VALIDITY OF A PECAN-ENRICHED MEAL TO ACCESS POSTPRANDIAL CHANGES IN PLASMA UROLITHINS AND Γ-TOCOPHEROL

by

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(Under the Direction of Chad M. Paton)

ABSTRACT

There has been no consistent plasma biomarker confirms pecan consumption. Traditionally, γ-tocopherol, a vitamin E isoform in pecans, has been previously used as a marker, peaking 5-8 hours post-consumption. However, with typical 18-24-hour testing gaps, γ-tocopherol is unsuitable as a biomarker. This single-blind, randomized crossover trial aimed to confirm whether a pecan-enriched meal provides enough substrate to possibly increase plasma urolithins (Uro), gut-metabolized products of ellagitannins. Fifteen healthy participants consumed a pecan-enriched meal and a control meal with concentrations of 16,5331 mg/mL ellagic acid (EA) 1,815.2 ng/mL γ-tocopherol and 0 mg/mL EA, 593.4 ng/mL γ-tocopherol ng/mL respectively. We measured these meals micronutrients with liquid chromatography–tandem mass spectroscopy (LC–MS/MS). This study purposefully matched both meals macronutrients', but not micronutrients, to be able access if the shake recipes are adequate to examine a possible plasma Uro increase. According to our results both shakes were designed perfectly for the long run and future direction of this study.

INDEX WORDS: ELLAGITANNIN, PECAN, PLASMA BIOMARKER, POLYPHENOLS, UROLITHINS, VITAMIN E

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Table of Contents

Introduction

Obesity affects more than 30% of the world's population and the numbers are expected to increase to over 50% by 2050. Among obese individuals, over 1.2 million are diabetic or prediabetic, increasing the risk for cardiovascular disease (CVD), stroke, and cancer (1). With the increasing incidence and burden of obesity and related diseases, methods to combat weight gain, in addition to physical activity and caloric restriction are needed. A promising approach to offsetting the detrimental effects of obesity is through bioactive foods and food components, such as antioxidants and polyphenolics. We and others have recently shown that either chronic consumption of pecans can provide protection from saturated fat-containing meals, improve postprandial lipemia (2), and energy expenditure (5), and can be protective against post-meal oxidative stress (3).

Numerous tree nuts have been studied for their nutritional quality, antioxidant potential, and/or ability to reduce disease risk; however, compared to other tree nuts, there is limited data on how pecan consumption can impact human health. Chronic feeding trials have demonstrated pecan intake to be linked to reduced risk of occurrence of CVD due to its chemical composition (2-5). Pecan's proximate composition consists of a high lipid content with over 72% of their weight from fat, 9% from protein, 14% from carbohydrates (4% simple sugars and 10% fiber), 2% ash and 4% water (6). In addition to their macronutrients, pecans have a high antioxidant potential due to their phytochemicals like polyphenols and tocopherols (6). One of the most well studied antioxidant polyphenols in pecans is ellagic acid (EA) originated from the hydrolysable tannins,

ETs (17, 20, 21), however, it is also known that they have poor bioavailability in its native form and does not significantly contribute to in vivo health outcomes in clinical models (7). Despite ETs not being absorbed efficiently through the intestinal epithelium, recent work has shown the role of gut bacteria in catabolizing ellagitannin into urolithins (Uro) which can cross the gut epithelial barrier (10). The serial dihydroxylation reactions yield different metabolic products/isoforms of Uro, such as urolithin-A (Uro-A), urolithin-B (Uro-B), urolithin-C (Uro-C). Once they are in circulation, Uro-A, -B, and -C are converted into glucuronide conjugates (UroA-G, Uro-B-G, Uro-C-G) (20) through phase II/glucuronide metabolism (20).

Regardless of whether it is free or conjugated, Uro appearance in plasma is relatively long-lived, as a dose-dependent clinical trial with Uro-A supplementation concluded that Uro-A and Uro-A-G were eliminated from plasma circulation 72-96 hours post-intervention (19). Single dose studies with other ellagitannin-containing sources, such as wild berries (*Eugenia Brasiliensis Lam)* (147), jaboticaba (8), red and black raspberry (182, 183, 184) pomegranate (146, 155, 156), blackberry (141), strawberry (145) and walnuts (3) have a reported significant an increase in plasma Uro beginning at 24 hours, peaking at 32 hours (143) and remained elevated at 48 hours postprandially. Although Uro appearance in plasma is long-lasting, it is a dose and source dependent metabolite (2), opposed to ellagic acid (EA) that regardless of dose intake, plasma levels hardly ever elevate due to its low bioavailability (121, 125, 126). Therefore, chronic feeding trials with EA and/or ETs intake reported health benefits that were associated with their metabolite, free circulating Uro and their prolonged stay increase on plasma (56, 142, 153, 157, 132, 152).

Chronic dietary interventions of polyphenol containing foods are often limited when assessing compliance during feeding studies due to the inaccuracy of dietary self-report tools, such as food frequency questionnaires and 24-hour dietary recalls (12,13,15). A more objective method of estimating pecan intake would be to measure its metabolites in plasma, serum, or

urine. However, previous studies have used plasma levels of γ-tocopherol after 4 weeks of daily pecan intake as an attempt to assess pecan consumption, and both studies reported a high level of variance in tocopherols (4,5). Furthermore, acute γ-tocopherol interventions reported peak concentrations at 8-9 hours post-meal and returning to baseline around 24 hours post-meal (15, 16, 18). If a biomarker is used to access pecan consumption, it would likely need to remain elevated in plasma for more than 24 hours given that most feeding studies report consumption of the last pecan-containing meal between 8-24 hours post ingestion (4, 5, 14).

We recently demonstrated that chronic pecan consumption leads to a significant increase in plasma Uro and its glucuronide conjugates levels in humans (8), which led us to question whether Uro might be a valid biomarker for pecan consumption. Even though an increase of Uro and their glucuronide conjugates in plasma was reported after pecan consumption (8), their rate of appearance in plasma after a single pecan-enriched meal has not been determined. It is important to determine both the time of appearance and the relative amount of Uro in plasma after consuming pecans to better determine their ability to serve as biomarkers of pecan consumption. Accessing the patterns of Uro levels in plasma after a single dose of pecans is needed to determine their ability to serve as an indicator of consumption beyond 24-hours after ingestion. However, it is essential to design a study that can clearly answer our questions. This study aimed to validate the feasibility of this project in order to successfully determine plasma changes in Uro and vitamin E post pecan-enriched meal. We hypothesized that a single meal containing pecans would be sufficient to provide adequate levels of EA and γ-tocopherol to examine postprandial changes in plasma Uro. Therefore, we tested a 2x2, randomized control trial with two different breakfast meals matched in macronutrients, but different phytochemicals. Our glucose and triglycerides results showed no difference between the metabolic perception o

both meals (p=0.7, p= 0.3 respectively). Thus, we can confirm the meals and study design are adequate to possibly show changes in Uro and tocopherols are due to the phytochemicals of the pecan-enriched meal. Once we can confirm the changes of these metabolites in plasma, we will be able to establish a biomarker of compliance not only for pecans only, but for a great range of EA/ETs-containing foods.

Methods

Study Design

All procedures have been approved by the University's Institutional Review Board (IRB). This study was a single-blinded randomized cross-over design in humans conducted in the Human Nutrition Laboratory (HNL) at the University of Georgia (Athens, GA, USA). Participants were recruited and screened by research personnel before participating in the study. After being screened for inclusion criteria (**Table 1**), eligible subjects came to the lab on three separate occasions. The first visit (v1) was a screening visit that consisted of a 1-hour, in-person meeting at the HNL to obtain informed consent, followed by anthropometric measurements (height, weight, and BMI kg/m^2). Also, on v1, research personnel provided participants with oral and printed dietary recommendations to be followed prior to v2 and v3 and provide the subjects with their lead-in meals for the evening before v2 and v3. At the two testing visits (v2 and v3), each participant was given a different meal for breakfast: a pecan-containing shake or a control shake, with heavy whipping cream. Both v2 and v3 each required subjects to stay in the HNL for 8 hours, then left the lab after the 8-hour blood draw but return to the lab for blood draws at 12, 24, and 48 hours after each test meal. The schedule of the blood draws is further explained under "*Testing visits – (v2) and (v3)"* section.

Participants were required to fast (overnight for 12 hours) after consuming the provided pre-visit dinner meal. Detailed expectations of how subjects were instructed to prepare and report to HNL on testing days under *Pre-testing visit recommendations.* At the start of v2 and v3, an intravenous catheter (IV) was placed in the participant's arm throughout the 8-hour visit, and blood was collected at fasting, then every 2 hours up to 8 hours postprandially, then at 12, 24 and 48 hours post shake feeding. More details on blood drawing, blood processing, and sample analysis are in the *Testing visits – (v2) and (v3),* and *Sample analyses* sections below. Before v2 day, participants were randomly allocated in two groups by research personnel to receive either pecan (intervention) or control meal as breakfast on the first testing day. On v3, each participant received the opposite shake of the one assigned on v2 (**Figure 1**).

The pecan breakfast shake contained 68g of raw frozen pecan pieces, 244.0 ml of 2% fat milk, 15g of soy lecithin, and 25g of chocolate-flavored powder. In contrast, the control breakfast shake contained 138ml of heavy whipping cream, 152.50 ml of 2% fat milk, 5.7g of fiber supplement, 15g of soy lecithin granules, and 31g of the same chocolate powder. Pecans were acquired unshelled, in pieces and halves, raw, at room temperature, and packaged by South Georgia Pecan, Valdosta, GA (31603). The pecans used in this study belonged to two different harvests: November/2022 and November/2023. In the lab, they were stored in the –20° C freezer in the dark before being added frozen to the blender with other fresh ingredients of the intervention breakfast shake. Further information regarding the ingredient brands, and nutritional content of each breakfast shake is provided in **Tables 2-5**.

Participants and Recruitment

Fifteen participants were recruited to adequately power the study (see *Statistical Analysis* section). Both men and women were recruited for this study through printed flyers (**Figure 2**), emails to departments' listservs (See recruitment email script in **Appendix 1**), and word of

mouth through the University of Georgia's (UGA) Athens campus. After prospective participants contacted research personnel upon expressing interest in participating in the study, a screening call was scheduled (see **Appendix 2** for email/phone script), and each participant was screened by phone to determine eligibility to participate in the study (see **Appendix 3** for screening call form). The eligibility criteria for this study were being between 18 and 30 years old, body mass index (BMI) between 18.5 and 29.9 Kg/ m^2, healthy gastrointestinal system without any lipid-related, or digestion issues (i.e. Celiac disease, Chron's disease), no lactose, dairy and/or nut intolerance or allergy. Individuals on psychiatric medications that do not interfere with metabolism were eligible if the medication intake remained the same throughout the study. Individuals that take nutritional supplements, such as multivitamins, and fish oil were instructed and expected to resume intake of supplements for at least 2 weeks prior to v2.

Exclusion criteria included individuals with compromised swallowing or gastrointestinal functions or who have previously submitted for gastrointestinal surgeries, as well as individuals with food allergies or intolerances, such as dairy/lactose intolerance or nut allergies. Individuals on medications that affect the metabolism of lipids, such as cholesterol-lowering medications, were excluded from the study, as well as those who were or had been taking antibiotics for 60 days prior to or during testing. Individuals' users of nicotine or tobacco products, or that drink > 2 alcoholic drinks/day were excluded from the study. For all blood samples collected and stored, there were no names or other personal identifiers on the sample tubes. Only the subject ID (name acronym and assigned number) were shown on tubes.

Protocol

Screening Visit and Consent Visit (v1)

If a potential subject was deemed eligible to participate in the study, a v1 date was scheduled. The v1 meeting took place at the HNL and lasted no longer than 40-60 minutes. At

this meeting, research personnel explained to the subject what to expect from the study, and its timeline and presented to them the informed consent form. No information was retained without the consent of the subject, and if they chose to participate in the study, both research personnel and the subjects have signed informed consent form copies signed by both parties. If after reading the consent form, the subject decides they would abstain from the study, any information retained up until this point was not be kept. After the consent form was signed by both parties, the research personnel engaged in a conversation with the subject, to clarify possible questions about the study, and to provide the expectations for the upcoming testing visits (v2 and v3). On v1, participants were provided with study food for the evening meal for the nights prior to v2 and v3. The food provided by research personnel consisted of: Marie Callender's Macaroni and Cheese meal, and the snack was pre-packaged Animal Crackers (Stauffer's) (See **Table 6** for nutritional content of crackers and mac and cheese). Details of when and how to consume those were given during the v1 meeting and were clearly explained verbally and in writing from the supplemental written material provided by study personnel.

By the end of v1, the subject's height, weight, waist and hip circumference were measured at HNL, and recorded on a data collection screening visit form (see **Appendix 4**). Height was recorded by a stadiometer, and participants were asked to stand up straight, barefoot, and place their heels together. For weight, a standard standing scale was used, and participants were weighted with clothes on but no shoes. For waist and hip circumferences, an anthropometric tape was used. The measurements of each participant were recorded on screening visit form and kept with research personnel.

Pre-testing visits recommendations

The pre-testing visit instructions consisted of a written 48-hour low polyphenol diet protocol, samples of suggested meal plans, and specific instructions for v2 and v3. Participants were asked to reproduce what was previously done prior to v2 for preparation and during v3. The specific instructions (See **Appendix 5**) for testing days v2 and v3 consisted of consuming the provided meals Marie Callender's Macaroni and Cheese, with optional crackers (portioned into 27g snack bags), as dinner. This dinner was instructed to be consumed as the last meal prior to the testing visit, and participants were expected to arrive at the HNL after fastening for 12 hours and yet remaining hydrated. Subjects were asked to avoid caffeine for at least 16 hours prior to each testing visit or single blood draw, avoid physical exercise, over-the-counter medications, and alcohol for at least 24 hours before the visits (v2 and v3). A food list of 'Foods to Avoid 48-Hours Before Each of Your Testing Visits' **(Appendix 5)** sheets were handled available to aid participants in following a low-polyphenol diet for 48 hours prior to each of v2 or v3 visits and single blood draws. Also, participants were given a food dairy form (**Appendix 6**) to be filled out with the respective food intake 48 hours before visiting the lab for v2 and/or v3, what they eat after they leave on v2 and v3, and what they ate the next day of testing days.

Testing Visits (v2) and (v3)

Testing procedures for v2 and v3 were identical, with the only difference between the visits being the assigned breakfast meal order. Before the test meal, an IV catheter was inserted into the antecubital vein for a fasting blood draw. Next, the participants received their breakfast shake and were instructed to consume the entire contents within 5 minutes. At each time point, 10 mL of blood were collected, including at 0-, 2-,4-, 6-, 8-, 12-, 24-, and 48 hours postprandial. Blood was collected in ® EDTA-vacutainers, that were immediately placed and transported on ice, then centrifuged at 4º Celsius (C) at 3000 rpm, for 15 minutes. Separated plasma was

promptly aliquoted into separate 1.6ml microtubes and stored at –80º C until assayed. The IV catheter line was kept patent by administering 10ml of saline solution into the line every hour along with 200ml of water provided orally, every hour. Each participant remained in the HNL for the first 8 hours postprandial, then after the 8-hour time-point blood draw, the participants had the IV catheter removed and were instructed to continue the low polyphenol diet for the remainder of the visit outside of the HNL. Participants returned to the HNL for the 12-, 24- and 48-hour blood draws which were collected by butterfly needles.

Blood draws occurred every two hours, and ~ 10ml.

Blood glucose assay

The enzyme-based glucose assay is a colorimetric method, and a standard curve was run to each plate of samples. Plasma samples were thawed on ice and all used buffers will be made fresh on the day of assay. For this assay 4 buffers were made in the lab: 0.2M Na-P buffer A, 0.2M Na-P buffer B, 0.2% Dimethylaniline ($C_6H_5N(CH_3)_2$) and 20mM 4-Aminoantipyrine ($4(NH_2)C_5H_4N$) (See **Appendix 7**) by lab personnel. For the 0.2M Na-P buffer A sodium phosphate monobasic monohydrate (NaH_2PO_4) from Sigma-Aldrich (St. Louis, MO) was mixed with DI, and for 0.2M Na-P buffer B sodium phosphate dibasic anhydrous (Na_2HPO_4) from AMRESCO (Solon, OH) was mixed with DI. The 20mM 4-Amioantipyrine was made fresh with 4-Aminoantipyrine from Sigma Aldrich (St. Louis, MO), and mixed with DI water. The 0.2% dimethylaniline solution was made by diluting 37%, or 12M hydrochloric acid (HCl) from Sigma Aldrich (St. Louis) into 1M HCl solution with DI. Then, dimethylaniline by Sigma Aldrich (St. Louis, MO) was diluted in HCL, forming 0.2% dimethylaniline solution. The Deionized (DI) water, the enzymes peroxidase (p8250), and glucose oxidase (g2133) from Sigma-Aldrich (St. Louis, MO, USA) were used as well. Enzymes were stored at –20°C. Standard analytes were

prepared with glucose solutions in concentrations of 150mg/dL and 300mg/dL so a standard curve could be plotted.

