BMJ Open

Actions of annatto-extracted tocotrienol supplementation on obese postmenopausal women: study protocol for a double-blinded placebo-controlled randomized trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-034338
Article Type:	Protocol
Date Submitted by the Author:	15-Sep-2019
Complete List of Authors:	Aryaie, Amir; Texas Tech University Health Sciences Center, Surgery Tinsley, Grant; Texas Tech University, Kinesiology and Sport Management Lee, Jaehoon ; Texas Tech University, Educational Psychology and Leadership Watkins, Bruce; University of California Davis, Nutrition Moore, Lane; Texas Tech University, Kinesiology and Sport Management Alhaj-Saleh, Adel ; Texas Tech University Health Sciences Center, Surgery Shankar, Kartik; University of Colorado Denver School of Medicine, Pediatrics Wood, Sarah; Texas Tech University Health Sciences Center, Clinical Research Institute Wang, Rui; Texas Tech University Health Sciences Center, Pathology Shen, Chwan-Li; Texas Tech University Health Sciences Center, ;
Keywords:	tocotrienols, lipid, inflammation, women, microbiome

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Actions of annatto-extracted tocotrienol supplementation on obese postmenopausal women: study protocol for a double-blinded placebo-controlled randomized trial

Amir Aryaie, M.D.[a], Grant Tinsley, Ph.D.[b,e], Jaehoon Lee, Ph.D.[b,f], Bruce A. Watkins, Ph.D.[h], Lane Moore, Ph.D.[e], Adel Alhaj-Saleh, Ph.D.[a], Kartik Shankar, Ph.D.[i], Sarah Wood, Ph.D.[c], Rui Wang, Ph.D.[d], Chwan-Li Shen, Ph.D.[b,d,g,*]

[a] Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[b] Center of Excellence for Integrative Health, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[c] Clinical Research Institute, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[d] Department of Pathology, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[e] Department of Kinesiology and Sport Management, Texas Tech University, Lubbock, TX, 79409

[f] Department of Educational Psychology and Leadership, Texas Tech University, Lubbock, TX, 79409

[g] Obesity Research Cluster, Texas Tech University, Lubbock, TX, 79409

[h] Department of Nutrition, University of California-Davis, Davis, CA, 95616

[i] Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, 80045

Email:

amir.aryaie@ttuhsc.edu; grant.tinsley@ttu.edu; jaehoon.lee@ttu.edu; bawatikins@ucdavis.edu; lane.moore@ttu.edu; adel.alhaj-saleh@ttuhsc.edu; Kartik.Shankar@Cuanschutz.edu; sarah.r.wood@ttuhsc.edu; rui.wang@ttuhsc.edu; leslie.shen@ttuhsc.edu

[*] Corresponding author.
Trial sponsor. Chwan-Li Shen. E-mail Leslie.Shen@ttuhsc.edu. Phone: 1 (806) 743-2815. Fax: 1 (806) 743-2117.

Trial registration: ClinicalTrials.gov identifier: NCT03705845. Title: Tocotrienols for obesity of postmenopausal women. The trial registration is prospective and data collection is not expected to be completed until the end of 2020.

ABSTRACT

Obesity is a major health concern in postmenopausal women, and chronic low-grade inflammation contributes to the development of obesity. Cellular studies and high-fat-diet induced obese mouse model mimicking obesity show the anti-obesity effect of tocotrienols (TT) with antioxidant capability. We aim to assess the safety and efficacy of TT consumption for lipid-related parameters in obese postmenopausal women. A 24-week randomized double-blinded placebo-controlled trial will be conducted to evaluate the effects of dietary TT supplementation in obese postmenopausal women. Eligible participants will be randomly assigned to placebo group (430 mg olive oil) and TT group (430 mg of 70% pure TT) for 24 weeks. The primary outcome is total fat mass. The secondary outcomes include serum lipid profile, adiponectin, leptin, c-reactive protein, urine 8-hydroxy-2'-deoxyguanosine, lipid metabolism-related gene expression in adipose tissue, concentrations of endocannaboinods and oxylipins in plasma and adipose tissue, and abundance and composition of intestinal microbiota. Outcomes are assessed by blinded evaluators at baseline and 24 weeks. Lipid profiles and liver function will be assessed at baseline, 12, and 24 weeks. "*Intent-to-treat*" principle is employed for data analysis. Hierarchical linear modeling is utilized to estimate the effects of dietary TT supplementation while properly accounting for dependency of data and identified covariates. To our knowledge, this is the first randomized, placebo-controlled, double-blinded study to determine dietary TT supplementation on obese population. If successful, this study will guide the future efficacy TT interventions and TT can be implemented as an alternative for obese population in anti-obesity management.

Keywords: tocotrienols; lipid; inflammation; women; microbiome

Strength and limitations of this study

- This is the first randomized, double-blind, placebo-controlled trial to investigate the effects of dietary annatto-extracted tocotrienol supplementation on obesity-related outcomes in obese postmenopausal women.
- This study will be performed at a single research center with experience in conducting independent, investigator-initiated, randomized controlled trials in nutrition research.
- Annatto-extracted tocotrienol supplement is not available over the counter worldwide.
- There is no long-term follow-up.

Introduction

Obesity is now recognized as a worldwide epidemic disease, which leads to the development of several comorbidities such as insulin resistance, dyslipidemia, and metabolic syndrome (WHO 2011 [1]). With advances in basic redox biology, emerging evidence indicates that chronic low-grade inflammation paves the way for the development of comorbidities in obesity [2]. Obesity-induced chronic low-grade inflammation, called metaflammation, is initiated by excess nutrients in metabolic cells [2]. The inflammatory signaling conducted by these metabolic cells eventually causes activation of specialized immune cells and leads to an unresolved inflammatory response within the adipose tissue [2]. Therapeutic interventions to inhibit inflammatory pathways in obesity have shown beneficial effects on insulin sensitivity in mouse models and human trials [2].

Oxylipins (OxLs) are oxygenated fatty acid metabolites derived from n-6 polyunsaturated fatty acids (PUFA) (i.e., linoleic acid, dihomo-γ-linolenic acid, and arachidonic acid (AA)) or n-3 PUFAs (i.e., α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) through the actions of cyclooxygenases (COX), lipoxygenase (LOX), and cytochrome P450 enzymes [3]. In postmenopausal women at risk of metabolic syndrome, the amounts of OxLs were modified by dietary n-3 PUFA such that the levels of pro-inflammatory were reduced and anti-inflammatory were enhanced [4]. Particularly, DHA feeding reduced plasma levels of various inflammatory cytokines and OxLs (5-lipoxygenase-dependent inflammatory mediators and vasoconstrictive 20-HETE) in a rodent model of obesity [5]. Emerging evidence suggests that AA-derived OxLs contribute to obesity-associated inflammation [6, 7]. For example, 5-LOX products of AA (e.g., 5-hydroxy-eicosatetraenoic acid (HETE) or 5-oxo-eicosatetraenoic acid) exert pro-inflammatory effects by increasing the production of pro-inflammatory cytokines and inducing chemotaxis to attract inflammatory cells in the blood vessels in adipose tissue from obese mice [8, 9]. 12-LOX products of AA (e.g., 12-HETE, 12-hydroperoxyeicosatetraenoic acid (12-HPETE)) have also been shown to play a direct role in obesity-induced-inflammation [10] and insulin resistance [11-13]. Obese subjects with low-grade inflammation had elevated levels of several AA-derived OxLs (e.g., 5-, 8-, 11-, 12-, 15-, and 20-HETE) compared to those with no

inflammation [7]. On the other hand, OxLs that originate from n-3 PUFA (e.g., EPA and DHA) have demonstrated their ability to suppress obesity-associated inflammation as seen in inhibiting macrophage recruitment and increasing adiponectin secretion in adipose explants isolated from obese mice [14]. Supplementation of n-3 PUFA for 8 weeks significantly decreased AA-derived OxLs and increased EPA- and DHA-derived OxLs in hypertriglyceridemic subjects along with mitigated inflammatory and vascular status [15]. Since chronic low-grade inflammation in obesity is a systemic problem, administration of dietary supplements with potent anti-inflammatory function can be a good strategy to combat inflammation in obesity.

Tocotrienols (TT) and tocopherol are two subclasses of vitamin E, which can be further classified into four isomers (α, β, δ, and γ). The unsaturated side chain of TT allows for more efficient penetration into tissues compared with the completely saturated side chain of tocopherol. TT is lipophilic in nature and it is found in association with lipoproteins, fat deposits, and cellular membranes [16, 17]. TT has been shown to protect the PUFAs from peroxidation [16, 17]. In animal studies, dietary TT (palm TT-rich fraction, δ-TT, and γ-TT) decreased body weight [18] and fat mass [19, 20], reduced plasma concentrations of free fatty acids, triglycerides and cholesterol [21-25], and improved glucose and insulin tolerance [18-20, 25, 26]. Many epidemiology studies showed that dietary TT intake is associated with decreased serum concentrations of total cholesterol and lipoproteins of study subjects [23, 27-31]. In humans, TT supplementation (34.6% α-TT+43.5% γ-TT) reduced microalbuminuria and C-reactive protein (CRP), indicators of acute inflammation, in type 2 diabetic patients [32]. Intake of grape seed oil with high levels of TT was shown to attenuate serum levels of CRP and TNF-α and improve insulin resistance in overweight/obese women [33]. The review of the supporting evidence strongly suggests that TT can offer benefits to control obesity due to its potent anti-inflammatory-dependent biological activities. In addition, although δ-TT and γ-TT are considered as the most potent isomers among 8 vitamin E isomers [34], their clinical relevance to combat obesity along with mechanisms has never been investigated in humans. Furthermore, our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen et al. unpublished findings). Yet, it is not known whether supplementation with TT would benefit total fat mass reduction,

improve lipid metabolism, and modify OxL in plasma and adipose tissue of humans. Additionally, there is not a single study yet to evaluate how TT supplementation affects gut microbiome in humans, particularly obese postmenopausal women (PMW). Therefore, the objective of this pilot study is to test a mixture of 90% δ-TT+10% γ-TT dietary supplementation for feasibility, and to quantitatively assess its potential anti-obesity effects on obese PMW.

In this paper, we present the design and the detailed protocol of a double-blinded, placebo-controlled, and randomized trial, as well as a discussion of the overall challenges when conducting this trial. The results from this trial will be reported at the completion of the study in accordance with the Consolidation of Standards for Reporting Trials guidelines.

METHODS

Study design

This is a 24-week double-blinded, placebo-controlled, and randomized intervention trial with allocation 1:1 for two treatment arms. Women with at least 1 year after menopause will be recruited for this study. After screening, qualified participants will be randomly assigned to one of the two treatment groups: placebo and TT. The participants in the placebo group will receive one 430-mg olive oil softgel per day for 24 weeks. The TT participants will receive one 430-mg, 70% pure TT softgel (representing 300 mg TT) for 24 weeks. The primary outcome measure is total fat mass and the secondary outcome measures are serum lipid profile, leptin, adiponectin, lipid metabolism-related gene expression in adipose tissue, inflammation-associated biomarkers (serum c-reactive protein, plasma and adipose tissue OxL), and gut microbiome analysis in feces. Both primary and secondary outcome measures will be taken from participants at baseline and 24 weeks, except for serum lipid profiles and C-reactive protein, which will be assessed at baseline, 12, and 24 weeks. Liver function will also be monitored by assessing the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at baseline, 12, and 24 weeks. Physical activity and food frequency questionnaires will be assessed at baseline and 24 weeks. Investigators and outcome assessors evaluating the endpoints will be blinded to

intervention assignment. The study received ethical approval from the Texas Tech University Health Sciences Center Institutional Review Board. The time points of all participant-related actions to be taken during the study period are presented in Table 1.

