Regulation of Postprandial Nitric Oxide Bioavailability and Vascular Function By Dairy Fat

Research Protocol

To ensure an effective review by the Institutional Review Board, a full description of the planned research must be submitted with the Application for Initial Review. A research protocol provides the reader with background information of the problem under study, including the study rationale, a detailed plan for conducting the research involving human research participants, and a discussion of the potential importance of the research.

I. Objectives

The *objective of this application* is to define mechanisms by which dairy milk, and its protein and lipid fractions, protect against postprandial vascular dysfunction by reducing oxidative stress responses that limit nitric oxide ($NO^{\bullet}$) bioavailability to the vascular endothelium. Our *central hypothesis is that dairy milk ingestion by prediabetic adults will protect against PPH-induced vascular dysfunction through oxidative stress-dependent mechanisms that increase* $NO^{\bullet}$ *bioavailability, independent of changes in blood pressure.* To test this, we will complete the following objectives: 1) define $NO^{\bullet}$-mediated vasoprotection of non-fat milk on PPH-induced vascular dysfunction, 2) define the independent vasoprotective mechanisms of milk proteins on postprandial vascular function, and 3) define vasoprotection by dairy fat on PPH-induced vascular dysfunction. Upon completing these studies, we expect to provide the first direct evidence that dairy milk, mediated through its vasoactive protein and lipid fractions, protects against vascular dysfunction otherwise induced by PPH. Thus, these studies will extend epidemiological evidence supporting cardioprotective activities of dairy while also facilitating translational messages that encourage dairy consumption to mitigate metabolic insults leading to vascular dysfunction.milk.

II. Background and Rationale

Background. Cardiovascular disease (CVD) is the leading cause of death in the US, accounting for ~830,000 deaths annually.[2] Oxidative stress and inflammation are central mechanisms leading to vascular dysfunction due to their role in reducing nitric oxide ($NO^{\bullet}$) bioavailability. Although greater intakes of low-fat dairy foods have been associated with a lower incidence of CVD-related morbidity,[3, 4] perhaps by decreasing blood pressure,[3, 5-7] this is unlikely to fully explain the cardioprotective activities of dairy. Thus, a critical need exists to comprehensively define mechanisms by which dairy protects against CVD, consistent with our controlled studies and those of others demonstrating that dairy attenuates vascular dysfunction independent of any changes in blood pressure.[8-10] In the absence of this knowledge, the development and validation of dairy-based strategies to improve vascular homeostasis and mitigate CVD risk will remain limited, and its successful management will continue to be problematic.

Dairy Improves VEF Independent Of Blood Pressure. Observational studies support greater consumption of dairy foods to lower CVD risk. In support, a meta-analysis shows an inverse relation indicating 6% lower CVD risk per 200 mL/d of milk intake.[15] Although greater dairy intakes are linked to lower blood pressure,[16-19] its blood pressure-lowering activities do not fully explain its role in reducing CVD-related morbidity.[15, 20] Indeed, our clinical studies[8, 11, 21] and others[22, 23] show that dairy and its bioactive constituents may lower CVD risk by regulating vascular endothelial function (VEF), glucose homeostasis, inflammation, and oxidative stress independent of changes in blood pressure.

VEF Can Be Evaluated Non-Invasively. Vascular dysfunction is an early atherosclerotic event[26-30] that promotes vasoconstriction, platelet aggregation, thrombosis, and inflammatory cell adhesion to the endothelium.[31] Its early detection is critical for preventing CVD, as the degree of vascular dysfunction increases CVD risk.[32] Fundamental knowledge showing that the vascular endothelium centrally regulates CVD progression led to validating techniques that quantify VEF. Brachial artery flow-mediated dilation

(FMD) has emerged as a *validated ultrasound technique that non-invasively* assesses VEF to predict CVD,[34, 35] even after controlling for various risk factors.[36] FMD is responsive to lifestyle modifications,[37, 38] supporting its use for defining incremental changes in vascular health and CVD risk. FMD assessment involves causing brief reactive hyperemia using cuff occlusion distal to the ultrasound probe, as this cuff position better reflects endothelium-dependent vasodilation.[39] Shear stress following cuff release stimulates luminal mechanoreceptors to initiate a signaling cascade that increases endothelial nitric oxide synthase (eNOS)-mediated $NO^{\bullet}$ biosynthesis and vasodilation. Percent change in vessel diameter is calculated relative to baseline vessel diameter to define FMD.

Acute Hyperglycemia Increases CVD Risk. Chronic hyperglycemia is known to increase CVD risk.[41] *However, PPH better predicts CVD-related mortality compared to fasting glucose regardless of the presence of diabetes.*[42] PPH, but not fasting glucose, also correlates with carotid intima media thickness (cIMT),[43-45] an index of atherosclerosis. Our clinical trials and those of others support PPH as a risk factor for CVD as evidenced by PPH transiently impairing VEF in normo- and hyper-glycemic adults.[1] That PPH is implicated in CVD provides clear rationale for validating dairy-based strategies for attenuating PPH-induced vascular dysfunction.