Plasma triglyceride assay

The enzyme-based colorimetric triglycerides assay was purchased from Fujifilm Wako Chemicals (Richmond, VA), and kept at -4°C until use. Samples were thawed on ice. Then, 2μL of samples were allotted to the 96-well plate, and the first enzyme, R1 (10752-444) was added to the plate. The plate was incubated at 37°C for 5 minutes and taken to the plate reader. Then, the second enzyme, or R2 (10752-442) was added to the 96-well plate, which was taken once again for incubation at 37°C for 5 minutes. Then, the plate was taken to the plate reader and set absorbance at 600 nm. The same procedure of the glucose assay was previously described for quantification of glucose in the plasma samples in mg/dL. Different volumes of an 88mg/dL triglycerides solution were aliquoted into each 96-well plate, treated just like the samples, and taken to the plate reader. A linear regression equation was generated from the standard curve and the unknown concentrations calculated in mg/dL.

Liquid chromatography /mass- spectrometry measurement of ellagic acid and urolithins

Uro-A, -B, -C, and ellagic acid, were purchased from Sigma Aldrich (St. Louis, MO, USA), as well as Acetonitrile (ACN), and methanol. Water and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Uro-A-G, Uro-B-G, Uro-C-G were from Toronto Research Chemicals Inc. (North York, ON, Canada). ACN, methanol, water, and formic acid were LC-MS grade. The standards of Uro-A and Uro-A-G were prepared in dimethyl sulfoxide (DMSO)-ACN (1:4, v/v) and Uro-C in DMSO-ACN (1:1, v/v). Uro-B, Uro-B-G were dissolved in ACN. EA was dissolved in DMSO. Stock solutions were stored at -80°C until used. Standard

mixture solution was prepared by diluting stock solutions with ACN. Stock solutions of individual compounds were prepared at a concentration of 1,000 µg/mL.

LC-MS analyses were carried out using Agilent 1260 Infinity II UHPLC system coupled to a 6470 Triple quadrupole (OqO) mass spectrometer (Agilent Technologies, Santa Clara, CA USA). Analytes were separated using a Zorbax Eclipse Plus C18 column (2.1 x 50 mm, particle size 1.8 µm) at a column temperature of 30°C using a gradient elution. Water: formic acid (99.9:0.1, v/v) and ACN: formic acid (99.9:0.1, v/v) will compose the mobile phase A and B, respectively, with a flow rate of 0.4mL/min. The linear gradient will start with 5% of solvent B, reaching 95% solvent B in 4 minutes. The column was re-equilibrated in 3 minutes using the initial composition of the mobile phase (5% solvent B). The injection of sample volume was 2 µL. The mass spectrometer was equipped with electrospray ionization (ESI) interface, operating in the negative ionization mode. The following ESI parameters were employed: gas temperature 325°C, gas flow 10 L/minutes, nebulizer pressure 20 psi, sheath gas temperature 400°C, and sheath gas flow 11 L/minutes. All compounds were monitored in the multiple reaction monitoring mode (MRM) with dwell time 50 msec. The optimization of MRM conditions was conducted via flow injection analysis of individual standards. The optimum MRM conditions were tested and reported. MassHunter software (Agilent Technologies, Waldbronn, Germany) was used for data acquisition and data processing.

Liquid chromatography /mass- spectrometry measurement of vitamin E

LC-MS analysis was carried out using an Agilent 1260 Infinity II UHPLC system coupled to a 6470 Triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Analytes were separated using a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, particle size 1.8 µm) at a column temperature of 40°C using a gradient elution. Water:

formic acid (99.9:0.1, v/v) and ACN:formic acid (99.9:0.1, v/v) were used as mobile phase A and B, respectively, with a flow rate of 0.3 mL/minute. The linear gradient will start with 50% eluent B for 1 minute, reaching 98% solvent B in 4 minutes. 98% solvent B was maintained for 9 minutes. The column was re-equilibrated for 8 minutes using the initial composition of the mobile phase (50% solvent B). The injection volume was 3 µL. The mass spectrometer was equipped with electrospray ionization (ESI) interface, operating in the negative ionization mode. The following ESI parameters were employed: gas temperature 325°C, gas flow 10 L/minutes, nebulizer pressure 20 psi, sheath gas temperature 375°C, and sheath gas flow 11 L/minutes. All compounds were monitored in the multiple reaction monitoring mode (MRM) with dwell time 100 msec. The optimization of MRM conditions was conducted via flow injection analysis of individual standards. MassHunter software (Agilent Technologies, Waldbronn, Germany) was used for data acquisition and data processing.

Liquid chromatography / mass- spectrometry measurement of ellagic acid and vitamin E in food samples

Both breakfast shakes, heavy whipping cream, 2% fat milk, and pecan kernels were analyzed for EA and vitamin E. In a 1.5 mL microtube, 200 µl of liquid sample were extracted with 600 µL ACN: formic acid (98:2, v/v). Samples were vortexed for 10 minutes followed by sonification for 10 minutes. Then, samples were centrifuged at 17,000g per 10 minutes, and the supernatant was dried under a nitrogen stream. The dried residue was reconstituted with 100 µL of methanol and was filtered through a 0.22 µm PVDC membrane filter before LC-MS analysis. For solid, and semi-solid samples, such as heavy whipping cream and pecan kernels a defatting step was added and performed by vertexing the sample with 1ml of hexane for 10 minutes. This procedure was repeated three times and then the samples were left to dry under the hood for 12

hours. After that, 200 µg of sample were added to 1.5 mL microtube, and extraction was done the same way as for liquid samples.

For vitamin E, γ- and α-tocopherols were analyzed in all food samples. In a 1.5 mL microtube, 50 µl of liquid sample were extracted with 1 ml of hexane: methyl tert-butyl ether (4:1, v/v), containing 15 mg/L of butylated hydroxytoluene (BHT, antioxidant). The samples were vortexed for 30 min at 5°C. After centrifugation (12,000 g, 5 min, 5°C), the (supernatant) was dried under a nitrogen stream. The dried residue was reconstituted with 200 µL of methanol containing 0.2 mg/L of BHT, followed by vortexing for 10 min. The samples were filtered through a 0.22 µm PVDF membrane filter before LC–MS analysis. For solids or semi-solids, 50 µg of sample were added to 1.5 mL microtube, and extraction was done the same way as for liquid samples.

LC-MS analyses were carried out using Agilent 1260 Infinity II UHPLC system coupled to a 6470 Triple quadrupole (OqO) mass spectrometer (Agilent Technologies, Santa Clara, CA USA). Detailed analytical conditions for EA and vitamin E are described above. See **Figure 5 (A, B, C)** and **Table 12 & 13** for the calibration curves, linearity and optimal MS/MS parameters for EA, γ- and α-tocopherols.

Statistical Analysis

Samples sizes, and all data statistical analyses were estimated with 95% confidence and power of 80% using SAS version 9.4 statistical package (SAS Institute Inc, Cary, NC). The study by Istas et al (182) was used for Uro, and sample sizes ranging from 5 to 10 subjects were estimated for each individual Uro metabotype and Total Uro (sum of Uro-A-C/timepoint). Radosavac et al (194) and Galli et al (176), for □- and □- were used to reference tocopherols, respectively. For the two vitamin E isoforms, sample sizes of 8-12 subjects were calculated for

both timepoints. Outcomes were reported in absolute values as mean ± standard error of the mean (SEM), unless other stated. Repeated measures ANOVA with post-hoc comparisons were used to analyze glucose and triglycerides.

Results

Participants

Fifty-six participants were screened, and 36 have given consent. Twenty-one subjects dropped out after signing consent, causing an attrition rate of 58.3%. Fifteen adults (n = 9 female, n = 6 male) completed all testing visits (**Table 8**). They averaged BMI of 22.9 ± 0.9 kg/m². Baseline (fasting) samples were collected for both testing visits, and within each subject none of the analytes were significantly different between visit days (**Table 9**). Furthermore, all subjects were administered the same caloric amount for pre-visit meals and breakfast meal. We provided a range of 47.9 to 81.5% of total required calories of subjects.

Plasma Glucose and Triglyceride

Glucose and triglycerides were measured at each time point to comply with the standard practice of clinical trials to evaluate metabolic impacts of interventions and were graphically portrayed 0-48 hours postprandially in **Figure 4 (A, B)**. This approach not only informs primary outcomes but also elucidates the broader metabolic responses to given breakfast meals, enhancing the understanding of treatment effects thoroughly. Subsequent measures beyond 8 hours post prandial reflect differences associated with pecan or control consumption after mixed meals in free-living (post 8 hours) humans. For plasma triglycerides ($p=0.3$) and glucose levels ($p=0.7$), there was not a significant difference between the effect of two treatments throughout the 48 hours postprandial whilst subjects are still present in the lab and after they left, as seen in **Figure 4 (A, B)**. This means the breakfast shakes had a similar macronutrient composition, and

thus a similar metabolic impact when consumed and digested by the participants (**Tables 2, 3 and 5).**

Food Samples

The total concentration of EA, □- and □- tocopherols per shake can be found on **Table 10**, as well as the ingredient amounts fort each shake on **Tables 2** and **3.** The control shake included ingredients, such as 2% fat milk and heavy whipping cream that although had no EA contributed to the total vitamin E content of the control shake. We have detected the concentrations of □-tocopherol for 2% fat milk to be 124.9 ng/ml. For heavy whipping cream we detected 1,556.6 ng/mL of □-tocopherol, and 320.5 ng/mL γ-tocopherol. Finally, the actual control shake concentrations were: 617.6 ng/mL □-tocopherol, 593.4 ng/mL γ-tocopherol, and no EA at all.

The pecan shake included ingredients listed on **Table 10** and within them are pecan kernels, and 2% fat milk that contributed to the micronutrient count. Pecan and control shakes received different amounts of 2% fat milk, so milk did contribute with different amounts for both shakes micronutrients, each. The pecan kernels though had 1,069 ng/g of □-tocopherol, 22,899,06 ng/mL γ-tocopherol and 483,721 ng/g EA. The actual pecan shake micronutrients were the following: 16,533 ng/mL EA, 93.53 ng/mL □-tocopherol, 1,815 ng/mL γ-tocopherol.

Discussion

In order to successfully quantify Uro in plasma, it is important to ensure the presence of ellagitannins (ETs) and/or ellagic acid (EA) on the diet/meal (144, 146, 149). In this study, we have purposefully differentiated the micronutrients between meals and pecan kernels were the ETs/EA source. It was detected 32.9mg of free EA (**Table 10)** in the pecan shake although not directly accounting for ETs it's future hydrolysis that occurs on the stomach and will yield more

EA. Pecans are expected to have between 0.96-86.20 mg·g-1 ETs/g pecan, and the main ETs in pecans is pedunculagin (128). Since we added 68g of pecans total to the shake, it possibly contained a range of 65.28-5,861 mg total. Other ETs-containing food feeding trials administered 30mg of ETs and 23mg of EA from 400g red raspberry (184), 47.14mg/100mL of grumixama juice of total EA (190), 191.12 mg ETs, 2.39 mg EA of pomegranate juice (188). Furthermore, the degree of polymerization of each ETs might affect its hydrolysis, and consequently the amount of free EA released. EA will not be absorbed by the epithelium barrier of the intestine because of its poor solubility, yet it will be catabolized by the gut microbiome, producing Uro. Plasma is the biological specimen with the lowest concentrations of Uro, behind breast milk and urine (146), which might play a role in the absence of plasma Uro post ETs/EA intake as reported by others (149, 153, 182, 185).

For vitamin E, we have detected a relatively elevated presence of □-tocopherols in heavy whipping cream, 1,556.6 ng/mL concentration, and 215,000 ng total, which should not be a limitation to this study because heavy whipping cream is only part of the control shake (**Tables 2-5**). Therefore, the two vitamin E isoforms will not compete for absorption within subjects. The tocopherol transporter protein (α-TTP) has a high and specific affinity for α-tocopherol, consequently the other isoforms are metabolized and excreted faster (170, 171, 181). The fractional disappearance rate (FDR) of γ-tocopherol is 13-15 hours, but susceptible to change if α-tocopherol is present and consumed together, which is not the case. Previously, a study with acute pecan intake (~2,130,882 ng □- tocopherol) has shown significant increase of plasma □-tocopherol at 5-, and 8-hour postprandial. Therefore, this pecan shake provided 1,610,000ng □-tocopherol, whilst control provided 63,300ng, due to milk and heavy whipping cream.

The study personnel provided oral and written instructions for a 48-hour low polyphenol diet protocol to ensure that subjects would not consume sources of ETs/EA or vitamin E foods within the period of study **(see Appendix 5**). We also provided pre-visit dinner and snack (Table 6) to enhance compliance with fasting requirements and minimize variability in the fasting state of participants. The eligibility criteria of this study included individuals 18-30 years old, 18.5≤ BMI ≤29.9 kg/m2 and excluded individuals that were taking lipid-metabolism related medications and/or antibiotics that could possibly affect Uro and vitamin E metabolism (**Table 1)**.

We designed a cross-over, single-blinded, 2x2 study design so each subject would be their own control in order to achieve power with a small sample size, such as 15 subjects, in a cost and time-effective way. Since the study was single-blinded, pecans were blended into the intervention shake, and the control meal was also provided as a shake, in non-translucent cups, so subjects would not be aware of which breakfast shake they were consuming at each visit. The pecans kernels halves were kept in -20°C and in the dark to avoid oxidation and nutrient loss until they were blended within the shake. As seen on **Tables 2-5** and **10**, both shakes' macronutrients were matched purposefully, but not the micronutrients.

In order to test the metabolic impact of both shakes, glucose and triglycerides plasma levels were measured at each timepoint. There was no significant difference between plasma glucose ($p=0.7$) and triglycerides ($p=0.3$) levels measured 0-48 hours post both meals. This means that we have successfully designed a study in which two meals have the same metabolic impact within subjects, but do not contain the same amounts of micronutrients, which are expected to be the substrate, vitamin E and EA, for possible expected plasma changes in Uro and γ- tocopherol post ETs/EA intake.

Conclusion

We concluded that this study design and both shakes are adequate to provide the micronutrients that will be the substrate to possibly cause changes in plasma. Since the plasma samples collected in this study are still going to be analyzed for Uro and □- tocopherol levels, it is not possible to make remarks on their levels yet. However, we successfully demonstrated that both shake recipes are adequate to provide different levels of substrates such as EA, and vitamin E, while keeping the same metabolic impact for both meals within subjects. Therefore, this is an appropriate approach to both validate the study and shake recipes, and in the future make reliable conclusions on plasma γ-tocopherol and Uro levels.

Literature Review

Impact of Diet in Health

The influence of diet on human health and the aggravated incidence of diseases has shown that there is a causal correlation between chronic diseases and imbalanced diet, such as high sodium intake and hypertension. Not only diet but also lifestyle improvement plays an essential role in cardiovascular disease prevention (23). Inadequate diets are one of the reasons for chronic lifestyle diseases, but additional modifiable behaviors also play a key role in chronic disease development. A notable example is the lack of physical activity, which is another key factor in the development of chronic diseases (23). The greater the physical activity, the better the individual's overall health. In 1950s, the first reliable scientific evidence of the causal relationship of physical inactivity and the risk of chronic diseases began to emerge, and sparked interest in the three basic aspects of the problem: Increased use of technology to accomplish daily activities, difficulty in motivating individuals into being more active, and the physiological evidence of specific effects, such as the relationship between coronary disease mortality and physical activity levels (24). However, this correlation failed to induce changes in the behavior of the general population. It was not increased until increased morbidity related to sedentary behavior that concerns started to rise, and in 2004 (updated in 2010), the World Health Organization (WHO) ruled sedentary lifestyle as the 4th most common risk factor for cause of death worldwide. The most recent report estimated that the lack of physical activity is responsible for 6% of the burden of coronary heart disease, 7% of Type 2 Diabetes (T2D), 10% of breast cancer, and 10% of colon cancer (23). Later in 2014, the WHO reported that adults that

do not engage in sufficient physical activity regularly, have a higher risk of death than the ones who do. In addition, the WHO reported that women tend to be more sedentary than men, which increases their risk for chronic diseases. Another concerning fact reported by the WHO is that worldwide, 81% of adolescents did not engage in sufficient physical exercise, and that girls tend to be less active than boys.

Not only was the lack of physical activity recognized as prejudicial to health, but also an imbalanced diet has been linked to increased incidence of chronic diseases or non-communicable diseases (NCDs). Chronic diseases tend to last long and are a result of a combination of genetic, physiological, environmental, and behavioral factors, the main ones being cardiovascular diseases (CVD) (WHO, 2023). The development of NCDs is linked to changes in global nutrition and the food industry, and to the spread of unhealthy dietary behavior across different world regions (26). For instance, the evolution of technologies in the food industry as seen in the edible oils market enabled an increase in the availability of cheap vegetable oils (26), and consequently consumption of saturated and *trans*-fat. The aggravated consumption of these fats causes inadequate consumption of n-3 and n-6 polyunsaturated fatty acids (PUFAs), which have been related to cardiovascular benefits (27).

