.e. levels equal to sucrose with up to 45 g per serving in some products such as artificially sweetened pie fillings). It is therefore appreciated by the food industry as a bulking sugar substitute that confers texture, moisture, and increases shelf life of processed foods without aftertaste. Consequently, xylitol is sold in grocery stores in bulk (like sugar) where its 1:1 replacement is suggested. It is marketed as a ‘natural sweetener’, ‘keto-friendly’, or ‘low-carb’ and is generally recommended as a sugar substitute for patients with diabetes to improve glycaemic control. Despite the growing market share and use of xylitol in processed foods and oral care products, the impact of xylitol on cardiovascular event risk has not been reported. Here, we provide human clinical observational, interventional, and mechanistic studies linking xylitol to CVD event risk and both heightened platelet reactivity and a pro-thrombotic state in vivo.

Methods

More extensive details for all methods can be found in Supplementary data online, Methods.

Human subjects

Study approvals

We performed three distinct clinical studies with non-overlapping subjects. All human subjects provided written informed consent, and all human studies were approved by the Institutional Review Board of the Cleveland Clinic approved all human study protocols (GenoBank IRB 4265; IRB 21-005 (xylitol ingestion related studies), healthy volunteer blood donors for platelet-related studies IRB 09-506). Detailed information about the clinical observational cohorts (discovery cohort and validation cohorts) and the xylitol intervention cohort can be found in Supplementary data online, Methods.

Animals

Mice were used to causally test whether elevation in xylitol plasma levels results in enhancement in thrombosis potential. All mice were C57BL/6J and 12–14 weeks of age. All animal studies were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic (IRB 2019-2251).

Agreggometry studies in platelet-rich plasma

Agreggometry in platelet-rich plasma (PRP) was performed as previously described.

Intracellular calcium measurements

Measurement of intracellular calcium release in washed platelets was performed as previously described.

Platelet flow cytometry assay

Antibody staining of washed platelets for flow cytometry was performed as described previously.

Imaging flow cytometry in whole blood

Imaging flow cytometry was performed on an Amnis ImageStreamX MK II two-camera system using anti-CD45 AF488, anti-CD41 AF647, and anti-CD62P-PE. Details and the gating strategy are described in Supplementary data online, Methods.

Whole blood in vitro thrombosis assay

Shear flow experiments of whole blood were performed with a Cellix Microfluidics System (Cellix, Dublin, Ireland) as previously described and outlined in Supplementary data online, Methods. The extent of platelet activation and adhesion to the collagen matrix was quantified using computer-assisted tomographic analyses, as previously described.

Carotid artery FeCl3 injury model

The FeCl3-induced carotid artery injury model was performed using intravital fluorescence microscopy with continuous image capture monitoring, as previously described. Time to cessation of blood flow through thrombus formation for all experiments was determined by visual inspection by two independent investigators.

Untargeted and targeted mass spectrometry analyses of human plasma

For untargeted mass spectrometry analyses, subject plasma samples were derivatized and run on gas chromatography–mass spectrometry (GC-MS) analyses as previously described. Raw data files were processed using the metabolomics BinBase database.
Stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) was developed to specifically quantify xylitol in human and mouse plasma, urine, and faeces (faecal pellets were dissolved in water and supernatant subjected to LC-MS/MS). The chromatographic separation of xylitol from its structural isomers is shown in Supplementary data online, Figure S1. Accuracy and precision of the method to quantify xylitol (separated from its structural isomers arabinol and ribitol) were assessed by a standard addition method of pure synthetic standard into three different plasma pools. Accuracy was 2.4%–2.8%, and intra- and inter-day coefficients of variation (CVs), were 1.2%–8.9% and 10.9%–11.5%, respectively. Urinary creatinine was similarly analysed by stable isotope dilution LC-MS/MS using D$_3$-creatinine as internal standard as previously described. Erythritol was quantified by stable isotope dilution LC-MS/MS as previously described. Further details for all assays can be found in Supplementary data online, Methods.