The mechanism by which PPH impairs VEF is incompletely understood, but as we reviewed,[1] oxidative stress decreases $NO^{\bullet}$ bioavailability.[46-48] Using FMD, we show that PPH impairs VEF by decreasing $NO^{\bullet}$ status in an oxidative stress-dependent manner.[9] In a cross-over design, healthy men ingested glucose or fructose (75 g) prior to assessing VEF for 3 h postprandially. Glucose ingestion, but not fructose, increased plasma glucose and decreased FMD. Decreases in FMD correlated highly to postprandial glycemia ($r = -0.82$), supporting that reducing PPH protects against vascular dysfunction. Malondialdehyde (MDA) increased to a greater extent following glucose ingestion. It also correlated to glycemic responses ($r = 0.87$), and inversely correlated to FMD ($r = -0.80$), supporting that PPH impairs VEF in an oxidative stress-dependent manner. We also show that oxidative stress impairs VEF by increasing asymmetric dimethylarginine (ADMA) relative to arginine (ARG).[9] ADMA is an endogenously produced competitive inhibitor of eNOS that outcompetes ARG for eNOS-mediated synthesis of $NO^{\bullet}$.[1] Increases in basal ADMA (~0.5 μM) by as little as 0.1 μM increase CVD risk up to 5.3-fold[49] due to lower $NO^{\bullet}$ status and greater vascular dysfunction.[50] Our novel evidence shows that PPH-induced vascular dysfunction is accompanied by greater increases in ADMA/ARG.[9] Consistent with PPH, glucose ingestion decreases ARG to a greater extent than fructose. Although postprandial ADMA was unaffected, glucose caused greater time-dependent increases in ADMA/ARG than fructose. No time or trial effects occurred for blood pressure. Thus, PPH reduces $NO^{\bullet}$ synthesis by limiting ARG availability and increasing ADMA relative to ARG, which limits substrate for $NO^{\bullet}$ biosynthesis while competitively inhibiting eNOS.

Oxidative stress also decreases $NO^{\bullet}$ bioavailability by oxidizing tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2), which limits BH_4 binding to eNOS and prevents $NO^{\bullet}$ synthesis.[51] In isolated endothelial cells, hyperglycemia decreases the BH_4/BH_2 ratio.[52] In addition, other oxidative stressdependent mechanisms exist by which PPH impairs VEF. For example, low endothelin-1 (ET-1) levels are considered vasodilatory, but their increase during acute hyperglycemia[56] triggers oxidative stress by activating NADPH oxidase, resulting in eNOS uncoupling that reduces $NO^{\bullet}$ synthesis.[54, 55] Similarly, thromboxane A_2 (TXA_2) increases oxidative stress via protein kinase C-dependent activation of NADPH oxidase, which impairs eNOS-dependent $NO^{\bullet}$ synthesis and VEF.[57] Emerging evidence also implicates the highly reactive dicarbonyl and glycolytic byproduct methylglyoxal (MGO), a precursor of advanced glycation end-products, in increasing CVD risk.[56, 57] MGO is higher in diabetics[58] and increases during PPH.[59, 60] It also promotes mitochondrial dysfunction that increases free radical generation.[61] This impairs $NO^{\bullet}$ synthesis by uncoupling eNOS and oxidizing BH_4,[62] resulting in vascular dysfunction.[59, 60] Indeed, we show that a glucose challenge in healthy men increases postprandial plasma MGO,[59] consistent with PPH-mediated oxidative stress and decreases in FMD shown to occur in this same study population.

Milk Mitigates PPH-Associated Vascular Dysfunction. Milk induces a low glycemic response,[63] highlighting its potential to limit PPH and downstream responses that impair VEF.[1] Our double-blind, cross-over trial in metabolic syndrome (MetS) adults examined the extent to which low-fat milk limits PPH-mediated disturbances in oxidative stress and NO• bioavailability.[8] Participants ingested low-fat milk (2 cups) or an isocaloric volume of rice milk prior to evaluating FMD and metabolic responses for 3 h postprandially. Plasma glucose increased by 17-38% at 30-120 min relative to baseline following rice milk ingestion.[8] In comparison, low-fat milk increased plasma glucose by 11% at 30 min, which returned to fasting levels by 60 min. MDA increased by 17-33% at 30-120 min relative to baseline while FMD decreased by 37-47% at 30-60 min following rice milk ingestion. In contrast, MDA and FMD were unaffected by low-fat milk. Relative to baseline, ADMA/ARG increased at 60-180 min following rice milk suggesting a decrease in NO• bioavailability. In comparison, ADMA/ARG decreased at 30 min and returned to levels no different than baseline by 60 min following low-fat milk ingestion. No time or trial effects were observed for blood pressure. These data support that low-fat milk ingestion at a volume consistent with the US Dietary Guidelines[37] maintains VEF, without affecting blood pressure, by limiting PPH-dependent oxidative stress responses that reduce NO• bioavailability.

Milk Peptides Improve VEF. Dairy contains bioactive constituents that improve VEF.[12] Whey and casein represent ~20% and 80% of milk proteins,[70] and both have vasoactive properties, but whey has received more extensive study.[71-73] We showed that NOP-47, a whey-derived peptide, improves postprandial VEF.[11] In a placebo-controlled cross-over study in adults, whey peptide (5 g) ingestion increased FMD at 30-90 min compared to fasting levels. Improved VEF was accompanied by an attenuation in the decline of NO• metabolites, suggesting that NOP-47 improves VEF in a NO• dependent manner. In support, mesenteric arteries incubated with whey peptide have greater endothelium-dependent relaxation that is abolished by inhibiting NOS.[74] In contrast, little is known about the vasoactivities of casein. Supplementation of a casein-derived lactotripeptide compared to sodium caseinate improved FMD in normotensive postmenopausal women.[81] Further complicating are the lack of studies directly comparing vasoactivities of whey protein and casein, a knowledge gap this application will address by defining the relative potencies of these major milk proteins on VEF.