Study setting, study population, and recruitment

The subjects, ambulatory women with at least 1-year postmenopausal history, for this present study will be recruited from Lubbock and surrounding areas in Texas. Ethnicity or race is not a factor in the inclusion of subjects. Direct person-to-person solicitation in the OB/Gyn clinic and health fairs, flyers, non-solicited email system, campus announcements, local radio, newspapers, senior newsletters, and TV scripts will be used to recruit potential subjects. In addition, we plan to recruit the minority participants who have limited access to such intervention due to social and cultural factors. Advertisement in minority newspaper and radio will also be implemented. According to our past experience in subject recruitment, it is not difficult to recruit qualified participants within a reasonable time frame from our existing subject pool with the methods described above. If a longer period is needed to recruit the subjects, the study supplement intervention may take place in a staggered fashion that is a block randomization strategy will place participants into subgroups for treatments on a first-come, first-served basis.

Screening

Prescreening will be conducted through a phone interview and will cover age, body weight, height, menstrual history, status of any serious chronic diseases, and availability for the study period. Subjects who pass the prescreening will attend an informed consent session and sign consents and Health Insurance Portability and Accountability Act (HIPAA) forms before completing a detailed questionnaire with demographic, health, and dietary information. Visits for fasting blood screening will follow afterwards.

Inclusion criteria

1. Postmenopausal women with body mass index ≥30 kg/m^2.
2. Normal function of thyroid, liver, and kidney.

Sedentary using International Physical Activity Questionnaire (IPAQ-short form) [35]

Exclusion criteria

1. Unstable body weight (more than 5% change in body weight) within 3 months before intervention begins.
2. Changes in medications or supplements (i.e., steroids, statins) within 3 months of baseline study visit that could affect lipid metabolism.
3. Taking anticoagulants that may interact with TT.
4. Serious chronic disease (e.g., unstable CVD, uncontrolled diabetes and hypertension, and active cancer).

Sample size

Sample size is determined to provide adequate power for the study. Data from previous studies suggest that the TT intervention would yield moderate to large changes (median f=0.31) [20, 36-38] in total fat mass and body weight. The power analysis for the present study is considered to observe similar changes in other previous studies [20, 36-38] and assumed 0.20 correlations among repeated measures. The analysis results revealed that the sample size of N=46 will produce 80% power while controlling Type I error under 5%. Conservatively assuming a high attrition rate of 20%, therefore, we plan to recruit 60 participants (30 in each group) at baseline—i.e., anticipated final N≥46 (power ≥80%) at the end of the study with up to 20% attrition. Missing data either attrition or nonresponse will be fully recovered via multiple imputation as described later, which will remove or minimize (if present) confounding effects of missingness on our statistical power.

Randomization and allocation concealment

Participants who meet the inclusion criteria will be randomly assigned 1:1 to placebo and TT groups using a stratified randomization with BMI (≥35 or <35 kg/m^2), age (≥60 or <60 yr), use of hormone replace

therapy (yes or no), and use of cholesterol-lowing drug (yes or no). All participants, investigators, coordinators, and assessors will be blinded to the treatments. De-identified data with codes will be used to facilitate blinded randomization and data analysis. The blind on a participant will be removed in the case of an adverse event or other legitimate reason.

Intervention

Purchasing and masking of study agents

Placebo and TT supplements of the same lot, respectively, will be provided by American River Nutrition, Inc. (Hadley, MA; Investigational New Drug (IND) number 120761 by FDA). Each placebo capsule of 430 mg olive oil contains no TT ingredient at detectable levels. Each TT capsule (DeltaGold® Tocotrienol 70%) contains 430 mg TT (90% delta- and 10% gamma-tocotrienol) with a 70% purity, representing 300 mg TT. The use of placebo to mask the control group is desirable in this study to keep respondents blinded to the TT treatment assignment. Placebo softgels will be made of the same size and color as the TT softgels for identical appearance and taste. The placebo group will provide a comparison of blood and urinary outcome measures and serve as a basis to assess TT's effect.

Treatment arms

The study agent (TT, DeltaGold Annatto Tocotrienol) has GRAS (Generally Recognized as Safe) status. Based on (i) TT fed at 400 mg/kg diet to high-fat-diet induced obese mice showed reduced total fat mass relative to the no-TT group [19], and (ii) the use of body surface area for dose translation from mouse to human [39], the estimated effective dose of TT in humans for anti-obesity is approximately 300 mg daily. Therefore, we will use a dosage of TT (300 mg TT daily) for 24 weeks in this study. The current RDA value of vitamin E, 15 mg/d, for this study population is largely focused on α-tocopherol. No specific recommendation exists for TT. However, no adverse effects were observed with daily intake of 3.2 g or less TT [40]. Qualified participants will be randomly assigned into one of the two study groups (placebo and TT). Participants in the

placebo group will receive 430 mg of olive oil daily for 24 weeks. Participants in the TT group will receive a 430-mg TT softgel for 24 weeks.

Dietary intake, physical activity, and concomitant medication assessment

A food frequency questionnaire and a physical activity log will be collected at the baseline and 24-week visits to account for any drastic changes in the intake of macro- and micro- nutrients and deviations from the usual activities that could affect outcome measures. Prescription and over-the-counter medications and dietary supplement, as well as the reasons for taking these substances, will be recorded for the study period.

Blinding and unblinding

Study participants and investigators including biostatistician, coordinators, and measurement and site personnel will be blinded to intervention allocation throughout this study. The investigators will be supplied with a blind code-breaker envelope for each subject. The blind code will not be broken except in a medical emergency or a potential study-related adverse event determined by the principal investigator. Medical emergencies may include abnormal elevation in liver function (ALT, AST) according to the regulation of FDA for TT intake.

Sample collection

Fasting blood will be drawn from a superficial arm vein and blood will be allowed to clot in a vacutainer at room temperature. Within 2 hr of collection, blood samples will be centrifuged at 1500x g for 10 min and aliquoted. Urine and stool specimen will also be collected for this study. The collected blood, urine, and stool specimen will be stored in -80°C freezers until outcome measures.

Evaluation of adherence and compliance

We will measure adherence to the intervention by counting the softgels consumed and determine compliance by the percentage of all softgels ingested. We will also measure serum concentrations of TT and tocopherol at baseline and 24-week visit as additional evidence for adherence and compliance.

Evaluation of adverse events

Despite the generally recognized as safe (GRAS) reports that TT at the doses used in this study has minimal liver toxicity in humans, we will monitor liver function by measuring AST and ALT at 0, 12 and 24 weeks. Adverse effects associated with dietary supplement treatments, if any, will be self-reported by the participants and will be monitored by observing liver function every 12 weeks in the course of the intervention trial. All adverse events, whether observed by investigators or voluntarily disclosed by subjects, will be recorded on the adverse event form throughout the study.

Outcome measures

In the present study, the primary outcome is total fat mass. The secondary outcomes include serum lipid profile, adiponectin, leptin, and CRP, urine 8-hydroxy-2'-deoxyguanosine (8-OHdG), lipid metabolism-related gene expression in adipose tissue, concentrations of OxL and eCB in plasma and adipose tissue, and abundance and composition of intestinal microbiota. Every participant will be evaluated at 0 (prior to starting intervention) and 24 weeks of intervention, except for serum lipid profile and CRP at 0, 12, and 24 weeks.

Fat mass

Rationale δ-TT supplementation has been shown to improve glucose tolerance, insulin sensitivity, and lipid profile, and to reduce total fat mass, abdominal circumference, and visceral adiposity index in obese rats [38]. Such an improvement in glucose utilization and insulin sensitivity contributes to the reduced pro-inflammatory microenvironement and subsequent reduction in total fat mass and VAT [19]. Our animal study

further confirmed δ-TT's anti-obesity potential in obese mice [19]. These animal studies indicate that δ-TT plays an important role in reducing VAT in obese population.

Methods Total and regional fat mass, relative body fat, and VAT will be quantified via dual-energy X-ray absorptiometry (DXA; GE Lunar Prodigy with enCore software v. 16.2) [41, 42]. Body regions for analysis include the trunk, legs and arms. VAT will be estimated using the built-in functionality of the enCore software. In the same DXA report, we will also collect data of bone mineral mass/density and lean mass. Additional total fat and VAT estimates will be obtained via multi-frequency bioelectrical impedance analysis (MFBIA; Seca 514/515).

Serum lipid profile, adiponectin, leptin, and CRP, and urine 8-OHdG

Rationale In our previous animal study, we have shown that TT supplementation to diet reduced serum triglycerides and proinflammatory adipokines in obese mice [19]. In a randomized double-blinded, placebo-controlled trial, we reported that 12-week TT supplementation (90% δ-TT+10% γ-TT) at 300 mg/day lowered urine 8-OHdG, an oxidative stress biomarker in osteopenic PMW [43].

Methods We will ship blood samples to Covenant Medical Laboratory, Lubbock, TX for lipid profile (total cholesterol, triglycerides, LDL, and HDL) and CRP analysis. The level of serum adiponectin and leptin, and urine 8-OHdG (oxidative stress biomarker) will be measured at Dr. Shen's lab using ELISA.

Lipid metabolism-related gene expression in adipose tissue

Rationale Obesity is characterized by altered mitochondrial activity and lipid metabolism, promoting accumulation of triglycerides in tissues, mainly white adipose tissue [44]. Defective mitochondrial biogenesis and fatty acid oxidative metabolic pathways in subcutaneous adipose tissue during acquired obesity, leading to the metabolic disturbance in obesity [45]. Based on our animal findings [19], we will focus on gene expression associated with lipid β-oxidation and fatty acid synthesis of subcutaneous adipose tissue sampled before and after TT regime.

Methods Total RNA will be isolated from frozen adipose samples using Qiagen RNeasy Lipid Kit (Qiagen, Valencia, CA) and reversely transcribed into cDNA using iScript Reverse Transcription Supermix. Conventional quantitative real-time PCR will be performed using SYBR Green with respective primers for CPT-1, FOXA2, FAS, and ACC (Human ProbeLibrary Gene) [46]. The relative gene expression will be assessed using the ΔΔCT method [47] and GAPDH will be used as internal control for normalization [46]. In addition, total RNA will be extracted from de-identified serum and adipose tissue for later stranded mRNA-sequencing.

Concentrations of OxL and endocannabinoid (eCB) in plasma and adipose tissue.

Rationale Aging and decline of estrogen are factors that contribute to weight gain in PMW, and approaches, such as anti-inflammatory potential in TT, to reduce inflammation may likely combat obesity. Oxylipins (OxLs) and endocannabinoids (eCBs) are classes of bioactive fatty acid metabolites with many structural members that influence insulin signaling, adipose function and inflammation through autocrine, paracrine and endocrine mechanisms [4, 5]. In addition, physical activity is reported to increase specific levels of eCB [48] leading to responses of well-being. In our recent animal study, male mice fed with TT-supplemented diet experienced reduced thromboxane B2 (TXB2) and 13 HODE, and increased 12HEPE (anti-platelet) and some essential amino acids (i.e., lysine and methionine) (Shen et al. unpublished findings). The former exerting complex functions like 13-HODE but lowering TXB2, a product of TX2, could have implications on the function of TXA2 (target of aspirin). Thus, higher TXB2 means increased catabolism of TXA2 and less thrombosis may result in reducing the risk for atherosclerosis. Our animal study suggests that TT may influence macronutrient metabolism in obese PMW (Shen et al. unpublished findings). Identification of novel metabolites and metabolic pathways, especially antioxidant and oxidative stress metabolites, of TT, in serum and adipose tissue of subjects will help to understand the composition and roles of these bioactive compounds in management of obesity. However, there has been no study to evaluate the effect of TT on any

metabolomics profiling in obese population. Herein, we will use the same targeted metabolomics to measure OxL and eCB.