Statistical analysis
Continuous variables are summarized as median [interquartile range (IQR)], and categorical variables are presented as %. Difference between groups (e.g. tertiles) was examined using Kruskal–Wallis test for continuous variables and χ$^2$ test for categorical variables. Kaplan–Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratios (HR) and 95% confidence intervals (CI) for incident major adverse cardiovascular events (MACE). Adjustments included traditional cardiovascular risk factors (age, sex, diabetes mellitus, systolic blood pressure, low-density lipoprotein cholesterol levels, high-density lipoprotein cholesterol levels, triglyceride levels, smoking status) and high-sensitivity C-reactive protein (hsCRP). In some analyses, estimated glomerular filtration rate (eGFR) was used in addition to the aforementioned variables in the adjustment. We confirmed that the proportionality hazards assumptions were met using the Schoenfeld residuals against the transformed time. Mann–Whitney U-test or Wilcoxon matched-pairs signed rank test were applied to continuous variables to examine differences between two groups. Kruskal–Wallis test with Dunn’s post hoc test was used for pairwise multiple comparisons of ranked data. Two-way analysis of variance (ANOVA) with Sidák’s multiple comparison post hoc test was used for multiple-group comparisons of aggregometry responses using different concentrations of agonists. For analysis of collagen-dependent platelet adhesion in whole blood, a two-way repeated measures ANOVA with Sidák’s multiple comparison post hoc test was used. All reported measurements represent distinct samples. Data analyses were performed with R software (version 4.2.2) and GraphPad Prism software (version 9.1.2). A two-sided $P < .05$ was considered statistically significant.

Results
Untargeted metabolomics analyses in a discovery cohort suggested xylitol was associated with adverse cardiovascular outcomes
We have previously utilized untargeted metabolomics as a discovery platform to identify metabolites and pathways linked to residual CVD risks beyond traditional established risk factors. During the conduct of those untargeted mass spectrometry studies, we examined plasma from sequential stable subjects undergoing elective diagnostic cardiac evaluations (discovery cohort, n = 1157) and observed that numerous polyols, including a metabolite with presumed pre-derivatization elemental composition of C$_9$H$_{12}$O$_3$ and tentatively classified as xylitol, showed significant difference in level between those who experienced an incident (3-year) adverse cardiovascular event vs. those who did not. In new studies, we performed further analyses of the raw untargeted metabolomics data from this discovery cohort (Table 1 and Table S1 show baseline characteristics), this time utilizing a fragment ion more selective for xylitol as quantifying ion (Methods). As shown in Figure 1A, left, levels of the analyte tentatively annotated as xylitol showed enhanced relative abundance in subjects who experienced (vs. not) a MACE during the ensuing 3 years of follow-up. Further examination showed subjects with increased levels of the analyte [tertile 3 (T3) vs. either T1 or T2] displayed both poorer event-free survival in Kaplan–Meier survival analyses (Figure 1A, middle) and significant increased risk for incident (3-year) MACE [T3 vs. T1, HR (95% CI), 1.63 (1.04–2.53), $P < .05$] in Cox proportional hazards regression analyses, including following adjustments for traditional CVD risk factors and hsCRP [adjusted(a)HR (95% CI), 1.64 (1.05–2.56), $P < .05$; Figure 1A, right].

Stable isotope dilution tandem mass spectrometry analyses of xylitol in the validation cohort
Untargeted metabolomics studies are both semi-quantitative and not designed to necessarily distinguish between structural isomers. For example, xylitol and its isomers arabinol and ribitol all share identical elemental composition (C$_{5}$H$_{10}$O$_{5}$) and overall structure (see Supplementary data online, Figure S1) and have similar physicochemical properties; the isomers also have similar mass spectrometry fragmentation patterns and often co-chromatograph under routine separation protocols employed during untargeted metabolomics (Methods). We therefore developed a stable isotope dilution LC-MS/MS assay with conditions that provided baseline separation of xylitol from its structural isomers (see Supplementary data online, Figure S1) and then applied the method to unequivocally quantify xylitol in an independent (non-overlapping) validation cohort (n = 2149) comprised of stable subjects undergoing elective diagnostic cardiac evaluation (Table 1 and Table S2). Higher plasma levels of xylitol were again observed among subjects who experienced MACE (Figure 1B, left). Kaplan–Meier analysis similarly revealed higher levels of circulating xylitol were associated with poorer event-free survival over the follow-up period (Figure 1B, middle). In time-to-event Cox proportional hazards analysis, subjects with higher plasma xylitol levels had significantly increased risk of incident (3-year) MACE [HR (95% CI), 1.81 (1.29–2.53), $P < .001$], including following adjustments for traditional cardiovascular risk factors and hsCRP [aHR (95% CI), 1.57 (1.12–2.21), $P < .01$; Figure 1B, right]. Elevated levels of xylitol were similarly associated with incident risk in males and females alike, with no sex-specificity noted ($P$ interaction for sex $>.51$). Further, in Cox regression models where xylitol was treated as a continuous variable, xylitol remained independently associated with MACE following adjustments for traditional cardiovascular risk factors and hsCRP [aHR (95% CI), 1.06 (1.01–1.11) per SD of xylitol, $P = .023$]. In further analyses, elevated xylitol levels (T3 vs. T1) were also associated with incident thrombotic event risks [aHR 1.80 (1.05–3.08), $P = .03$]. In parallel analyses, we looked at long-term risk of MACE amongst validation cohort subgroups defined by multiple platelet-related phenotypes (see Supplementary data online, Figure S2). The significant association between xylitol levels with incident MACE risk was observed amongst subjects on anti-platelet drugs and, in general, amongst all subgroups examined where subjects were stratified by multiple different platelet functional metrics ($P$ for interaction in all subgroups >.35; Supplementary data online, Figure S2). When kidney function (eGFR) was included into the fully adjusted model, the association of xylitol with MACE remained significant [aHR 1.50 (1.06–2.11), $P = .02$].
Examination of post-prandial circulating levels of xylitol following ingestion of a xylitol-sweetened drink