In ***conclusion***, evidence shows that: 1) PPH increases CVD risk, 2) lowering PPH and oxidative stress mitigates vascular dysfunction in a NO•-dependent manner, and 3) dairy attenuates oxidative stress and PPH, which likely protects against vascular dysfunction by improving NO• bioavailability. This provides a compelling basis to study vasoprotective activities of dairy milk. In addition, our team has the expertise needed to perform controlled interventions that assess VEF, oxidative stress, and NO• homeostasis, thereby positioning us ideally to undertake these innovative studies that will advance the understanding of bioactive components of dairy milk.

Anticipated Results, Significance, and Potential Benefit

Most studies examining dairy-dependent vasoprotection have focused on its anti-hypertensive activities with limited regard for other mechanisms that could improve VEF. While important, and as we discuss,[12] blood pressure-lowering activities of dairy are unlikely to fully explain its role in reducing CVD risk. Thus, expected findings from the proposed randomized controlled, cross-over trials are anticipated to be paradigm-shifting by mechanistically linking improvements in VEF by dairy milk, and its protein and lipid fractions, to reductions in PPH-induced oxidative stress responses that otherwise limit NO• bioavailability and increase CVD risk. Findings of this application are also expected to provide the first evidence attributing the relative vasoprotective activities of whey protein, casein, and dairy fat on PPHinduced vascular dysfunction in association with improvements in oxidative stress and NO• homeostasis. These collective findings are also directly translatable into evidenced-based recommendations supporting forthcoming revisions to the US Dietary Guidelines regarding dairy consumption.

Pitfalls and Alternatives

Based on our preliminary studies, we do not anticipate any conceptual or technical difficulties in this application.

III. Procedures Study Design

This IRB protocol addresses three objectives by completing two separate randomized cross-over studies in two separate cohorts of participants. For clarity, *Study 1* is a 4-arm cross-over study that addresses the work under *Objectives 1 and 2* and will utilize one cohort of participants. *Study 2* is a separate 3-arm cross-over study that addresses the work under *Objective 3* and will utilize a different cohort of participants. Each study aims to enroll 24 participants for a total of 48 participants for all of the anticipated work.

Studies under Objectives 1 and 2 will be accomplished through a common 4-arm crossover study. For **Objective 1**, which will examine the inhibition of non-fat milk on PPH-induced vascular dysfunction, prediabetic men and women (n = 24) in the fasted state will ingest 75 g glucose dissolved in water or nonfat milk (2 cups; 473 mL) on two occasions separated by ≥7 d. We will then assess FMD and collect blood samples prior to and at 30 min intervals for 3 h following test beverage ingestion to examine VEF and biomarkers regulating $NO^{\bullet}$ bioavailability. In **Objective 2**, which will define independent contributions of dairy proteins in regulating VEF, participants will ingest glucose (75 g) with isonitrogenous quantities of whey protein isolate or sodium caseinate (16.5 g in 473 mL water) on two occasions separated by ≥7 d. Identical to Objective 1, FMD and blood samples will be obtained at 30 min intervals for 3 h. For **Objective 3**, which will define vasoprotective activities of dairy fat, a separate cohort of prediabetic men and women (n = 24) will complete a 3-arm cross-over study by ingesting glucose (75 g), or glucose with non-fat milk or whole milk (2 cups; 473 mL) on three occasions separated by ≥7 d. Identical to Objectives 1-2, FMD and blood samples will be obtained at 30 min intervals for 3 h.

Sampling Criteria and Methods

Enrollment Criteria. Prediabetic men and women are an ideal study population because of their underlying vascular dysfunction and exacerbated PPH.[82] They will be required to meet these ***inclusion criteria***: a) prediabetes (fasting glucose 100-125 mg/dL and hemoglobin A1c 5.7-6.4%[83]), b) 18-50 y of age, c) non-dietary supplement user, c) no medications affecting vasodilation, inflammation, or energy metabolism, e) no CVD, and f) nonsmoker. To improve homogeneity, we will only enroll individuals having blood pressure <140/90 mmHg and total cholesterol <240 mg/dL. Those having any of these ***exclusion criteria*** will not be enrolled: a) unstable weight (±2 kg), b) vegetarian or dairy allergy, c) alcohol intake >3 drinks/d or >10 drinks/wk), d) ≥7 h/wk of aerobic activity, or e) women who are pregnant, lactating, or have initiated or changed birth control in the past 3-mo. For all studies, the washout period will be 7 d for men and 1 mo for women because women require vascular testing at the same time during their menstrual cycle to account for known changes in vascular reactivity.[84]

Recruitment. We will recruit participants through posted flyers, e-mail, electronic and newsprint advertisements (e.g. campus student and faculty/staff newspapers, local and regional newspapers), word of mouth, and through use of ResearchMatch (OSU CCTS). The posted advertisements will instruct interested participants to call the study center (Bruno Laboratory, Department of Human Sciences) to obtain further information. During the phone-in hours, a trained individual (i.e. project coordinator or graduate assistant) will be available to describe the study and determine preliminary qualification by conducting a scripted phone interview (e.g. do you take vitamin supplements?, are you healthy?; see attached *Phone Script*). The individual will record answers and assess whether or not the person calling is likely or not to be an acceptable study participant. If the caller and the interviewer agree that the caller should participate, the prospective participant will be invited to a screening meeting, where the study will be fully described and the individual will be provided a consent form to complete prior to any involvement in the study procedures.