Methods Targeted metabolomics analyses of OxL and eCBs The levels of OxL and eCB in plasma and adipose tissue will be quantitatively analyzed using UPLC tandem mass spectrometers based on our previously published work [4]. Briefly, 60 mg Oasis-HLB solid phase extraction columns (SPE) (Waters Corp., Milford, MA) are placed on a vacuum manifold, cleaned and conditioned, then spiked with deuterated OxLs and eCBs internal standards. The serum samples are thawed on ice and a 200 µL volume transferred to the SPE, up-diluted to 3 mL with 5% MeOH/0.1% acetic acid, and gravity extracted. Columns are then washed with 3 mL the 5% MeOH solution. OxLs and eCBs are eluted from the SPE with 0.2 mL MeOH followed by 1.5 mL ethyl acetate, by gravity. Solvent is removed by vacuum and samples are reconstituted in 50 µL MeOH containing the internal standard 1-cyclohexyl-3-dodecyl-urea (Sigma, Aldrich, St. Louis, MO) then, filtered at 0.1 µm. Analytes are chromatographically separated on a 2.1×150 mm, 1.7µm Acquity BEH C18 (Waters Corp.) column using 0.1% acetic acid and acetonitrile gradient. The OxLs and eCBs profiles are acquired using electrospray ionization and tandem mass spectrometry by back-to-back (+)-mode/(-)-mode injections on a Sciex API 4000-QTRAP (Pleasanton, CA). A complete list of measured analytes with retention times, acquisition parameters, and detection limits are shown for the OxLs and eCBs in a previously published article [4], respectively.

Abundance and the composition of intestinal bacteria in feces.

Rationale There has been a growing body of evidence to implicate altered intestinal microbiota in the development of obesity. The commensal microflora of the human GI actively participates in the processes of energy harvest and expenditure. Biochemical crosstalk between gut bacteria and the host has a critical impact on the metabolic status (OxLs and eCBs) of the human body. In our previous animal study, we reported that relative to the high-fat diet (HFD) control group, the HFD+TT at 800mg/kg diet group increased abundance of *Akkermansia g* (of the phylum *Verrucomicrobia*) and *S24-7 family (class Bacteroidetes)*, and decreased that of

Clostridiales order, and *Oscillospira genus* (of the phylum *Firmicutes*). Our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen et al. unpublished findings). Therefore, in this pilot study, we would like to explore how dietary TT supplement affects the abundance and composition of gut microbiome in PMW.

Methods 16S Metagenome analysis of fecal samples Bacterial DNA will be isolated from feces using a MoBio PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA). The microbial diversity of fecal samples will be evaluated using metagenomic 16S rDNA gene sequencing technique. Variability in the bacterial V3-V4 region will be used to identify bacterial abundances in both treated and untreated subjects. Quantitative insights into microbial ecology (QIIME, version 1.8.0) will be used for data analysis according to previous published method [49]. In brief, one μL of DNA sample (20 ng/μL) will be used for a 25 μL PCR reaction. The V3-V4 regions of the 16S rRNA gene region will be amplified using gene specific primer pairs. PCR amplicons from all samples will be normalized using SequalPrep plate Normalization kit (Thermo Fisher Scientific, MA). Equal volumes of samples will be pooled and quantified using Qubit 2.0 (Thermo Fisher Scientific, MA) and quality will be checked using TapeStation (Agilent Technologies, CA). Libraries will be sequenced on MiSeq (Illumina Inc., CA) using a 600 cycle v3 sequencing kit. Post sequencing, reads will be filtered based on quality score and aligned on 16S database using our custom pipeline involving PEAR, and QIIME software's. Final OTUs will be taxonomically classified using BLASTn against the GreenGenes database, and compiled into each taxonomic level into both "counts" and "percentage" files.

Statistical Analysis

Sample demographics and all outcome measures will be summarized by descriptive statistics and bivariate tests within the whole sample and between treatment groups (placebo vs. TT) and demographic subgroups (e.g., age, BMI). The correlations among outcomes and potential covariates (e.g., demographic variables, dietary and lifestyle changes, concomitant medications) will be examined in a preliminary analysis. In order to examine the effects of the TT intervention, hierarchical linear modeling will be conducted to examine

overall treatment group difference across time (i.e., group effect), linear or nonlinear change over time (i.e., time effect), and group difference in this change (i.e., group-by-time interaction). Models will be adjusted for identified covariates, thereby providing unbiased effect estimates. If present, missing data will be handled by Monte Carlo Markov Chain (MCMC) multiple imputation [50]. A large number of (e.g., 200) imputed datasets will be generated and then analysis results from each imputed dataset will be combined together to make valid statistical inference. Gut microbiome data will be analyzed designed to pinpoint similarities and differences of bacterial communities between the treated and untreated subjects.

Data for OxL and eCB will be expressed by descriptive statistics. If necessary, OxL and eCB data will be transformed to normality by an iterative process using imDEV v1.4.2 [51] and missing data will be handled by MCMC multiple imputation. Statistical significance of the means will be evaluated using 2-tailed t-test at 0.05 alpha level. To account for multiple testing, the false discovery rate will be computed and reported for each comparison using the q-value method [52]. A summary of the numbers of biochemical (or called metabolite) that achieved statistical significance will be reported. OxL and eCB data will be combined with measurements for partial-least squares discriminate analysis. To improve clustering performance, the reduced data matrix variables (complete data set) will be subjected to orthogonal signal correction using the procedures of Wehrens [53] and partial least squares discriminate analysis of Variable Importance Plot filtered data set using the orthogonal scores PLSR component of the R 'pls' package [54]. Iterative rotations for uniform projection of discriminate variables onto a single latent variable will be done.

Data of microbiota OTU reads will be imported into R version 3.4.3, and all statistical analyses will be performed using the vegan and phyloseq packages unless specifically noted. OTU richness will be determined by Chao1 and the OTU evenness will be determined by several diversity indices (i.e., Shannon, Simpson, Inverse Simpson, and Fisher). Group differences in α-diversity (richness and diversity) will be evaluated by ANOVA. Between-specimen diversity (β-diversity) will be assessed by the Bray–Curtis method and then visualized using nonmetric multidimensional scaling. Group differences in β-diversity will be measured using permutational multivariate analysis of variance with 500 permutations. Group differences among taxa- and

genus-level OTUs will be assessed by pairwise comparisons on read counts using Negative Binomial Wald Tests from the DESeq2 package. All tests will be corrected for multiple comparisons using the false discovery rate correction by Benjamini and Hochberg. Associations among selected variables will be assessed with Spearman's correlations. The statistical significance is as determined at $P \leq .05$.

Data management and data collection

Researchers will keep information obtained in this study confidential except as required by law. All participant questionnaires, records, and data will be coded and properly stored in locked cabinets. Only study investigators will have access to those files linking a person's study number to his/her name. Those forms will include Informed Consent Form, HIPAA Authorization form, all recruitment questionnaires, and all other related materials.

The principle investigator will be responsible for oversight of data management of the trial. A certified monitor from Clinical Research Institute, independent from research team and sponsor, will assist the principle investigator in monitoring data collection before the datasets are delivered to study biostatistician. Questionnaire data will be entered on various forms and then entered into the Excel database. Laboratory data will also be verified and entered into an Excel database. Data of eligibility, medical records, attrition rate, and compliance rate will all be entered into an Excel database. Data queries including missing values will be referred to the principle investigator. The principle investigator will have access to the final trial dataset and disclosure of contractual agreements. The principle investigator will also incorporate any correction or addition into the datasets. Finally, a clean dataset without any identification will be generated and delivered to the biostatistician for statistical analysis.

Ethics

The study protocol and template consent forms have been reviewed and approved by the Bioethics Committee of the Texas Tech University Health Sciences Center. An informed consent form will be signed by a participant before enrolling in the study. Any modifications to the protocol, which may affect the conduct of the

study, potential benefits to the study participants, or their safety will be reported to the ethics committee for all necessary amendments. All study-related information will be stored securely at the study site in local cabinets, in an area with limited access (databases will be secured with a password-protected access system).

Patient and Public Involvement

The patients and the public will not be involved in the design, assessment or conduction of this trial. Recruitment information is available on local newspaper, senior newsletter, and clinics. This information will allow postmenopausal women to contact research coordinator if they are willing to participant. Postmenopausal women with adverse outcomes will be followed up throughout their intervention and will be referred to experts. Feedback will be sought from all participants at the end of the study to assess burden of intervention and to help develop future trials.

Dissemination

The findings of this study will be submitted to a peer-referred journal in the areas of obesity or nutrition/functional food. Abstracts will be submitted to relevant national and international conferences.

CONCLUSIONS

The effectiveness of annatto-extracted tocotrienols for obesity-associated parameters in postmenopausal obese women is still unknown. Our study, carried out at a single research center with experience in conducting independent, investigator-initiated FDA-IND clinical trial, is intended to address a gap in the field and will test the safety and efficacy of annatto-extracted tocotrienols supplementation for anti-obesity capacities in postmenopausal obese women.

COMPETING INTERESTS

This study is funded by School of Medicine, Texas Tech University Health Sciences Center, Laura W. Bush Institute for Women's Health, and American River Nutrition, Inc. None of the authors declared any conflicts of financial interest.

AUTHOR'S CONTRIBUTIONS

AA and CLS conceptualized the study. AA, GT, JL, LM, BAW, AAS, KS, SW, RW, and CLS designed this trial. CLS developed the first draft of the manuscript and the rest of coauthors participated in the revision of subsequent drafts. All authors approved the final draft of the manuscript. CLS made final decision to submit the report for publication.

Funding This study is funded by School of Medicine, Texas Tech University Health Sciences Center, Laura W. Bush Institute for Women's Health, and American River Nutrition, Inc. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of American River Nutrition, Inc.

References

[1] New WHO report: deaths from noncommunicable diseases on the rise, with developing world hit hardest, Cent Eur J Public Health 19(2) (2011) 114, 120.
[2] M.F. Gregor, G.S. Hotamisligil, Inflammatory mechanisms in obesity, Annu Rev Immunol 29 (2011) 415-45.
[3] W.S. Powell, J. Rokach, Biosynthesis, biological effects, and receptors of hydroxyeicosatetraenoic acids (HETEs) and oxoeicosatetraenoic acids (oxo-ETEs) derived from arachidonic acid, Biochim Biophys Acta 1851(4) (2015) 340-55.
[4] B.A. Watkins, J. Kim, A. Kenny, T.L. Pedersen, K.L. Pappan, J.W. Newman, Circulating levels of endocannabinoids and oxylipins altered by dietary lipids in older women are likely associated with previously identified gene targets, Biochim Biophys Acta 1861(11) (2016) 1693-1704.
[5] J. Kim, M.E. Carlson, G.A. Kuchel, J.W. Newman, B.A. Watkins, Dietary DHA reduces downstream endocannabinoid and inflammatory gene expression and epididymal fat mass while improving aspects of glucose use in muscle in C57BL/6J mice, Int J Obes (Lond) 40(1) (2016) 129-37.
[6] M.M. Heemskerk, M. Giera, F.E. Bouazzaoui, M.A. Lips, H. Pijl, K.W. van Dijk, V. van Harmelen, Increased PUFA Content and 5-Lipoxygenase Pathway Expression Are Associated with Subcutaneous Adipose Tissue Inflammation in Obese Women with Type 2 Diabetes, Nutrients 7(9) (2015) 7676-90.
[7] K. Moller, A.I. Ostermann, K. Rund, S. Thoms, C. Blume, F. Stahl, A. Hahn, N.H. Schebb, J.P. Schuchardt, Influence of weight reduction on blood levels of C-reactive protein, tumor necrosis factor-alpha, interleukin-6, and oxylipins in obese subjects, Prostaglandins Leukot Essent Fatty Acids 106 (2016) 39-49.
[8] R. Horrillo, A. Gonzalez-Periz, M. Martinez-Clemente, M. Lopez-Parra, N. Ferre, E. Titos, E. Moran-Salvador, R. Deulofeu, V. Arroyo, J. Claria, 5-lipoxygenase activating protein signals adipose tissue inflammation and lipid dysfunction in experimental obesity, J Immunol 184(7) (2010) 3978-87.
[9] D. Poeckel, C.D. Funk, The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease, Cardiovasc Res 86(2) (2010) 243-53.
[10] S.K. Chakrabarti, B.K. Cole, Y. Wen, S.R. Keller, J.L. Nadler, 12/15-lipoxygenase products induce inflammation and impair insulin signaling in 3T3-L1 adipocytes, Obesity (Silver Spring) 17(9) (2009) 1657-63.
[11] A.D. Dobrian, D.C. Lieb, B.K. Cole, D.A. Taylor-Fishwick, S.K. Chakrabarti, J.L. Nadler, Functional and pathological roles of the 12- and 15-lipoxygenases, Prog Lipid Res 50(1) (2011) 115-31.
[12] C.S. Nunemaker, M. Chen, H. Pei, S.D. Kimble, S.R. Keller, J.D. Carter, Z. Yang, K.M. Smith, R. Wu, M.H. Bevard, J.C. Garmey, J.L. Nadler, 12-Lipoxygenase-knockout mice are resistant to inflammatory effects of obesity induced by Western diet, Am J Physiol Endocrinol Metab 295(5) (2008) E1065-75.
[13] D.D. Sears, P.D. Miles, J. Chapman, J.M. Ofrecio, F. Almazan, D. Thapar, Y.I. Miller, 12/15-lipoxygenase is required for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice, PLoS One 4(9) (2009) e7250.
[14] E. Borgeson, C. Godson, Resolution of inflammation: therapeutic potential of pro-resolving lipids in type 2 diabetes mellitus and associated renal complications, Front Immunol 3 (2012) 318.
[15] J.W. Newman, T.L. Pedersen, V.R. Brandenburg, W.S. Harris, G.C. Shearer, Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects, PLoS One 9(11) (2014) e111471.
[16] Y.J. Suzuki, M. Tsuchiya, S.R. Wassall, Y.M. Choo, G. Govil, V.E. Kagan, L. Packer, Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: implication to the molecular mechanism of their antioxidant potency, Biochemistry 32(40) (1993) 10692-9.
[17] M. Meydani, Vitamin E, Lancet 345(8943) (1995) 170-5.
[18] G.C. Burdeos, K. Nakagawa, F. Kimura, T. Miyazawa, Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats, Lipids 47(5) (2012) 471-81.

