Cover Page

Document: Study Protocol

Official Study Title: Achieving Nutritional Adequacy Of Vitamin E With An Egg/Plant-Based Food Pairing

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Achieving Nutritional Adequacy of Vitamins E and K With An Egg/Plant-Based Food Pairing

Executive Summary

The initial intention of this protocol was to conduct Study 1 and Study 2 as separate investigations, with each study entailing a cross-over design for 4 different 72-h pharmacokinetic (PK) trials. Prior to initiating these plans, the Sponsor provided additional support to conduct an ancillary investigation under *Study 1*, which resulted in a total of 6 different 72-h PK trials; no changes were made to *Study 2 (i.e., 4 PK trials)*. If participants consented separately to complete both Study 1 and Study 2, this resulted in a total of 10 PK trials. Both during and post-pandemic, our progress to conduct and complete Study 1 and 2 has been impacted. Thus, this revised protocol (submitted Aug 2022) reflects our efforts to gain efficiency in our work and address the study's primary objectives by combining a subset of Study 1 and Study 2 PK trials into a new Study 3 that only involves 6 PK trials. Our intent here is to complete all procedures in participants who are actively involved in *Study 1* and *Study 2*. Those who complete *Study 1* will be invited to reconsent for enrollment in *Study 2*. Thereafter, no new participants will be enrolled in *Study 1* or *Study 2*; all future participants will be enrolled only in *Study 3*. With this plan, we will enroll 5 participants in *Study 1* to ensure fulfillment of primary and ancillary objectives. A maximum of 5 persons will complete *Study 2* and up to 10 participants will complete *Study 3*. Thus, total enrollment across Study 1, 2, and 3 (n = up to 20) does not increase the number of total participants targeted for enrollment compared with our initial protocol that only involved *Study 1* and *Study 2*. We can therefore achieve study all objectives without increasing overall enrollment, but will be able to reduce participant burden and potential risk.

I. Objectives

The *objective* of this study is to use deuterium-labeled spinach (containing stable isotopes of vitamin E (α-tocopherol; α-T) and vitamin K (phylloquinone; PQ)) to validate eggs as a dietary tool to improve α-T and PQ bioavailability directly from a model plant food, and hence achieve nutrient adequacy. Our *hypothesis* is that the bioavailability of α-T and PQ from deuterium-labeled spinach will be potentiated by egg intake in a dose- and time-dependent manner by increasing their secretion in intestinal-derived chylomicrons. Further, phospholipid-rich egg yolk lipid will enhance nutrient bioavailability compared with vegetable oil. To test this, our team with expertise in bionutrition, plant sciences, and advanced mass spectrometry will fulfill these *specific aims*:

1) Study 1: Assess egg-mediated improvements in αT and PQ bioavailability

The *working hypothesis* is that, compared with deuterium-labeled spinach alone, co-ingestion of eggs will dose- and time-dependently increase plasma bioavailability of spinach-derived deuterium-labeled αT and PQ (based on $AUC_{0\text{-}72\ h}$, C_{max}, and % estimated absorption) without affecting time to maximal concentrations or half-lives. Further, eggs will dose-dependently improve chylomicron enrichment of deuterium-labeled αT and PQ in association with their greater absorption.

2) Study 2: Potentiation of α-T and PQ bioavailability by egg yolk lipid

The *working hypothesis* is that phospholipid-rich whole eggs will enhance spinach-derived αT and PQ bioavailability compared with vegetable oil, and will be most functionally responsible for the benefits of eggs to enhance lipophilic nutrient absorption. We further hypothesize that egg whites will more greatly promote spinach-derived nutrient bioaccessibility compared with spinach alone.

3) Study 3: Combination of primary objectives in Study 1 and 2

This newly added Study 3 consists of select trials from *Study 1* and *Study 2* to allow us to assess dose-dependent effects of eggs and food matrix-dependent effects in a succinct study design involving only 6 pharmacokinetic trials.

The *rationale* for these studies is that, by establishing the efficacy of eggs to potentiate the bioavailability of plant-derived fat-soluble nutrients, a strong framework will exist for an easily implementable health-promoting food pairing strategy to overcome malnutrition of α-T and PQ. The planned research is *innovative* because it will be the first study to establish a novel function of eggs to improve plant-derived α-T and PQ bioavailability by enhancing their intestinal absorption. These quantifiable outcomes, made possible with the use of a deuterium-labeled plant, are *significant* because they will establish: (1) critical knowledge for evidence-based dietary requirements of α-T and PQ, and (2) an egg-based food pairing that can enhance the health benefits of plant-centric dietary patterns.

	Study 1	**Study 2**	**Study 3**
Arm 1	Spinach + 0 hardboiled eggs (Time 0)	Spinach + 0 hardboiled eggs (Time 0)	Spinach + 0 hardboiled eggs (Time 0)
Arm 2	Spinach + 1 hardboiled egg (Time 0)	Spinach + 2 hardboiled egg whites (Time 0)	Spinach + 1 hardboiled egg (Time 0)
Arm 3	Spinach + 2 hardboiled eggs (Time 0)	Spinach + 2 hardboiled eggs (Time 0)	Spinach + 2 hardboiled eggs (Time 0)
Arm 4	Spinach + 3 hardboiled eggs (Time 0)	Spinach + vegetable oil (Time 0)	Spinach + 3 hardboiled eggs (Time 0)
Arm 5	Spinach + 0 eggs (Time 0) + 1 egg (at 3 h)	Not applicable	Spinach + 2 hardboiled egg whites (Time 0)
Arm 6	Spinach + 1 egg (Time 0) + 1 egg (at 3 h)	Not applicable	Spinach + vegetable oil (Time 0)

II. Background and Rationale

Americans Have Poor Vitamin E and Vitamin K Status. Malnutrition, which manifests from nutrient deficiencies, excesses, or imbalances, impacts ~2.4 billion adults worldwide.[1] In the US, inadequate status of the fat-soluble nutrients vitamin E (α-tocopherol; α-T) and vitamin K (phylloquinone; PQ) are problematic. Indeed, >92% of Americans fail to meet the Estimated Average Requirement (12 mg/d[2]) for α-T[3] and up to 81% of adults have suboptimal circulating levels.[4-5] Similarly, PQ intakes are poor with only 43% of men and 63% of women meeting gender-specific Adequate Intakes (120 or 90 μg/d, respectively).[6] Thus, a substantial proportion of Americans are at-risk for inadequate status of these health-promoting nutrients that alleviate oxidative distress and tissue injury (i.e. α-T) and post-translationally modify proteins essential for blood coagulation, bone metabolism, and lowering atherosclerotic risk (i.e. PQ).[2, 7] Few foods are rich in α-T and/or PQ, and the leading sources are of plant origin. Thus, emphasis has been placed on effective food pairings to potentiate their bioavailability, which is essential for optimal health.