Data collected during this phase will include participant's age, health status, physical activity, and contact information (see *Informed Consent*). Women will be asked to complete a short questionnaire regarding their menstrual history (see *Menstrual History Questionnaire*).

Recruiting efforts through ResearchMatch will utilize a strategy whereby registered individuals in the ResearchMatch database can be searched for against their non-identifiable volunteer profile in the system. Unidentified individuals meeting search criteria will then be forwarded an electronic recruitment message (see attached *ResearchMatch Recruitment Message*) that identifies them as a potential match for study participation. The secure ResearchMatch clearinghouse will route this standard notification that provides specific study content (i.e. content similar to that of a posted advertisement) to each of these potential ResearchMatch volunteers who will then have the option of replying "yes", "no", or not respond through a set of quick links available in this notification. ***Note:*** This message will not include the study's direct contact information (e.g. email, phone) as ResearchMatch will measure the response rate through the clearinghouse's quick links made available in this electronic message. The response rate metrics will be made available to researchers through their ResearchMatch dashboard as well as the Institutional Liaison dashboards. By responding "yes", the volunteer has authorized ResearchMatch to release their contact information to the researcher responsible for the study. This information will be made available on the researcher's ResearchMatch study dashboard. The researcher will be responsible for managing this contact information as specified in the IRB-approved study protocol. ResearchMatch will also be collecting aggregate data regarding the status of ResearchMatch volunteers within the study. ResearchMatch volunteers consent to this within the ResearchMatch volunteer agreement. The ResearchMatch enrollment continuum will allow researchers to indicate where the volunteer currently stands within the recruitment process and thus helps researchers monitor the utility and effectiveness of this recruiting tool. Research access to recruit through ResearchMatch will last only as long as the duration of IRB-study approval with the expiration date of ResearchMatch being identical to the end-date of OSU IRB approval. Researchers will be able to submit current IRB-approval letters for the lifetime of the study and thus provide evidence of successful continuing review applications. If an unintentional lapse in time occurs and the research is not able to submit this continuing review evidence via ResearchMatch, stored ResearchMatch data will not be deleted, but the researcher will not have access to this information until a current IRB-approval letter is uploaded and routed to the Institutional Liaison for review. A complete description of ResearchMatch along with the most current IRB approval from Vanderbilt University (i.e. this is the site where ResearchMatch was developed and its secure computer servers are housed) has been attached.

Power Calculation and Statistics. A power calculation was performed to determine the fewest participants needed to complete the two separate proposed cross-over studies (i.e. *Objectives 1-2* and *Objective 3*). We used FMD data from our studies[9, 10] and predicted that non-fat milk will protect against PPH-mediated decreases in FMD. Assuming no gender differences, which is consistent with our earlier studies in this area,[8] 11 prediabetic adults are needed to reject the null hypothesis with 80% power ($P<0.05$). Thus, 24 male and female prediabetics will be enrolled to improve statistical power to >90% while also accounting for study attrition that may occur due to women requiring a longer washout period between trials (1 mo vs. 7 d). Although participants will complete all arms of each cross-over study, statistical analysis will be handled discretely to examine differences between trials within each *Objective*. Baseline comparisons between trials will be performed a paired t-test (*Objectives 1-2*) or 1-way RM ANOVA (*Objective 3*) to ensure no changes in participant health status. Other analyses will be performed by 2-way RM ANOVA to define time and treatment effects, and their interaction. In the presence of any significant effects, Bonferroni's post-test will be used to examine pair-wise differences. To better define postprandial responses between treatments, area under the curve ($AUC_{0\text{-}3\ h}$) will be calculated for each biomarker to enable more sensitive comparisons of postprandial responses between treatments and simplify analyses to a paired t-test (*Objectives 1-2*) or 1-way ANOVA (*Objective 3*) as we described.[9, 10] Lastly, to define correlations between study variables, we will perform multiple linear regression, controlling for within-subject repeated measures.[85]

Justification of Test Meals. In *Objective 1*, beverages will consist of 75 g glucose in 473 mL water vs. 75 g glucose in 473 mL non-fat milk. This volume of milk was chosen to be consistent with our work[8] and recommendations of the US Dietary Guidelines for Americans. The amount of glucose administered (75 g) is equivalent to that of a standard oral glucose challenge. We fully acknowledge that test beverages in *Objective 1* are not isocaloric. While this might be viewed as a limitation, this approach is being specifically undertaken to determine the vasoprotective effects of non-milk mediated through an attenuation of PPH-dependent vascular dysfunction rather than through the displacement of carbohydrate. In *Objective 2*, protein is being administered at 16.5 g to reflect total protein of non-fat milk, rather than their proportional contributions in milk, to pharmacologically define each protein in regulating VEF. In *Objective 3*, beverages will consist of glucose (75 g; positive control) vs. glucose (75 g) ingested with non-fat milk or whole milk. Despite associations between dietary fat and CVD risk, experimental evidence is lacking to support: 1) widespread restrictions of fat-containing dairy foods, and 2) that dairy fat, which is predominately saturated fat, is causatively responsible for increasing CVD risk. This approach will enable an interpretation of the contribution of dairy fat in protecting against PPH-induced VED while providing findings to fill this research knowledge gap.