[19] L. Allen, L. Ramalingam, K. Menikdiwela, S. Scoggin, C.L. Shen, M.D. Tomison, G. Kaur, J.M. Dufour, E. Chung, N.S. Kalupahana, N. Moustaid-Moussa, Effects of delta-tocotrienol on obesity-related adipocyte hypertrophy, inflammation and hepatic steatosis in high-fat-fed mice, J Nutr Biochem 48 (2017) 128-137.
[20] W.Y. Wong, H. Poudyal, L.C. Ward, L. Brown, Tocotrienols reverse cardiovascular, metabolic and liver changes in high carbohydrate, high fat diet-fed rats, Nutrients 4(10) (2012) 1527-41.
[21] T.W. Chou, C.Y. Ma, H.H. Cheng, Y.Y. Chen, M.H. Lai, A rice bran oil diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes, J Clin Biochem Nutr 45(1) (2009) 29-36.
[22] S. Das, S. Mukherjee, I. Lekli, N. Gurusamy, J. Bardhan, U. Raychoudhury, R. Chakravarty, S. Banerji, A.A. Knowlton, D.K. Das, Tocotrienols confer resistance to ischemia in hypercholesterolemic hearts: insight with genomics, Mol Cell Biochem 360(1-2) (2012) 35-45.
[23] A.A. Qureshi, N. Qureshi, J.O. Hasler-Rapacz, F.E. Weber, V. Chaudhary, T.D. Crenshaw, A. Gapor, A.S. Ong, Y.H. Chong, D. Peterson, Dietary tocotrienols reduce concentrations of plasma cholesterol, apolipoprotein B, thromboxane B2, and platelet factor 4 in pigs with inherited hyperlipidemias, Am J Clin Nutr 53(4 Suppl) (1991) 1042S-1046S.
[24] T. Watkins, P. Lenz, A. Gapor, M. Struck, A. Tomeo, M. Bierenbaum, gamma-Tocotrienol as a hypocholesterolemic and antioxidant agent in rats fed atherogenic diets, Lipids 28(12) (1993) 1113-8.
[25] R.S. Wong, A.K. Radhakrishnan, T.A. Ibrahim, S.K. Cheong, delta- and gamma-tocotrienols induce classical ultrastructural apoptotic changes in human T lymphoblastic leukemic cells, Microsc Microanal 18(3) (2012) 462-9.
[26] S.B. Budin, F. Othman, S.R. Louis, M.A. Bakar, S. Das, J. Mohamed, The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats, Clinics (Sao Paulo) 64(3) (2009) 235-44.
[27] E.C. Heng, S.A. Karsani, M. Abdul Rahman, N.A. Abdul Hamid, Z. Hamid, W.Z. Wan Ngah, Supplementation with tocotrienol-rich fraction alters the plasma levels of Apolipoprotein A-I precursor, Apolipoprotein E precursor, and C-reactive protein precursor from young and old individuals, Eur J Nutr 52(7) (2013) 1811-20.
[28] A.A. Qureshi, B.A. Bradlow, L. Brace, J. Manganello, D.M. Peterson, B.C. Pearce, J.J. Wright, A. Gapor, C.E. Elson, Response of hypercholesterolemic subjects to administration of tocotrienols, Lipids 30(12) (1995) 1171-7.
[29] A.A. Qureshi, D.A. Khan, W. Mahjabeen, N. Qureshi, Dose-dependent modulation of lipid parameters, cytokines and RNA by ¦Ä-tocotrienol in hypercholesterolemic subjects restricted to AHA Step-1 diet, 2015.
[30] A.A. Qureshi, D.A. Khan, W. Mahjabeen, A.M. Trias, N. Silswal, N. Qureshi, Impact of δ-Tocotrienol on Inflammatory Biomarkers and Oxidative Stress in Hypercholesterolemic Subjects, Journal of Clinical and Experimental Cardiology 6(4) (2015).
[31] N. Zaiden, W.N. Yap, S. Ong, C.H. Xu, V.H. Teo, C.P. Chang, X.W. Zhang, K. Nesaretnam, S. Shiba, Y.L. Yap, Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion, J Atheroscler Thromb 17(10) (2010) 1019-32.
[32] N. Haghighat, M. Vafa, S. Eghtesadi, I. Heidari, A. Hosseini, A. Rostami, The Effects of Tocotrienols Added to Canola Oil on Microalbuminuria, Inflammation, and Nitrosative Stress in Patients with Type 2 Diabetes: A Randomized, Double-blind, Placebo-controlled Trial, Int J Prev Med 5(5) (2014) 617-23.
[33] P. Irandoost, M. Ebrahimi-Mameghani, S. Pirouzpanah, Does grape seed oil improve inflammation and insulin resistance in overweight or obese women?, Int J Food Sci Nutr 64(6) (2013) 706-10.
[34] B.B. Aggarwal, C. Sundaram, S. Prasad, R. Kannappan, Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases, Biochem Pharmacol 80(11) (2010) 1613-31.
[35] C.L. Craig, A.L. Marshall, M. Sjostrom, A.E. Bauman, M.L. Booth, B.E. Ainsworth, M. Pratt, U. Ekelund, A. Yngve, J.F. Sallis, P. Oja, International physical activity questionnaire: 12-country reliability and validity, Med Sci Sports Exerc 35(8) (2003) 1381-95.

[36] S. Choquette, E. Riesco, E. Cormier, T. Dion, M. Aubertin-Leheudre, I.J. Dionne, Effects of soya isoflavones and exercise on body composition and clinical risk factors of cardiovascular diseases in overweight postmenopausal women: a 6-month double-blind controlled trial, Br J Nutr 105(8) (2011) 1199-209.
[37] M.A. Pervez, D.A. Khan, A. Ijaz, S. Khan, Effects of Delta-tocotrienol Supplementation on Liver Enzymes, Inflammation, Oxidative stress and Hepatic Steatosis in Patients with Nonalcoholic Fatty Liver Disease, Turk J Gastroenterol 29(2) (2018) 170-176.
[38] W.Y. Wong, L.C. Ward, C.W. Fong, W.N. Yap, L. Brown, Anti-inflammatory gamma- and delta-tocotrienols improve cardiovascular, liver and metabolic function in diet-induced obese rats, Eur J Nutr 56(1) (2017) 133-150.
[39] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J 22(3) (2008) 659-61.
[40] A. Mahipal, J. Klapman, S. Vignesh, C.S. Yang, A. Neuger, D.T. Chen, M.P. Malafa, Pharmacokinetics and safety of vitamin E delta-tocotrienol after single and multiple doses in healthy subjects with measurement of vitamin E metabolites, Cancer Chemother Pharmacol 78(1) (2016) 157-65.
[41] G.M. Tinsley, Proportional bias between dual-energy x-ray absorptiometry and bioelectrical impedance analysis varies based on sex in active adults consuming high- and low-carbohydrate diets, Nutr Res 42 (2017) 85-100.
[42] G.M. Tinsley, Reliability and agreement between DXA-derived body volumes and their usage in 4-compartment body composition models produced from DXA and BIA values, J Sports Sci 36(11) (2018) 1235-1240.
[43] C.L. Shen, S. Yang, M.D. Tomison, A.W. Romero, C.K. Felton, H. Mo, Tocotrienol supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: a 12-week randomized double-blinded placebo-controlled trial, Osteoporos Int 29(4) (2018) 881-891.
[44] C. Christodoulides, C. Lagathu, J.K. Sethi, A. Vidal-Puig, Adipogenesis and WNT signalling, Trends Endocrinol Metab 20(1) (2009) 16-24.
[45] S. Heinonen, J. Buzkova, M. Muniandy, R. Kaksonen, M. Ollikainen, K. Ismail, A. Hakkarainen, J. Lundbom, N. Lundbom, K. Vuolteenaho, E. Moilanen, J. Kaprio, A. Rissanen, A. Suomalainen, K.H. Pietilainen, Impaired Mitochondrial Biogenesis in Adipose Tissue in Acquired Obesity, Diabetes 64(9) (2015) 3135-45.
[46] J.P. Walhin, J.D. Richardson, J.A. Betts, D. Thompson, Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men, J Physiol 591(24) (2013) 6231-43.
[47] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25(4) (2001) 402-8.
[48] B.A. Watkins, Endocannabinoids, exercise, pain, and a path to health with aging, Mol Aspects Med 64 (2018) 68-78.
[49] M.A.I. Al-Obaide, R. Singh, P. Datta, K.A. Rewers-Felkins, M.V. Salguero, I. Al-Obaidi, K.R. Kottapalli, T.L. Vasylyeva, Gut Microbiota-Dependent Trimethylamine-N-oxide and Serum Biomarkers in Patients with T2DM and Advanced CKD, J Clin Med 6(9) (2017).
[50] C.K. Enders, Applied missing data analysis, Guilford Publications, New York, 2010.
[51] D. Capitanio, M. Vasso, S. De Palma, C. Fania, E. Torretta, F.P. Cammarata, V. Magnaghi, P. Procacci, C. Gelfi, Specific protein changes contribute to the differential muscle mass loss during ageing, Proteomics 16(4) (2016) 645-56.
[52] G.R. Adams, B.M. Hather, K.M. Baldwin, G.A. Dudley, Skeletal muscle myosin heavy chain composition and resistance training, J Appl Physiol (1985) 74(2) (1993) 911-5.
[53] R. Wehrens, Chemometrics with R Multivariate Data Analysis in the Natural Sciences and Life Sciences, (2011).
[54] B.-H. Mevik, R. Wehrens, The pls Package: Principal Component and Partial Least Squares Regression in R, 2007 18(2) (2007) 23.