Plasma levels monitored in both the discovery cohort and the validation cohort were following overnight (>12 h) fast. Since ‘naturally sweetened’ or ‘keto-friendly’ processed foods and beverages can contain large amounts of xylitol and thus might substantially increase circulating levels in the post-prandial setting, before performing platelet function studies, we assessed how high circulating levels of xylitol reach following a commonly observed dietary exposure. Post-prandial levels of xylitol were measured in healthy volunteers (n = 10) following ingestion of a xylitol-sweetened (30 g) water, an exposure comparable with a pint of numerous xylitol-sweetened ice creams, a xylitol-sweetened bakery good, or several pieces of xylitol-sweetened candy (clinicaltrials.gov identifier NCT04731363; Methods, Supplementary data online, Table S3). At baseline (following overnight fast), plasma levels of xylitol were low [median (IQR), 0.30 (0.27–0.34) µM] and comparable with quartile 1 (Q1) values observed in the validation cohort (Figure 1B, right). However, 30 min following ingestion, 1000-fold increases in plasma levels were noted [median (IQR), 312 (134–629) µM], with concurrent and subsequent excretion in the urine (Figure 2A and Supplementary Table 1).
Plasma xylitol levels returned to low-micromolar levels within 4 to 6 h [median (IQR), 1.87 (1.43–2.80) µM and 0.67 (0.57–1.25) µM, respectively], with a plasma half-life of ∼13 (± 4) min. The rapid rate of xylitol excretion observed in healthy volunteers with return to near baseline (fasting) levels within hours following ingestion of a significant dietary exposure suggests that the plasma levels observed in our observational (validation) cohort represent variations in endogenous production/levels and not food intake.