Dietary Control. Participants' basal diets will be controlled for 3 d leading into each trial, consistent with our other successful postprandial studies.[8-10] A 3-d energy adequate, but dairy-free diet will be developed to provide 50-60% of energy from carbohydrate, 15-20% from protein, and 25-30% from fat. To normalize physiologic responses to test beverages, diets will contain >150 g of carbohydrate/d. Diets will also contain 1.5 servings per day of fruit and 1.5 servings/d of vegetables to improve homogeneity of participants' antioxidant status while also being consistent with American dietary patterns in that ~7080% of adults do not consume the recommended 5 servings/d of fruits and vegetables.[87, 88] Fruit/vegetable choices by participants will also be limited to better control antioxidant intakes (e.g. standardizing citrus vs. non-citrus foods due to differences in vitamin C). All foods and beverages will be provided to participants for 3 d preceding each trial. In addition to controlling fruit/vegetable intake, participants' dietary nitrate/nitrite will also be restricted to <100 mg/d by eliminating foods high in these compounds.[89, 90] To control physiologic responses between trials, participants will replicate their prescribed 3-d diet for each subsequent trial. To ensure compliance, participants will be instructed to complete a food record for the 3 d preceding each trial. Accuracy will be confirmed by a dietitian and nutrient intakes assessed using NDSR dietary analysis software (University of Minnesota). Consistent with our work,[8-10] and our experience in assessing FMD,[1] VEF assessments will be standardized by having participants abstain from exercise and foods affecting vasodilatory responses (e.g. caffeine, alcohol, niacin supplements) for 48 h preceding each trial.

Detailed Study Procedures

Overview of Study Procedures. Potential participants who call the study center for more information as well as those identified through ResearchMatch will be given a brief description of the study and asked a few questions to determine their eligibility (*see Phone Script Study 1 (Objectives 1 and 2); Phone Script Study 2 (Objective 3)*). If they meet the initial study criteria, they will be invited to the study center for a screening meeting. During this meeting, the Informed Consent (attached) will be explained and provided to them for their review. The participant will then have the opportunity to review the Informed Consent, and if he/she chooses to participate in the study, will be asked to provide written consent. Women choosing to participate will also be asked to self-administer a urinary pregnancy test. If women self-report that the pregnancy test is negative, they will fill out a short questionnaire regarding their menstrual history (*see Menstrual History Questionnaire*), so that each controlled intervention is completed at the same phase of the female participant's menstrual cycle, consistent with standardized guidelines for vascular testing.[104] Then, blood pressure and a small blood sample will be collected for blood chemistry analysis. These results will be used to determine eligibility. Eligible participants who agree to proceed with the study will be invited to meet with a Registered Dietitian who will instruct them on how to complete a dietary food record. Participants under Objectives 1 and 2 will then complete four 3 h postprandial trials in a randomized order;

participants under Objective 3 will then complete three 3 h postprandial trials, also in a randomized order. We estimate that completion of all study procedures will take ~4-6 weeks for men and ~4-6 months for women. Each step of the study procedure will be discussed in detail below:

Screening Meeting. Potential participants who have met the initial criteria of the study (based on the telephone interview) will be invited to the study center at a mutually convenient time. During this time, the participant and a member of the research team will meet in a private, quiet conference room or office. The individual will be provided the informed consent form, and its contents will be described to the potential participant. The participant will then have the opportunity to review it, and if they choose to participate in the study, they will be asked to provide written consent. Although the participant will be asked to sign the informed consent, the participant will be told that they will not be asked to participate if their fasting serum chemistries do not meet the study criteria. If the participant has provided consent and is fasted for 12 h, we will request if a trained individual can draw a small blood sample (15 ml; 1 T) so that we may measure blood chemistries (glucose and cholesterol) as well as perform a finger prick to obtain a small drop of blood to measure hemoglobinA1c (HbA1c). All samples will be coded to maintain participant anonymity. We will also assess blood pressure. If the blood pressure of the participant is >140/90 mm Hg, they will be told that their blood pressure is too high for participation in our study. Blood chemistries will be used to define whether individuals meet the criteria of our study. All female participants will be asked to undergo a pregnancy test using a pregnancy urine test strip to ensure that only non-pregnant women are included in this study.

Potential participants will be called within a few days of their screening meeting to provide them with their blood results and inform them of their eligibility to participate in the study. Consistent with our CLIA exemption, blood results will be provided in a categorical manner (i.e. “normal”, “marginally high”, “high”) rather than providing actual blood concentrations of lab values. Potential participants having any blood values outside of the “normal range” will be directed/encouraged to follow-up with their own physician. Those having blood values within acceptable limits (see Inclusion/Exclusion criteria), will be invited to participate in the study. Subjects will be included or excluded based on a best fit of the inclusion and exclusion criteria (an example of best fit would be if a potential subject says they exercise 8 h/wk, which is close to our exclusion criteria of 7 h/wk, they might still be included in the study if they meet all other inclusion and exclusion criteria more closely than other potential subjects). Participants will be read one phone script if they qualify and another phone script if they do not qualify (see *Participant Eligibility Phone Script*). Potential participants who qualify for the study will be communicated a message as follows: I have your screening results. Congratulations, you meet the eligibility criteria for our clinical trial. Your fasting blood chemistries of glucose, hemoglobin A1c, and cholesterol were all within the specified range. Your glucose falls in the [category], hemoglobinA1c falls in the [category], and cholesterol falls in the [category]. A summary of your results has been prepared for you, which you will receive when you come in for your trials. We look forward to and are thankful for your participation. Potential participants who do not qualify will be provided the following message: I have your screening results. Unfortunately, you do not meet the eligibility criteria for our clinical trial. Your glucose, hemoglobin A1c, and cholesterol were not within the eligible range. Your glucose falls in the [category], hemoglobinA1c falls in the [category], and cholesterol falls in the [category]. A summary of your results has been prepared for you. Please let me know if you would like to drop by the study center to collect a hard copy of your information or if you would like them emailed to you. Thank you so much for your time and effort. All individuals regardless of study eligibility will be encouraged to consider sharing these results with their physician.