Eggs: A Mediator of Nutrient Adequacy. Although some hypothesis-generating observational studies suggest that higher egg intakes increase cardiovascular-related risks,[8-9] evidence from controlled interventions, including those from the Bruno Lab, directly support cardioprotective activities of eggs (e.g.[10-14]). While dense with micronutrients and phytochemicals, which directly promote favorable health outcomes, an often dismissed benefit of eggs is their yolk-derived lipids (e.g. emulsification-promoting phospholipids; ~1.3 g/egg[15]). These anti-inflammatory bioactive lipids[15] can also help to address nutritional inadequacies when co-ingested with plant foods that are rich in lipophilic nutrients but limited in fat. Indeed, α-T and PQ are biosynthesized abundantly in certain plants (e.g. spinach, collards). Both nutrients require co-ingestion with fat to facilitate their absorption and bioavailability that is otherwise limited.[2, 7] Nonesterified lipid, bile acids and pancreatic secretions are critical for α-T and PQ micellularization within the intestine. Subsequently, αT and PQ are transferred to enterocytes and secreted in chylomicrons. Chylomicron remnants are then taken up at the liver prior to secretion of α-T and PQ in VLDL for target tissue delivery. Thus, the co-ingestion of dietary fat is critical to α-T and PQ bioavailability, especially when α-T and PQ are derived from plants that are largely devoid of accessible lipid.

Eggs have a highly digestible lipid matrix that potentiates the bioavailability of a lipophilic carotenoid.[16] Separate from triglyceride and cholesterol, egg yolk is especially rich in phosphatidylcholine, which accounts for 72% of the total phospholipid content.[15] While neutral lipids (e.g. triglyceride) are important for micelle formation, phosphatidylcholine- and bile acid-containing micelles are demonstrated to enhance intestinal permeation of poorly absorbed compounds.[17] Phospholipid micellar content is also critical for the binding to and dissociation from intestinal brush border scavenger receptors (e.g. cluster determinant-36; SR class B type I)[18] that facilitate enterocyte uptake of αT and PQ.[19-20] Eggs have been demonstrated to increase the absorption of α-T among adults who ingested a mixed-vegetable salad alone (prepared with 3 g canola oil) or the same salad with a high-egg or low-egg meal (150 vs 75 g eggs).[21] Co-ingestion of the high-egg meal with salad increased α-T appearance in the triglyceride-rich lipoprotein fraction compared with the salad alone or the salad with the low-egg meal. PQ bioavailability also has been examined in relation to fat co-ingestion (0-32 g).[22] Although the reported evidence supported dietary fat to dose-dependently increase chylomicron PQ, individual responses varied extensively and the predictive benefit of fat on PQ bioavailability was lost at lower fat intakes.

Studies examining α-T and PQ bioavailability support the premise that fat, including that from eggs, is an important mediator of absorption. However, isotopes of α-T or PQ were not used. This precluded an understanding of: 1) whether eggs increased absorption of αT that was derived from a mixed-vegetable salad (2.1 mg), eggs themselves (1.14 mg/150 g eggs), or both;[21] and 2) whether dietary fat at lower intakes dose-dependently increased PQ absorption.[22] Addressing these knowledge gaps is a focus of our proposed study. We plan a pharmacokinetics trial in which eggs (0-3 large) will be ingested with deuterium-labeled spinach prior to assessing plasma and chylomicron deuterium-labeled αT and PQ. We will also address a secondary hypothesis that the "timing" of egg ingestion can potentiate nutrient bioavailability. Relative to the *co-ingestion* of eggs with deuterium-labeled spinach on nutrient bioavailability, we will also assess whether plasma and chylomicron deuterium-labeled αT and PQ bioavailability increases when an 'egg snack' is consumed 3 hours after the initial ingestion of spinach with or without an egg. This hypothesis is supported by reports detailing that dietary fat increases the bioavailability of plant-derived αT and PQ,[7-8] as well as recent evidence that a "second meal" a few hours post-ingestion of a "first meal" mobilizes αT that is otherwise "trapped" in enterocytes (Fig 1).[23] Our investigative approach will provide the sensitivity needed to assess dose- and time-response benefits of eggs while also quantifying the extent to which eggs enhance absorption of these nutrients directly from a representative plant food. Thus, we expect to establish clear evidence to support eggs as part of "plant-centric" healthy eating patterns and recommendations (e.g. Dietary Guidelines for Americans;[24] Mediterranean Diet[25]).

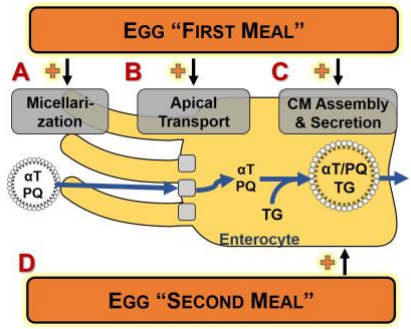


Fig 1. Putative benefits of egg phospholipid (PL) to improve intestinal trafficking of αT and PQ. PL from an Egg "First Meal" may promote: ***A)*** αT and PQ micellarization and ***B)*** transporter-mediated uptake of αT and PQ. PL from both an Egg "First Meal" ***C)*** and "Second Meal" ***D)*** may promote secretion of αT and PQ in chylomicrons (CM). TG, triglyceride.