Physiological levels of xylitol augment platelet responsiveness

The observed positive association between xylitol and incident thrombotic event risk (Figure 1) suggested a potential impact of xylitol on platelet function. In initial studies, we assessed whether xylitol can impact platelet responsiveness using physiological concentrations of xylitol (i.e. at levels observed in overnight fasted subjects from our validation cohort, which is comprised of subjects with relatively...
Figure 2 Xylitol levels following oral challenge and effect of xylitol platelet responsiveness. (A) Study participants \((n = 10)\) were given 30 g of xylitol dissolved in water to ingest. Xylitol levels were quantified via LC-MS/MS in the blood before and at the indicated times after the xylitol challenge in the first four subjects. In the remainder of subjects, xylitol levels were measured before and 30 min after xylitol challenge. Values listed above data points at each time point represent median (IQR). The distribution of fasting \((\geq 12 \text{ h})\) plasma xylitol levels observed in the validation cohort is also shown; the dashed lines represent the upper and lower range, and the dotted lines indicate the bottom boundaries at quartiles \((Q)\) 2, 3, and 4 in the validation cohort. (B and C) PRP was isolated from healthy volunteers and used to study the effects of varying levels of xylitol on agonist-induced platelet aggregometry. Scatter plots show aggregometry responses for fixed concentrations of xylitol \((30 \mu M, \text{ red circles})\) vs. vehicle \((\text{blue circles})\) with different concentrations of ADP \((A)\) or thrombin receptor activator peptide \((TRAP6, B)\) with line representing medians. Global \(P\)-values (for xylitol effect) were calculated with two-way ANOVA and Šídák’s multiple comparisons test to compare groups. * \(P < .05\), ** \(P < .01\), *** \(P < .001\). Bar graphs (magnified areas) show submaximal ADP-stimulated \((2 \mu M, A)\) and TRAP6-stimulated \((5 \mu M, B)\) platelet aggregometry responses of human PRP following incubation with xylitol \((30 \mu M, \text{ red})\) vs. normal saline (vehicle, \text{blue}), with line and whiskers representing means \((\pm \text{SD})\). \(P\)-values were calculated by two-tailed Mann–Whitney test. (D and E) Aggregometry responses of human PRP with varying concentrations of xylitol and fixed submaximal concentration of ADP \((2 \mu M, D)\) and TRAP6 \((5 \mu M, E)\) with lines and whiskers representing medians (IQR). \(P\)-values were calculated by two-sided Kruskal–Wallis (K.W.) test with Dunn’s post hoc test. * \(P < .05\), ** \(P < .01\), *** \(P < .001\). Bar graph data are represented as means \((\pm \text{SD})\). \(P\)-values were calculated by two-tailed Mann–Whitney test.
preserved renal function; Table 1). Brief incubation of human PRP recovered from healthy volunteers with a fixed physiological (Q4) level of xylitol (30 µM) showed no effect. However, when platelets were exposed to the same fixed xylitol concentration in the presence of submaximal levels of known agonists such as adenosine diphosphate (ADP) or the thrombin receptor activator peptide TRAP6, marked enhancement in stimulus-dependent platelet activation and aggregation was observed (Figure 2B and C). Employing a different study design, we next instead used a fixed submaximal level of each of the platelet agonists (ADP or TRAP6) and varying levels of pre-incubation with xylitol. Notably, a dose-dependent enhancement in the extent of platelet responsiveness was observed across both the physiological range of xylitol in fasting subjects in our clinical (validation) cohort (e.g. 30 µM) and extending to the post-prandial levels observed in healthy volunteers following consumption of xylitol [e.g. we also examined 300 µM (Figure 2D and E), though post-prandial levels of up to ~1000 µM were observed; Figure 2A]. Collectively, these data show that within the range of fasting plasma (endogenous) levels of xylitol observed in our cohort, xylitol augments stimulus-dependent platelet responsiveness (i.e. shifts the dose response curve for known agonists to the left). Moreover, post-prandial levels of xylitol were observed to enhance platelet reactivity to an even greater extent.

Platelet aggregation responses can involve factors beyond direct interaction with platelets. We therefore isolated washed human platelets from healthy volunteers to directly test whether xylitol interacts with the platelets and impacts agonist-induced intracellular cytosolic calcium release. Brief (15–30 min) pre-incubation of Fura 2, loaded platelets with varying levels of xylitol, dose-dependently enhanced submaximal (0.02 U) thrombin-evoked intracellular Ca\(^{2+}\) release in multiple different platelet preparations (Figure 3A and B). In parallel experiments, exposure of washed human platelets to physiologic levels of xylitol caused dose-dependent enhancement in multiple examined platelet activation phenotypes including ADP-stimulated P-selectin surface expression (Figure 3C and D). Activated platelets bind to leucocytes—a process that leads to mutual changes in cellular effector functions and is associated with various CVD phenotypes.\(^{45}\) We therefore also examined the effect of xylitol on platelet–leucocyte aggregates. For these studies, we used imaging flow cytometry since it is optimized for cell–cell interaction and, in contrast to conventional flow cytometry, distinguishes between tethered platelets (genuine platelet–leucocyte aggregates) from coincidental, un tethered platelets near leucocytes.\(^{46}\) Following brief pre-incubation with a range of physiological levels of xylitol, whole blood from healthy volunteers showed a dose-dependent increase in TRAP6-stimulated platelet–leucocyte aggregates (Figure 3E).

**Xylitol enhances platelet clotting in whole blood and thrombosis potential in vivo**

To investigate the effect of xylitol on the initial step in thrombus formation, platelet adhesion to a collagen surface, we examined platelet adhesion in whole blood under physiological shear flow using a microfluidics device.\(^{35}\) Xylitol substantially accelerated the rate of collagen-dependent platelet adhesion and spreading under physiological shear flow (Figure 4A). In additional studies, the impact of xylitol on in vivo thrombosis potential was assessed using a FeCl\(_3\)-induced carotid artery injury model.\(^{39}\) In preliminary studies, mice showed poor oral absorption of xylitol (in comparison with erythritol) in both feeding studies (when provided in drinking water or food) and in studies where the sugar alcohol was delivered by gastric gavage (see Supplementary data online, Figure S4). Thus, an i.p. injection model was performed to recapitulate physiological plasma levels of xylitol in mice that paralleled those observed in humans. Compared with vehicle (saline) control, mice with elevated plasma levels of xylitol exhibited both a marked increase in the rate of clot formation (Figure 4B, left) and a significant reduction of the time to cessation of blood flow following arterial injury (Figure 4B, right).