In the event that a participant is telephoned and is unavailable, a message will be left requesting a callback at a convenient time or that at a member of the study team will try calling again at a later time. No confidential or sensitive information will be shared with third parties or left on answering machines.

Test Trials. Each participant in studies under Objectives 1 and 2 will complete a 4-arm, randomized, cross-over study. On 4 occasions separated by ≥7 d for men and 1 mo for women (women require vascular testing at the same time during their menstrual cycle to account for known changes in vascular reactivity), participants will report in the fasted state (10-12 hours) to the study center. In Objective 1, participants will ingest 75 g glucose dissolved in water or non-fat milk (2 cups; 473 mL). In Objective 2, participants will ingest 75 g glucose with isonitrogenous quantities of whey protein isolate of sodium caseinate (16.5 g in 473 mL water). In Objective 3, a separate cohort of men and women will complete a 3-arm, randomized, cross-over study. On 3 occasions separated by intervals identical to those in Objectives 1 and 2, participants will ingest 75 g glucose alone or 75 g glucose dissolved in non-fat milk or whole milk (2 cups; 473 mL). This volume of milk was chosen to be consistent with our work and recommendations of the US Dietary Guidelines for Americans. For Objective 2, protein is being administered at 16.5 g to reflect total protein of non-fat milk, rather than their proportional contributions in milk. The amount of glucose included in each mixed beverage (75 g) is equivalent to the dose of a clinical oral glucose challenge, which we and others have routinely shown to induce PPH, oxidative stress, and VED. Then, at 30 minute intervals during the 3 hour postprandial period, we will assess brachial artery flow-mediated dilation (FMD) via non-invasive ultrasonagraphy and blood biomarkers. To ensure that the order by which each participant completes each trial is randomized, a random sequence generator (http://www.random.org/sequences/) will be used to determine the order by which each participant will undergo the respective trials. While the trials will be conducted in a randomized order, for simplicity, they have been listed as Trials 1 – 4 below for Objectives 1 and 2 and Trials 1 – 3 below for Objective 3. Because participants will be consuming moderate volumes of fluid, they will have access to use the restroom as needed in-between collection points.

Objectives 1 and 2

The 4-arm, randomized, cross-over study under these objectives will be completed by a separate cohort of prediabetic men and women from those described under Objective 3.

Trial 1: On the morning of testing, subjects will arrive at the laboratory after abstaining from food for 10-12 hours. Height, weight and two blood pressure measurements will be obtained using standard procedures. A finger prick will be performed to measure baseline hemoglobinA1c (HbA1c) and a flexible catheter will be inserted into a forearm vein. After a 15 min supine stabilization period, a blood sample will be obtained from a 3-way stopcock connected to the end of the catheter. Blood will be collected with a syringe and transferred to appropriate tubes for processing and subsequent determination of fasting biochemical measurements. We will then perform fasting measurements of flow-mediated dilation (FMD) and carotid intima media thickness (cIMT) using ultrasound (described below). Following these baseline measurements, participants will ingest 75 g glucose dissolved in water. FMD measurements will be performed intermittently post-ingestion at 30, 60, 90, 120, 150, and 180 minutes. Blood samples will be collected at 0 min (immediately prior to eating) and at 30, 60, 90, 120, 150, and 180 minutes following the ingestion of the beverage. After each blood sample is obtained, the catheter will be flushed with saline in order to prevent the formation of clots and to minimize the likelihood of having to insert a needle again. Subjects will remain supine in a comfortable position for the entire duration of the test. A trained phlebotomist will perform all cannula insertions, needle sticks, and blood collections.

Trial 2: Participants will visit our study center again, and will complete the procedure as described above (Trial 1) with the exception that they will ingest glucose (75 g) dissolved in non-fat milk.

Trial 3: Participants will visit our study center again, and will complete the procedure as described above (Trial 1) with the exception that they will ingest glucose (75 g) with isonitrogenous quantities of whey protein isolate (16.5 g in 473 mL water).

Trial 4: Participants will visit our study center again, and will complete the procedure as described above (Trial 1) with the exception that they will ingest glucose (75 g) with isonitrogenous quantities of sodium caseinate (16.5 g in 473 mL water).

Objective 3

The 3-arm, randomized, cross-over study under this objective will be completed by a separate cohort of prediabetic men and women from those described under Objectives 1 and 2.

Trial 1: A separate cohort of participants will visit our study center for Objective 3. On the morning of testing, subjects will arrive at the laboratory after abstaining from food and beverage except water for 10-12 hours. Height, weight and two blood pressure measurements will be obtained using standard procedures. A finger prick will be performed to measure baseline HbA1c and a flexible catheter will be inserted into a forearm vein. After a 15 min supine stabilization period, a blood sample will be obtained from a 3-way stopcock connected to the end of the catheter. Blood will be collected with a syringe and transferred to appropriate tubes for processing and subsequent determination of fasting biochemical measurements. We will then perform fasting measurements of flow-mediated dilation (FMD) and carotid intima media thickness (cIMT) using ultrasound (described below). Following these baseline measurements, participants will ingest 75 g glucose dissolved in water. FMD measurements will be performed intermittently post-ingestion at 30, 60, 90, 120, 150, and 180 minutes. Blood samples will be collected at 0 min (immediately prior to eating) and at 30, 60, 90, 120, 150, and 180 minutes following the ingestion of the beverage. After each blood sample is obtained, the catheter will be flushed with saline in order to prevent the formation of clots and to minimize the likelihood of having to insert a needle again. Subjects will remain supine in a comfortable position for the entire duration of the test. A trained phlebotomist will perform all cannula insertions, needle sticks, and blood collections.