Deuterium-labeled Plants: A Tool for Evaluating Nutrient Bioavailability. The food matrix, nutrient quantity, and other determinants of bioavailability have been examined in humans and animals. However, research tools have evolved in their sophistication to better interpret experimental outcomes. For example, when eggs were co-ingested with a mixed-vegetable salad, there was insufficient sensitivity to detect a dose-response effect of eggs (75 g vs 150 g) on α-T absorption.[21] The use of unlabeled α-T also precluded a determination of which food source(s) accounted for the increase in α-T absorption, which was further confounded by endogenous α-T that is detectable in intestinal- and liver-derived chylomicrons and VLDL, respectively.[26] Thus, an accurate measure of the proportion of the orally administered α-T that was absorbed and available for target tissue delivery could not be quantified. To address these limitations, the Bruno Lab and others use non-radioactive or stable isotopes of α-T and PQ to assess bioavailability in a scientifically rigorous manner. These tools enable precise nutrient assessments in plasma and isolated lipoproteins using LC-MS and detailed pharmacokinetic analyses to define gut-to-liver nutrient trafficking. Notable examples demonstrating this expertise of Dr Bruno (PI) include studies examining cigarette smoke-induced oxidative stress on dietary αT requirements,[27-28] vitamin C interactions that improve α-T status,[29] the detriment of metabolic syndrome on gut-to-liver trafficking of α-T,[26, 30] and the dose-response effect of dietary fat on α-T absorption (**Fig 2**).[31]

We provided the most accurate evidence in humans that α-T absorption, when administered in an apple matrix, is only 10% when ingested in the absence of fat, and that its absorption increases in a dietary fat-dependent manner up to 33% when co-ingested with 11 g of fat (**Fig 2**).[31] Pharmacokinetic analysis indicated that the time to maximal plasma concentrations of αT (T_{max}) and its half-life ($t_{1/2}$) were unaffected regardless of the quantity of dietary fat ingested. Higher maximal concentrations (C_{max}) of α-T in response to increasing dietary fat (**Fig 2**) were therefore attributed to greater intestinal absorption rather than altered gastric emptying. We also observed that fat intakes, within the range of 0-11 g ingested, linearly increased α-T bioavailability such that 0.43 mg of α-T was absorbed for each additional gram of fat ingested (**Fig 2C**). Thus, our planned studies will exploit the highly digestible lipid content of eggs (4.8 g total fat/egg) to demonstrate that eggs dose- and time-dependently improve plant-derived αT and PQ bioavailability, and more substantially than phospholipid-free vegetable oil. Further, because co-PI Blakeslee is enlisted to hydroponically grow deuterium-labeled spinach, pharmacokinetic responses of both deuterium-labeled α-T and PQ can be assessed independently of the confounding effects of the low-level amounts of unlabeled α-T and PQ present in egg yolks or those already present in intestinal or liver tissue.

Deuterium-labeled (i.e. stable isotope) plants have distinct advantages for assessing nutrient bioavailability. While supplements containing isotopically labeled nutrients can be administered, α-T bioavailability from pills is poorer and has greater inter-subject variability than that from a food matrix.[32] Further, studies involving plant-derived α-T and PQ are more physiologically relevant. This is because they examine the whole food matrix and low nutrient doses (compared with pills), which enable translational messages of greater public health importance. Indeed, we reported that a small dose of deuterium-labeled α-T was well-absorbed without affecting circulating unlabeled αT concentrations (**Fig 2A**).[31] Because the total plasma α-T pool (i.e. sum of unlabeled and labeled α-T) was essentially unchanged during and between each pharmacokinetic trial, the absorbed hexadeuterium (d_6-) α-T did not significantly change plasma vitamin E elimination kinetics. This outcome provided a novel opportunity to model the αT kinetics data and calculate evidence-based dietary α-T requirements that consider the amount of α-T absorbed relative to daily tissue needs and bodily elimination.

Deuterium-labeled collard greens have been grown to study plasma PQ transport following PQ depletion and repletion, including its time-dependent incorporation into lipoprotein fractions.[33] Studies with deuterium-labeled collard greens also show that age-dependent increases in PQ bioavailability are attributed to increased circulating triglycerides in older adults.[34] Using

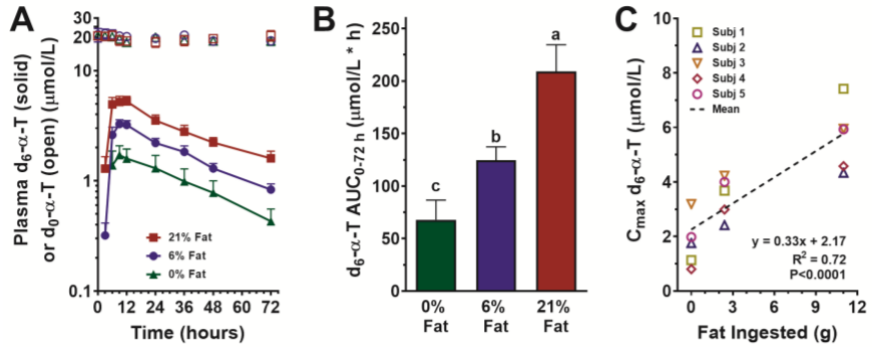


Fig 2. α-T bioavailability increases in a dietary fat-dependent manner. In a randomized, cross-over study, healthy adults ingested 80 g of apple pieces impregnated with 22 mg d_6-αT alone (0 g fat; 0% energy from fat), with a low-fat breakfast (2.4 g fat; 6% fat), or with a regular fat breakfast (11.0 g fat; 21% fat). Plasma collected in timed intervals over 72 h was analyzed by LC-MS for unlabeled (d_0) and labeled (d_6)-αT. **A-B)** Co-ingestion of fortified apples with fat dose-dependently increased d_6-αT concentrations (C_{max} and $AUC_{0\text{-}72\ h}$) without altering time to maximal concentration, rates of elimination, or half-lives. **B)** Dietary fat quantity ingested predicted d_6-αT absorption such that C_{max} increased by 0.33 μmol/L (equivalent to 0.43 mg absorbed) for each g of fat ingested.

isotopically-labeled kale, PQ bioavailability was determined to be only 4.7% with a plasma half-time of 8.8 h.[35] Deuterated collard greens have also been applied to the study of αT pharmacokinetics in younger and older persons.[36] The outcomes showed that αT fractional disappearance rates (0.63 pools/d), half-lives (30.6 h), and absorption (24%) did not differ by age or gender, but that lipidemia slowed αT elimination without affecting αT absorption.

In summary, α-T and PQ absorption, distribution, metabolism, and elimination are best evaluated with the use of stable isotope vitamers. A critical step towards human health is validating food pairings, and by extension dietary patterns, that can enhance the otherwise poor absorption of α-T and PQ. In the planned investigation, we expect that spinach-derived α-T and PQ bioavailability will be poor, but that eggs will dose-dependently and substantially improve intestinal α-T and PQ absorption and to a greater extent than that by vegetable oil. This novel evidence will support eggs as an important "dietary tool" to achieve nutrient adequacy in the context of health-promoting dietary patterns. Secondary outcomes will provide evidence, based on pharmacokinetic assessments, to help fill scientific gaps that preclude the ability to establish an RDA for PQ, in place of an Adequate Intake.[7] Further, current vitamin E requirements are based on limited data from the 1950s from only 7 institutionalized men and do not consider the effects of food-nutrient interactions. Validated, modern technologies carried out with a high degree of scientific rigor, as proposed herein, are critical for moving the nutrition field forward, and supporting human health by identify health-promoting dietary strategies to overcome malnutrition.