**Xylitol dietary challenge in subjects enhances multiple indices of platelet reactivity**

Since our studies with isolated washed human platelets, PRP, whole blood, and murine in vivo thrombosis models all suggested that xylitol can impact platelet responsiveness, we next sought to examine whether dietary exposure to xylitol impacted platelet phenotypes in humans following ingestion of xylitol-sweetened water. For these studies, we rapidly isolated PRP from healthy volunteers (n = 10) before and 30 min after ingestion of xylitol (clinicaltrials.gov identifier NCT04731363, Methods) and assessed agonist-induced aggregation responses. As noted before, xylitol ingestion significantly increased postprandial (30 min) plasma levels [median (IQR), 312 (134–629) µM]. In parallel, platelet functional analyses revealed xylitol exposure provoked a substantial (multiple-fold) increase in aggregation responses to either ADP or TRAP6 (P < .0001 at all doses examined; Figure 5), in line with our *in vitro* studies using similar concentrations of xylitol (Figure 2D and E). When responses of individual subjects to submaximal agonist (ADP and TRAP6) concentrations were examined, a significant increase in platelet responsiveness was observed following xylitol ingestion in every subject (Figure 6, Supplementary data online, Figures S5 and S6). Further, subjects showing the largest increases in post-xylitol responsiveness post-xylitol challenge also tended to have the highest post-prandial xylitol levels, and a strong positive correlation was noted between postprandial xylitol levels and either ADP-induced or TRAP-induced aggregation responses (Spearman rho 0.71 and 0.74, respectively; P < .0001 for each; Supplementary data online, Figure S7).

**Discussion**

Many studies, including data analyses from the National Health and Nutrition Examination Survey (NHANES), have confirmed a dramatic increase of low-calorie sweetener use over the past decades.\(^{47}\) Meanwhile, the consumption of sweeteners is likely underestimated because of the lack of itemized listing of specific low-calorie sweeteners on labels in many reduced- and low-calorie processed foods and the lack of disclosure requirements (e.g. for the quantity used) in policies for food labelling by both the FDA and EU.\(^{46}\) Remarkably, artificial sweeteners have even been detected in presumed ‘non-consumers’ who were counselled extensively to avoid artificial sweetener exposure before they were enrolled into randomized clinical trials.\(^{39}\) Moreover, the increases in artificial sweetener use have even reached levels where they are readily detected within ground water and waste effluent, where their detection and quantification has been recommended as ‘ideal chemical markers of domestic wastewater in groundwater’.\(^{50}\) Although non-nutritive and low-calorie sweeteners have historically been GRAs by public agencies (e.g. EU\(^{33}\) and the FDA\(^{43}\)) several cohort studies,\(^{13–17}\) but not all,\(^{51,52}\) have linked ingestion of artificially sweetened foods with cardiometabolic adverse phenotypes. This recently prompted authorities, including the European Food Safety Authority (EFSA) and the World Health Organization, to re-evaluate exposure...
and toxicity of sweeteners, in particular with respect to potential long-term effects on health. In light of the substantial rise in the incorporation of artificial sweeteners into our food chain and the present studies identifying both clinical and mechanistic links between xylitol and CVD risks and relevant phenotypes, further studies that assess their long-term cardiovascular safety seem warranted. Polyol sweeteners like xylitol are difficult to quantify since they possess multiple structural isomers that differ only in the spatial orientation of the hydroxyl groups on the molecule. These difficulties, coupled with limited regulatory requirements for disclosure, have hampered their quantification in epidemiological studies to explore links between levels of sweeteners in blood and both metabolic and CVD risks. In the present studies, an unambiguous link between plasma levels of xylitol and incident MACE risks was observed in our validation cohort, where xylitol was separated from its structural isomers and quantified using isotope dilution LC-MS/MS. Further, comparisons between xylitol levels that elicit increases in platelet reactivity and in vivo thrombosis potential suggest even at elevated plasma levels among overnight fasted subjects (e.g. Q4), significant increases in platelet responsiveness are observed. By performing human clinical intervention studies exploring the physiological effects of xylitol ingestion, a marked enhancement in multiple indices of platelet responsiveness was observed in every subject examined (Structured Graphical Abstract). Collectively, the body of evidence accrued, including in vitro, in vivo (animal model), and xylitol