Another change that has drastically impacted the population's health is the aggravated consumption of Sodium (Na). Although Na is a necessary nutrient for the regulation of fluids and transport of molecules in the human body, it has a well-established causal relationship with blood pressure levels. Consequently, in 2010, high blood pressure (BP) was ranked as the main risk factor for the global burden of diseases, and in 2019, among all the factors, the largest increases in exposure were in high fasting plasma glucose and high body-mass index (28).
Adding salt to food has been part of food processing for centuries, due to its flavor-enhancing properties, antimicrobial capacity, and consumer acceptance. Despite the health consequences

from high sodium intake, it has been an essential ingredient for the food industry to enhance flavor, texture, and aroma. Several studies have shown that a high intake of Na causes high blood pressure values and a high rate of hypertension (29, 30), consequently contributing to the risk of CVD.

An imbalanced diet is the worst of the risk issues for stroke in the USA, and in other high-income countries (31). An excessive intake of food goes against the maintenance of healthy weight in healthy individuals, which can lead to obesity, an established risk factor for stroke and mortality (33). In fact, lowest mortality rates were associated with BMI values of 22·5–25 kg/m². Within 25-50 kg/m² range, each 5 kg/m² higher BMI was on average associated with 30% higher overall mortality, 40% for vascular mortality, 60-120% for diabetes, renal and hepatic mortality (33). Atherosclerosis is the dominant cause of CVDs, such as myocardial infarction (MI), heart failure, stroke and claudication (32). Despite the impact diet has on one's cardiovascular health, at cellular level overnutrition can also cause negative effects.

Impact of Diet on Health

A feature newly related to sedentary lifestyle and poor dietary habits is metabolic inflexibility. Metabolic inflexibility consists of the inability to switch between the oxidation of lipids and carbohydrates based on which substrate is available, and it is commonly seen in T2D and obesity (34). Obesity-related cardiometabolic diseases are increasingly categorized as a disorder of metabolic inflexibility. The mitochondrion of the cell has been pointed out as a key part of the cell's capacity to adapt through different stages of metabolic demands. Therefore, if the mitochondrial capacity is compromised it might lead to the inability to adapt fuel preferences and induce morphological changes to respond to different stimuli. Nutrient overload and heightened substrate competition might lead to mitochondrial indecision, resulting in impaired fuel switching, and energy dysregulation (35). The onset of insulin resistance is usually seen

early in the disease and might lead to further etiology of late-stage complications. A healthy individual without any impairment should be able to switch between the fuels according to hormones and nutrient availability and be able to oxidize both carbohydrates and lipids for energy. However, a healthy individual living with continuous availability of energy goes against the most common environmental conditions in nature, which is the scarcity of energy (35).

The nutrient overload caused by an excessively caloric and nutrient-dense diet will lead to mitochondria. When individuals eat voraciously and often, a state of gridlock develops because carbon traffic and competition for substrate intensifies. Chronic overnutrition causes metabolic confusion, and failure in signaling, which leads to unabated influx of surplus nutrients fuel and ensuing "traffic" of convergent metabolic pathways. As the mitochondria reaches a state of gridlock, and electron imbalance generates towards reactive oxygen species (ROS) and protein modifications (PTMs) such as glutathionylation and lysine acetylation, which further disrupts nutrient signaling and sensing (35). The mitochondrion needs repairing systems to manage the hazardous molecules capable of causing irreversible damage to cellular macromolecules and possibly leading to organ dysfunction (35). In a larger scale and, in addition to the cellular impact overnutrition can have, the excessive consumption of lipids, especially saturated fatty acids (SAFs), most likely will cause a negative impact to one's health being reported as possible contributors for atherosclerosis and possibly CVDs (36-38).

Atherosclerosis and diet

As previously stated, atherosclerosis is considered a dominant facilitator for CVDs such as MI and stroke (32). According to the American Heart Association (AHA) atherosclerosis consists of a hardening of the arteries due to the formation of plaques on the wall of the arteries. The calcification within large arteries starts with the activation of the epithelium, that is

susceptible to the atherogenic hemodynamics variations of the blood, such as tensile stress due to blood pressure and wall shear stress (WSS), a tangential force to the artery walls induced by blood flow (32, 39, 40). The vascular epithelium of vessel walls controls the diffusion of plasma molecules and regulate vascular tone, inflammation and prevent thrombus formation. These functions are with dysfunctional epithelium (41). Increased blood lipids, such as low-density lipoprotein (LDL) are linked to decreased arterial WSS, which causes the endothelial cells to change from flattened shape to a cobblestone shape, remodulating the underlying vascular smooth muscle. This anatomical change though will be responsible for a cascade of events. Accumulation of LDL in plasma favors trans endothelial infiltration of circulating LDLs to the intima (41), as well as retention and deposition of oxidized LDL (OxLDL) molecules on the altered epithelium sites, causing the modification of them within the intima. In the intima space, there is no availability of protective plasma antioxidants, such as tocopherols and ascorbate, to protect the LDLs from oxidation (41). Regardless of the mechanism of initiation of LDL oxidation, any possible LDL-bound antioxidants, such as α -tocopherols and carotenoids, are lost. Injuries to the endothelial-related antithrombotic properties enhance platelet adhesion and activation to the dysfunctional area, leading to secretion of growth factors. Consequently migration, accumulation and proliferation of vascular smooth cells (VSMCs) and leukocytes, promoting plaque progression, (42). Also, a small degradation of PUFAs such as linoleic and arachidonic acids take place, generating hydroperoxides ($_{H}2_{O}2$)) and forming conjugated dienes that upon further oxidation, results in small chain aldehydes and more modifications to OxLDL(41, 43). The binding of the aldehydic products to free amino groups of Apolipoprotein B (Apo B) causes macrophages uptake, enhancing the immune response. Macrophages will be responsible for the release of inflammatory cytokines, producing nitric oxide (NO) and reactive

oxygen species (ROS), which promote monocyte recruitment and inflammatory response propagation (41). The OxLDL leads to increased atherogenic factors circulating, enhancing the cascade of immune response to the pro-inflammatory stimuli, such as the attracted macrophages scavenge OxLDLs, and convert into foam cells. The accumulation of foam cells evolves into fatty streaks during the early stages of the atherosclerotic process and later will evolve to a fibrous plaque, considered the hallmark of established atherosclerosis (41, 44). Ultimately, the atherosclerotic lesions are composed of three components: smooth muscle cells (SMCs), and macrophages. Then, connective tissue matrix and extracellular lipids. Lastly, intracellular lipid that is stored inside of macrophages, converting them into foam cells. The atherosclerotic lesion may contain significant amount of lipids, that if unstable may lead to denudation of overlying endothelium or plaque rupture, resulting in thrombotic occlusion of the artery (32, 41, 44).

The calcification of the fibrous plaques consists of a bone-like formation within the plaque, that continuously advances as the disease progresses. As the latter stages of the disease progress, the now-called atheroma plaque is most likely formed in areas of lower WSS, such as the branched arteries (41). Of many issues these atheroma plaques might bring, when they rupture of fissure, they develop a coagulation process to cover the formed wound. Consequently, pro-thrombotic elements are released, encounter coagulation factors of plasma and lead to thrombin production. The coverage of the wound is called thrombus, and it increases the rate of reactions that make the plaque thicker, increasing chances of vessel obstruction, lowering the blood flow in coronary arteries, leading to ischemic cardiopathies. The consequences of the formation of thrombus are increased chances of cardiac insufficiency, angina pectoris, myocardial infarction or stroke. The detachment of the thrombus from the artery wall produces a clot that circulates within the cardiovascular system, and lodges in distal arteries where it can

obstruct blood flow, and consequently an infarction (41,44). Therefore, it is safe to say CVDs are enrooted in the pathogenic advancing process of an atherosclerotic condition in arteries.

The deposition of LDLs requires accumulating lipids in the intima of the vessel wall, allowing for definitive formation of the plaque (44). Certain health conditions can strengthen the deposition, such as dyslipidemia and hypertension (45). These conditions are strongly related to obesity (46-50), whilst obesity is related to diet and sedentary lifestyle (51-54). Therefore, significant data available in the literature suggests a connection of CVDs occurrence and development with sedentary lifestyle, added to a caloric surplus intake throughout life (5557). The chronic surplus of calorie intake, especially if calories sources are lipids or carbohydrates, will contribute to increased LDLs levels, as well as triglicerydes (TAGs), and sugar in blood, contributing directly with development of atherosclerosis. Nonetheless, occurrence and progression of other diseases and conditions have been previously linked to diet and food choices, such as inflammatory bowel disease (IBD), Chron's disease (CD), osteoporosis (57-59). For this reason, research on nutrition plays such an important role in preventing and taking care of health issues.

Nutritional Research

Enough significant data suggests that diet and food choices have great impact on one's health, as previously discussed (see Section *Impact of Diet on Health*) and for this reason the interest in nutritional research has been increasing since the early 20th century (60). It was not until 1926 that the first 'vital amine' was discovered, but by mid-20th century all the vitamins have been fully established and were recommended in dietary guidelines (60). Then, through 1950s to 1970s the interest of nutritional research shifted to fat *versus* sugar and the protein gap. From 1970s to 1990s, it switched to diet related NCDs and supplementation (60, 61). The burden

of non-communicable diseases (NCDs) or chronic diseases led the government to invest in nutrition science, and in response in 1980 the *Dietary Guidelines for Americans* was published. In the 1990's there were significant advances in scientific development, such as completion of multiple, complementary, large nutrition studies like prospective observational cohorts, and randomized clinical trials (RCTs). The latter allowed to further test of specific questions in targeted population in supplements, and a specific diet pattern. As of now the focus and main development of nutritional research has been the consolidation of the design and completion of multiple, complementary, nutrition studies including prospective observational cohort studies, clinical trials and more recently, genetics. A novelty for nutrition research was the insertion of genetic consortiums. The access to effect of food intake in genetic level has brought more insight to genetic influences in dietary sources, gene-diet interactions affecting disease risk factors and endpoints, as well as causal effects of dietary biomarkers (62, 63).

Clinical nutrition research played and still plays a pivotal role in establishing causality between diet and health outcome measures and in the determination of dietary and supplemental levels to achieve determined outcome (63). Nonetheless, the field developed exclusive assessment challenges such as: background intake, nutrient status, effective delivery, effective control, length of study, compliance, nutrient bioavailability and lack of specific guidelines for designing, performing, documenting and reporting. In 1996, the U.S Food and Drug Administration (FDA) published the first Consolidated Standards for Reporting Trials (CONSORT) document, that aimed to set standardized procedures to help improve the quality of RCTs and its reports (63). However, by that time, CONSORT was directed to rule exclusively pharmacological interventions, in which nutritional intervention does not fit. Despite the lack of standards, there was a high incidence of non-pharmacological treatments (NPTs) publications in 2000, in which one in every four publications were about NPTs. In 2008, an extension was

added to the original statement, and now CONSORT addressed not only drug intervention, NPTs such as nutrition trials, surgery, rehabilitation or psychotherapy (64).

NPT term is an umbrella term for several types of trials that do not involve intervention with drugs, but that assess surgery and procedures (64). Although there is no specificity for RCTs based on nutritional intervention on the CONSORT standards for NPTs, these standards can still be applied in feeding trials (64). For instance, it is crucial to provide mechanisms for internal validity of the study, such as adherence to intervention by the subjects/participants. Currently, nutritional research is focused on investigating the effects of dietary patterns, instead of focusing solely on single nutrient interventions (62). However, to investigate dietary patterns in free living human beings can be challenging, which deteriorates internal validation of participant adherence to intervention (65, 66). To accurately reach one's dietary pattern and reliable intake during a certain period allows for truthful conclusions about one's dietary pattern. However, there are challenges within nutritional research field to assess intake during chronic interventions (65).
A challenge of chronic dietary interventions in free-living human beings is to measure the dietary adherence during intervention, and to assess the timeframe of intake. Especially studies with a short menu cycle, long periods of feeding, dietary restrictions that demand greater participant commitment are hard to ensure compliance of participants with food intake (65). However, there is no consensus on the optimal approach to measuring dietary adherence in feeding interventions (66). The most common resources available to track subjects' compliance are food frequency questionnaires (FFQ), 24-hour diet recall (24HR), and food diaries (67), that rely on individual's capacity of reporting. Self-reporting food intake can be very challenging since it relies on one's memory, and capacity of reporting correct products and amounts. Memory-based methods to assess intake have been deemed as irrelevant to the physiologic effects of consumed foods,

beverages, and diet-disease relations (68). For this reason, biological markers for specific foods or food components are considered a great tool and resource for nutritional research, as discussed under 'Nutritional Biological Markers'.

Clinical Trials

As previously mentioned under the 'Nutritional Research' section, it is imperative to investigate the effect of food on health, especially due to the aggravated risk of CVDs. From an epidemiological point of view the study design can be observational or experimental. Observational studies are hypothesis-generating and can be further divided into descriptive and analytic (70). Descriptive studies provide descriptions of exposure or outcome, while analytic studies can provide a measurement between the exposure and the outcome. Experimental studies are hypothesis-testing and involve an intervention that tests the link between exposure and outcomes. In experimental studies the risk factor/exposure of interest/treatment is controlled by the investigator (70). It can be divided into two categories: controlled, in which there is a comparison, and uncontrolled, with no comparison. Furthermore, experimental studies design can be subdivided into three categories: clinical trials, field trials and community trials. Field trials are preventative or prophylactic and community trials are also known as clusterrandomized trials, because they involve two groups, with and without diseases (70). Clinical trials are also known as therapeutic trials, and that will be further divided into randomized and non-randomized trials. Randomized control trials (RCTs) are considered the most adequate for epidemiological research and consists of randomizing subjects with similar characteristics to two groups (or multiple groups): one group receiving the intervention/experimental treatment and another group that will receive the placebo or standard care treatment. Also, identical dependent outcome variables are measured in all groups in the trial, with outcomes compared between groups, or between participants, to determine treatment effectiveness. Furthermore, if it is a randomized

trial, each individual or group is randomly assigned to receive treatment A or B first (70). The independent variable has at least two levels: both being a unique dietary intervention or with one level being a control comparison. The dependent variable is the health outcome to be observed after treatments and analyzed. Non-randomized trials involve an approach to selecting controls without randomization (70). Human nutrition RCTs can be relatively short or long duration according to the study goals and might need or not of a washout period (69).

In human nutrition, RCTs are the gold standard to determine causal relations between exposure to nutrients, foods, or dietary patterns and prespecified health outcomes, such as event rates and biomarkers (69). RCTs are considered the highest level of evidence to establish causal associations between the exposure or intervention and the outcome. Nutrition intervention trials examine the effect of a randomly assigned exposure on an outcome such as disease occurrence, risk factor for disease, or a biomarker (70-73). In an RCT, one or more treatments are compared with a control group, and patients are assigned to treatment or control by chance. The randomization of participants to groups is an important tool to minimize the differences in characteristics of the groups that may influence the outcome (72). Each group in a RCT is called an 'arm', so a two-arm study compares an experimental treatment vs a control group, respectively called, 'treatment arm' and 'control arm' (72). A non-randomized control trial there are two groups that undergo the same treatments, and a pattern can be adopted, such as selection of subjects and controls on certain days of the week. If the selection of subjects becomes predictable, there is bias regarding the selection of subjects and controls, which would question the validity of the results obtained (70).

RCTs can be designed differently depending on the study's aims and target populations. RCTs will affect the utility or the generalizability of the study results. The choice of study design for RCTs should be aligned with the study goals (70, 71). Crossover study design is commonly

used to examine the effects of dietary intervention by examining groups or individuals that undergo the same intervention/experiment at different time periods of the study. The crossover design is applicable for interventions in which the treatment effect is considered reversible, since participants receive a set of treatments in a random order or fixed sequence (70, 73). Each subject goes through both treatments in a random order, which allows each participant to serve as their own control (70, 73). Treatment effects are based on within-participant variation, which consolidates a higher statistical power relative to the sample size of other study designs. A crossover study design might cause higher participant burden due to multiple interventions and washout periods, that can extend the duration of the study. As the washout period aims to not allow for carryover effects in the study, it can also lead to a longer duration of the study might lead to increased risk of dropouts, or low adherence to the protocol (70, 73). Insubordination to the study requirements is a major threat to its viability. Furthermore, treatments that cause longterm outcomes are unsuitable for the crossover study design (69).

The parallel study design consists of each participant being randomly assigned to a single study intervention. This is participants are assigned to be part of either control or intervention group (69). Comparisons between groups are based on between groups/among-subject variation. Furthermore, this study design allows for multiple treatments to be tested simultaneously, resulting in shorter periods of duration. This design eliminates potential risk of carryover effects between treatments, and extraneous differences such as unanticipated in food composition or as light exposure are minimized. Moreover, this design enables absence of washout period due to no risk for carryover effects to happen (70-73). A limitation of this design is the requirement for larger sample sizes than a crossover study design would be due to participant variability.

A factorial study design aims to test two or more drugs with independent effects on the same population (70). Typically, the population is divided into 4 groups and receive different

treatments a different combination of treatments, which allow for multiple interventions being tested at the same time, which saves time and allows for both drugs to be tested in the same population (70). A cluster study design consists of interventions randomly assigned to entire groups rather than individuals. It can be an efficient way of administering intervention in a population with natural groupings such as households, and community centers (70-73).