Anticipated Results and Significance of the Research

Trials in Study 1 are expected to provide the first detailed evidence that eggs are an effective dietary tool to potentiate the bioavailability of plant (spinach)-derived α-T and PQ by mediating their intestinal absorption. Several novel outcomes are expected: (1) eggs co-ingested with deuterium-labeled spinach will dose-dependently increase the bioavailability of deuterium-labeled α-T and PQ ($AUC_{0\text{-}72\ h}$, C_{max}, % absorption) without affecting half-lives or T_{max}, and (2) eggs consumed 3 hours post-ingestion of a "first meal (spinach with or without an egg)" will improve αT and PQ bioavailability by mobilizing the α-T and PQ that is otherwise "trapped" in enterocytes (3) total egg lipid will strongly predict ($P<0.05$) improvements in bioavailability parameters, and (4) % absorption and chylomicron enrichment of deuterium-labeled α-T and PQ will increase with increasing egg intakes, which will indicate that eggs promote fat-soluble nutrient bioaccessibility from spinach. These outcomes will be utilized to define the extent to which egg-mediated improvements in αT bioavailability contribute to achieving adequate α-T status. Because a well-defined cut-off for circulating PQ concentrations does not currently exist, the findings will be used to calculate daily turnover of PQ (based on elimination kinetics) in relation to bioavailability responses. This knowledge will complement anticipated outcomes of future studies that establish biomarkers of PQ status. Ultimately, our findings concerning both α-T and PQ will provide evidence to help redefine the Estimated Average Requirement (EAR) for α-T and the first-ever EAR for PQ, which is currently limited to an Adequate Intake. Thus, our outcomes will establish evidence-based dietary recommendations in relation to an effective food pairing that helps to achieve adequate status of these nutrients.

Trials in Study 2 are expected to establish that phospholipid-rich egg lipid can potentiate α-T and PQ absorption and bioavailability. Because phospholipids are largely removed during oil refinement, the expected outcomes will support a novel benefit of an egg component that receives

limited attention. Further corroborating, pharmacokinetics assessments of α-T and PQ in response to their co-ingestion with whole egg vs. egg white are expected to demonstrate an added value of whole eggs (by way of its egg yolk lipids) to promote adequate nutrient absorption. In addition, provided that our secondary hypothesis is correct, that egg whites can increase the formation of simple micelles (i.e. bile salts without dietary fat), additional translational messages can be established to support a fat-free alternative in egg whites to improve lipophilic nutrient absorption and bioavailability. These findings, along with those under *Study 1*, would be of significance because lipophilic nutrients can be "trapped" in enterocytes and lost in the feces as enterocytes are sloughed off if not efficiently trafficked for secretion in chylomicrons. Thus, outcomes of these studies will serve as the foundation for easy-to-implement messages of public health importance in support of whole eggs ***and*** egg whites as part of a plant-based dietary pattern.

The newly added Study 3 is hybrid approach to address the major objectives of Study 1-2 in an efficient manner. Thus, participants who complete Study 3 (instead of Study 1 and/or Study 2), will enable our understanding of X and Y simultaneously and avoid some scientific redundancies that were previously occurring under the separate conduct of Study 1 and Study 2.

Lastly, our innovative approach with deuterium-labeled spinach will establish a new investigative tool to examine egg-mediated benefits on the bioavailability of other bioactive components present in spinach. For example, spinach is a leading dietary source of lutein (~12 mg/100 g). While the planned studies focus on α-T and PQ, which are dietary essential nutrients, future studies are immediately possible to examine lutein pharmacokinetics in relation to egg intake from the biospecimens (plasma and chylomicrons) that will be archived in the planned studies. Similarly, outcomes from the planned work will inform large scale food-based interventions regarding the appropriate delivery of α-T and PQ as part of a mixed-diet. This is important because, while current recommendations strongly encourage diets rich in fruits and vegetables, limited guidance is available concerning how consumers should effectively pair plant foods with other dietary matrices to potentiate micronutrient and phytochemical bioavailability to achieve optimal health.

Pitfalls and Alternatives

These studies utilize techniques that are well-established in our hands. Thus, we do not expect any technical problems to complete these analyses. Deuterated-spinach will be grown in several cycles. This may increase variability of the α-T and PQ content. If the dose is more variable than expected (±3%), or the 4:1 α-T:PQ ratio cannot be maintained, we will standardize the dose to PQ (500 μg). This is because α-T pharmacokinetics are unaffected by doses ranging from ~1-50 mg α-T (e.g.[26, 29, 31, 36]). Should complex pharmacokinetic modeling be required to consider unanticipated influences of the gastrointestinal tract, plasma, and body tissue pools, we will consult the OSU Pharmacoanalytical Core on a fee-for-service basis.

In light of the pandemic, which has significantly impacted the progress of the study, we are adding a new Study 3, which encompasses the primary objectives of Study 1 and 2. This newly added Study 3 will allow us to focus on the primary interest with a 1 × 6 trial rather than doing 2 separate trials having 1 × 6 and a 1 × 4 designs (i.e., 10 total pharmacokinetic trials). In this way, we will be able to comprehensively examine the initial proposed objectives and complete the project in a timely manner while also reducing participant burden.

We hypothesize that the lipid content of eggs (e.g. emulsification-promoting phospholipids) will substantially increase chylomicron enrichment of αT and PQ. However, we have considered the possibility that total bioavailability of α-T and PQ could be improved by whole eggs (compared with oil) without increasing their enrichment in chylomicrons. Should that occur, we will assess deuterium-labeled α-T and PQ from VLDL, as we described,[26] to assess the extent to which eggs promote hepatic secretion of these vitamins. Studies in HepG2 cells could also be performed to define hepatocyte trafficking in response to egg lipids.

III. Procedures

A. Research Design

Our initial objective was to conduct two separate trials having 1 × 4 study designs (Study 1 (4-arms) & Study 2 (4-arms). Arms 5 and 6 in Study 1 were added to address a secondary objective through an ancillary study. Due to the significant delay in progress caused by the global pandemic, we plan focus the remainder of work on the primary objectives by enrolling only 5 participants, instead of 10, for Study 1 and create a new Study 3 that combines select arms of Study 1 and Study 2 into a succinct 1 × 6 study design. This will avoid the need to re-consent persons to complete Study 2 and overall reduce participant burden.