Figure 3 Xylitol increases stimulus-dependent intracellular calcium release and markers for activation in human platelets. (A) Representative fluorescent signal showing thrombin (0.02 U)-induced changes in intracellular calcium release in Fura-2-filled washed human platelets incubated with xylitol. (B) Fold change (relative to vehicle) in peak Fura 2 fluorescence following submaximal (0.02 U) thrombin stimulation at the indicated concentrations of xylitol in washed human platelets. Bars show mean with SEM indicated by whiskers. P-values were calculated by two-sided Kruskal–Wallis test with Dunn’s post hoc test. *P < .05; **P < .01; ***P < .001. (C) ADP-induced changes in P-selectin surface expression in washed human platelets pre-incubated with the indicated concentrations of xylitol. Plotted are interquartile ranges (boxes). The line in the box is the median, and whiskers represent minimum and maximum values. P-values were calculated by two-sided Kruskal–Wallis test with Dunn’s post hoc test. *P < .05; **P < .01; ***P < .001. (D) ADP-induced changes in GP IIb/IIIa (PAC-1 antibody staining) in washed human platelets pre-incubated with the indicated concentrations of xylitol. Plotted are interquartile ranges (boxes). The line in the box is the median, and whiskers represent minimum and maximum values. P-values were calculated by two-sided Kruskal–Wallis test with Dunn’s post hoc test. *P < .05; **P < .01; ***P < .001. (E, left) Representative fluorescent images of platelet–leucocyte aggregates (BF, bright field, CD45 in green, P-selectin in yellow, CD41 in red, merged image) in human whole blood stimulated with TRAP6 (7.5 μM). (Right) Numbers of platelet–leucocyte aggregates quantified by image stream in human whole blood incubated with indicated concentrations of xylitol at baseline (blue circles) and stimulated with 7.5 μM TRAP6 (red circles) relative to vehicle control with TRAP6. N numbers shown for donors for TRAP-stimulated blood samples, for unstimulated samples n = 6–8. P-values were calculated by two-sided Kruskal–Wallis test with Dunn’s post hoc test. *P < .05; **P < .01; ***P < .001
human ingestion studies, argues for a direct effect of the low-calorie sweetener xylitol on platelet function and thrombosis potential in vivo.

Interestingly, during the conduct of these and prior studies, we note inter-species differences in the absorption and metabolism of different sugar alcohols, arguing for both the necessity of monitoring circulating levels of specific molecular species in studies (to confirm what is ingested is actually absorbed) and the need for human clinical investigations. For example, we found xylitol, like erythritol, is readily absorbed following oral ingestion by humans, with plasma levels increasing over 1000-fold in the post-prandial setting. However, our present studies also revealed that in mice, in contrast to erythritol, which is rapidly absorbed by oral route, xylitol is poorly absorbed, with the majority of the ingested sugar alcohol recovered in faeces (see Supplementary data online, Figure S4). We therefore used intraperitoneal injection to elevate xylitol plasma levels in mice to the concentrations observed in humans, allowing for testing the effect of systemic exposures in rodent models analogous to those experienced in humans (Methods). Another example of inter-species differences in metabolism of sugar alcohols like xylitol within mammals occurs with dogs. While xylitol does not induce glucose or insulin secretion in humans or rodents, it is highly toxic to dogs, where it provokes extreme insulin secretion, and numerous reports list the extensive use of xylitol in human processed foods as a warning for pet (dog) owners.

Despite the clear evidence for xylitol inducing a direct physiological effect on isolated human platelets, the molecular participants involved (i.e. receptor) transmitting the effect on platelet function remain unknown. Indeed, little is known about how polyol sweeteners are recognized by cells. In the present studies, when added at submaximal levels, xylitol enhanced stimulus-induced platelet aggregation responses to multiple agonists (e.g. ADP, thrombin, collagen) and in vivo thrombosis formation. Collectively, when coupled with recent observations with erythritol, our data suggest that xylitol and erythritol (and possibly other sugar alcohol polyol sweeteners) act on converging pathways to enhance platelet responsiveness and in vivo thrombosis potential. They thus argue for a potential adverse class effect of some sugar alcohol sweeteners, a finding that is highly relevant for both the processed food industry and the diets of the more vulnerable subjects most likely to consume multiple artificial sweeteners (diabetics, obese, those with CVD).