Biological Markers

a. *Overview of Biological Markers*

Many definitions have been presented for the term 'biological marker' (74-76). A biological marker is a broad term for objective indicators of medical or nutritional status measured from outside of an individual and that can be measured accurately and reproducibly (74). Biomarkers are objective, quantifiable characteristics of biological processes measured in biological specimens (76). Historically, they have been mainly used in medical practices and for research purposes such as a novel therapeutic drug or as early warning systems for disease development (77). In clinical practice, biomarkers have been used to personalize medication or health care and analyze the safety of pharmaceuticals, leading to the need of regulations and definitions. The Food and Drug Administration (FDA) together with the National Institutes of Health (NIH) initiated a continuously updated online document "Biomarkers, Endpoints, and Other Tools" (BEST) as a resource for the scientific community (74). The purpose of BEST is to improve the collective ability to pair biomarkers with their specific processes, which enables greater speed, efficiency, and precision to develop useful diagnostics and therapeutic drugs and strategies, as well as implement public health policies (78).

There is a range of classifications for biomarkers used in the medical field, one of them being BEST. Their available material for biomarkers divides and defines them succinctly into

several categories according to their identity, biological plausibility, measurement methods and its subcategories (78). For instance: Kidney Injury Molecule 1 (KIM-1), source: Urine, type: Molecular. Biologic plausibility category consists of the biological association of the biomarker with the condition of interest. In this case, KIM-I has been observed to increase in the presence of drug-induced acute tubular kidney injury, and its measurement method is ELISA (78). The BEST resource also subdivides the medical biomarkers into subtypes and emphasizes that a single biomarker may meet multiple criteria for different uses, nonetheless, each definition requires scientific evidence. The subtypes categories of BEST are diagnostic, monitoring, pharmacodynamic/response, safety, susceptibility/risk, and predictive, and prognostic biomarkers (74, 78). However, this division is not set in stone. Various sources available in the literature report variables of the medical biomarkers classification system, as seen in (77, 79). The first study elucidates the diverse ways in which subcategories of biomarkers can be organized according to their genetic and molecular biology, characteristics and clinical applications (78). The clinical application subcategory is branched out into three classes: diagnostic, prognostic and therapeutic biomarkers. Differently, the latter, Jain, K. K., 2017, suggests a different division of categories based on what type of diseases and conditions the different biomarkers should be applied to, such as biomarkers of infectious diseases and biomarkers of musculoskeletal disorders.

Regardless of how the biomarkers are subdivided, their determination and application allow for precision of measurement, less bias, reliability, and study of mechanism of the biological processes of a certain molecule in the human organism. Consequently, assessing changes through established biomarkers serves not only as a guide for decision-making and consequently changes in the clinical course, but also to detect initial response in dietary or

pharmacological interventions (78). There is a well-established clinical importance for the efforts directed to establishing and applying biomarkers: They facilitate the research and development of areas such as biomedical, pathophysiological and nutritional sciences, as well as pharmacology, diagnostic and preventative research (73-77)

b. *Food Biological Markers*

As previously discussed in the section Nutritional Research, there is great importance in accessing reliable and accurate information on diet intake, especially when a hypothesis is being tested, such as in scientific research. Observational, cohort studies, and other nutritional approaches for research tend to rely a lot on self-reported diet, questionnaires, and food records, which is reported not to be the most accurate dataset (80). The Observing Protein and Energy Intake (OPEN) study conducted to assess dietary measurement error using two self-reported dietary instruments: a food frequency questionnaire (FFQ) and the 24-hour dietary recall (24HR), and an unbiased biomarker of energy and protein intake (doubly labeled water and urinary nitrogen). On average, men underreported energy intake by 12-14% on 24HRs and 3136% on FFQs and underreported protein intake by 11-12% when compared to protein biomarker on 24HRs and 30-34% on FFQs. Women underreported energy intake on 24HRs by 16-20% and in FFQs by 34-38% and underreported protein intake by 11-15% in 24HRs and 27-32% on FFQs (81). The use of biomarkers is very common dietary intervention studies, in which the biological fluid used is urine, and plasma, and involves participants consuming specific food(s) (81).

Another study conducted with 450 women in the Women's Health Initiative resulted in underreport of dietary self-intake (82). From 2007 to 2009, a 4-day food record, three 24-hour dietary recalls, and a food frequency questionnaire were collected from each of the participants

along with biomarkers of energy and protein consumption. In this study, a more 'flexible' measurement model was implemented, as the researchers combined the measurements provided individually by the 4-day food record, 24HR recalls, the food frequency questionnaire and biomarkers into a calibration equation. A calibration equation can show a more 'calibrated' consumption estimative (82) due to its capacity to allow for systemic and random measurement errors in the self-report assessments. Those measurements involve linear regression of the variables, which allows for a more thorough approach of data collection, such as disease association analyses. Each of the 3-self-report procedures underestimates energy substantially (20-70%), protein (4-10%), and overestimated protein density compared to biomarker (16-25%). The application of biomarkers for nutritional research has overgrown its only use as 'recovery' marker, instead, it seems to be promising, since biomarkers are believed to be useful in measuring food intake (82).

The use of biomarkers for food allows researchers to establish firm evidence between the health effects associated with foods and food components (81). A food- or nutrient specific biological marker can aid in tracking its physiological pathway, and therefore, understand its mechanisms, absorbance rate, nutrient interaction, protocol compliance, and kinetics (82). A series of putative acute intervention biomarkers have been developed for foods, such as the citrus fruits (84-86), cruciferous vegetables (84, 87), red meat (89, 90) coffee (91, 92), tea (92, 93), sugar-sweetened beverages (94) and wine (82, 96). There has been reported the need for biological markers of food polyphenols (1, 59) since these compounds have been associated with prevention of human disease (29, 81). However, there are some particularities to those compounds that aggravate the need for a marker: a. there is a great diversity of types of polyphenols among foods b. there is limited data for the content of polyphenols in foods c. there

are challenges in characterizing and quantifying habitual food intake d. there is a limited understanding regarding the extent of absorption and metabolic fate of individual polyphenols from particular foods (58). Previously, Uro-B B has been suggested as an intake marker of ETs containing foods (1, 59).

Pecans and Health

a. *Pecan Biochemical Composition*

Pecans [Carya illinoinensis (Wangenh.) K. Koch] are tree nuts native of the southeast of North America and the U.S is responsible for producing more than 80% of the world's total production (83). During 1999-2005 the U.S had a market value for pecan production of $201-401 million, with Georgia being one of the major producing states and having 11 commercially viable cultivars: including Cape Fear, Curtis, Desirable, Elliot, Gloria Grande, Kiowa, Oconee, Pawnee, Schley, Stuart, and Sumner (183). The Desirable and Stuart are the most important commercial cultivars, and Schley, Stuart and Wichita are commonly grown in the southern eastern of USA (83). The United States Department of Agriculture (USDA) National Nutrient Database reports averages of the nutritional composition of raw pecans halves. In 100g of raw pecan halves there are 9.96g of protein, 73.3g of total lipids (22.9g of polyunsaturated fatty acids, 39.3g of monounsaturated fatty acids, and 6.46g of saturated fat) and 12.7g of carbohydrates with 5.8g being fiber (83, 84). Wells et al. 2009 reported that orchard management practices can produce considerable variations within the same cultivar, just like the soil and weather of cultivation place can cause changes within the same cultivar as shown by Siebeneichler et al. (2022). Each cultivar genetic factor also affects this composition variability, and there are more than 500 cultivars of pecans available in nature (83).

Triacylglycerols (TAGs) were reported to compromise ~97% of total pecan lipidic fraction (83). A report on 70 different cultivars of pecans by Rudolph et al. (1992), in 6 cultivars

by (80) concluded that there are differences in the fatty acid composition profile depending on cultivar, and environmental factors (83). The variation of lipid content according to the pecan cultivar have been reported as 60.3%-76.6% (Rudolph et al. 1992), 72%-75% (Senter & Horvat 1976), 70%-75% (Toro-Vasquez 1998), 52.70-78.07% (Siebeneichler et al. 2022). Rudolph et al. (1992) suggested the main fatty acids (FA) of pecans in descending order of concentration to be: oleic (18:1n-9) ranging from 48.5-73.1%, linoleic acid 16.9-34.3% (18:2n-6), palmitic (4.9-6.6%(16:0), stearic 1.7-3.5% (18:0) and linolenic acid 1.0-2.6% (18:3n-3), in accordance with Siebeneichler et al. (2022) Villarreal-Lozoya et al. (2006) and Alasalvar, C & Shahidi, F (2008). The sum of 18:1n-9 and 18:2n-6 can reach up to 90% of total lipids, and the content of SAFs is smaller than 11%, which is associated with the reduced levels of LDL cholesterol and the risk for CVDs (75). Also, 18:2n-6 and α -18:3n-3 are respectively omega-6 (ω6) and omega-3 (ω3), which are essential fatty acids for human beings, and therefore should be consumed through the diet (75).

Protein is the third main constituent of the pecan, and its amounts can range from 5.00-17.84% (75, 83). The protein content of the pecans contains all the essential amino acids for human diet, and high contents of glutamine and arginine, which have been associated with positive vascular benefits (78). It is estimated there's 12.45mg of arginine in 100g pecans, and 21.06% in 100g pecans of glycine. However, Alasalvar, C & Shahidi, F (2008) reported lysine was the first limiting amino acid and very low levels of arginine in pecans, such as 0.953mg arginine per 100g pecans. Furthermore, carbohydrates of pecans have been unspecified according to the cultivar origins, it's content is of 13.86g in 100g of pecans, and the total dietary fiber content is 9.6g/100g, total sugars 3.97g/100g, sucrose 3.90g/100g. Minerals present in pecans are Cu,Fe,Cr, Mn, B, Zn, Ba, P, K, Ca, Mo, Sr and Mg (82), and each mineral level is

variable according to the cultivar and nut tree site. Fe was reported to range between 23.258.8mg and Zn 25.1 to 59.6mg in 100g pecans (82). One portion of pecans of 1.5 ounces (Oz.) or 42.5g provides 56% of adequate intake (AI) of Cu, 14% of Fe of AI for men, 17% AI of P, 83106% AI of Mn, and 17-24% AI of Zn for women and men, respectively. The vitamin content per 100g of pecans consists of thiamin 0.546-0.66mg, niacin 0.946-1.17mg, vitamin B6 0.171mg, pantothenic acid 0.86mg, vitamin C 1.1mg, biotin 22.7ug, vitamin K (phylloquinone) 4.1ug, and vitamin E 21.8-32.0 mg. (75, 83).

The tocopherol content and composition of pecans is very important because tocopherols are the first protective barrier against oxidative events, that can lead to off-flavor of pecans and darkening. Changes were observed in the tocopherol content of pecan nuts due to storage for 16 months at 30°C. Also, changes in tocopherol content can also happen due to cultivar, site of plantation, crop year and storage (73). The four isomers tocopherol content of pecans can vary respectively: α-tocopherol 1.1-1.3mg, γ-tocopherol 19.6-29.0mg, β-tocopherol 0.1-1.3mg, and δtocopherol 0.1-0.3mg. Pecans do not have any tocotrienols in them. Furthermore, pecan nuts were reported to contain 108mg of phytosterols, and to contain respectively amounts: Δ5-Avenasterol 12.6-14.6mg, Campesterol 5.9-6.0mg, β-Sitosterol-116.5-130mg, and Stigmasterol 2.4-2.6mg per 100g pecans (73). Moreover, pecans also contain xanthophylls in small amounts such as lutein: 3.05mg/kg and zeaxanthin 3.13mg/kg pecan oil (75).

Pecans also have secondary metabolites called phytochemicals that provide biological antioxidant activity usually due to the phenolic constituents such as phenolic acids and tannins. These hydrophilic compounds mainly include phenolic compounds with oligomers and polymers of proanthocyanidins (75). Up to 40 phenolic compounds have been identified in pecans yet, and pecans have a significantly high antioxidant capacity. ‘Phenolic compounds’ is an umbrella term for many smaller categories of phenol-containing compounds and are present in different

amounts in tree nuts. Phenolic acids content in pecans and it's ranges are listed below (ug/g): phydroxybenzoic acid (28-90ug/g), protocatechuic acid (13.3-30.5ug/g), gallic acid (182274ug/g), ellagic acid (EA) (54-2505 ug/g), hydroxy cinnamic aids and derivatives (0.7-11ug/g), flavonoids (10.6-231.55ug/g), flavanols (1.67-10.01 mg/g), anthocyanins (0.14mg/g). EA was identified as the main component of the fraction of free polyphenols in pecans for both high and low cultivars and total phenolic content (TPC) can be quantified and EA equivalents (74). In addition, the presence of hydrolysable tannins such as ellagitannins (ETs) and 3-O-gallate derivatives in the pecan kernel influences the amount of ellagic and gallic acid equivalents, due to hydrolyzation, resulting in free ellagic and gallic acid (74,75).

b. *Pecan Dietary Interventions*

Nutrition intervention studies with tree nuts or a combination of them such as have been going on for at least 20 years, and multiple health benefits have been attributed to them (9097). The current *Dietary Guidelines for Americans 2020-2025* recommends 4-6 ounces (oz) of nuts and seeds a week for a healthy dietary pattern due to its association with cardio-metabolic health benefits (87). The chronic consumption of pecans has been and still is investigated and provides significant evidence of its beneficial effects on risk of chronic diseases. However, it is of extreme importance to ensure subjects are compliant with the dietary recommendations during the study period so reliable evidence is yielded. For that reason, the importance of biomarkers for food has been implicated in dietary interventions (91).

Currently, twenty nutrition interventions with pecans have been conducted and published (98-117), and none reported a descent biomarker to track the actual intake of pecans in freeliving human beings in the study. Of the 20 interventions, only three (101, 102, 109) of them were based on acute consumption of pecans, meaning that intake of treatments happened on set, and therefore dietary compliance is easily tracked. However, the other 17 nutritional trials that

required free-human livings to consume pecans for weeks on their own, do require at least the minimum compliance reinforcement to guarantee integrity and reliability of the study. Despite the previous discussion on the low efficacy of 24HR recalls of diet, food frequency questionnaire (FFQs), and food diary/records (under '*Nutrition Biological Marker*') those methods were used whether combined or singularly, in 12 of 17 studies (100, 103-108, 94, 97-102, 110-118). In those trials, participants were expected to report their accurate food intake during the study period, as free-living humans, through FFQs, food log, 24HR diet recalls, so research personnel can evaluate dietary intake outside the lab. This way, it is possible to investigate if possibly other dietary factors could be interfering with the results, or if the subjects are lacking compliance with the diet consumption when outside the lab.

On the other hand, if the self-report is the unique way of checking subjects' acquiescence of diet protocol, research personnel stays limited to granting accuracy to their report, without any resources to confirm the information. On this note, some authors tried electing a possible pecancontaining compound that could be used as a biomarker of compliance for chronic pecan interventions (105, 106, 111, 113). Two different authors, Rajaram et al. (2001) and McKay et al. (2018) suggested tracking common fatty acids of pecans in human plasma postprandially. Whilst Rajaram et al. (2001) was a cross-over 4-week chronic feeding of pecan-enriched diet followed off to a control diet (no pecans). To ensure participants followed the dietary protocol, SAFs such as caprylic acid (C8:0) - to stearic (C16:0) and palmitic acid (C18:0), MUFAs such as oleic acid (18:1(n-9)), and PUFAs such as linoleic and linolenic acid (18:2(n-6)), 18: 3(n-3). They also measured ratios of SFA:MUFA, SFA:PUFA and 18:2(n-6): 18: 3(n-3), which makes sense knowing the pecan fatty acid composition. They concluded that the fatty acid composition of plasma before and after intervention was in accordance with the pecan composition (as discussed in *Pecan Biochemical Composition*) and blood draws occurred with participants at fast at the end

of each assigned diet (107). More recently McKay et al. (2018) also attempted to use fatty acids to investigate and confirm the pecan intake throughout the intervention. Differently from the previous study, McKay et al. (2018) elected as a possible biomarker the red blood cells' fatty acid (RBC fatty acids), and participants were also expected to report to the laboratory fasted prior to blood draws. Mckay et al. (2018) reported significant increases in levels of MUFAs and oleic acid after pecan-enriched diet, the predominant fatty acid in pecans, when compared to the control diet. A decrease in palmitic acid after the pecan-enriched diet was also reported, which is associated with the consumption of PUFAs and MUFAs (111). Nonetheless, many other foods are composed of MUFAs and PUFAs, which lowers the credibility of these biomarkers, because the source of the fatty acids could have been pecans or another lipidic food. Furthermore, individuals with impaired lipid metabolism, or with any condition that could affect fatty acids levels, or RBCs, would not fit these biomarkers category, limiting the eligibility of the study. Especially pecans trials, that have been associated with lowering CVD risk and improving cholesterol profiles, including subjects in risk for these conditions in clinical trials is essential.