Trial 2: Participants will visit our study center again, and will complete the procedure as described above (Trial 1) with the exception that they will ingest glucose (75 g) dissolved in non-fat milk.

Trial 3: Participants will visit our study center again, and will complete the procedure as described above (Trial 1) with the exception that they will ingest glucose (75 g) dissolved in whole milk.

Brachial Artery Flow-Mediated Dilation (FMD). FMD will be measured to assess vascular endothelial function as a functional index of the impact of each beverage's ingestion on arterial blood flow. In brief, FMD is determined by a blinded, trained research assistant using an Acuson 13 MHz linear array transducer and a cardiac ultrasound system. Echocardiogram (ECG) is used to monitor heart rate throughout the procedure. Prior to testing, the participant is resting in the supine position for 10 min. Using ultrasound, the brachial artery will be located and the transducer will be secured in place to ensure consistent image acquisition. FMD measurements will be conducted by placing an inflatable cuff just below the antecubital fossa (crease of the elbow). The procedure is initiated by obtaining resting images (30 sec) of the brachial artery, followed by arterial occlusion (5 min) using the aforementioned inflatable cuff. The cuff is then rapidly deflated and post-occlusion ultrasound images are collected for 300 heart beats, which are analyzed using semi-automated software to calculate the ECG-gated measurement of dilation responses. Post-occlusion vessel diameters at 1 and 3 min, and peak diameter, will be used to calculate percent dilation relative to the pre-occlusion diameter. Post-occlusion peak blood flow velocity will also be collected simultaneously to normalize FMD responses to shear-stress (i.e. post-occlusion blood flow).

Carotid Intima Media Thickness (cIMT). cIMT will be measured at baseline of each trial to assess vascular endothelial function. While anticipated to be unchanged during the study, its assessment will allow for its consideration as a statistical co-variate in defining physiologic responses to test beverages. Images of the right- and left-sided far wall of the common carotid artery will be recorded for ~10 heartbeats and analyzed using edge-detection software to obtain mean, maximum, and minimum lumen diameter, and cIMT. Plaque

is defined by the presence of focal wall thickening that is ≥50% greater than that of the surrounding vessel wall or ≥1.5 mm cIMT protrusion that is distinct from the adjacent boundary.

Sample Handling. Ultrasound images of the brachial and carotid arteries and blood flow data will be stored on the computer that is used to collect the data. Only a study code will be used to identify participants. At each time point, a blood sample (15 mL; 1 tablespoons x 7 time points = 105 mL or ~0.5 cups) will be collected into evacuated blood collection tubes. Throughout the span of the study which consists of the acute ingestion of 4 types of beverages for Objectives 1 and 2 and 1 screening day, we will be collecting a total of ~435 mL or ~2 cups of blood. For Objective 3, occurring in a separate cohort, consists of the acute ingestion of 3 types of beverages and 1 screening day, we will be collecting a total of ~330 mL or ~1.5 cups of blood.

During each trial, plasma will be obtained by centrifugation, and then transferred to cryogenic storage tubes. Tubes will be stored at -80° C until analysis can be completed. Analyses will include plasma glucose, insulin, triglyceride, total cholesterol, metabolites of NO (nitrite/nitrate), vasoactive markers (arginine, asymmetric dimethylarginine, tetrahydrobiopterin, dihydrobiopterin, endothelin-1, 11dehydro thromboxane B_2), antioxidants (vitamins E and C) and markers of oxidative stress (malondialdehyde and methylglyoxal). Remaining plasma sample aliquots not used for these analyses will be archived for 5 years at -80 C in the event we decide to measure additional inflammatory or antioxidant related markers. Appropriate notation has been made in the informed consent to alert participants that we will be archiving specimens and that they have the right to refuse our use of these specimens for future analyses. Lastly, approval from OSU IRB will be sought via a protocol amendment prior to the analysis of any additional biomarkers not specified herein.

Privacy/Confidentiality. For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant, and will only be available to research personnel. Any records containing the names of participants will be stored in a locked filing cabinet or on a password protected computer in the PI's laboratory or office. Research personnel under the supervision of the PI and the PI himself will be the only individuals that have access to this information. The names of participants will not be used for publication in any form. The records will be maintained until the data are published, up to a maximum of five years. All archived samples will be coded, but the key linking the code to each participant's identifiable information will have been destroyed. In addition, participants will be instructed that their participation in this study is voluntary and that they may withdraw at any time without prejudice. Data (food records and biochemical values) obtained from this study will stored on a computer in the PI's laboratory. In addition, a back up of digital data will be stored on the PI's computer in his office. Both computers are password protected and both doors are locked when they are not in use.

Measurement / Instrumentation

For all *Objectives,* the below procedures will be utilized. All biomarkers and functional indices of vascular health will be assessed prior to (0 min) and at 30 min intervals for 3 h postprandially following ingestion of each test beverage unless otherwise noted. Other parameters will be measured during participant screening and/or at baseline (0 min) for each trial, as indicated.