One topic that deserves further discussion is the relationship in the large-scale clinical observational studies performed associating plasma levels of xylitol with incident risks of MACE and dietary exposures to xylitol. The present studies, we believe, argue that the observed associations noted in the discovery and validation cohorts reflect endogenous xylitol levels (steady state between endogenous production and excretion) and not recent dietary exposure. First, while a

Figure 4 Xylitol enhances in vivo clot formation. (A) Human platelet adhesion in whole blood to a collagen-coated microfluidic chip surface under physiological shear conditions ± xylitol. Representative images of platelet (green) adhesion at the indicated times (scale bar, 50 μm). P-values were calculated by two-way repeated measures ANOVA with Šidák’s post hoc test. Overall, P-value (xylitol effect) is shown in black, and Šidák’s post hoc test P-values are shown in red over the three follow-up times. Data is represented as means (±SEM). (B) Representative micrographs of carotid artery thrombus formation at the indicated time points following FeCl₃-induced carotid artery injury (scale bar, 200 μm) and time to cessation of blood flow in mice from indicated groups i.p. injected with vehicle or xylitol. Bars represent means, and two-sided P-values were calculated by Mann–Whitney test. Plasma xylitol concentrations in both groups are indicated as means (±SEM).
As far as we are aware, the glucuronic acid pathway has not yet been linked with CVD pathogenesis. However, we note that in recent studies, glucuronic acid levels were reported to be associated with health span and longevity in both population-based cohort studies and in mice. Yet, other studies have linked glucuronic acid pathway intermediates with cell migration and metastasis. Finally, we also think it important to mention that throughout the evolution of humans, Homo sapiens have not experienced micromolar levels of xylitol in plasma, i.e. until its introduction into our food chain within the past decade or so. The present studies show, however, that even at plasma xylitol levels as low as 19 µM in animal models of thrombosis (Figure 4B), well within the fourth quartile of our fasting plasma levels from a cohort (validation) of subjects with largely preserved renal function, we observe significant heightened thrombosis potential in vivo. Based on our additional human intervention study data (Figures 2, 5, and 6), much higher post-prandial levels of xylitol (driven by consumption of dietary xylitol from artificially sweetened processed foods) can be superimposed on endogenous levels, substantially further enhancing thrombotic risk (especially in the post-prandial setting).

Our studies have several limitations. We only measured baseline fasting levels of xylitol in our clinical cohort studies; thus, whether serial measures of xylitol provide enhanced clinical prognostic value for CVD risk assessment remains unknown. In addition, as noted, our observational cohorts do not have dietary information. Moreover, our subjects have a high burden of CVD risk factors, and whether the results can be translated to a community-based setting needs to be determined. However, we note that in countries like the USA, most adults in their 40s have at least one chronic disease and in their 60s two or more chronic diseases, with cardiometabolic diseases including obesity, diabetes, and CVD among the most prevalent. A further limitation of our studies is that the initial observational cohort results are only associative in nature and there is the possibility for residual confounding. However, we also note that we used the observational cohort findings as hypothesis generating and supplemented these findings with numerous in vitro and in vivo studies, as well as an interventional human xylitol challenge study. Collectively, all results point to xylitol exerting a direct effect on platelet function and thrombosis potential in vivo, not only at levels observed in the post-prandial setting but also across the distribution of overnight fasting (and presumed endogenous) plasma xylitol levels observed. Our mechanistic studies also focused on a relatively brief exposure of xylitol, and the effects of chronic exposures need to be explored in future studies. We also note that the receptors involved in recognizing xylitol remain unknown. However, our studies show that xylitol directly elicited enhancement in stimulus-dependent intracellular calcium release and activation in isolated washed human platelets, including for various receptor systems (those recognizing ADP, thrombin, and collagen). Finally, beyond direct platelet effects of xylitol, further studies on different processes that contribute to coagulation are of interest, given the strong clinical association of xylitol levels with thrombotic event risk.

With increased availability and reduced production costs, artificial sweetener use in processed food has expanded in general and xylitol consumption specifically. Industrial production of xylitol has recently been advanced through new biotechnological methods that reduce cost and energy requirements (e.g. microbial and enzymatic fermentation with the substrate (xylose) obtained from abundant sources, such as an intermediate of the glucuronic acid pathway—an alternative route of glucose utilization that provides biosynthetic precursors and involves detoxification through glucuronidation with an estimated endogenous production of 15 g daily in subjects. As far as we are aware, the glucuronic acid pathway has not yet been linked with CVD pathogenesis. However, we note that in recent studies, glucuronic acid levels were reported to be associated with health span and longevity in both population-based cohort studies and in mice. Yet, other studies have linked glucuronic acid pathway intermediates with cell migration and metastasis. Finally, we also think it important to mention that throughout the evolution of humans, Homo sapiens have not experienced micromolar levels of xylitol in plasma, i.e. until its introduction into our food chain within the past decade or so. The present studies show, however, that even at plasma xylitol levels as low as 19 µM in animal models of thrombosis (Figure 4B), well within the fourth quartile of our fasting plasma levels from a cohort (validation) of subjects with largely preserved renal function, we observe significant heightened thrombosis potential in vivo. Based on our additional human intervention study data (Figures 2, 5, and 6), much higher post-prandial levels of xylitol (driven by consumption of dietary xylitol from artificially sweetened processed foods) can be superimposed on endogenous levels, substantially further enhancing thrombotic risk (especially in the post-prandial setting).