Another pecan compound has been used previously as an attempt to investigate compliance with the intervention protocol: γ-tocopherol in chronic interventions with pecan by Guarneiri et al. 2021. Coincidentally, Haddad, E et al. 2006 hypothesized a pecan-enriched diet would influence the levels of γ-tocopherol. Whilst Guarneiri et al. 2021 measured free γtocopherol at fasting before and after intervention, Haddad et al. 2006 also made fasting measurements of not only free γ and α-tocopherol and adjusted their values for cholesterol ratio. Guarneiri et al. 2021 reported 17-29% increase in fasting γ-tocopherol from pre-, to mid-, to post- intervention within groups assigned to eat pecans every day, and Haddad et al. 2006 also reported significant increase in cholesterol adjusted γ-tocopherol. However, γ-tocopherol levels

in plasma can be very transient within individuals, it's known for a half-life of 13+/-4 hours and leveling off at 24 hours postprandially (79). An acute trial by Hudthagosol et al. 2011 reported γtocopherol to peak after a pecan-enriched meal at 5 and 8 hours postprandially. Another study by Sundl et al. 2006 has shown γ-tocopherol to If γ-tocopherol were to be established as a biological marker of chronic pecan intake, it would be too difficult to guess a timeframe to have participants come in fasted for blood draws since their patterns in plasma are not uniform. Moreover, Leonard et al. 2004 has shown that γ-tocopherol demonstrates faster plasma disappearance and greater γ-metabolite production than α-tocopherol. This is due to the preference of liver for α-tocopherol, leading the other isoforms to excretion route, leading the two isoforms to overlap each other: lower intake of α -tocopherol.

Ellagitannins

Ellagitannins (ETs) are dietary polyphenols, part of the "hydrolysable tannins", containing an EA subunit (120), hexahydroxydiphenoyl (HHDP) moieties esterified to a polyol (usually α-D-glucopyranose or β-D-glucose) and a galloyl residue. ETs and EA are examples of non-extractable polyphenols, which are phenolic compounds that are not extracted with the solvents of choice, and therefore are not assessed in polyphenols analyses (126). EA, a dimeric derivative of gallic acid, is present in the plant vacuole, either in its free forms as EA or EA derivatives, or else bound as water-soluble ETs (131). Regardless of the structure of ETs, they all share the same core, the HHDP, even if the amount of monomers residues varies. Possible variations in position, frequency, stereochemistry of the HHDP units, galloylation extent and/or anomeric stereochemistry if sugar moieties can cause ETs to vary, which makes it very difficult to elucidate their native structures (126). According to the number of HHDP groups linked to sugar moiety, ETs can be classified into monomeric, oligomeric, and polymeric ETs (125). They

are phenolic compounds and possess one of the largest molecules, and relatively high polarity, which decreases its bioavailability (121). To date, more than 1000 natural ETs have been identified in nature but most of them are not preponderant in foods (120, 121, 124). ETs present an important structural diversity depending on where their matrix origins but the main ones available in diets are: punicalagin ($C_{48}H_{28}O_{30}$) in pomegranates, pedunculagin ($C_{34}H_{24}O_{22}$) in pecans, sanguiin H6 ($C_{82}H_{54}O_{52}$), and lambertianin C ($C_{123}H_{80}O_{7}$) in berries, vescalagin ($C_{41}H_{26}O_{26}$), castalagin ($C_{41}H_{26}O_{26}$) in wood-aged wine, casuarictin or sanguiin 11 ($C_{41}H_{28}O_{26}$) and potentillin ($C_{41}H_{28}O_{26}$) in oak-barrel stored wine (124, 125). **Table 7.** shows food sources, their ETs and content.

Due to poor bioavailability ETs have never been reported to be circulating in human systemic circulation system or in urine, even after consumption of high amounts of dietary ETs (121, 126). ETs are large molecules, with high polarity and presence of C-C linkage (123, 125). ETs can be hydrolysable in acidic or basic conditions and appears to be less stable in alkali conditions (121,125). In the human organism, ETs can bind to some proteins in saliva and cause astringency, and in this case, they may not be metabolized further (123, 125). It might happen that the ETs are resistant to pH changes and reach the large intestine without any metabolic influence. However, most ETs are sensitive to acidic or basic hydrolysis in the stomach, leading to the release of EA structures, which are still poorly bioavailable. Furthermore, the degradation of ETs in the upper GI depends on their chemical structure, the food matrix, and their susceptibility to acid or base hydrolysis (125). The bioavailability of EA derivatives depends on the part of gastrointestinal tract (GI) in which these compounds are released. In stomach or small intestine (SI) only low levels of absorption could occur, and EA can be detected in plasma between 1 and 5 hours after ingestion of dietary ETs as methyl and dimethyl esters or glucuronic

acid conjugates in very low concentrations (125). EA has a weak nature of its four phenolic groups that around neutral pH leads it to be deprotonated, while above pH 9.6 its lactone rings open to give carboxyl derivative (127). EA low oral bioavailability is mostly due to its poor water solubility, which increases with the pH.. However, in basic solutions, phenolic compounds lack stability and undergo extensive transformations or are converted into quinones and a result of oxidation (127). Also, EA can bind to cellular DNA irreversibly, or form poorly soluble complexes with calcium and magnesium, which affects transcellular absorption (125). Despite of their low availability recent studies have shown that although they are not readily absorbed on the small intestine, ETs and EA will be transformed into hydroxydibenzopyran-6-one derivatives further on the gastrointestinal tract by gut microbiota (120-128, 134).

The hydrolysis and changes of ETs is not only due to GI tract and happen within the food matrix, leading to subsequent changes in the bioavailability and bioactivity of these compounds. The spontaneous hydrolysis of ETs into EA within food matrix causes decrease in amounts of ETs but increase in total phenolic content (TPC) due to formation of EA and derivatives. Some studies have investigated the stability of ETs and EA during long periods of storage and concluded that the processing of the food product and size/structure of ETs will influence the extent of degradation during storage. Canning, pureeing and freezing of blackberries have shown to little effect on ETs content, but processing berries into clarified and nonclarified juices resulted in total ETs loss ranging from 70-82% (10). Also, temperature and humidity also influence degradation; frozen blackberries kept the same amounts of ETs throughout 6 months (129), and loss of 7% of lambertianin C in red raspberry jam kept at 20°C in the dark for 6 months (130).

It depends on food source and its preparation to understand whether ETs, EA or both will be in the food matrix. Regardless, both compounds have the same fate (120). ETs release EA due

to the physiological condition of GI and can also happen spontaneously on the intestine lumen without an enzyme (128). ETs are hydrolyzed with bases or acids, by microbial enzyme tanninhydrolase in stomach, and the HHDP group, through lactonization spontaneously or through the enzyme lactonase, releases the galloyl-glucose residue from its structure and yields EA. The galloyl-glucose is eventually transformed by gut microbiota to gallic acid, resorcinol and pyrogallol. The latter were found to increase significantly during in vitro human fecal incubation and raspberry extract (120, 121). Free EA is released in the upper GI, and it can be directly absorbed. EA appears quickly in the systemic recirculation system and has a relatively short residence time, indicating absorption could have happened in either stomach or/and duodenum. EA and other direct derivatives of ETs, such as methyl and dimethyl ethers or glucuronic acid conjugates have been found in human plasma and urine 1 to 5 hours after ingestion of dietary ETs (128). Furthermore, a study reported that healthy individuals consumed a standardized extract of pomegranate, and plasma peak concentration of EA ranged from 0.06 to 0.1 $\mu mol\ l^{-1}$ one hour postprandial (146). On the other hand, another study reported in the literature showed maximum peak concentration of 0.66 $\mu mol\ l^{-1}$ after orally ingesting pure EA (153). By the time dietary ETs reach the duodenum, it is almost completely transformed into EA, which has a low rate of absorption. EA will be further metabolized into polyhydroxylated dibenzopyranones, or urolithins (Uro). EA is insoluble in aqueous solution which can aid to explain its low bioavailability, also its ability to bind to intestinal epithelial cells aggravates insolubility (138).

The gut microbiome can transform EA into Uro by lactone-ring cleavage, decarboxylation, and dehydroxylation recations, and the main Uro production occurs in in the distal colon region (123). In some cases, EA can suffer bacterial dehydroxylation without the action of lactonase-decarboxylase bacterial enzymes, without the opening of lactone ring,

forming a compound known as nusitin (134). Nonetheless, the metabolism and production of Uro is given the encounter of the gut microbiome with ETs and EA (134). In the lumen, lactone ring cleaving enzymes will open the lactone ring of EA molecule, yielding a polyhydroxylated dibenzopyranone, the 3,4,8,9,10-pentahydroxy-6H-dibenzo[*b,d*]pyran-6-one or Urolithin M5 (Uro-M5). Dehydroxylases will continue the process and generate 3,8,9,10-tetrahydroxyurolithin or urolithin M6 (Uro-M6), 3,4,8,9-tetrahydroxy urolithin or urolithin D (Uro-D) and 3,4,7,9 – tetrahydroxy urolithin or urolithin E (Uro-E). Sequentially, other dehydroxylases will remove – OH ligand at carbon 4, generating 3,8,9-trihydroxy-urolithin, or urolithin C (Uro-C), and 3,7,9-tihydroxy-urolithin, or urolithin M7 (Uro-M7). Finally, new dehydroxylases remove the third – OH ligand from Uro M7 and C, generating 3,8-dihydroxy-urolithin or urolithin A (Uro-A) and 3,7-dihidroxy-isourolithin A (Iso-Uro-A). Iso-Uro-A can be transformed into 3monohidroxy-urolithin B (Uro-B), whilst Uro-A and Uro-B are interchangeable (123). The removal of hydroxyl from urolithins and their variability is dependent on the time of exposure to gut microbiota, the composition of the microbiota, and the type of dehydroxylase . The dehydroxylation process is progressive as the structure goes from penta-to-mono-hydroxydibenzopyranone, and it increases its lipophilicity and adsorption ability (123).

Urolithins

In vitro experiments with variety of fecal suspension showed that up to 80% of Uro were produced from EA, while other suspensions did not present formation of any Uro, which shows the role that the intestinal microbiota plays in the fate of dietary ETs (121). Since the gut microbiota composition varies immensely within individuals, the inter variability of microbiomes within individuals most likely will yield different metabolites upon degradation of ETs and EA (133), therefore individuals can also be categorized according to their urilithins metabotype

(UM). UM is a broad term that can involve the gut microbial and host metabolism and subsequent biological activity of either endogenous or exogenous compounds. In the case of polyphenols, a metabotype associated with the metabolism of polyphenols by the gut microbiota refers to a metabolic phenotype characterized by specific metabolites of ETs and EA (133). It is possible to qualitatively categorize individuals as producers vs. non-producers of a metabotype 123). Subjects ruled as UM-0 cannot produce Uro but Uro-M5 has been detected (123). The UM are characterized by the subjects' epigenetics, physiology, gut microbiota composition and functionality (120, 121,123, 124, 135). Yang & Tomas-Barberan, 2019 tested 10 different teas in 10 participants of known UM to be able to associate each UM to type of ETs present in the respective teas. Metabotype 0 (UM-0) was used to categorize the 'nonproducers' of Uro, metabotype A (UM-A) consists of producers of Uro-A and its derivatives, and metabotypes B (UM-B), producers of Uro-A, Iso-Uro-A and Uro-B. They reported the Uro content postprandially in urine was in accordance with their pre-categorization of participants (134). The distribution of M within adults varies probably due to geographic changes, but in a Western adult population UM-A is the most abundant metabotype at 55%, followed by UM-B at 34% and UM0 at 11% (136). Although the three UM standards worked consistently across other studies, a large cohort among Caucasian elderly adults showed that age could determine the individual's capacity to metabolize EA into Uro-A, Uro-B (135, 136).

The formation of these catabolic metabotypes is subjective to every individual since it depends on one's colonic microflora composition, BMI, medication intake as well as the assay conditions, such as amount of ET-food eaten, concentration and type of ETs, and composition of food matrix (121). A study conducted with six participants in which they consumed 1 liter of pomegranate juice daily for 5 days showed two subjects did not produce any UM, while others

reached peak concentration of plasma Uro metabolite between the third and fourth day of study (120). In another study conducted within ten subjects, urine samples were collected from 7 to 48 hours post ET-containing meal. It was reported that 5 out of the 10 participants excreted relatively high Uro concentration, 4 excreted very low amounts, and 1 subject did not excrete any Uro (148). These results enabled an outdated concept within individuals: they could be rated as 'high-urolithin' producers and 'low-urolithin' producers, as well as non-urolithin' producers. This is not the most appropriate way to categorize individuals because there has not been an establishment of the cut-off between high and low Uro producers, therefore this criterion is questionable and arbitrary (123). Moreover, only two bacteria strains that catabolize EA into Uro have been previously elucidated isolated from human microbiota: *Gordonibacter urolithinfaciens* and Gordonibacter pamelaeae showed ability in vitro to transform EA into UroC (123, 125, 135, 136).

The Uro are highly reactive and for this reason, tend to undergo phase II metabolism, enhancing their solubility in plasma and consequently excretion through urine (Villalgordo et al., 2022). Each Uro metabotype has its own glucuronide or sulfate conjugate, and those are usually found in urine and feces but also in plasma. Concentration of Uro in human tissues has been studied and in plasma was reported in a range of 0.003−5.2 μM and in urine up to 50 μM (123). Uro can be also found in colonic tissue and can reach systemic organs such as prostate, and maternal breast milk (123). In-vivo studies with rats detected that the degree of polymerization of the ETs affected metabolite formation (124,132). Rats fed with monomers and dimers ETs produced higher cecal metabolite concentration, mainly nasutin and Uro-A, whereas rats fed with dimers and trimers ETs successfully fermented them into short chain fatty acids, such as butyrate

(132). Furthermore, rats fed with EA had Uro-A as the predominant metabolite, and nasutin was the main metabolite produced by rats supplemented with ETs (122).

In the literature, the intake and metabolism of ETs and EA in vivo have been investigated for at least 15 years (137-139, 140). The patterns of Uro in humans' plasma, urine and feces have been investigated after different sources of ETs-containing foods such as strawberry (47), Brazilian fruits such as jaboticaba (153) and grumixama (152) blackberry (145), raspberry (148, 150) pomegranate (137, 146, 147) walnuts (143,144), and pecans (142). A study conducted in which two subjects ingested walnuts and provided feces samples to be incubated clarified the time course of Uro production by the gut microbiota (154) to investigate the time course production of Uro reported that Uro-M5 peaked at 5-12 hours, followed of Uro-C at 14 hours, Uro-M7 at 16 hours. Uro-A was detected after 14 hours of incubation and reached peak concentration at 42 hours, hitting a plateau. Iso-Uro-A appeared at 24 hours of incubation, and Uro-B increased up to 60 hours of incubation (154).

Although Uro are a product of the gut microbiome; to investigate thoroughly these metabolites, it is essential to study their appearance in other biological samples such as urine and blood (92, 96). Acute intake of ETs and EA studies have shown the appearance of Uro in urine starting at 8-12 hours and Uro-A reaching peak at 24-36 hours and Uro-A-G at 48 hours postprandially (153), urinary Uro peaking at 24 and 48 hours (143), Uro-A and B are only found in traceable amounts 4 days post-ETs/EA ingestion (145), Uro-A mainly peaking at 24 and 48 hours (147), Uro peaked in urine at 24-32 hours, and 32-48 hours (148). A chronic intervention of 30g daily intake of tree nuts as hazelnuts, walnuts and almonds for 12 weeks led to significant increase in Uro concentrations in urine (144). The kinetics of Uro in plasma upon single and chronic doses of ETs-containing foods have been investigated and shown to match the

appearance rates of Uro in urine (137, 142, 143, 146, 155, 156, 157). Uro is reported to rise over 32 hours postprandially after acute ETs and EA ingestion, declining over the next 12 hours (143). Seeram, P et al. 2006 reported a shallow concentration of Uro in plasma, but detectable, at 6 up to 48 hours after a single dose of pomegranate juice (146). Another acute intervention with pomegranate juice concluded the appearance of Uro in plasma only after 24 hours postprandially (155). Furthermore, chronic intervention trials conducted with nuts showed an increase in circulating Uro that persists of longer than 48 hours postprandially (143). A chronic intervention of 4-week daily intake of 68g of pecans concluded a significant increase after 4-week of daily pecan intake in fasting Uro concentrations in plasma (142). Furthermore, 8-week intervention of 300mg extract of French oak wood extract reported to increase concentrations of Uro in serum, but not much in red blood cells (56). Other chronic studies conducted with ETs or EA-containing foods have shown consistent increase in fasting Uro concentration in plasma seen at 45 days of intervention (159).