Table 1. Timetable of activities planned during the course of the study directly related to participants

	Weeks										
Activity	-4	-2	0	3	6	9	12	15	18	21	24
Enrollment	x										
Screening (liver function, TSH)		x									
Randomization			x								
Intervention			x	x	x	x	x	x	x	x	x
Body composition			x								x
Fat biopsy (optional)			x								x
Blood for outcomes			x				x				x
Liver function assessment			x				x				x
Pill counts			x				x				x
Food intake assessment			x								x
Physical activity survey			x								x
Self-report adverse event			x	x	x	x	x	x	x	x	x

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number of manuscript
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
	2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	3	Date and version identifier	n/a
Funding	4	Sources and types of financial, material, and other support	17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1, 17
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	n/a
Introduction			

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3-5
	6b	Explanation for choice of comparators	5
Objectives	7	Specific objectives or hypotheses	5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5-6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6-7
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8-9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	9-10
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n/a
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	10-14
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	14

Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	9
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	9
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	13-15
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	n/a
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	16
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	14-15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	14
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14-15

Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	17
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n/a
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	10
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	16
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	6
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	16
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	6
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	16-17
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	17
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n/a
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	17

	31b	Authorship eligibility guidelines and any intended use of professional writers	n/a
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	n/a
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	n/a

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

Actions of annatto-extracted tocotrienol supplementation on obese postmenopausal women: study protocol for a double-blinded placebo-controlled randomized trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2019-034338.R1
Article Type:	Protocol
Date Submitted by the Author:	04-Dec-2019
Complete List of Authors:	Aryaie, Amir; Texas Tech University Health Sciences Center Tinsley, Grant; Texas Tech University, Kinesiology and Sport Management Lee, Jaehoon ; Texas Tech University, Educational Psychology and Leadership Watkins, Bruce; University of California Davis, Nutrition Moore, Lane; Texas Tech University, Kinesiology and Sport Management Alhaj-Saleh, Adel ; Texas Tech University Health Sciences Center Shankar, Kartik; University of Colorado Denver School of Medicine, Pediatrics Wood, Sarah; Texas Tech University Health Sciences Center Wang, Rui; Texas Tech University Health Sciences Center Shen, Chwan-Li; Texas Tech University Health Sciences Center
Primary Subject Heading:	Complementary medicine
Secondary Subject Heading:	Complementary medicine, Nutrition and metabolism
Keywords:	tocotrienols, lipid, inflammation, women, microbiome

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Actions of annatto-extracted tocotrienol supplementation on obese postmenopausal women: study protocol for a double-blinded placebo-controlled randomized trial

Amir Aryaie, M.D.[a], Grant Tinsley, Ph.D.[b,e], Jaehoon Lee, Ph.D.[b,f], Bruce A. Watkins, Ph.D.[h], Lane Moore, Ph.D.[e], Adel Alhaj-Saleh, Ph.D.[a], Kartik Shankar, Ph.D.[i], Sarah Wood, L.V.N.[c], Rui Wang, Ph.D.[d], Chwan-Li Shen, Ph.D.[b,d,g,*]

[a] Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[b] Center of Excellence for Integrative Health, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[c] Clinical Research Institute, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[d] Department of Pathology, Texas Tech University Health Sciences Center, Lubbock, TX, 79430

[e] Department of Kinesiology and Sport Management, Texas Tech University, Lubbock, TX, 79409

[f] Department of Educational Psychology and Leadership, Texas Tech University, Lubbock, TX, 79409

[g] Obesity Research Cluster, Texas Tech University, Lubbock, TX, 79409

[h] Department of Nutrition, University of California-Davis, Davis, CA, 95616

[i] Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, 80045

Email:

aryaiemd@gmail.com; grant.tinsley@ttu.edu; jaehoon.lee@ttu.edu; bawatkins@ucdavis.edu; lane.moore@ttu.edu; adel.alhaj-saleh@ttuhsc.edu; Kartik.Shankar@Cuanschutz.edu; sarah.r.wood@ttuhsc.edu; rui.wang@ttuhsc.edu; leslie.shen@ttuhsc.edu

[*] Corresponding author.
Trial sponsor. Chwan-Li Shen. E-mail Leslie.Shen@ttuhsc.edu. Phone: 1 (806) 743-2815. Fax: 1 (806) 743-2117.

Trial registration: ClinicalTrials.gov identifier: NCT03705845. Title: Tocotrienols for obesity of postmenopausal women. The trial registration is prospective and data collection is not expected to be completed until the end of 2020.

ABSTRACT

INTRODUCTION: Obesity is a major health concern in postmenopausal women, and chronic low-grade inflammation contributes to the development of obesity. Cellular studies and high-fat-diet induced obese mouse model mimicking obesity show the anti-obesity effect of annatto-extracted tocotrienols (TT) with antioxidant capability. We aim to assess the safety and efficacy of TT consumption for lipid-related parameters in obese postmenopausal women.

METHODS AND ANALYSIS: Eligible obese postmenopausal women will be randomly assigned to placebo group (430 mg olive oil) and TT group (DeltaGold® Tocotrienol 70%) for 24 weeks. In the present study, the primary outcome is total/regional fat mass and visceral adipose tissue. The secondary outcomes include lipid profile in serum, mRNA expression of fatty acid synthase and carnitine palmitoyltransferase 1A in fat tissue, oxylipins and endocannabinoids in plasma and adipose tissue, abundance and composition of intestinal microbiome in feces, hs-CRP in serum, leptin in serum. Every participant will be evaluated at 0 (prior to starting intervention) and 24 weeks of intervention, except for serum lipid profile and hs-CRP at 0, 12, and 24 weeks. "*Intent-to-treat*" principle is employed for data analysis. Hierarchical linear modeling is utilized to estimate the effects of dietary TT supplementation while properly accounting for dependency of data and identified covariates. To our knowledge, this is the first randomized, placebo-controlled, double-blinded study to determine dietary TT supplementation on an obese population. If successful, this study will guide the future efficacy TT interventions and TT can be implemented as an alternative for obese population in anti-obesity management.

ETHICS AND DISSEMINATION: This study has been approved by the Bioethics Committee of the Texas Tech University Health Sciences Center, Lubbock. An informed consent form will be signed by a participant before enrolling in the study. The results from this trial will be actively disseminated through academic conference presentation and peer-reviewed journals.

Keywords: tocotrienols; lipid; inflammation; women; microbiome

Strength and limitations of this study

- This is the first randomized, double-blind, placebo-controlled trial to investigate the effects of dietary annatto-extracted tocotrienol supplementation on obesity-related outcomes in obese postmenopausal women.
- This study will be performed at a single research center with experience in conducting independent, investigator-initiated, randomized controlled trials in nutrition research.
- Annatto-extracted tocotrienol supplement is not available over the counter worldwide.
- There is no long-term follow-up.

Introduction

Obesity is now recognized as a worldwide epidemic disease, which leads to the development of several comorbidities such as insulin resistance, dyslipidemia, and metabolic syndrome (WHO 2011 [1]). With advances in basic redox biology, emerging evidence indicates that chronic low-grade inflammation paves the way for the development of comorbidities in obesity [2]. Obesity-induced chronic low-grade inflammation, called metaflammation, is initiated by excess nutrients in metabolic cells [2]. The inflammatory signaling conducted by these metabolic cells eventually causes activation of specialized immune cells and leads to an unresolved inflammatory response within the adipose tissue [2]. Therapeutic interventions to inhibit inflammatory pathways in obesity have shown beneficial effects on insulin sensitivity in mouse models and human trials [2].

Oxylipins (OxLs) are oxygenated fatty acid metabolites derived from n-6 polyunsaturated fatty acids (PUFA) (i.e., linoleic acid, dihomo-γ-linolenic acid, and arachidonic acid (AA)) or n-3 polyunsaturated fatty acids (PUFAs) (i.e., α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) through the actions of cyclooxygenases (COX), lipoxygenase (LOX), and cytochrome P450 enzymes [3]. In postmenopausal women at risk of metabolic syndrome, the amounts of OxLs were modified by dietary n-3 PUFA such that the levels of pro-inflammatory were reduced and anti-inflammatory were enhanced [4]. Particularly, DHA feeding reduced plasma levels of various inflammatory cytokines and OxLs (5-lipoxygenase-dependent inflammatory mediators and vasoconstrictive 20-hydroxy-eicosatetraenoic acid (HETE) in a rodent model of obesity [5]. Emerging evidence suggests that AA-derived OxLs contribute to obesity-associated inflammation [6, 7]. For example, 5-LOX products of AA (e.g., 5- HETE or 5-oxo-eicosatetraenoic acid) exert pro-inflammatory effects by increasing the production of pro-inflammatory cytokines and inducing chemotaxis to attract inflammatory cells in the blood vessels in adipose tissue from obese mice [8, 9]. 12-LOX products of AA (e.g., 12-HETE, 12-hydroperoxyeicosatetraenoic acid (12-HPETE)) have also been shown to play a direct role in obesity-induced-inflammation [10] and insulin resistance [11-13]. Obese subjects with low-grade inflammation had elevated levels of several AA-derived OxLs (e.g., 5-, 8-, 11-, 12-, 15-, and 20-HETE)

compared to those with no inflammation [7]. On the other hand, OxLs that originate from n-3 PUFA (e.g., EPA and DHA) have demonstrated their ability to suppress obesity-associated inflammation as seen in inhibiting macrophage recruitment and increasing adiponectin secretion in adipose explants isolated from obese mice [14]. Supplementation of n-3 PUFA for 8 weeks significantly decreased AA-derived OxLs and increased EPA- and DHA-derived OxLs in hypertriglyceridemic subjects along with mitigated inflammatory and vascular status [15]. Since chronic low-grade inflammation in obesity is a systemic problem, administration of dietary supplements with potent anti-inflammatory function can be a good strategy to combat inflammation in obesity.

Tocotrienols (TT) and tocopherol are two subclasses of vitamin E, which can be further classified into four isomers (α, β, δ, and γ). The unsaturated side chain of TT allows for more efficient penetration into tissues compared with the completely saturated side chain of tocopherol. TT is lipophilic in nature and it is found in association with lipoproteins, fat deposits, and cellular membranes [16, 17]. TT has been shown to protect the PUFAs from peroxidation [16, 17]. In animal studies, dietary TT (palm TT-rich fraction, δ-TT, and γ-TT) decreased body weight [18] and fat mass [19, 20], reduced plasma concentrations of free fatty acids, triglycerides and cholesterol [21-25], and improved glucose and insulin tolerance [18-20, 25, 26]. Many epidemiology studies showed that dietary TT intake is associated with decreased serum concentrations of total cholesterol and lipoproteins of study subjects [23, 27-31]. In humans, TT supplementation (34.6% α-TT+43.5% γ-TT) reduced microalbuminuria and high-sensitivity C-reactive protein (hs-CRP), indicators of acute inflammation, in type 2 diabetic patients [32]. Intake of grape seed oil with high levels of TT was shown to attenuate serum levels of hs-CRP and TNF-α and improve insulin resistance in overweight/obese women [33]. The review of the supporting evidence strongly suggests that TT can offer benefits to control obesity due to its potent anti-inflammatory-dependent biological activities. In addition, although δ-TT and γ-TT are considered as the most potent isomers among 8 vitamin E isomers [34], their clinical relevance to combat obesity along with mechanisms has never been investigated in humans. Furthermore, our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen et al. unpublished findings). Yet, it is not known whether supplementation with TT would benefit total fat mass

reduction, improve lipid metabolism, and modify OxL in plasma and adipose tissue of humans. Additionally, there is not a single study yet to evaluate how TT supplementation affects gut microbiome in humans, particularly obese postmenopausal women (PMW). We previously reported that 12-week annatto-extracted TT supplementation suppressed bone resorption and oxidative stress in postmenopausal women with low bone mass [35]. However, there is no study to evaluate how TT supplementation would affect obese postmenopausal women. Therefore, the objective of this pilot study is to test a mixture of 90% δ-TT+10% γ-TT dietary supplementation for feasibility, and to quantitatively assess its potential anti-obesity effects on obese PMW.

In this paper, we present the design of a 24-week double-blinded, placebo-controlled, and randomized trial as well as a discussion of the overall challenges when conducting this trial. The results from this trial will be reported at the completion of the study in accordance with the Consolidation of Standards for Reporting Trials guidelines.