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These advances contribute to increasing global production rates and market penetration with more than a 40-fold increase of xylitol production over the last four decades. For example, xylitol has even been proposed by some as a public health intervention for use in children to prevent dental caries, with recommendation of at least 6–10 g of xylitol daily. Large amounts of xylitol are typically found in numerous processed foods designed for a diabetic diet, like baked pastries, keto ice cream, and confectioneries, often exceeding 30 g per serving size. Despite the recent guidance from the WHO recommending avoidance of artificial sweeteners for weight reduction, the acceptable daily xylitol intake assigned by the WHO/FAO Expert Committee on Food Additives remains ‘not specified’. Our studies suggest that xylitol will likely confer heightened thrombosis potential in the same vulnerable patients that it is marketed towards and intended to protect (e.g. subjects with diabetes, obesity, and CVD).

**Figure 6** Effect of dietary xylitol exposure on platelet responsiveness in individual subjects. (A and B) Platelet aggregation responses in PRP from each subject in response to submaximal concentration of ADP (2 μM, A) and TRAP6 (7.5 μM, B) before and after xylitol exposure. Shown are aggregation responses of paired samples (baseline and post-xylitol) that were analysed together. Boxes represent interquartile ranges (IQR) with the notch indicating the median. Lower whiskers represent the smallest observation (≥25% quantile – 1.5 x IQR) and upper whiskers the largest observation (≤75% quantile + 1.5 x IQR). The total number of replicates per individual donor is indicated. All subjects showed significant differences in agonist-induced aggregation (P < .05) for pairwise comparison (pre- vs. post-xylitol exposure) except for Subject 1 with only three replicates, and Subject 10 with four replicates showed P = .07 for TRAP6 stimulation. *P*-values were calculated with the Kruskal–Wallis (K.W.) test with a Dunn post hoc analysis.
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Supplementary Data
Supplementary data are available at European Heart Journal online.

Declarations
Disclosure of Interest
S.L.H. and Z.W. report being named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. S.L.H. and Z.W. also report having received royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab, a fully owned subsidiary of Quest Diagnostics, and Proctor & Gamble. S.L.H. is a paid consultant for Zehna Therapeutics and formerly for Proctor & Gamble; has received research funds from Zehna Therapeutics, Proctor & Gamble, Pfizer, and Roche Diagnostics; and is eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics and therapeutics from Zehna Therapeutics. W.H.W.T. reports being a consultant for Sequana Medical A.G., Owkin Inc., Relaypsa Inc., and PreCardiac Inc. and having received honorarium from Springer Nature for authorship/editorship and American Board of Internal Medicine for exam writing committee participation—all unrelated to the subject and contents of this paper. The other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Data Availability
Where permissible, the data sets generated and/or analysed during the present studies are available from the corresponding author on request. There are restrictions to the availability of some of the clinical data generated in the present study, because we do not have permission in our informed consent from research subjects to share data outside our institution without their authorizations. Custom R codes used in this manuscript can be accessed at https://doi.org/10.5281/zenodo.6780497. Where permissible, source data are made available in the public data sharing repository (https://doi.org/10.5281/zenodo.10106620). Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

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Ethical Approval
We performed three distinct clinical studies. All human subjects provided written informed consent, and all human studies abided by the Declaration of Helsinki. The Institutional Review Board of the Cleveland Clinic approved all human study protocols [GeneBank IRB 4265; IRB 21-005 (xylitol ingestion related studies), healthy volunteer blood donors for platelet-related studies IRB 09-506]. All animal studies were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic (IRB 2019-2251).

Pre-registered Clinical Trial Number
The pre-registered clinical trial number for GeneBank at the Cleveland Clinic is NCT00592000. The trial number for the xylitol intervention study is NCT04731363.

References