For the planned studies, we will conduct three separate cross-over studies. The first study consists of a 6-arm cross-over study (Study 1), the second in a separate cohort will be 4-arm crossover study (Study 2), and the third study consists of a 6-arm cross-over study (Study 3). For all studies, healthy adults who fulfill the enrollment criteria will be enrolled from the Columbus, OH area. Studies will be completed in the OSU Human Nutrition Research Center.

For *Study 1*, fasted participants will ingest ~100 g of deuterium-labeled steamed spinach with 0, 1, 2, or 3 hardboiled eggs (Study 1 Arms 1-4, respectively). In Study Arm 5, participants will ingest spinach alone followed by 1 egg 3-hours later. In Study Arm 6, participants will ingest spinach with 1 egg followed by another egg 3-hours later. Thus, Study Arms 1-4 will test the dose-dependent effects of eggs on nutrient bioavailability and Study Arms 5-6 (with comparison to Study Arms 1 and 2) will test the 'timing'-dependent effects of eggs on nutrient bioavailability. For all studies, ~100 g of deuterium-labeled steamed spinach (to provide 2 mg α-T and 500 µg PQ[37]) will be administered. Hardboiled eggs will be administered at a dose of 0-3 eggs (~50 g each) to provide 0-14.4 g fat. For each cross-over trial, blood will be collected prior to (0 h) and at 3, 4.5, 6, 7.5, 9, 12, 24, 36, 48, and 72 h post-test meal ingestion to ensure that blood samples are collected at times corresponding to the T_{max} of α-T between 10-12 h[23] and T_{max} of PQ at either 3-5 h[22, 38] or 6-8 h[34-35]. Plasma and chylomicrons will be collected and archived as we described.[26]

For *Study 2*, a separate cohort of healthy adult participants will ingest ~100 g of deuterium-labeled steamed spinach alone (0 g fat), or with 2 egg whites (0 g fat), 2 whole eggs (9.6 g fat), or vegetable oil (9.6 g fat) (Study 2 Arms 1-4, respectively). Blood will be collected from 0-72 h as indicated above, processed, and archived until analyzed.

For Study 3, a separate cohort of healthy adult participants will ingest ~100 g of deuterium-labeled steamed spinach alone (0 g fat), or 1, 2, or 3 hardboiled eggs (Study 1 Arms 1-4), or with 2 egg whites (0 g fat), or vegetable oil (9.6 g fat) (Study 2 Arms 2 & 4). Blood will be collected from 0-72 h as indicated above, processed, and archived until analyzed.

For each study, a 7-d washout period between study arms is sufficient, based on our reports, to fully eliminate labeled compounds and prevent "carry-over" to the subsequent trial.[26, 31] To standardize postprandial responses and accurately define pharmacokinetic parameters under *Studies 1-3*, all foods will be controlled for 3-d preceding each trial and for the initial 24-h of each trial. The controlled diet will be eucaloric and standardized for α-T and PQ intakes. For all of these planned studies, only blood will be collected for the isolation of plasma and chylomicrons. No fecal samples, urine samples, or genetic material is being collected.

B. Sample

Total Number of Participants For Studies 1-3. For both planned studies, up to 40 persons in total will be screened in order to identify 15 persons (n = 5 per study) who meet the below inclusion/exclusion criteria. 10 persons in each study are needed to enhance statistical power and consider potential effects due to gender as described below. The newly added Study 3 will allow us to still have 10 persons under the arms that fulfill the primary objectives. Maximum 15 persons will be formally enrolled in the study.

Inclusion/Exclusion Criteria. Participants (18-65 y) will be required to meet the following ***inclusion criteria***: a) BMI = 19-25 kg/m^2; b) normolipidemic (total cholesterol <240 mg/dL; triglyceride <150 mg/dL); c) glucose <100 mg/dL; d) normal hematocrit level (41%-50% for men and 36%-48% for women); e) normal hemoglobin level (13.5-17.5 g/dL for men and 12.0-15.5 g/dL for women); f) no use of dietary supplements for >1 mo; g) no use of medications that affect lipid or glucose metabolism; h) non-smoker; and i) no history of gastrointestinal disorders. Participants having any of these criteria will be ***excluded***: a) egg allergy; b) >2 alcoholic drinks/d; c) >7 h/wk of aerobic activity; d) >2 kg body mass change in the past 1 mo; e) women who are pregnant, lactating, or initiated or changed birth control in the past 3 mo, f) history of iron deficiency anemia; g) vegetarian. We will also not enroll any participants using any medications to manage diabetes, hypertension, or hyperlipidemia (e.g. statins, metformin, ACE inhibitors) or any blood thinning medications [Warfarin]).

Power Calculation. The quantity of egg-derived fat is similar to that in our report demonstrating a dose-response increase in d_6-α-T bioavailability when ingested with 0-11 g of fat derived from cream cheese.[31] Using the smallest difference in d_6-α-T C_{max}, which occurred between the 0 g and 2.4 g fat levels (**Fig 2A**), our power analysis indicated that only 5 participants would be needed to detect statistically significant differences (90% power, P<0.05; PS Power and Sample Size). Similar powering is also obtained when using d_6-α-T $AUC_{0\text{-}72\ h}$ or absorption (%). Studying so few participants is impractical for establishing dietary recommendations. Thus, for studies 1-3 separate cohorts of 5 adults will be enrolled. By combining participants from all 3 studies, we will have 10 adults under each primary objectives to consider egg-dependent increases in *primary outcomes* (C_{max}, AUC, estimated absorption of α-T or PQ). This increases statistical power to >97% (P<0.01) while allowing for consideration of potential differences between men and women.

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 social media (e.g. Facebook). The posted advertisements will instruct interested participants to complete an online eligibility survey or 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 dietary supplements?, do you smoke?; see *Phone Script* attachment). 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* attachment).

Anticipated Data Analysis Plan. Primary outcomes (C_{max}, $AUC_{0\text{-}72\ h}$, absorption) will be analyzed initially using 2-way repeated measures ANOVA to assess effects due to treatment, gender, and their interaction. If no gender effects are observed, consistent with prior reports,[29, 31, 39] we will reassess outcomes using 1-way RM ANOVA and Bonferonni's post-test, as appropriate, to focus on egg-dependent effects on pharmacokinetics. Multiple linear regression, controlling for within-subject repeated measures,[40] also will be performed to define relations among study variables.

C. Measurement/Instrumentation

Anthropometric Parameters and Blood Pressure. At screening, participants will rest for 15 minutes prior to determining blood pressure using an automated cuff. 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.