METHODS

Study design

This is a 24-week double-blinded, placebo-controlled, and randomized intervention trial with allocation 1:1 for two treatment arms. Women with at least 1 year after menopause will be recruited for this study. After screening, qualified participants will be randomly assigned to one of the two treatment groups: placebo and TT. The participants in the placebo group will receive one 430-mg olive oil softgel per day for 24 weeks. The TT participants will receive one 430-mg, 70% pure TT softgel (representing 300 mg TT) for 24 weeks. The primary outcome measure is total fat mass and the secondary outcome measures are serum lipid profile, leptin, adiponectin, lipid metabolism-related gene expression in adipose tissue, inflammation-associated biomarkers (serum c-reactive protein, plasma and adipose tissue OxL), and gut microbiome analysis in feces. Both primary and secondary outcome measures will be taken from participants at baseline and 24 weeks, except for serum lipid profiles and C-reactive protein, which will be assessed at baseline, 12, and 24 weeks. Liver function will also be monitored by assessing the activity of aspartate aminotransferase (AST) and alanine aminotransferase

(ALT) at baseline, 12, and 24 weeks. Physical activity and food frequency questionnaires will be assessed at baseline and 24 weeks. Investigators and outcome assessors evaluating the endpoints will be blinded to intervention assignment. The study received ethical approval from the Texas Tech University Health Sciences Center Institutional Review Board (protocol number L18-194). The time points of all participant-related actions to be taken during the study period are presented in Table 1.

Study setting, study population, and recruitment

The subjects, ambulatory women with at least 1-year postmenopausal history, for this present study will be recruited from Lubbock and surrounding areas in Texas. Ethnicity or race is not a factor in the inclusion of subjects. Direct person-to-person solicitation in the OB/Gyn clinic and health fairs, flyers, non-solicited email system, campus announcements, local radio, newspapers, senior newsletters, and TV scripts will be used to recruit potential subjects. In addition, we plan to recruit the minority participants who have limited access to such intervention due to social and cultural factors. Advertisement in minority newspaper and radio will also be implemented. According to our past experience in subject recruitment, it is not difficult to recruit qualified participants within a reasonable time frame from our existing subject pool with the methods described above. If a longer period is needed to recruit the subjects, the study supplement intervention may take place in a staggered fashion that is a block randomization strategy will place participants into subgroups for treatments on a first-come, first-served basis.

Screening

Prescreening will be conducted through a phone interview and will cover age, body weight, height, menstrual history, status of any serious chronic diseases, and availability for the study period. Subjects who pass the prescreening will attend an informed consent session and sign consents and Health Insurance Portability and Accountability Act (HIPAA) forms before completing a detailed questionnaire with demographic, health, and dietary information. Visits for fasting blood screening will follow afterwards.

Inclusion criteria

1. Postmenopausal women with body mass index (BMI) ≥30 kg/m^2.
2. Normal function of thyroid, liver, and kidney.
3. Sedentary using International Physical Activity Questionnaire (IPAQ-short form) [36]

Exclusion criteria

1. Unstable body weight (more than 5% change in body weight) within 3 months before intervention begins.
2. Changes to medications or supplements (i.e., steroids, statins) within 3 months of the baseline study visit that could affect lipid metabolism. • If they change any medications/supplements after the baseline visit that will affect lipid metabolism, their study participation will end.
3. Taking anticoagulants that may interact with TT.
4. Serious chronic disease (e.g., unstable cardiovascular disease, uncontrolled diabetes and hypertension, and active cancer).

Sample size

Sample size is determined to provide adequate power for the study. Data from previous studies suggest that the TT intervention would yield moderate to large changes (median f=0.31) [20, 37-39] in total fat mass and body weight. The power analysis for the present study is considered to observe similar changes in other previous studies [20, 37-39] and assumed 0.20 correlations among repeated measures. The analysis results revealed that the sample size of N=46 will produce 80% power while controlling Type I error under 5%. Conservatively assuming a high attrition rate of 20%, therefore, we plan to recruit 60 participants (30 in each group) at baseline—i.e., anticipated final N≥46 (power ≥80%) at the end of the study with up to 20% attrition.

Missing data either attrition or nonresponse will be fully recovered via multiple imputation as described later, which will remove or minimize (if present) confounding effects of missingness on our statistical power.

Randomization and allocation concealment

Participants who meet the inclusion criteria will be randomly assigned 1:1 to placebo and TT groups using a stratified randomization with BMI (≥35 or <35 kg/m^2), age (≥60 or <60 yr), use of hormone replace therapy (yes or no), and use of cholesterol-lowing drug (yes or no). All participants, investigators, coordinators, and assessors will be blinded to the treatments. De-identified data with codes will be used to facilitate blinded randomization and data analysis. The blind on a participant will be removed in the case of an adverse event or other legitimate reason.

Intervention

Purchasing and masking of study agents

Placebo and TT supplements of the same lot, respectively, will be provided by American River Nutrition, LLC (Hadley, MA; Investigational New Drug (IND) number 120761 by FDA). Each placebo capsule of 430 mg olive oil contains no TT ingredient at detectable levels. Each TT capsule contains 430 mg DeltaGold® Tocotrienol 70% (90% delta- and 10% gamma-tocotrienol) with a 70% purity, representing 300 mg TT. In the present study, we will include the placebo group to mask the control group in order to keep participants and evaluators blinded to the treatment assignment. Placebo softgels will be made of the same size and color as the active TT softgels for identical appearance and taste. We will compare the results of blood and urinary outcome measures in the placebo group with those in the TT group.

Treatment arms

The study agent (annatto-extracted TT, DeltaGold® Tocotrienol 70%) has Generally Recognized as Safe (GRAS) status. A tocotrienol has an unsaturated isoprenoid side chain, which is different from tocopherols. Both tocotrienols and tocopherols exist in 4 different forms in nature: α-, β-, γ-, and δ-isomers. Our study agent, the DeltaGold® Tocotrienol 70% consists of 90% delta-TT and 10% gamma-TT with 70% purity.

Based on (i) TT fed at 400 mg/kg diet to high-fat-diet induced obese mice showed reduced total fat mass relative to the no-TT group [19], and (ii) the use of body surface area for dose translation from mouse to human [40], the estimated effective dose of TT in humans for anti-obesity is approximately 300 mg daily. Therefore, we will use a dosage of TT (300 mg TT daily) for 24 weeks in this study. The current RDA value of vitamin E, 15 mg/d, for this study population is largely focused on α-tocopherol. No specific recommendation exists for TT. However, no adverse effects were observed with daily intake of 3.2 g or less TT [41]. Qualified participants will be randomly assigned into one of the two study groups (placebo and TT). Participants in the placebo group will receive 430 mg of olive oil daily for 24 weeks and participants in the TT group will receive a 430-mg DeltaGold® Tocotrienol 70% softgel for 24 weeks.

Dietary intake, physical activity, and concomitant medication assessment

We will take the same strategy that we used in our previous TT for bone health study [42] into this obesity study to monitor participants' dietary intake using a food frequency questionnaire, physical activity using a log, and over-the-counter medications/dietary supplement at the baseline and 24-week visit.

Blinding and unblinding

Study participants and investigators including biostatistician, coordinators, and measurement and site personnel will be blinded to intervention allocation throughout this study. The investigators will be supplied with a blind code-breaker envelope for each subject. The blind code will not be broken except in a medical emergency or a potential study-related adverse event determined by the principal investigator.

Sample collection

Fasting blood will be drawn from a superficial arm vein at Clinical Research Institute, Texas Tech University Health Sciences Center, Lubbock, and blood will be allowed to clot in a vacutainer at room temperature. After centrifugation of blood samples, serum samples will be aliquoted and stored in -80°C freezers for later analysis. Urine and stool specimen will also be collected and stored in -80°C freezers for later analysis. Under local anesthesia, the fat biopsy specimen will be obtained from visceral fat with Bard® Magnum® instrument and needle (C.R. Bard, Inc., Covington, GA).

Evaluation of adherence and compliance

We will count the softgels consumed and measure serum TT concentration at baseline and 24 weeks for adherence and compliance of participants. We will not use the results of the percentage of softgels consumed or serum concentration of TT and tocopherol as our exclusion criteria.

Evaluation of adverse events

We will measure the AST and ALT at 0, 12, and 24 week to monitor the liver function of participants. In addition to abnormal liver function, all self-reported adverse events and medical emergencies will be recorded throughout the study period. In our previous study [43], we reported TT supplementation at both 300 mg and 600 mg TT daily for 12 weeks did not affect liver or kidney function parameters and no adverse event due to treatments were reported by study participants.

Outcome measures

In the present study, the primary outcome is total/regional fat mass and visceral adipose tissue. The secondary outcomes include lipid profile (total cholesterol, high-density lipoprotein, and triglycerides) in serum, mRNA expression of fatty acid synthase (FAS) and carnitine palmitoyltransferase 1A (CPT-1) in fat tissue, OxL and endocannabinoids (eCB) in plasma and adipose tissue, abundance and composition of intestinal

microbiome in feces, hs-CRP in serum, leptin in serum. Every participant will be evaluated at 0 (prior to starting intervention) and 24 weeks of intervention, except for serum lipid profile and hs-CRP at 0, 12, and 24 weeks.

Fat mass

Rationale δ-TT supplementation has been shown to improve glucose tolerance, insulin sensitivity, and lipid profile, and to reduce total fat mass, abdominal circumference, and visceral adiposity index in obese rats [39]. Such an improvement in glucose utilization and insulin sensitivity contributes to the reduced pro-inflammatory microenvironement and subsequent reduction in total fat mass and visceral adipose tissue (VAT) [19]. Our animal study further confirmed δ-TT's anti-obesity potential in obese mice [19]. These animal studies indicate that δ-TT plays an important role in reducing VAT in obese population.

Methods Total and regional fat mass, relative body fat, and VAT will be quantified via dual-energy X-ray absorptiometry (DXA; GE Lunar Prodigy with enCore software v. 16.2) [44, 45]. Body regions for analysis include the trunk, legs and arms. VAT will be estimated using the built-in functionality of the enCore software. In the same DXA report, we will also collect data of bone mineral mass/density and lean mass. Additional total fat and VAT estimates will be obtained via bioimpedance analysis (SC-331S Body Composition Analyzer, Tanita Corporation of America, Inc., Arlington Height, IL, USA).

Serum lipid profile, adiponectin, leptin, and hs-CRP, and urine 8-OHdG

Rationale In our previous animal study, we have shown that TT supplementation to diet reduced serum triglycerides and proinflammatory adipokines in obese mice [19]. In a randomized double-blinded, placebo-controlled trial, we reported that 12-week TT supplementation (90% δ-TT+10% γ-TT) at 300 mg/day lowered urine 8-OHdG, an oxidative stress biomarker in osteopenic PMW [46].

Methods We will ship blood samples to Covenant Medical Laboratory, Lubbock, TX for lipid profile (total cholesterol, triglycerides, low-density lipoprotein, and high-density lipoprotein) and hs-CRP analysis. The

level of serum adiponectin and leptin, and urine 8-OHdG (oxidative stress biomarker) will be measured at Dr. Shen's lab using ELISA.

Lipid metabolism-related gene expression in adipose tissue

Rationale Obesity is characterized by altered mitochondrial activity and lipid metabolism, promoting accumulation of triglycerides in tissues, mainly white adipose tissue [47]. Defective mitochondrial biogenesis and fatty acid oxidative metabolic pathways in subcutaneous adipose tissue during acquired obesity, leading to the metabolic disturbance in obesity [48]. Based on our animal findings [19], we will focus on gene expression associated with lipid β-oxidation and fatty acid synthesis of subcutaneous adipose tissue sampled before and after TT regime.

Methods Total RNA will be isolated from frozen adipose samples using Qiagen RNeasy Lipid Kit (Qiagen, Valencia, CA) and reversely transcribed into cDNA using iScript Reverse Transcription Supermix. Conventional quantitative real-time PCR will be performed using SYBR Green with respective primers for FAS and CPT-1 (Human ProbeLibrary Gene) [49]. The relative gene expression will be assessed using the ΔΔCT method [50] and GAPDH will be used as internal control for normalization [49]. In addition, total RNA will be extracted from de-identified serum and adipose tissue for later stranded mRNA-sequencing.

Concentrations of OxL and eCB in plasma and adipose tissue.