Vitamin E

Sources, RDAs, structure, supplements

The term 'Vitamin E' stands for a range of fat-soluble compounds chemically similar, tocopherols and tocotrienols and its isoforms: α, β, γ, and δ, which are present in food in different amounts. Tocopherols and tocotrienols differ by the level of methylation of the ring, and by the chemical structure of the 16-carbon side chain, saturated on tocopherols and unsaturated on tocotrienols (159-161). Each hydrophobic isoprenoid side chain is linked to a chromanol head structure of two rings, one phenolic and one heterocyclic. Tocopherols have three chiral centers on its isoprenoid side chain which is responsible for the existence of eight stereoisomers (162) and the arrangements around these chirality centers for naturally occurring form of *RRR*-α-

tocopherol is the 2*R*, 4'*R*, 8'*R* stereoisomer. Tocotrienols though lack of chirality centers in its isoprenoid side chain due to three double bounds at positions 3', 7' and 11', so each isoform has only two stereoisomers. Although multiple isoforms can be present in food, they are not interconvertible in animals in human tissues and primarily α-tocopherol that holds vitamin E property in humans (162). There is a structure-activity relationship between the conformation of each vitamer and its biological activity within the human body (162). A biological assay in vitamin E deficient rats (159) for fetal resorption of a variety of vitamin E vitamers has shown *RRR* – configuration of α- isomer of tocopherol to have the highest relative activity rate amongst them all, and the stereoisomers *SRR-, RRS-, SRS-, RSS-, RSR-, SSS-* tocopherol to have lowered bioactivity. The reason behind it is that the main determinant for the biological activity of tocopherol is the chirality of C-2, the four isomers with the 2*R* configuration demonstrated more activity than the corresponding 2*S* isomers (159). The 2*S* isomers are not kept in plasma, and do not bind to the α-tocopherol transfer protein (159), which will be further discussed in this study.

The eight naturally occurring biosynthesized isoforms are distributed in different amounts in food and can be found non-esterified in the food matrix as free alcohols, or as esters. Plant foods such as seeds, vegetable oils and nuts are good sources of naturally occurring vitamin E and might offer up to 130% of the daily value established by the USDA for Vitamin E. As shown in **Table 8,** the natural food sources of vitamin E have different contents of its isoforms, and although γ-tocopherol is not the preferred isoform used by the body, it is the main isoform present in the American diet, and the second most abundant in blood and other tissues (Jiang Q et al. 2022). γ -tocopherol is abundant in soybean, corn and canola oil, pecans, and walnuts and accounts for more than half of the regional intake (162). The Vitamin E composition of the diet varies with food intake, and although plant oils, seeds and their products are the best source, animal foods might contain some vitamin E, as seen on **Table 8.** some foods can be Vitamin E

enriched such as whole milk, breakfast cereals, peanut butter, eggs and potato chips, which will contribute to adequate intake. Tocotrienols are less widely distributed than tocopherols but are present in select foods such as barley and rice brans and in palm oil. Palm oil is reported to be composed of up to 70% tocotrienols, γ-tocopherol being the main isoform constituent (163). Interestingly, γ-tocopherol-rich nuts or oils often contain high levels of PUFAs, while αtocopherol-rich plant oils are more abundant in MUFAs.

Table 8. shows one ounce (oz) of sunflower seed kernels oil offers 7.4 mg of αtocopherol, which is almost 50% of the established Daily Value (DV) of 15 mg of vitamin E per day, according to the FDA. As mentioned, only α-tocopherol form is recognized to meet human requirements, so all the recommended intakes are based on the activity of this vitamer. Vitamin E is listed in milligrams (mg) on the new Nutrition Facts and Supplements Facts by the FDA, but prior to that food companies could still use International Units (IU) to quantify Vitamin E in their labels. One mg of (synthetic) DL-α-tocopherol acetate is equivalent to 1.49 IU, or 1mg of (RRR)-α -tocopherol. The FDA defined the recommended dietary allowances (RDAs) for vitamin E as: Children 7-12 months, 5 mg, 1-3 years, 6mg, 4-8 years, 7mg, 9-13 years 11mg, 14 + years and pregnant females 15mg, females in lactation, 19mg. Natural and synthetic preparations of Vitamin E are commercially available, and three main techniques are used for fortification of food. First, the byproduct of the soybean oil refining process, secondly chemical methylation of these preparations and third, the α-tocopherol is complete chemical synthesis (162). The chemically synthetic supplements of Vitamin E consist of mixture of all the possible eight stereoisomers, called all-racemic (all-rac)-α-tocopherol usually bounded to succinate or acetate group (esterified), due to their greater stability against oxidation (159, 162). For instance, α-tocopheryl acetate, in which the acetate group is replacing the hydroxyl group in C-6 of the phenolic ring (162). However, since the 6-hydroxyl group of the phenolic ring is the functional

unit for the antioxidant activity, the ester forms of α-tocopherol are biologically inactive until removal of acetate or succinate group (162). The synthetic and natural forms of Vitamin E differ in the chirality of the hydrophobic side chain, and approximately 50% of dietary vitamin E is absorbed with no major differences in absorption rates among all the other forms (162).

Metabolism

Vitamin E vitamers have a lipophilic character, which makes vitamin E a fat-soluble vitamin. The tocopherols and tocotrienols stay within the lipid portion of the food matrix, therefore will go through similar steps in the gastrointestinal tract as other dietary lipids. In the stomach, no digestive activity seems to happen upon vitamin E (164), yet gastric lipase starts the partial dissociation of triacylglycerols and esterified fat compounds (165). Once in the intestinal lumen, pancreatic enzymes, phospholipase A and carboxyl esterase are secreted and the digestion of dietary lipids continues (165) The vitamin E is totally released from the food matrix by then and emulsified into lipid droplets at both gastric and duodenal levels (164), forming a lipid phase of the meal. In the duodenum, bile is responsible for emulsifying the lipid droplets into micelles along with other lipid digestive products, which will allow the vitamin to be solubilized, diffused into the unstirred water layer and approach the brush border membrane of the enterocytes. This is an essential step for the absorption of vitamin E due to the transition from fat emulsion globules to water-soluble multi- and unilamellar vesicles and mixed micelles (165) If the vitamin E source is synthetic it will most likely be in an esterified form that cannot be absorbed by the mucosa prior to hydrolyzation, which will probably be carried by cholesteryl ester hydrolase, also known as bile-salt dependent lipase (164). When approaching the brush border membrane, the mixed micelles dissociate due to the pH gradient, and the released constituents will be taken up by the enterocytes through different mechanisms. Only in 2006 that the mechanism of uptake

of α- and γ-tocopherol absorption mediated at least partly by the scavenger receptor class B type I (SR-BI) (166). Also, it was shown that NPC1 like intracellular cholesterol transporter 1 (NPLC1) was involved in α-tocopherol and γ-tocotrienol absorption (164, 166, 167, 168), and the role of cluster determinant CD36 in absorption of α-tocopherol and γ-tocopherol (169). CD36 and SR-BI have recently been discovered to be intestinal lipid sensors, and that they play an essential role in chylomicron secretion. Across the enterocyte, the hydrophobic character of vitamin E enables three ways of crossing: into organelle membranes, cytosolic lipid droplets, or traffic bound to binding proteins (164). In the latter, vitamin E is transported from lumen to epithelium through SR-BI and NPLC1. In the luminal site the ATP-binding cassette (ABC) transporters ABCG5 and ABCG8, and at the apical site, ABC1 transporters are responsible for sterol efflux into the lumen, and later for taking vitamin E to the lymph system (165) High-density lipoprotein (HDL) also appeared to be involved in the absorption of vitamin E (171). It is important to note that all the forms of vitamin E are absorbed in the intestine and secreted into the circulation in chylomicrons (170). Vitamin E and the other fat-soluble compounds are then packed into chylomicrons with triacylglycerols, cholesterol and phospholipids. The chylomicron then travels through the lymphatic system and reaches peripheral tissues including bone marrow, adipose, muscle, skin and possibly brain (171). On these tissues, vitamin E is picked up by a lipoprotein-mediated process and although, not much is known about this process, it is believed that the chylomicron-associated uptake of this vitamin supports the retention of all the isoforms of tocopherol, especially non α-tocopherol forms. Subsequently, the chylomicron remnants are taken up by the liver where vitamin E will be metabolized. In the liver, the mobilization process is dependent on a cytoplasmic protein, αtocopherol-transfer protein (α-TTP), that binds preferably to α-tocopherol due to the number of

methyl groups and is expressed by the liver. The α-TTP is a member of the CARL-TRIO family, which is a lipid-binding protein in control of intracellular trafficking of hydrophobic molecules (171). The stereochemistry of the isoprenoid tail at the point where it meets the chromanol ring (position 2) determine the affinity of the α-TTP for the vitamin E molecule (113). Consequently α-TTP has an affinity percent of 100% to α-tocopherol and much lower affinity toward other vitamers, such as: 50, 10-30 or 1% affinity to α-tocopherol, β-tocopherol, γ-tocopherol, and δtocopherol (Jiang, Q., 2014). α-TTP together with ATP-binding cassette transporter A1 (ABCA1) incorporates α-tocopherol in lipoproteins which transports vitamin E through the circulation (113, 161, 170, 171). The main function of α -TTP is to maintain normal levels of αtocopherol in the plasma and extrahepatic tissues (165), and for that -tocopherol will be given back to blood. α-tocopherol is added into nascent very low-density lipoproteins (VLDL) particles, but the underlying mechanisms for that are not known yet (165) Then, the conversion of VLDL to LDL enriches the latter with α -tocopherol. High density lipoproteins (HDLs) also transport α -tocopherol through the bloodstream and distributed to other tissues. In the hepatocytes α-tocopherol is 'protected' from excretion pathway due to binding to α-TTP, but the other isomers, that lack affinity with α -TTP, are going to be catabolized by the cytochrome P450 (CYP42F) enzyme. Upon arrival in the hepatocyte the non- α -tocopherols and tocotrienols enter the hepatocytes through lysosome compartment and are less likely be bound to α -TTP due to low affinity, but α -TTP is still able to protect the side chains of the isoforms to be degraded through ω-hydroxylase-induced degradation (170). α-tocopherol is delivered to the organ tissues through VLDLs, then unesterified cholesterol and α-tocopherol translocate spontaneously from cellular membranes to lipoproteins.

In the hepatocytes, the non- α -tocopherol and tocotrienols isoforms will not bind to α TTP, yet they will be metabolized through an extensive postabsorptive process to become water soluble to be primarily excreted in the urine, then feces and bile. Instead of binding to α -TTP and going into circulation, the non-α-tocopherols are the remnant chylomicron that enters the hepatocytes through LDL-receptor-related proteins (LDLR), the low-density lipoproteins (LRP) and SR-B1. During lipoprotein catabolism in the circulation, α-tocopherol is redistributed among the various lipoproteins (170). The catabolic process starts with phase I enzymes of the liver causing a ω-hydroxylation of the phytyl side chain mediated by the P450 enzymes. In human beings the endoplasmic reticulum (ER) enzyme cytochrome P450-4F2 (CYP4F2 and CYP3A4) depends on NADPH and adds the first –OH (hydroxyl group) to a terminal methyl group from the phytyl chain. In humans, mainly CYP3A4 is responsible for oxidation and hydroxylation of tocopherols. In the ER, the hydroxylated intermediate is dehydrated and hydroxylated, to become hydroxy chromanols (13'-OH) or 13'- γ-hydroxy chromanol, 13'-δ-hydroxy chromanol, or 13'β-hydroxy chromanol, by alcohol dehydrogenase, followed by catalysis of aldehyde dehydrogenase, generating 13' carboxychromanols (13'-COOH)(170). Then at the peroxisomes, the 13'-COOHs go through a series of reactions: ω-oxidation, β -oxidation and saturation to become 11', 9', 7', 5'-carboxychromanol (-COOH), long-chain metabolites (LCM). In the mitochondria, three rounds of β-oxidation (five total) will lead to the formation of carboxyethyl hydroxy chromans, such as 2,5,7,8-tetramethyl-2-2'-carboxyehtil)-6-hydroxychroman (α-CEHC, LLU- α), which is the main metabolite of α-tocopherol metabolism. For γ-tocopherol, 2,7,8trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman (γ -CEHC) (170, 171, 172, 173). Then, these metabolites will be sulphated or gluconated during phase II metabolism of the liver enzymes increasing its solubility, to be excreted via biliary or renal route. The glucuronidation is

catalyzed by uridine 5'-diphospho-glucuronosyltransferase (UGT), and sulfidation by CEHC-Glu or 3'COOH-Glu, intermediate-chain metabolites (ICM), is the final product and enters the excretion paths through bile and urine through the multidrug-resistance protein 3 (MDR3). The excess αtocopherol goes through the same excretion pathway the other isoforms go through. It might go through a xenophobic pathway, including CYP3A and MDR1, since excess α-tocopherol upregulates these pathways.

Tocotrienols are comparatively metabolized like tocopherols starting with a ωhydroxylation followed by five cycles of β-oxidation and resulting in the end-product CEHC. The following metabolites were elucidated for tocotrienols: Carboxytrienol (13'), carboxydimethyldecadienylhydroxychromanol [CDMD(en)$_2$HC; 11'], carbodimethyloctenylhydroxychromanol (CDMOenHC; 9'), as well as carboxydimethyloctadienylhydroxychromanols [CDMO(en)$_2$HC; 9'], carboxymethylhexenylhydroxychromanol (CMHenHC; 7'), and carboxymethylbutadienylhydroxychromanol (CMBenHC; 5'). The main difference of metabolism between tocotrienols and tocopherols is due to the sidechain being saturated before shortening (170, 171, 172).

Vitamin E Kinetics & Assessment

As previously explained vitamin E is an important micronutrient in the diet and essential for life. To understand the kinetics of vitamin E and its bioactivity in humans, assessment tools are necessary. Currently, in the literature vitamin E status is assessed in humans by tracking its metabolite α-CEHC in urine and/or α-tocopherol in plasma (170, 175, 176). This urinary metabolite is an indicator of optimum vitamin E based on assumption of saturated binding capacity of vitamin E in plasma (170). However, α -tocopherol concentrations are not necessarily a good indicator of

vitamin E status although it is the preferred isoform by the body. As noted by Traber, M., 2014, α-tocopherol is not a good indicator of vitamin E because it's serum levels did not reflect faithfully the dietary intake (176). A clinical trial in which adolescents and relatives were tested for α-tocopherol levels, illustrated that although estimates for adolescents were higher, plasma αtocopherol concentrations were higher in adults and lower in adolescents (174). In fact, the serum or plasma levels of mineral and vitamins might not give reliable insight into nutrient status and consumption (175, 176). Furthermore, external factors can affect bioavailability and consequently serum levels of α-tocopherol (175, 176, 177). There is conflicting data on the age influence of vitamin E status: some studies report a decrease in vitamin E after the age of 80 years old (178), whereas other report increased concentrations of vitamin E in plasma of 60-year-olds given the age-dependent increase in serum cholesterol and lipoprotein medications (179, 180). Lifestyle factors such as alcohol consumption and smoking obesity and other nutritional factors also affect bioavailability of vitamin E (176). A study conducted with obese children measured levels of both plasma α- and γ-tocopherol, as well as its urinary metabolites, α-CEHC and γ-CEHC after 4 months of daily vitamin E intake (178). They concluded that although there was significant increase in fasting α-tocopherol, α -CEHC and γ-CEHC from baseline to endpoint in treatment group, γ-tocopherol plasma levels decreased (176).

The isoforms of vitamin E present in the food matrix or any dietary source are also capable of interfering with overall vitamin E content within individuals (181). γ-tocopherol is the most common form of vitamin E in the American diet (181), and its plasma levels are directly affected by intake of α-tocopherol because α-TTP is chemically prone to binding to α-tocopherol (170, 171). Studies have shown consistently that upon vitamin E intake γ-tocopherol will be metabolized by the hepatic enzyme cytochrome P450-4F2 into γ-CECH before α-tocopherol, consequently being

excreted faster (170, 181) The fractional disappearance rate (FDR) of γ-tocopherol is reportedly three times faster than α-tocopherol (181). The apparent half-life of plasma αtocopherol has been reported to be 48, up to 60 hours (170, 171), and 57 ± 19 hours (179), whereas the half-life of γ-tocopherol 13 ± 4 hours (170), and 15 hours (174) significantly shorter. The metabolites of α-CECH have been shown to persist in blood for 24 hours (172) Acute vitamin E dose studies have reported peak concentrations γ-tocopherols to happen in plasma anytime around 5-9 postprandially and peak urinary γ-CECH excretion after 12 hours (175, 176, 181). Urinary γCECH are reported to be significantly decreased at 24 hours tocopherol reportedly peaked starting at 9-12 hours postprandially after vitamin E supplementation (181).

Significant data shows that although vitamin E has been studied for many years there is relevant difference between the metabolism of γ- and α-tocopherols within metabolic and urinary metabolism. Nevertheless, only α-tocopherol has an established urinary marker indicating the saturation of vitamin E transporters (126, 176, 177, 180) but none of the other isoforms. However, to determine a marker of assessment for γ-tocopherol status it is necessary to fully investigate, and understand the kinetics of isoform, as well as its metabolites. The short-lived half-life of γtocopherol, added to its bioavailability and subjective fat-related metabolism of individuals, make it difficult to rule out γ-tocopherol as its own marker of assessment.