Blood Chemistries. At screening, fasting plasma glucose, triglyceride, cholesterol, hematocrit, and hemoglobin will be measured by spectrophotometry utilizing validated clinical assays (Pointe Scientific). In our hands, intra- and inter-assay coefficient of variance (CV) for these assays is <8%.

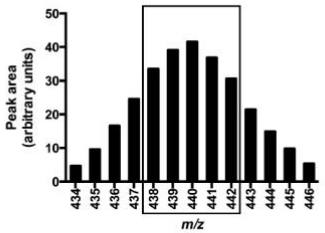
Diet Assessment/Control. To improve diet homogeneity and limit potential confounding effects of the basal diet on study outcomes, participants will be provided all foods and beverages consistent with our prior studies.[26] These controlled diets will be provided for the 3-days prior to each pharmacokinetics trial and during the initial 12-hours of each pharmacokinetics trial. All foods will be prepared in the Human Nutrition Metabolic Kitchen, packaged, and provided to participants in a storage cooler. A registered dietitian (PI Bruno) will prescribe the 3-d eucaloric diet to deliver 50-60% of energy from carbohydrate, 15-20% from protein, and 25-30% from fat. Dietary α-T will be standardized at 7 mg/d and PQ at 80 µg/d based on typical Americans intakes.[2, 7] Vitamin C will be provided at the gender-specific RDA[2] based on our reports that vitamin C status regulates α-T bioavailability.[28-29, 41] To control for between-trial physiological differences, participants will repeat the prescribed diet in an identical manner. For compliance assessment, participants will return all empty containers and/or uneaten portions to assess actual energy and nutrient intakes using NDSR dietary analysis software.

Deuterium-labeled Spinach. Spinach has been selected for all studies as a plant-rich source of α-T and PQ.[37] Spinach biosynthesizes α-T and PQ in quantities appropriate for pharmacokinetic studies, and is a member of the *Brassicaceae* family known for its diverse health benefits.[42] These features and its rapid maturation cycle (~6 wk) make it highly amenable to isotopic labeling for studies examining αT and PQ bioavailability.[33-34, 36] It also can be steamed and preserved frozen without any appreciable loss of αT or PQ, and is a vegetable commonly paired with eggs in the diets of Americans (e.g. salads, omelets), Europeans, and Asians.

Spinach will be grown hydroponically by Dr. Joshua Blakeslee (co-PI, Department of Horticulture and Crop Sciences, CFAES, OSU) in growth chambers located in Rightmire Hall as described for deuterium-labeled collard greens,[34, 36] with minor modifications. Plants will be grown using standard hydroponics techniques appropriate to generate cruciferous vegetable plants (e.g. spinach) for human consumption in other research efforts. Prior to spinach cultivation, controlled-environment plant growth chambers will be washed and sterilized. Briefly, chambers will be washed twice with soap, a bleach solution, and water prior to wiping down twice with 75% alcohol treating for 3 hours with UV light. Seeds will be sown on rock wool in a hydroponic growth system, and plants grown at 22ºC under fluorescent lights (200-220 µm/m/s), with an 18-h photoperiod. Spinach will be grown using a hydroponic nutrient solution supplemented with deuterated water (31 atom%; obtained from Sigma-Aldrich), as described.[34, 36] The plant nutrient solution is obtained commercially (MSSP06-50LT; Caisson Labs) and contains all the essential nutrients for plants (e.g. nitrogen, vitamins, minerals) to stimulate rapid growth. Under these growth conditions with this concentration of deuterated water, 100% of the α-T and PQ molecules present in leafy tissues become isotopically labeled. To generate sufficient spinach for the proposed studies, we plan 4-5 lots (growth cycles) of ~6-wk each to yield ~15 kg total spinach. For each cycle, spinach will be grown in 3-4 controlled-environment growth chambers, harvested, pooled, and analyzed for total α-T and PQ and their isotopomer distributions for use in the proposed clinical trial.

Spinach will be harvested at 6-wk post-germination, pooled, and analyzed by HPLC and LC-MS prior to steaming, preparing individual portions containing ~2 mg αT and 500 µg PQ (anticipated to be ~100 g pre-steamed spinach[37]), and freezing in a refrigeration/freezer unit dedicated for foods for human consumption. Each pooled sample will undergo LC-MS/MS analyses to assess the isotoper profile of both αT and PQ.[34-36] MS analyses are expected to reveal a normal distribution range of mass-to-charge ratios of labeled α-T and PQ (see example in **Fig 2**).[36, 43] The most abundant ions representing >70% of each deuterated vitamer (referred herein as d_x-αT and d_x-PQ) will inform the specific tracers assessed in the clinical trial. Total αT and PQ from liquid nitrogen-pulverized spinach will be quantified by routine HPLC procedures in the OARDC Metabolite Analysis Cluster (OMAC, located in the Blakeslee Lab). LC-MS/MS analyses will be performed by Dr. Blakeslee at both the OMAC (targeted metabolomics) and the OSU Campus Chemical Instrumentation Center if broad-spectrum metabolomics become needed.

Fig 3. Example of isotopomer distribution of deuterium-labeled α-T derived from deuterium-labeled collard greens (data from REF 35). The mass-to-charge ratio (m/z) of unlabeled (d_0)-α-T is 429. As shown, no d_0-α-T is detected and the isotopes comprising the majority of total α-T are at *m/z* of 438-442 (d_9- to d_{13}-α-T).



Plasma and Lipoprotein (chylomicron, VLDL, LDL, HDL) Vitamin E. Labeled (d_x-) and unlabeled (d_0-) αT will be measured in samples obtained at 0-72 h using our established LC-MS method,[26, 28, 31] with minor modification to use tocol as an internal standard. In brief, plasma is saponified, extracted with hexane, dried under nitrogen, reconstituted, and injected on the LC-MS in ESI mode at appropriate *m/z* ratios (informed in *Section 3.vi*).
Scientific Rigor – Intra- and inter-assay CV is <6% in our hands. NIST-traceable standards are used for quantification, and quality controls of known plasma αT concentration are included in each batch of analyzed samples to validate accuracy and precision.

Plasma and Lipoprotein (chylomicron, VLDL, LDL, HDL) Vitamin K. Labeled (d_x-) and unlabeled (d_0-) PQ will be assessed from samples obtained from the 72-h pharmacokinetics study,

as described,[35] with minor modifications. In brief, plasma is spiked with β-apo-carotenal (internal standard) prior to precipitating proteins with ethanol. Following hexane extraction, the extract is dried under nitrogen and reconstituted in 1:1 methyl-tert-butyl-ether (MTBE):methanol. The sample is then injected on the LC-MS/MS in APCI mode and separated on a YMC C_{30} column using a binary gradient of 1 mM ammonium acetate in methanol (mobile phase A) and MTBE (mobile phase B). *Scientific Rigor* – Procedures have been established using reference serum of known PQ concentration. Intra- and inter-assay CV is <9.2%. Quality control plasma has been procured, aliquoted in single-use vials, stored at -80°C, and is included with each analysis to ensure accurate quantification.