Rationale Aging and decline of estrogen are factors that contribute to weight gain in PMW, and approaches, such as anti-inflammatory potential in TT, to reduce inflammation may likely combat obesity. OxLs and eCBs are classes of bioactive fatty acid metabolites with many structural members that influence insulin signaling, adipose function and inflammation through autocrine, paracrine and endocrine mechanisms [4, 5]. In addition, physical activity is reported to increase specific levels of eCB [51] leading to responses of well-being. In our recent animal study, male mice fed with TT-supplemented diet experienced reduced thromboxane B2 (TXB2) and 13-hydroxyoctadecadienoic acid (13-HODE), and increased 12-HEPE (anti-

platelet) and some essential amino acids (i.e., lysine and methionine) (Shen et al. unpublished findings). The former exerting complex functions like 13-HODE but lowering TXB2, a product of TX2, could have implications on the function of TXA2 (target of aspirin). Thus, higher TXB2 means increased catabolism of TXA2 and less thrombosis may result in reducing the risk for atherosclerosis. Our animal study suggests that TT may influence macronutrient metabolism in obese PMW (Shen et al. unpublished findings). Identification of novel metabolites and metabolic pathways, especially antioxidant and oxidative stress metabolites, of TT, in serum and adipose tissue of subjects will help to understand the composition and roles of these bioactive compounds in management of obesity. However, there has been no study to evaluate the effect of TT on any metabolomics profiling in obese population. Herein, we will use the same targeted metabolomics to measure OxL and eCB.

Methods Targeted metabolomics analyses of OxL and eCBs The levels of OxL and eCB in plasma and adipose tissue will be quantitatively analyzed using UPLC tandem mass spectrometers based on our previously published work [4]. Briefly, 60 mg Oasis-HLB solid phase extraction columns (SPE) (Waters Corp., Milford, MA) are placed on a vacuum manifold, cleaned and conditioned, then spiked with deuterated OxLs and eCBs internal standards. The serum samples are thawed on ice and a 200 µL volume transferred to the SPE, up-diluted to 3 mL with 5% MeOH/0.1% acetic acid, and gravity extracted. Columns are then washed with 3 mL the 5% MeOH solution. OxLs and eCBs are eluted from the SPE with 0.2 mL MeOH followed by 1.5 mL ethyl acetate, by gravity. Solvent is removed by vacuum and samples are reconstituted in 50 µL MeOH containing the internal standard 1-cyclohexyl-3-dodecyl-urea (Sigma, Aldrich, St. Louis, MO) then, filtered at 0.1 µm. Analytes are chromatographically separated on a 2.1×150 mm, 1.7µm Acquity BEH C18 (Waters Corp.) column using 0.1% acetic acid and acetonitrile gradient. The OxLs and eCBs profiles are acquired using electrospray ionization and tandem mass spectrometry by back-to-back (+)-mode/(-)-mode injections on a Sciex API 4000-QTRAP (Pleasanton, CA). A complete list of measured analytes with retention times, acquisition parameters, and detection limits are shown for the OxLs and eCBs in a previously published article [4], respectively.

Abundance and the composition of intestinal bacteria in feces.

Rationale There has been a growing body of evidence to implicate altered intestinal microbiota in the development of obesity. The commensal microflora of the human GI actively participates in the processes of energy harvest and expenditure. Biochemical crosstalk between gut bacteria and the host has a critical impact on the metabolic status (OxLs and eCBs) of the human body. In our previous animal study, we reported that relative to the high-fat diet (HFD) control group, the HFD+TT at 800mg/kg diet group increased abundance of *Akkermansia g* (of the phylum *Verrucomicrobia*) and *S24-7 family (class Bacteroidetes)*, and decreased that of *Clostridiales* order, and *Oscillospira genus* (of the phylum *Firmicutes*). Our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen et al. unpublished findings). Therefore, in this pilot study, we would like to explore how dietary TT supplement affects the abundance and composition of gut microbiome in PMW.

Methods 16S Metagenome analysis of fecal samples Bacterial DNA will be isolated from feces using a MoBio PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA). The microbial diversity of fecal samples will be evaluated using metagenomic 16S rDNA gene sequencing technique. Variability in the bacterial V3-V4 region will be used to identify bacterial abundances in both treated and untreated subjects. Quantitative insights into microbial ecology (QIIME, version 1.8.0) will be used for data analysis according to previous published method [52]. In brief, one μL of DNA sample (20 ng/μL) will be used for a 25 μL PCR reaction. The V3-V4 regions of the 16S rRNA gene region will be amplified using gene specific primer pairs. PCR amplicons from all samples will be normalized using SequalPrep plate Normalization kit (Thermo Fisher Scientific, MA). Equal volumes of samples will be pooled and quantified using Qubit 2.0 (Thermo Fisher Scientific, MA) and quality will be checked using TapeStation (Agilent Technologies, CA). Libraries will be sequenced on MiSeq (Illumina Inc., CA) using a 600 cycle v3 sequencing kit. Post sequencing, reads will be filtered based on quality score and aligned on 16S database using our custom pipeline involving PEAR, and

QIIME software's. Final OTUs will be taxonomically classified using BLASTn against the GreenGenes database, and compiled into each taxonomic level into both "counts" and "percentage" files.

Statistical Analysis

Sample demographics and all outcome measures will be summarized by descriptive statistics and bivariate tests within the whole sample and between treatment groups (placebo vs. TT) and demographic subgroups (e.g., age, BMI). The correlations among outcomes and potential covariates (e.g., demographic variables, dietary and lifestyle changes, concomitant medications) will be examined in a preliminary analysis. In order to examine the effects of the TT intervention, hierarchical linear modeling will be conducted to examine overall treatment group difference across time (i.e., group effect), linear or nonlinear change over time (i.e., time effect), and group difference in this change (i.e., group-by-time interaction). Models will be adjusted for identified covariates, thereby providing unbiased effect estimates. If present, missing data will be handled by Monte Carlo Markov Chain (MCMC) multiple imputation [53]. A large number of (e.g., 200) imputed datasets will be generated and then analysis results from each imputed dataset will be combined together to make valid statistical inference. Gut microbiome data will be analyzed designed to pinpoint similarities and differences of bacterial communities between the treated and untreated subjects.

Data for OxL and eCB will be expressed by descriptive statistics. If necessary, OxL and eCB data will be transformed to normality by an iterative process using imDEV v1.4.2 [54] and missing data will be handled by MCMC multiple imputation. Statistical significance of the means will be evaluated using 2-tailed t-test at 0.05 alpha level. To account for multiple testing, the false discovery rate will be computed and reported for each comparison using the q-value method [55]. A summary of the numbers of biochemical (or called metabolite) that achieved statistical significance will be reported. OxL and eCB data will be combined with measurements for partial-least squares discriminate analysis. To improve clustering performance, the reduced data matrix variables (complete data set) will be subjected to orthogonal signal correction using the procedures of Wehrens [56] and partial least squares discriminate analysis of Variable Importance Plot filtered data set using the

orthogonal scores PLSR component of the R 'pls' package [57]. Iterative rotations for uniform projection of discriminate variables onto a single latent variable will be done.

Data of microbiota OTU reads will be imported into R version 3.4.3, and all statistical analyses will be performed using the vegan and phyloseq packages unless specifically noted. OTU richness will be determined by Chao1 and the OTU evenness will be determined by several diversity indices (i.e., Shannon, Simpson, Inverse Simpson, and Fisher). Group differences in α-diversity (richness and diversity) will be evaluated by ANOVA. Between-specimen diversity (β-diversity) will be assessed by the Bray–Curtis method and then visualized using nonmetric multidimensional scaling. Group differences in β-diversity will be measured using permutational multivariate analysis of variance with 500 permutations. Group differences among taxa- and genus-level OTUs will be assessed by pairwise comparisons on read counts using Negative Binomial Wald Tests from the DESeq2 package. All tests will be corrected for multiple comparisons using the false discovery rate correction by Benjamini and Hochberg. Associations among selected variables will be assessed with Spearman's correlations. The statistical significance is as determined at $P \leq 0.05$.

Data management and data collection

Researchers will keep information obtained in this study confidential except as required by law. All participant questionnaires, records, and data will be coded and properly stored in locked cabinets. Only study investigators will have access to those files linking a person's study number to his/her name. Those forms will include Informed Consent Form, HIPAA Authorization form, all recruitment questionnaires, and all other related materials.

The principle investigator will be responsible for oversight of data management of the trial. A certified monitor from Clinical Research Institute, independent from research team and sponsor, will assist the principle investigator in monitoring data collection before the datasets are delivered to study biostatistician. Questionnaire data will be entered on various forms and then entered into the Excel database. Laboratory data will also be verified and entered into an Excel database. Data of eligibility, medical records, attrition rate, and compliance rate will all be entered into an Excel database. Data queries including missing values will be

referred to the principle investigator. The principle investigator will have access to the final trial dataset and disclosure of contractual agreements. The principle investigator will also incorporate any correction or addition into the datasets. Finally, a clean dataset without any identification will be generated and delivered to the biostatistician for statistical analysis.

Ethics

The study protocol and template consent forms have been reviewed and approved by the Bioethics Committee of the Texas Tech University Health Sciences Center. An informed consent form will be signed by a participant before enrolling in the study. Any modifications to the protocol, which may affect the conduct of the study, potential benefits to the study participants, or their safety will be reported to the ethics committee for all necessary amendments. All study-related information will be stored securely at the study site in local cabinets, in an area with limited access (databases will be secured with a password-protected access system).

Patient and Public Involvement

The patients and the public will not be involved in the design, assessment or conduction of this trial. Recruitment information is available on local newspaper, senior newsletter, and clinics. This information will allow postmenopausal women to contact research coordinator if they are willing to participant. Postmenopausal women with adverse outcomes will be followed up throughout their intervention and will be referred to experts. Feedback will be sought from all participants at the end of the study to assess burden of intervention and to help develop future trials.

Dissemination

The findings of this study will be submitted to a peer-referred journal in the areas of obesity or nutrition/functional food. Abstracts will be submitted to relevant national and international conferences.

The effectiveness of annatto-extracted tocotrienols for obesity-associated parameters in postmenopausal obese women is still unknown. Our study, carried out at a single research center with experience in conducting independent, investigator-initiated FDA-IND clinical trial, is intended to address a gap in the field and will test the safety and efficacy of annatto-extracted tocotrienols supplementation for anti-obesity capacities in postmenopausal obese women.

COMPETING INTERESTS

This study is funded by School of Medicine, Texas Tech University Health Sciences Center, Laura W. Bush Institute for Women's Health, and American River Nutrition, LLC. None of the authors declared any conflicts of financial interest.

AUTHOR'S CONTRIBUTIONS

AA and CLS conceptualized the study. AA, GT, JL, LM, BAW, AAS, KS, SW, RW, and CLS designed this trial. CLS developed the first draft of the manuscript and the rest of coauthors participated in the revision of subsequent drafts. All authors approved the final draft of the manuscript. CLS made final decision to submit the report for publication.

Funding This study is funded by School of Medicine, Texas Tech University Health Sciences Center, Laura W. Bush Institute for Women's Health, and American River Nutrition, LLC. These three funding agencies have no role in the study conduct, dissemination, and interpretation of results. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of funders.

References

[1] New WHO report: deaths from noncommunicable diseases on the rise, with developing world hit hardest, Cent Eur J Public Health 19(2) (2011) 114, 120.

[2] M.F. Gregor, G.S. Hotamisligil, Inflammatory mechanisms in obesity, Annu Rev Immunol 29 (2011) 415-45.

[3] W.S. Powell, J. Rokach, Biosynthesis, biological effects, and receptors of hydroxyeicosatetraenoic acids (HETEs) and oxoeicosatetraenoic acids (oxo-ETEs) derived from arachidonic acid, Biochim Biophys Acta 1851(4) (2015) 340-55.

[4] B.A. Watkins, J. Kim, A. Kenny, T.L. Pedersen, K.L. Pappan, J.W. Newman, Circulating levels of endocannabinoids and oxylipins altered by dietary lipids in older women are likely associated with previously identified gene targets, Biochim Biophys Acta 1861(11) (2016) 1693-1704.