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TABLES AND FIGURES

Table 1. Eligibility criteria for the study. Overview of inclusion and exclusion criteria used in this study. The screening questionnaire can be found under **Appendix A.**

Inclusion criteria	**Exclusion criteria**
Age 18-30	17 ≥ Age ≥ 31
BMI 18.5-29.9 kg/m^2	18.5 > BMI > 29.9 kg/m^2
Men and Women	n/a
Health digestion and swallowing process	Digestion and swallowing impairments
Has a non-restricted diet (i.e. vegan, intermittent fasting, ketogenic)	Has a restricted diet (i.e. Abstain of any animal related products)
Does not consume tobacco/nicotine products	Consumes tobacco/nicotine products
Does not have any current gastro-intestinal condition/disorder (i.e.IBD, Celiac, Crohn's)	Does have a current gastro-intestinal condition/disorder (i.e. IBD, Celiac, Crohn's)
Does not have any food allergies or sensitives (i.e.	Does have food allergies (i.e. tree nuts allergies, lecithin, dairy)

dairy/lactose intolerance, nut allergies)	
Does not have any chronic or metabolic diseases (i.e. T2D, CVDs, hyper/hypothyroidism, endocrine disorders, kidney, lung, or liver disease)	Does have any chronic or metabolic diseases (i.e.T2D, CVDs, hyper/hypothyroidism, endocrine disorders, kidney, lung, or liver disease)
Has not gone through any gastro-intestinal surgeries (i.e. gastric bypass or banding)	Has gone through any gastro-intestinal surgeries (i.e. gastric bypass or banding)
Not taking any medications that could affect lipid digestion and metabolism (i.e. SSRIs* birth control allowed)	Taking any medications that could affect lipid digestion and/or metabolism (i.e. statins, carbamazepine)
Currently is not taking any antibiotics or in the past 60 days	Currently is or has been in the past 60 days on antibiotic cycle
Currently is not taking any dietary supplements (i.e.	Currently is taking dietary supplements (i.e.

fish oil, multivitamin) or in the past 14 days	Vitamin E, D, A, cranberry extract, pre- and probiotics)
Not currently pregnant nor breastfeeding	Currently pregnant or breastfeeding

Table 2. Pecan intervention shake breakdown of ingredients and its macronutrients.

Ingredient & Amount	**Kcals**	**Carbs (g)**	**Protein (g)**	**Fat (g)**	**SFA (g)**	**Unsaturated Fats (g)**
Pecans (68g)	469.9	9.6	6.2	48.9	4.4	44.6
1% Low-fat Milk (244.0g)	102.9	12.2	8.2	2.4	1.5	0.8
Nesquik (25g)	89.3	21.4	1.8	0.00	0.00	0.00
Lecithin Granules	80.00	0.00	0.00	8.00	2.1	5.9

(15g)						
Total	741	42.24	16.97	59.3	35.9	21.6

Table 3. Control (Heavy whipping cream) shake breakdown of ingredients and its macronutrients.

Ingredient & Amount	**Kcals**	**Carbs (g)**	**Protein (g)**	**Fat (g)**	**SFA (g)**	**Unsaturated Fats (g)**
Heavy Whipping Cream Liquid (138g)	469.2	3.8	3.9	49.8	32.8	15.2
1% Low-fat Milk (152.50g)	64.1	7.6	5.1	1.5	1.0	0.5

Fiber Supplement (5.7g)	21.4	4.3	5.7	0.0	0.0	0.0
Nesquik (31g)	110.7	26.6	2.2	0.0	0.0	0.0
Lecithin Granules (15g)	80.00	0.00	0.00	8.00	2.09	5.90
Total	745.3	42.2	16.9	59.3	35.9	21.8

Table 4. Ingredients of shake, brands, and other food will be provided in the study.

Ingredient	**Brand**
Pecans	American Pecan Council Co.
2 % low fat milk	Kroger ®
Nesquik	Nestle ®
Lecithin granules	The Vitamin Shoppe®

Fiber supplement	Target ®
Heavy whipping cream	Kroger®
Other food provided	
Mac and Cheese	Marie Callendar's ®
Animal Crackers	Stauffer's ®

Table 5. Nutritional table of each shake

Composition	**Intervention Liquid Shake (%)**	**Control Liquid Shake (%)**
Percentage of total energy (%)		
Protein	8.4	8.8
Carbohydrate	22.2	21.9
Fat	69.4	69.4
Percentage of energy from fatty acid		
SFA	13.5	60.5
PUFFA	33.8	11.4

MUFA	52.7	24.9
Trans	0.2	3.1

Table 6. Nutrient breakdown of evening meal/pre-visit l (pre v2 and v3) and additional snack provided by research.

	Marie Callender Mac'n'Cheese **1 serving size (369g)**	**Stauffer's Animal Crackers** **1 serving size (30g)**
Calories (Kcal)	470	120
Total Fat (g)	16g	2g
Saturated Fat (g)	8g	0g
Trans Fat (g)	0.5g	0g
Cholesterol (mg)	40mg	0mg
Total Carb. (g)	60g	25g
Dietary Fiber (g)	3g	Less than 1g
Protein (g)	20g	2g
Vitamin D (g)	0g	0g

Vitamin E (g)	0g	0g
Vitamin A (g)	0g	0g
Vitamin K (g)	0g	0g

Table 6. *Table provides the nutrient breakdown of both additional food resources this study provides.*

Table 7. ET-containing foods and each type of ETs

Food Source	**Ellagitannins**	**Content equ. EAE (mg/100g)**
Blackberries (*Rubus spp..*)	Saguiin H6, lambertianin D	150-270
Strawberries (*Fragaria ananassa*)	Casuaricitin, pedunculagin, sanguiin H6	71-83
Raspberries (*Rubus idaeus)*	Sanguiin H6, lambertianin C	312
Pomegranate (*Punica granatum*)	Punicalgin	58-177
Chestnuts (*Castanea sativa*)	Castalgin	149
Pecans (*Carya illinoinensis*)	Pedunculagin, Punicalgin	316
Walnut (*Juglans regi*a)	Pedunculagin, Casuaricitin	864

Table 8. Participants demographics on screening visit.

Age (y)	22.9 ± 0.9
Height	169.0 ± 0.1
Weight	68.5 ± 1.4
BMI kg/m^2	23.9 ± 0.7

Table 9. Fasting values for both control and pecan-enriched meal testing visits.

	Control	**Pecan**
Triglyceride (ng/mL)	78.9 ± 24.4	75.7 ± 30.1
Glucose (ng/mL)	67.8 ± 12.0	67.7 ± 17.0

Table 10. LC-MS/MS analysis of each shake for vitamin E and ellagic acid contents.

	Total EA (mg)	**Total Γ-TOH (**ng)	**Total A-TOH** (ng)
Control Shake	ND	6.3×10^4	2.2×10^5
Pecan Shake	32.9	1.6×10^6	7.3×10^4

Table 11. Content of α-tocopherol in milligrams (mg) per serving size of some plant foods. Food data retrieved from USDA'S FoodData Central and NIH Vitamin E fact sheet. Percent Daily Value calculated according to the FDA.

Food	**Measurement**	**Milligrams (mg)/ serving (α)**	**Milligrams (mg)/ serving (γ)**	**Percent DV**
Wheat germ oil	1 tbsp	20.3	6.1	130

Sunflower seed kernels, dry roasted	1 oz.	7.4	0	49
Almonds, dry roasted	1 oz.	6.8	0.2	45
Sunflower oil	1 tbsp	5.6	0.6	37
Canola oil	1 tbsp	2.4	3.8	16
Hazelnuts, dry roasted	1 oz.	4.3	0	28.7
Peanuts, dry roasted	1 oz.	2.4	1.8	14.7
Pecans, raw, unroasted	1 oz.	1.4	24.4	4.7
Walnuts, black, dried	1 oz.	2.1	28.8	13.8
Corn oil	1 tbsp	2.2	5.3	14.6
Soybean oil	1 tbsp	1.3	9.7	8.5
Safflower oil	1 tbsp	4.6	0.24	30.6
Tomato, canned	1 cup	4.9	*	32.6
Spinach, raw	1 cup	3.7	0	24.6

Kiwi, green, raw	1cup	2.6	*	17.3
Salmon, pink, canned, drained solids)	3 oz.	1.1	0	7.3
Turkey, all classes, roasted	1 cup	0.8	0.02	5.3

Table 12. Optimal MS/MS parameters for EA, alpha and gamma tocopherol.

Analytes	Retention time (min)	Polarity	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Fragmentor (V)	Cell accelerator voltage (V)
EA	3.05	Negative	301.0	283.6	33	176	4
Alpha tocopherol	11.80	Positive	431.7	165.1	25	135	4
Gamma tocopherol	10.66	Positive	416.7	151.0	25	135	4

Table 13. Calibration curves for EA, alpha and gamma tocopherol.

Analytes	Calibration range (ng/mL)	Linear regression	Correlation coefficient (r^2)
EA	203-15000	y = 0.0895x + 0.0296	0.999
Alpha tocopherol	0.93-120	y = 77.868x + 0.7452	0.999
Gamma tocopherol	1.87-60	y = 42.579x + 0.1486	0.999

Figure 1. General timeline for the acute meal challenges that happened on testing days v2 and v3. Each participant received a random breakfast shake on V2, and the opposite breakfast meal on v3. A washout period of 6-14 days between v2 and v3 was required. Oral and printed instructions for pre- washout period of 6-14 days between v2 and v3 were distributed. Oral and printed instructions for pre-visit intervention have been given on v1, and subjects were expected to report to HNL fasted for baseline measurements. No blood drawn on v1.

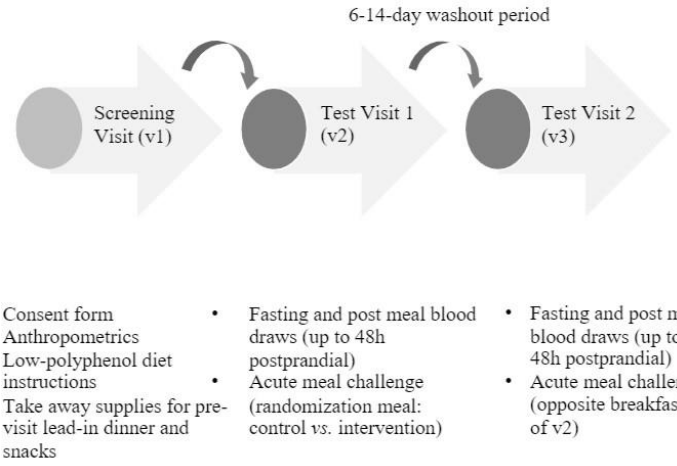


Figure 2. Recruitment flyer that was sent out through email to departments at the University of Georgia, as well as pinned on walls throughout campus.

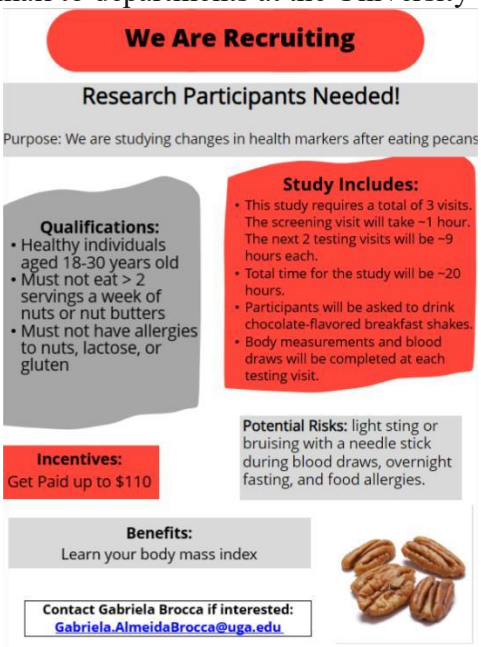


Figure 3. Metabolic pathway from ellagitannins to the formation of Uro

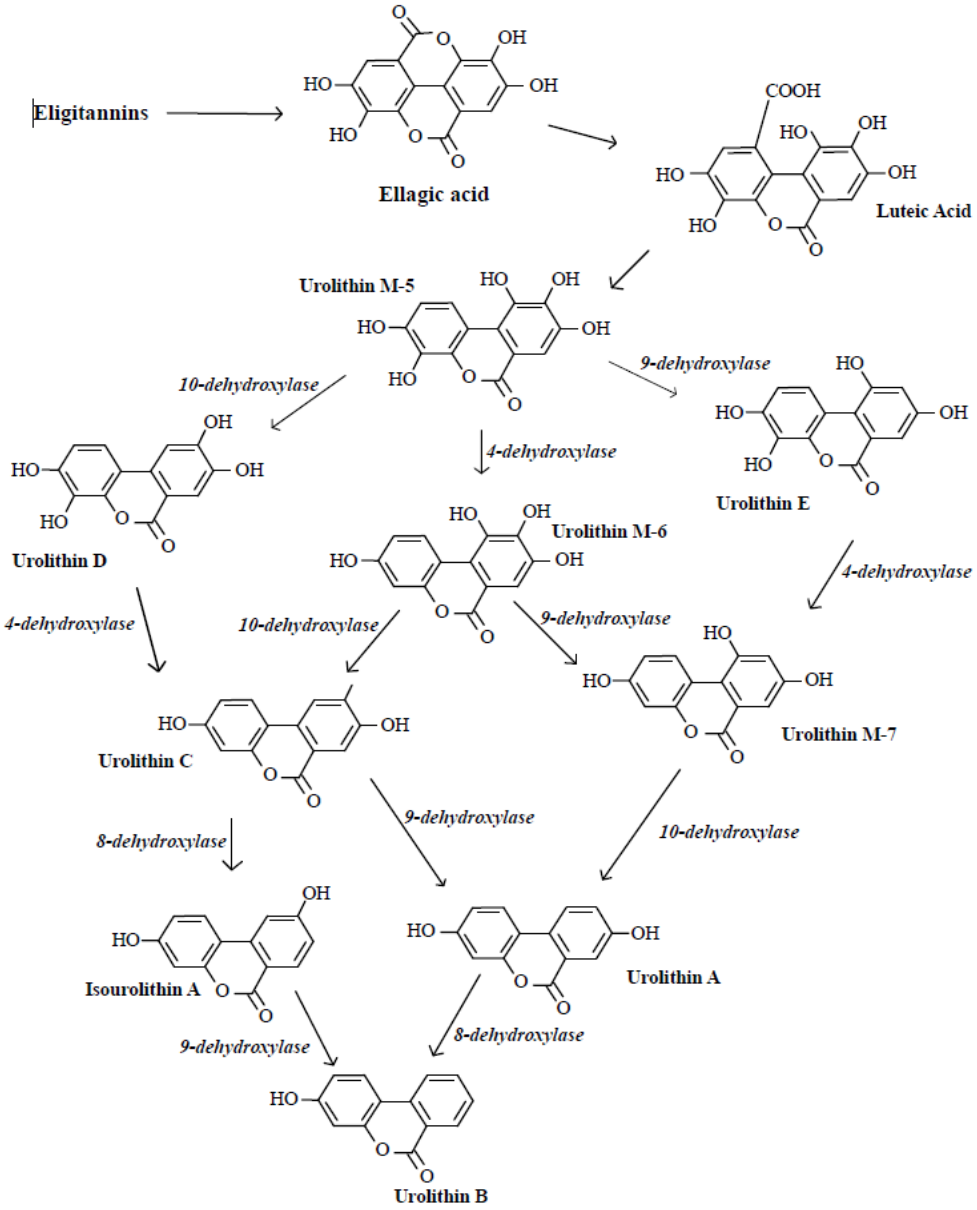

Eligitannins
Ellagic acid
Luteic Acid
Urolithin M-5
10-dehydroxylase
9-dehydroxylase
4-dehydroxylase
Urolithin D
Urolithin E
Urolithin M-6
4-dehydroxylase
4-dehydroxylase
10-dehydroxylase
9-dehydroxylase
Urolithin C
Urolithin M-7
8-dehydroxylase
9-dehydroxylase
10-dehydroxylase
Isourolithin A
Urolithin A
9-dehydroxylase
8-dehydroxylase
Urolithin B

Figure 4. Illustrates fasting and postprandial levels of glucose, triglycerides 0-48 hours on control and treatment days.