Anthropometric Parameters and Blood Pressure. At screening and postprandially, participants will rest for 15 min prior to determining blood pressure. BMI will be calculated from height determined from a wall-mounted stadiometer and weight from a calibrated scale. Waist circumference will be assessed at the level of the umbilicus using a nonflexible measuring tape.

Serum Chemistries. Plasma triglyceride, total cholesterol, glucose, and insulin will be measured by clinical assay (Pointe Scientific) or ELISA (ALPCO) at screening and throughout the 3 h postprandial period following ingestion of each test beverage. HbA1c will be measured at screening and baseline of each trial using an Alere Anfinion analyzer. This procedure involves pricking the finger for a small drop of blood to

be measured by the analyzer. Insulin resistance at baseline and screening will be calculated as: [(fasting glucose (mmol/L) * fasting insulin (μIU/mL)/22.5].[96]

Vascular Function Studies. High-frequency ultrasound will be used to assess postprandial brachial artery FMD, as we described,[11, 40] in response to each test beverage. In brief, the transducer is placed above the antecubital crease of the arm and anterior and posterior walls of the brachial artery are visualized. Anatomical markers will be noted to identically place the transducer during all visits. Baseline artery diameter will be recorded for 60 heartbeats. Then, a blood pressure cuff placed on the forearm immediately distal to the olecranon process will be rapidly inflated to occlude the artery (200 mmHg, 5 min). VEF will be measured following distal occlusion because this better reflects endothelial-dependent and $NO^{\bullet}$-mediated vascular dilation compared to upper arm occlusion.[97] Upon cuff release, arterial diameter will be assessed for 300 heartbeats (~5 min). Brachial artery images will be obtained using a 13 MHz linear array transducer and Terason T3000 ultrasound system. Image analysis will be performed using edge-detection software (Vascular Research Tools). Peak post-occlusion diameter will be calculated by identifying peak dilation, and averaging vessel diameters ±5 frames surrounding the peak. FMD is calculated as a percentage of baseline diameter and normalized to shear stress. FMD will be performed by the same technician and image analysis will be assessed independently by two technicians in a blinded manner. Our studies show that FMD responses between technicians are not different (P>0.05; CV <8%).

Ultrasound studies will also be performed as we describe[40] to define CVD risk by assessing carotid intima media thickness (cIMT) using established criteria.[98] cIMT is anticipated to be unchanged during the study, but its baseline assessment will allow for its consideration as a statistical co-variate in defining physiologic responses to test beverages. Images of the right- and left-sided far wall of the common carotid artery will be recorded for ~10 heartbeats and analyzed using edge-detection software to obtain mean, maximum, and minimum lumen diameter, and cIMT. Plaque is defined by the presence of focal wall thickening that is ≥50% greater than that of the surrounding vessel wall or ≥1.5 mm cIMT protrusion that is distinct from the adjacent boundary.[98]

Vasoactive Markers. Nitrite/nitrate, the stable end-metabolites of $NO^{\bullet}$, will be measured to define $NO^{\bullet}$ status using a spectrophotometric kit (Cayman Chemical).[11] Indirect assessment of eNOS activity in humans is not practical because of its degree of invasiveness. Instead, it will be assessed indirectly by measuring ADMA/ARG by HPLC-FL as we describe[11] and the BH_4/BH_2 ratio from deproteinized plasma by HPLC with sequential ECD-FL detection[99, 100] as described by our collaborating investigator, Dr. F. Villamena.[101] We will also assess inhibitors of eNOS, i.e. plasma ET-1 and 11-dehydro-TXB_2 by ELISA (Cayman Chemical).

Antioxidants/Oxidative Stress. We show that PPH does not affect plasma antioxidants, but increases lipid peroxidation.[9] However, antioxidant status regulates BH_4 recycling.[100, 102] To rule out potential antioxidant-BH_4 interactions on eNOS activity, plasma vitamin E and C will be measured at baseline of each trial using our routine HPLC methods.[9, 10] To define mitigating activities of dairy milk and its constituents on oxidative stress, MDA, a lipid peroxidation marker and MGO, a reactive glycolytic product that induces oxidative stress, will be measured as we described by HPLC-FL[9] and HPLC-UV[59] throughout the 3-h postprandial period for each trial.

Internal Validity

For all data and records that are a part of this study, a number (i.e. code) will be assigned to each participant. Test confections will be assigned a code as well. This will minimize measurement bias when performing analysis on dietary records, and biochemical markers because all samples/records will be coded. The codes will only be broken once data analysis has been completed and verified by the PI.

Data Analysis

Baseline comparisons between trials will be performed a paired t-test (*Objectives 1-2*) or 1-way RM ANOVA (*Objective 3*) to ensure no changes in participant health status. Other analyses will be performed by

2-way RM ANOVA to define time and treatment effects, and their interaction. In the presence of any significant effects, Bonferroni's post-test will be used to examine pair-wise differences. To better define postprandial responses between treatments, area under the curve ($AUC_{0\text{-}3\ h}$) will be calculated for each biomarker to enable more sensitive comparisons of postprandial responses between treatments and simplify analyses to a paired t-test (*Objectives 1-2*) or 1-way ANOVA (*Objective 3*) as we described.[9, 10] Lastly, to define correlations between study variables, we will perform multiple linear regression, controlling for within-subject repeated measures.[85]

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