Pharmacokinetic Analysis. Plasma triglyceride and total cholesterol will be measured at each time point from each subject using enzymatic assays. Pharmacokinetics will be calculated with and without d_x-α-T and d_x-PQ normalized to plasma lipids (i.e. sum of triglyceride and total cholesterol) to exclude the possibility of lipidemia influencing the analysis.[36, 44] Area under the time-concentration curve ($AUC_{0\text{-}72\ h}$) and other pharmacokinetic parameters (i.e. fractional rates of absorption and elimination, C_{max}, absorption T_{max}) will be determined as we described.[26, 29, 31]

Antioxidant Status & Oxidative Distress. Although the proposed studies are confined to healthy persons, and diet will be controlled, measures of antioxidant status and oxidative distress will define the *independent* benefits of eggs on α-T and PQ bioavailability. Thus, at baseline (0 h) of each 72-h trial, we will measure plasma vitamin C with our HPLC-ECD method[45] and carotenoids (lycopene, α- and β-carotene, lutein/zeaxanthin) by HPLC-PDA as we reported.[46] The lipid peroxidation biomarker malondialdehyde will be measured by HPLC-FL as we described.[47] Intra- and inter-assay CV for each of these assays is 6-14%

D. Detailed Study Procedures

Overview of Study Procedures. Potential participants who call the study center in an anonymous manner for more information will be given a brief description about the study and asked a few questions to determine their eligibility (*see Phone Script* attachment). If they meet the eligible criteria, they will be invited to the study center for a screening meeting. During the meeting, the Informed Consent (see *Informed Consent* attachment) will be explained and provided for them to review. The participant will then be given the opportunity to review the Informed Consent form. If he/she chooses to participate in the study, they will then be asked to provide written consent. After receiving informed consent, the participants' height, weight, waist circumference, and blood pressure will be measured. Additionally, a small fasting blood sample will be collected to blood chemistry analysis. If they are not fasted at least 10 hours), they will be asked to come back in the fasted state at time of mutual convenience. These blood results in combination with anthropometric parameters will determine the participant's eligibility. Eligible participants who agree to proceed with the study will then be formally enrolled to complete one of the three planned cross-over studies (i.e. Study 1 or Study 3 with 6 cross-over periods; or Study 2 with 4 cross-over periods). Participants may complete both cross-over trials provided that they wash-out between studies for >6 weeks. We estimate that all procedures to complete Study 1 and Study 3will require ~12 weeks per participant and that Study 2 will require ~8 weeks per participant.

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 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 body measurements or plasma chemistries (see *Enrollment Criteria*) do not meet the study criteria. If the participant has provided consent, we will then measure the participant's height, weight, waist circumference, and blood pressure. Next, if the participant is fasted for at least 10 hours, we will ask if a trained individual can draw a small blood sample (8 mL) so that we may measure blood chemistries (glucose, cholesterol, triglyceride, hematocrit, and hemoglobin). All samples will be coded to maintain participant anonymity. If the participant's anthropometrics or plasma chemistries do not meet the study criteria, they will be told that they do not meet the study criteria.

Potential participants who meet the criteria will be contacted within a few days after their screening meeting to provide them with their blood and body measurement 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 (see *Subjects Results Sheet*). Potential participants having any blood values outside of the "normal range" will be directed/encouraged to follow-up with their own physician. Those having body measurements and 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 he/she exercises 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* attachment). Potential participants who qualify for the study will be communicated a message as follows: "Congratulations! You have been selected for participation in our study based on your blood testing results. A study investigator will be telephoning you to invite you to the testing session. Would you like to know your blood chemistry results?" Subjects not selected for study will be told the following message: "You have not been chosen for our study, but thank you for your interest." This message will be followed by an explanation why they were not chosen, such as lab values outside of the range we are looking for: "Your blood testing data is…..we were looking for participants who had levels less than …..". "We can provide you a copy of your results if you would like…..how would you like us to provide them to you?". "You should also consider sharing these results with your physician."

If 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.

Intervention timeline. Each participant who meets formal study entry criteria will be expected to complete either Study 1 or Study 3 (6-arm cross-over study) or Study 2 (4-arm cross-over study). Both studies are designed identically with the exception that deuterium-labeled spinach will be consumed with different test meals to assess fat-nutrient bioavailability.

To complete the pharmacokinetics studies, each participant will visit the study center a total of 31 times (Study 1 or Study 3) or 21 times (Study 2) to complete all procedures of the respective cross-over trial. This includes 1 visit for screening and 5 visits (1 visit to pick-up food and 4 visits for blood collection) during each 72-h pharmacokinetics trial (x 6 cross-over trials = 30 visits; x 4 cross-over trials = 20 visits). Each 72-h cross-over study arm will be separated by at least 1-week.

Food Pick-Up Visit. On day -3 of each trial arm, participants will visit the study center to pick-up a cooler containing their foods and beverages. To ensure that foods meet participants' preferences, a study member will contact the participant by phone or email to share the menu of the 3-day diet. If specific foods or daily meals are unacceptable, the study team will substitute foods or complete daily meals from one of the other days on the diet.

Test trials. On ***day 0*** of each trial arm, participants will report to the study center in the fasted state (10-12 hours with no food or beverage except for water). They will return all empty food containers and/or uneaten portions. Arthrometric measurements will be performed. Then, a trained phlebotomist will collect a blood sample. Participants will then receive, in random order (determined with a random sequence generator; http://www.random.org/sequences/), one of the test meals to consume (see below table). Thereafter, blood samples will be collected at 3, 4.5, 6, 7.5, 9, and, 12 hours post-meal ingestion to isolate plasma and circulating lipoproteins for biochemical measurements (e.g. vitamin E, vitamin K). During each study visit, participants will have access to drinking water and can use the restroom as needed. A standardized lunch (after the 4.5-h blood draw) and dinner (after the 9-h blood draw) also will be provided.