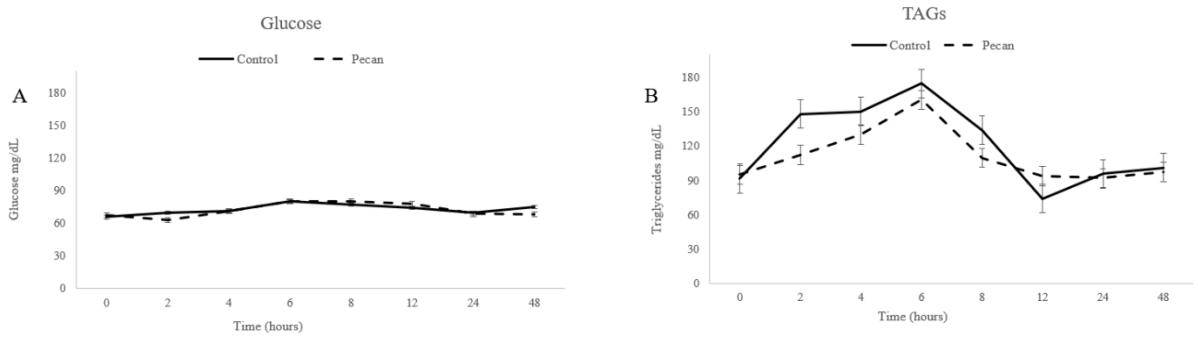
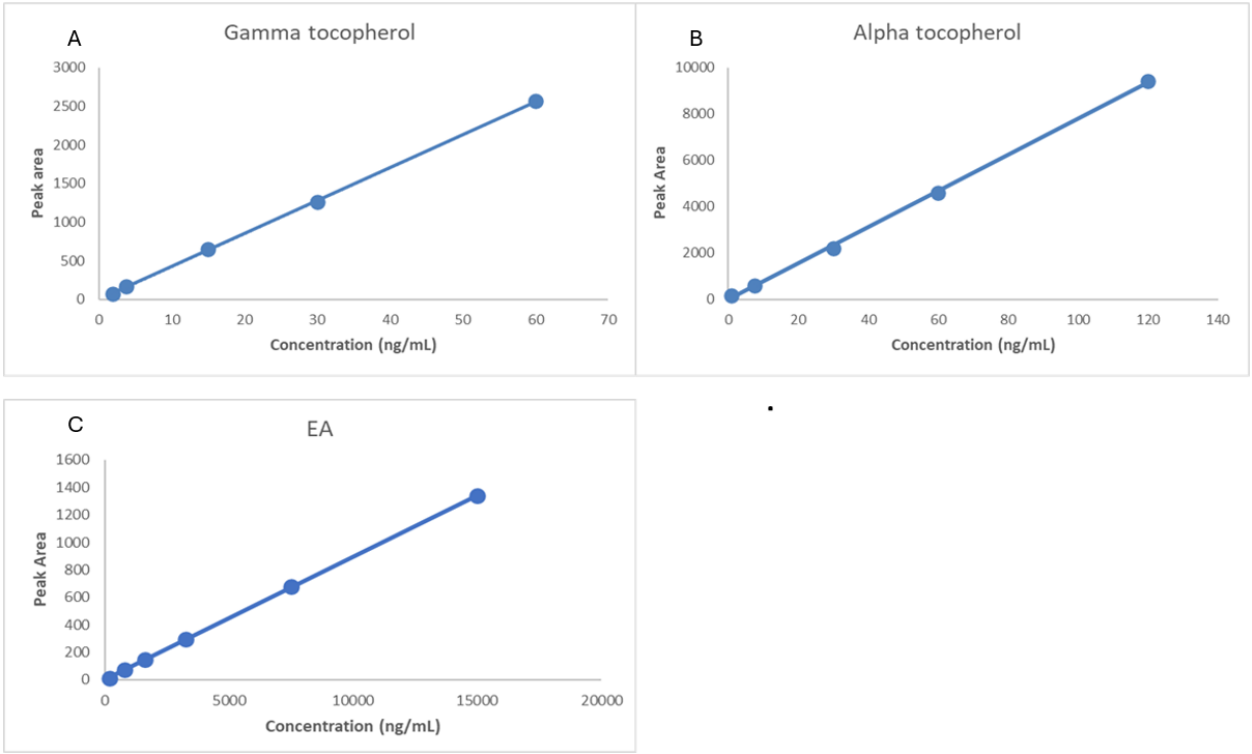


Figure 5. Calibration curves for α-tocopherol (A) and γ-tocopherol (B).



Appendixes

Appendix 1: Email of recruitment announcement

Paragraph for email announcement

Subject: Research Announcement

Are you interested in the health effects of food? If so, consider volunteering to be a research subject in a study looking at the effects of eating pecans on appearance of bioactive markers in blood.

The Departments of Food Science and Nutritional Sciences at the University of Georgia Seeks:

- Healthy men and women between the ages of 18-30
- Not taking medications (contraceptives are OK).
- do not eat nuts or nut butters more than 2 times per week
- are not allergic to nuts, gluten, or lactose

We are looking at the effects of eating pecans on appearance of bioactive markers in blood.

This study requires 3 visits for a total of about 20 hours. The first screening and consent visit will last approximately 45-min, and each of the 2 test visits will take about 8.5 hours on the first day, then approximately 30-min on day 2 and 3. This study involves the completion of questionnaires, body measurements, and blood draws. At each of the 2 testing visits, you will be provided with a chocolate-flavored breakfast shake.

What's in it for you?

- Monetary compensation: Study pays up to $110.
- Direct benefits: Learn your body mass index.

Potential Risks: light sting or bruising with a needle stick during blood draws, overnight fasting, and food allergies.

Can you stop participating after you start?

Yes, you may cease your participation at any time during the study.

If you are interested in participating and would like to see if you qualify for the study or request more information, please contact Gabriela Brocca *via email at* Gabriela.AlmeidaBrocca@uga.edu or text 706-363-5607.

Thank you for your interest; it is greatly appreciated!

Appendix 2: Telephone or email recruitment script

Phone/email scripts for participant eligibility:

Email script to inform participant that they qualify to continue participating

Hello [insert participant name],

I am excited to inform you that you qualify to continue to participate in our research study! To give you a little more information about the study, I have attached our informed consent document and food list (see attached). In short, this study is will take about 2-3 weeks to complete, with three visits to our lab. There is one screening and consent visit that will take about an hour to complete. Then, there are two separate 8-hour visits separated by approximately 7-10 days where you will come to the lab for a breakfast meal, several blood draws, a lunch meal with a few more blood draws. You would need to return to the lab the following two mornings for a single blood draw and after completing these steps for the second visit, your participation in the study will be complete. During the testing, you can do other tasks such as homework, reading, or remote work during these bigger visits if you stay at our lab. The foods we provide you will either have pecans or a control oil mixture incorporated, but you will not know which meal you are receiving.

The next step is for us to schedule a phone call so we can schedule your one-hour screening visit and so you can ask me any additional questions before your visit. **If you are interested in continuing, please let me know a good time and phone number to call.**

If you are not interested in continuing, please let me know.

If you are not eligible, your data will be immediately destroyed.

Kind regards,

Email script to inform participant that they DO NOT qualify to continue participating

Hello [insert participant name],

I am emailing to inform you that you do not qualify to continue to participate in our research study based on your responses to our preliminary screening questionnaire. If you have any questions about your responses, please let me know.

Kind regards,

Phone script to inform participant that they qualify to continue participating

Hello [insert participant name],

I am excited to inform you that you qualify to continue to participate in our research study! To give you a little more information about the study, I will email you our informed consent document and food list for you to review. In short, this study will take about 2-3 weeks to complete, with three visits to our lab. There is one screening and consent visit that will take about an hour to complete. Then, there are two separate 8-hour visits separated by approximately 7-10 days where you will come to the lab for a breakfast meal, several blood

draws, a lunch meal with a few more blood draws. You would need to return to the lab the following two mornings for a single blood draw and after completing these steps for the second visit, your participation in the study will be complete. During the testing, you can do other tasks such as homework, reading, or remote work during these bigger visits if you stay at our lab. The foods we provide you will either have pecans or a control oil mixture incorporated, but you will not know which meal you are receiving.

Is this a study you think you would be interested in participating in?

[if no] Thank you for your time, I hope you have a great rest of your day.

[if yes] {start here if following up from qualification email}

Great! Then the next step is for us to schedule your screening visit. This will be a one hour visit in the Human Nutrition Lab at the University of Georgia. It will include some questionnaires and body measures including height and weight.

If this sounds okay to you I need to schedule this screening and consent visit.

[if no] thank you for your time, I hope you have a great rest of your day.

[if yes]

Thank you, let's get your screening visit scheduled. [ask what days of the week might work best for a 1 hour visit in the morning fasted/ give some availability. Settle on a scheduled time.]

Alright, I have you schedule for [date] at [time]. I will email you a confirmation of this appointment including our address. We are at Dawson hall on Sanford drive and I am in room 161.

Thank you again for your time and interest. I will see you at your visit. Have a nice day!

[end call]

Phone script to inform participant that they DO NOT qualify to continue participating

Hello [insert participant name],

I am calling to let you know that you do not qualify to continue to participate in our research study based on your responses to our preliminary screening questionnaire.

[pause for questions]

[answer questions]

Thank you for your time and interest. I hope you have a nice rest of your day.

[end call]

Confirmation email for Screening visit

Hello [insert participant name],

I have you scheduled for your screening visit on [date] at [time]. We are located at Dawson Hall 305 Sanford drive, Athens GA in room 161.

Let me know if you have any questions.

Kind regards,

Appendix 3: Telephone or In Person Screening Form

ID: _____ **Date:** _____

1. What is your age? _____
2. What is your sex? _____
3. What is your ethnicity? _____
4. What is your height? _____

5. What is your current weight? _____
6. Calculated BMI: _____ (Research personnel will use given height and weight to calculate)

7. Do you consume alcohol? Yes

   No

   If so, how many drinks per day on average?

   _____

8. Do you use any tobacco or nicotine products? Yes

   No

9. Have you ever had any medical/surgical events that could affect digestion/swallowing? Yes

   No If so, what was the surgery and when did you have it?

   _____

10. Have you ever had any gastro-intestinal surgeries? Yes

    No

    (i.e. gastric bypass or banding)

    If so, what was the surgery and when did you have it?

    _____

11. Do you currently have any gastro-intestinal condition or disorders? Yes

    No

    (i.e. IBD, Ciliac's, Crohn's)

    If so, what condition do you have?

_____

12. Do you currently have any chronic or metabolic diseases? Yes

No

(i.e. Type I and II Diabetes, CVD, hyper/hypothyroidism,

endocrine disorders, kidney, lung, or liver disease)

If so, which ones?

_____

13. Are you currently taking any medications? Yes

No

If so, what medications?

{if yes to the above question}

Please specify:

_____

14. Are you currently taking any dietary supplements? Yes

No

(i.e., multivitamin or fish oil)

If so, which ones?

_____

15. Are you currently on a medically prescribed or special diet? Yes

No

(i.e., Atkins, Whole 30, Intermittent Fasting, Ketogenic,

Vegetarian, Vegan)
If so, please explain:

Appendix 4: Data collection screening visit form

Postprandial Urolithin Response to a Pecan-Enriched Meal

Data Collection Sheet – Screening Visit

If participant qualifies via phone screening and has given verbal consent, research personnel will schedule them for their screening visit. Participants will report to the Human Nutrition Lab in the Dept. of Nutritional Sciences at UGA at least 2h after their last meal. Informed written consent will be obtained prior to collecting the data below.

Participant ID: ---_____ Date: _____

Age: _____ Ethnicity: _____

Do you have a fever? _____

Have you recently had any viral infections? _____

Anthropometrics:

Height: _____ cm Calculated BMI: _____kg/m^2

Weight: _____ kg Use BMI formula: wt (kg) / ht (m^2)

Waist circumference: _____cm Hip circumference: _____cm

Waist circumference: _____cm Hip circumference: _____cm

Waist circumference: _____cm Hip circumference: _____cm

Averaged Waist circumference: _____cm Averaged Hip circumference: _____ cm

Instructions for prior to next visit

Visit 1 and Visit 2 scheduled: _____

Consulting on lead-in low polyphenol diet directions: _____

Lead-in diet instructions handout provided: _____

Lead-in meal and snack provided: _____

Consulting on fasting instructions: _____

Lead-in diet food diary provided: _____

Appendix 5: 24-48 Hour Pre-Visit Diet Protocol

24-48 Hour Pre-Visit Diet Protocol

General Guidelines:

For this study, it is important to follow the dietary instructions listed below and to document your diet as well. You will do this for the 24h prior to your testing visit. Please write down everything you eat and drink the day before your scheduled study visit (including medications) on the attached sheet labeled "Food Diary". It is important to include the time of your meals as well as accurate quantities. For instance, you can use measurements such as cups and ounces or object equivalents such as a deck of

cards or a baseball. **We ask that you replicate, to the best of your ability, your meals before each of your 2 scheduled visits.**

% Macronutrient Intake:

Please consume 50-55% carbohydrate, 15-20% protein, and 25-30% fat the day before each of your testing visits. Refer to the guidelines, and the sample meal plans below.

Sample Plate:

- 50-55% Carbohydrate
- 15-20% Protein
- 25-30% Fat

Evening Meal and Snack:

We will provide you with a frozen meal for you to consume as dinner, and a snack, the night before your testing visit. Please heat the meal as directed and eat this meal and snack and avoid any other foods or beverages (aside from water).

It is crucial that you are well-hydrated before beginning the study. Please drink plenty of water the night before and the morning of your visits. **Avoid caffeine consumption 16 hours prior to each of your visits.**

(Any time after _____). Avoid alcohol, exercise, and, if possible, avoid over-the-counter medications (not including birth control) for 24 hours before each visit. Finally, please follow a lowpolyphenol diet by avoiding the foods in the table below for 48 hours (2 days) before your scheduled visit. You will also be asked to avoid these foods from the start of visit 2 all the way through the 48hour blood draw (and then repeat it again for visit 3).

Foods to Avoid 48-hours Before Each of Your Testing Visits

Fruits- Avoid All	Vegetables- Avoid All	Whole Grains- Avoid All	Soy Products- Avoid All	Nuts & Seeds- Avoid All	Fish/ Seafood- Avoid All	Additional Foods to Avoid	Beverages to Avoid

Including:	*** The	Including:	Including:	For example:	For example:	All Spices,	Wine
Fresh fruits, Canned fruits, Dried fruits, and Fruit juices	ONLY form of vegetable allowed are white potatoes **without** the skin	Whole grain wheat, oat, rye & corn products, and whole grain flours	Tempeh, Tofu, Soy sauce, Soy milk/ yogurt, & Meats processed with soy	All nuts and nut butters (Peanut/ Almond butter etc.) Flaxseed Chia Seeds	Salmon, Oysters, Trout, Tuna, and Fish oils	Seasonings and Herbs (Salt & Pepper are o.k.) All Cooking Oils (Non-stick spray is o.k.) Cocoa Products (Dark or Milk Chocolate) Beans/Legumes	Tea Fruit juices Coffee or Caffeine containing drinks (just for 16h prior to visit)

						(Black/Kidney/Pinto beans, Lentils etc.)	

						Processed Meats (Deli Meats, Bacon, Pepperoni, Hot Dogs)	

Sample Meal Plans:

(Options/examples for breakfast and lunch on your own)

Breakfast:

- Egg whites and White Bread or Plain English Muffin Toasted
- Can add butter to the toast/muffin
- Original Cottage Cheese and White Bread, Toasted
- Can add butter to the toast
- Buttermilk Pancakes/Waffles:
- With corn syrup only (Aunt Jemima, Mrs. Butter-Worth's, Kroger Brand Original

Syrup)

- Can add butter
- Frozen Waffle's: Eggo Home-style or Buttermilk variety
- Not flavored (including blueberry, strawberry, chocolate, cinnamon)
- Danish Style Toaster Strudel (Pillsbury brand)

Lunch:

- Plain White Bagel with Original or low-fat Plain Cream Cheese
- Wrap (with Chicken, Beef, or Pork) with Sour Cream or Shredded Cheese:
- No added vegetables
- On a white flour tortilla
- **ONLY** use salt & pepper for seasoning
- Grilled Cheese Sandwich:
- On white bread

Snacks:

- Pretzels or Baked Potato Chips
- Rice Krispies (Original)

- Tortilla chips/Crackers & cheese
- Use unseasoned crackers (Club, Ritz, Original Premium, Carr's etc.)
- Salt is allowed
- White chocolate - plain

Beverages:

- Water
- Milk
- Pop/soda

Fast Food Options: (Not Required – just options for breakfast or lunch)

- Starbucks:
    - Breakfast or Lunch
        - Plain Bagel and Original Cream Cheese
- Chick-fil-A:
    - Breakfast
        - Egg White Grill

- Wendy's:
    - Lunch:
        - ✦ Cheese Baked Potato – do **NOT** eat the skin on the baked potato

          Do **NOT** order French fries

Dinner: (Required – Will be Provided by Research Personnel)

- Marie Callender's Creamy Vermont Mac & Cheese Bowl:
    - 1-2 servings
- Animal Crackers (original):
    - 2-4 servings

Appendix 6: Food record sheets

Participant ID: _____ Visit: _____ Date:

Time	Food/Drink	Quantity

Appendix 7: Recipes for Glucose assay buffers

0.1 M Na-P buffer

A. 0.2M NaH2PO4 (S9638-500g)

- Carefully weigh out 13.799g of NaH2PO4, and in a separate container weigh 500 ml of DI water. Mix until dissolved.

B. 0.2M Na2HPO4 (0404-500g)

- Carefully weigh out 14.196g of NaH2PO4, and in a separate container weigh 500 ml of DI water. Mix until dissolved.

0.2% Dimethylaniline (407275)

- Dissolve 200 ul of dimethylaniline in 2 ml of 1M of HCL. Then, dilute to 100ml with DI water to give 0.2% dimethylamine solution

20mM 4-Aminoantipyrine (A4382-50g)

-Maintain ratio of 4mg/ml DI water

-Weigh out 3-6mg of 4-aminoantipyrine into 1.6 ml microtube

- Calculate how much water is needed to keep the 4mg/ml ratio.

-Weigh out the DI water and vortex to mix

Glucose standard solution volumes and their known concentrations