For all test meals, deuterium-labeled spinach will be generated by Dr. Blakeslee as described above. Harvested spinach will be stored in vacuum-sealed bags in a freezer dedicated for human foods. For each trial ~100 g of spinach will used. In the Human Nutrition Metabolic Kitchen, spinach will be rinsed consistent with safe food handling practices and then steamed. Hardboiled eggs will also be prepared in egg cooker. These whole eggs in their entirety, or their egg white portion will be used to prepare the test meals. Whole eggs along with vegetable oil will be purchased from a local supermarket (e.g. Kroger).

	Study 1	**Study 2**	**Study 3**
Test Meal 1	Spinach + 0 hardboiled eggs (Time 0)	Spinach alone (Time 0)	Spinach + 0 hardboiled eggs (Time 0)
Test Meal 2	Spinach + 1 hardboiled egg (Time 0)	Spinach + 2 eggs whites (Time 0)	Spinach + 1 hardboiled egg (Time 0)
Test Meal 3	Spinach + 2 hardboiled eggs (Time 0)	Spinach + 2 whole eggs (Time 0)	Spinach + 2 hardboiled eggs (Time 0)
Test Meal 4	Spinach + 3 hardboiled eggs (Time 0)	Spinach + vegetable oil (Time 0)	Spinach + 3 hardboiled eggs (Time 0)
Test Meal 5	Spinach + 0 eggs (Time 0) + 1 egg (at 3 h)	Not applicable	Spinach + 2 hardboiled egg whites (Time 0)

Teat Meal 6	Spinach + 1 egg (Time 0) + 1 egg (at 3 h)	Not applicable	Spinach + vegetable oil (Time 0)

On ***day 1*** of each trial arm, they will report to the study center in the fasted state for blood collection at 24-h post-meal ingestion. They will return again for a non-fasting blood draw at 36-h post-meal ingestion.

On ***day 2*** of each trial arm, they will report to the study center in the fasted state for blood collection at 48-h post-meal ingestion.

On ***day 3*** of each trial arm, they will report to the study center in the fasted state for blood collection at 72-h post-meal ingestion. This is the terminal blood draw. Participants will then undergo at least 1-week washout before completing the remaining 3 trials within either study (see Table) in random order and in a manner identical to procedures described above.

Sampling Handling. Once at screening (8 mL), and 11-times during each pharmacokinetic trial (0, 3, 4.5, 6, 7.5, 9, 12, [16 mL/time point]; 24, 36, 48, and 72 h [8 mL/time point]), a blood sample will be collected. During each trial, a total volume of 144 ml (~0.6 cup) will be collected. Thus, 872 mL (~3.6 cups) will be collected in Study 1 (or Study 3) (screening + 6 trials) that spans at least 12 weeks. For Study 2, we calculate that 584 ml (~2.4 cups) of blood will be collected (screening + 4 trials) over a span of at least 8 weeks.

During each trial, plasma (0-72 h) and lipoproteins (0-72 h; chylomicrons, VLDL, LDL, HDL) will be obtained by centrifugation, and then transferred to appropriate storage tubes. Tubes will be stored at -80˚C until analysis can be completed. Analyses of blood samples will include labeled and unlabeled α-T and PQ cholesterol, triglycerides, vitamin C, carotenoids, and oxidative stress biomarkers (i.e. malondialdehyde). No genetic materials are being collected or archived as part of this study. However, remaining blood sample aliquots not used for these analyses will be archived for 5 y at -80˚C in the event we decide to measure additional biomarkers related to the study objectives. Archival of blood samples also includes those obtained at screening, both for persons meeting eligibility requirements as well as those who do not meet eligibility requirements. 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 the 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 backup 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 work areas are not in use.

E. 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. All collected blood samples will also be coded. This will minimize measurement bias when performing analysis on biochemical markers. The codes will only be broken once data analysis has been completed and verified by the PI.

F. Medical Safety Plan

All aspects of the clinical study will be conducted in Dr. Bruno's clinical lab located in Campbell Hall. Participants will be fasted for 10-12 hours prior to each study visit. We recognize that certain risks associated with fasting include: hypoglycemia, weakness, and fainting. This duration of fasting is consistent with guidelines set forth by the American Diabetes Association to minimize risk to the individual when determining fasting blood glucose concentrations.

Risks related to hypoglycemia are anticipated to be low due to their underlying "healthy" health status and a prescribed diet for the three preceding days that will supply ~50% carbohydrate. In addition, shortly upon arrival to our study center, they will be ingesting an plant/spinach-based test meal. Throughout each visit to the study center, participants will be closely monitored a member of the research team. These persons will monitor the safety and well-being of participants for any signs and symptoms of hypoglycemia including: confusion, dizziness, irritability, weakness, headaches, and fainting. Additionally, Dr. Bruno (PI; 325 Campbell Hall) has an academic office in close proximity to the study center and has significant experience coordinating clinical research studies involving overnight fasting, and other dietary-related meal challenges, thereby supporting the competency of our research team in managing potential adverse events relating to fasting glucose and glycemic responses.

Consistent with our prior studies of similar design, and to ensure participant safety, all blood collection procedures throughout each study visit will occur while positioned on a hospital bed in the prone position. In the event that a participant was to become weak, dizzy, or faint, they would already be ideally positioned to minimize risks associated with these symptoms. In the event that hypoglycemia-related symptoms occur, the study would be terminated to allow the participant to recover. We are prepared to provide pre-packaged beverages and snacks containing simple carbohydrates (e.g. Gatorade, fruit juice, apple sauce, crackers) that will allow for rapid restoration of blood glucose. These food items will be stored in a refrigerator that is located in close proximity and dedicated for foods used in research studies. Participant status (e.g. attentiveness, skin color) will be monitored in our clinical laboratory to ensure recovery. The clinical area where participants undergo blood draws is also equipped with first-aid measures (e.g. smelling salt) and all study team members are trained to assist with basic first-aid application if needed. Due to the amount of blood required for the study, participants will be advised not to donate blood and inform their doctor about the study if they need a blood draw at the doctor's office while they are on the study.

Any adverse hypoglycemic response that might occur during this study will receive care commensurate to the symptoms. For example, if a participant were to faint, then smelling salts would be administered along with a carbohydrate-containing food upon regaining consciousness. The study PI would also be contacted. Alternatively, should a more severe hypoglycemic response occur (e.g. contusion or laceration relating to fainting), the research team would immediately contact medical services (i.e. 911). For non-life threatening emergencies, any OSU students participating in these studies would be directed to the Wilce Student Health Center. For other emergencies or those participants who are not OSU students, individuals would be directed to the Wexner Medical Center either by transporting them directly or requesting ambulance service. Regardless of the complexity of the adverse events, the research team would monitor the participant in the interim, provide palliative care as appropriate, and follow-up after any medical care has been provided.

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