[5] J. Kim, M.E. Carlson, G.A. Kuchel, J.W. Newman, B.A. Watkins, Dietary DHA reduces downstream endocannabinoid and inflammatory gene expression and epididymal fat mass while improving aspects of glucose use in muscle in C57BL/6J mice, Int J Obes (Lond) 40(1) (2016) 129-37.

[6] M.M. Heemskerk, M. Giera, F.E. Bouazzaoui, M.A. Lips, H. Pijl, K.W. van Dijk, V. van Harmelen, Increased PUFA Content and 5-Lipoxygenase Pathway Expression Are Associated with Subcutaneous Adipose Tissue Inflammation in Obese Women with Type 2 Diabetes, Nutrients 7(9) (2015) 7676-90.

[7] K. Moller, A.I. Ostermann, K. Rund, S. Thoms, C. Blume, F. Stahl, A. Hahn, N.H. Schebb, J.P. Schuchardt, Influence of weight reduction on blood levels of C-reactive protein, tumor necrosis factor-alpha, interleukin-6, and oxylipins in obese subjects, Prostaglandins Leukot Essent Fatty Acids 106 (2016) 39-49.

[8] R. Horrillo, A. Gonzalez-Periz, M. Martinez-Clemente, M. Lopez-Parra, N. Ferre, E. Titos, E. Moran-Salvador, R. Deulofeu, V. Arroyo, J. Claria, 5-lipoxygenase activating protein signals adipose tissue inflammation and lipid dysfunction in experimental obesity, J Immunol 184(7) (2010) 3978-87.

[9] D. Poeckel, C.D. Funk, The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease, Cardiovasc Res 86(2) (2010) 243-53.

[10] S.K. Chakrabarti, B.K. Cole, Y. Wen, S.R. Keller, J.L. Nadler, 12/15-lipoxygenase products induce inflammation and impair insulin signaling in 3T3-L1 adipocytes, Obesity (Silver Spring) 17(9) (2009) 1657-63.

[11] A.D. Dobrian, D.C. Lieb, B.K. Cole, D.A. Taylor-Fishwick, S.K. Chakrabarti, J.L. Nadler, Functional and pathological roles of the 12- and 15-lipoxygenases, Prog Lipid Res 50(1) (2011) 115-31.

[12] C.S. Nunemaker, M. Chen, H. Pei, S.D. Kimble, S.R. Keller, J.D. Carter, Z. Yang, K.M. Smith, R. Wu, M.H. Bevard, J.C. Garmey, J.L. Nadler, 12-Lipoxygenase-knockout mice are resistant to inflammatory effects of obesity induced by Western diet, Am J Physiol Endocrinol Metab 295(5) (2008) E1065-75.

[13] D.D. Sears, P.D. Miles, J. Chapman, J.M. Ofrecio, F. Almazan, D. Thapar, Y.I. Miller, 12/15-lipoxygenase is required for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice, PLoS One 4(9) (2009) e7250.

[14] E. Borgeson, C. Godson, Resolution of inflammation: therapeutic potential of pro-resolving lipids in type 2 diabetes mellitus and associated renal complications, Front Immunol 3 (2012) 318.

[15] J.W. Newman, T.L. Pedersen, V.R. Brandenburg, W.S. Harris, G.C. Shearer, Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects, PLoS One 9(11) (2014) e111471.

[16] Y.J. Suzuki, M. Tsuchiya, S.R. Wassall, Y.M. Choo, G. Govil, V.E. Kagan, L. Packer, Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: implication to the molecular mechanism of their antioxidant potency, Biochemistry 32(40) (1993) 10692-9.

[17] M. Meydani, Vitamin E, Lancet 345(8943) (1995) 170-5.

[18] G.C. Burdeos, K. Nakagawa, F. Kimura, T. Miyazawa, Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats, Lipids 47(5) (2012) 471-81.

[19] L. Allen, L. Ramalingam, K. Menikdiwela, S. Scoggin, C.L. Shen, M.D. Tomison, G. Kaur, J.M. Dufour, E. Chung, N.S. Kalupahana, N. Moustaid-Moussa, Effects of delta-tocotrienol on obesity-related adipocyte hypertrophy, inflammation and hepatic steatosis in high-fat-fed mice, J Nutr Biochem 48 (2017) 128-137.
[20] W.Y. Wong, H. Poudyal, L.C. Ward, L. Brown, Tocotrienols reverse cardiovascular, metabolic and liver changes in high carbohydrate, high fat diet-fed rats, Nutrients 4(10) (2012) 1527-41.
[21] T.W. Chou, C.Y. Ma, H.H. Cheng, Y.Y. Chen, M.H. Lai, A rice bran oil diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes, J Clin Biochem Nutr 45(1) (2009) 29-36.
[22] S. Das, S. Mukherjee, I. Lekli, N. Gurusamy, J. Bardhan, U. Raychoudhury, R. Chakravarty, S. Banerji, A.A. Knowlton, D.K. Das, Tocotrienols confer resistance to ischemia in hypercholesterolemic hearts: insight with genomics, Mol Cell Biochem 360(1-2) (2012) 35-45.
[23] A.A. Qureshi, N. Qureshi, J.O. Hasler-Rapacz, F.E. Weber, V. Chaudhary, T.D. Crenshaw, A. Gapor, A.S. Ong, Y.H. Chong, D. Peterson, Dietary tocotrienols reduce concentrations of plasma cholesterol, apolipoprotein B, thromboxane B2, and platelet factor 4 in pigs with inherited hyperlipidemias, Am J Clin Nutr 53(4 Suppl) (1991) 1042S-1046S.
[24] T. Watkins, P. Lenz, A. Gapor, M. Struck, A. Tomeo, M. Bierenbaum, gamma-Tocotrienol as a hypocholesterolemic and antioxidant agent in rats fed atherogenic diets, Lipids 28(12) (1993) 1113-8.
[25] R.S. Wong, A.K. Radhakrishnan, T.A. Ibrahim, S.K. Cheong, delta- and gamma-tocotrienols induce classical ultrastructural apoptotic changes in human T lymphoblastic leukemic cells, Microsc Microanal 18(3) (2012) 462-9.
[26] S.B. Budin, F. Othman, S.R. Louis, M.A. Bakar, S. Das, J. Mohamed, The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats, Clinics (Sao Paulo) 64(3) (2009) 235-44.
[27] E.C. Heng, S.A. Karsani, M. Abdul Rahman, N.A. Abdul Hamid, Z. Hamid, W.Z. Wan Ngah, Supplementation with tocotrienol-rich fraction alters the plasma levels of Apolipoprotein A-I precursor, Apolipoprotein E precursor, and C-reactive protein precursor from young and old individuals, Eur J Nutr 52(7) (2013) 1811-20.
[28] A.A. Qureshi, B.A. Bradlow, L. Brace, J. Manganello, D.M. Peterson, B.C. Pearce, J.J. Wright, A. Gapor, C.E. Elson, Response of hypercholesterolemic subjects to administration of tocotrienols, Lipids 30(12) (1995) 1171-7.
[29] A.A. Qureshi, D.A. Khan, W. Mahjabeen, N. Qureshi, Dose-dependent modulation of lipid parameters, cytokines and RNA by ¦Ä-tocotrienol in hypercholesterolemic subjects restricted to AHA Step-1 diet, 2015.
[30] A.A. Qureshi, D.A. Khan, W. Mahjabeen, A.M. Trias, N. Silswal, N. Qureshi, Impact of δ-Tocotrienol on Inflammatory Biomarkers and Oxidative Stress in Hypercholesterolemic Subjects, Journal of Clinical and Experimental Cardiology 6(4) (2015).
[31] N. Zaiden, W.N. Yap, S. Ong, C.H. Xu, V.H. Teo, C.P. Chang, X.W. Zhang, K. Nesaretnam, S. Shiba, Y.L. Yap, Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion, J Atheroscler Thromb 17(10) (2010) 1019-32.
[32] N. Haghighat, M. Vafa, S. Eghtesadi, I. Heidari, A. Hosseini, A. Rostami, The Effects of Tocotrienols Added to Canola Oil on Microalbuminuria, Inflammation, and Nitrosative Stress in Patients with Type 2 Diabetes: A Randomized, Double-blind, Placebo-controlled Trial, Int J Prev Med 5(5) (2014) 617-23.
[33] P. Irandoost, M. Ebrahimi-Mameghani, S. Pirouzpanah, Does grape seed oil improve inflammation and insulin resistance in overweight or obese women?, Int J Food Sci Nutr 64(6) (2013) 706-10.
[34] B.B. Aggarwal, C. Sundaram, S. Prasad, R. Kannappan, Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases, Biochem Pharmacol 80(11) (2010) 1613-31.
[35] C.L. Shen, S. Yang, M.D. Tomison, A.W. Romero, C.K. Felton, H. Mo. Tocotrienols supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: a 12-week randomized double-blinded placebo-controlled trial. Osteoporosis International 2018; 29(4):881-9.

[36] C.L. Craig, A.L. Marshall, M. Sjostrom, A.E. Bauman, M.L. Booth, B.E. Ainsworth, M. Pratt, U. Ekelund, A. Yngve, J.F. Sallis, P. Oja, International physical activity questionnaire: 12-country reliability and validity, Med Sci Sports Exerc 35(8) (2003) 1381-95.
[37] S. Choquette, E. Riesco, E. Cormier, T. Dion, M. Aubertin-Leheudre, I.J. Dionne, Effects of soya isoflavones and exercise on body composition and clinical risk factors of cardiovascular diseases in overweight postmenopausal women: a 6-month double-blind controlled trial, Br J Nutr 105(8) (2011) 1199-209.
[38] M.A. Pervez, D.A. Khan, A. Ijaz, S. Khan, Effects of Delta-tocotrienol Supplementation on Liver Enzymes, Inflammation, Oxidative stress and Hepatic Steatosis in Patients with Nonalcoholic Fatty Liver Disease, Turk J Gastroenterol 29(2) (2018) 170-176.
[39] W.Y. Wong, L.C. Ward, C.W. Fong, W.N. Yap, L. Brown, Anti-inflammatory gamma- and delta-tocotrienols improve cardiovascular, liver and metabolic function in diet-induced obese rats, Eur J Nutr 56(1) (2017) 133-150.
[40] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J 22(3) (2008) 659-61.
[41] A. Mahipal, J. Klapman, S. Vignesh, C.S. Yang, A. Neuger, D.T. Chen, M.P. Malafa, Pharmacokinetics and safety of vitamin E delta-tocotrienol after single and multiple doses in healthy subjects with measurement of vitamin E metabolites, Cancer Chemother Pharmacol 78(1) (2016) 157-65.
[42] C.-L. Shen. H. Mo, S.Yang, S. Wang, C.K. Felton, M.D. Tomison, I.M. Soelaiman. Safety and efficacy of tocotrienol supplementation for bone health in postmenopausal women: protocol for a dose-response double-blinded placebo-controlled randomized trial. BMJ Open. 2016; 6(12): e012572.
[43] C.-L. **Shen, S.** Wang, S. Yang, M.D. Tomison, M. Abbasi, L. Hao, S. Scott, M.S. Khan, A.W. Romero, C.K. Felton, H. Mo, A 12-week evaluation of annatto tocotrienol supplementation for postmenopausal women: safety, quality of life, body composition, physical activity, and nutrient intake. BMC Complementary and Alternative Medicine (2018). 18(1):198.
[44] G.M. Tinsley, Proportional bias between dual-energy x-ray absorptiometry and bioelectrical impedance analysis varies based on sex in active adults consuming high- and low-carbohydrate diets, Nutr Res 42 (2017) 85-100.
[45] G.M. Tinsley, Reliability and agreement between DXA-derived body volumes and their usage in 4-compartment body composition models produced from DXA and BIA values, J Sports Sci 36(11) (2018) 1235-1240.
[46] C.L. Shen, S. Yang, M.D. Tomison, A.W. Romero, C.K. Felton, H. Mo, Tocotrienol supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: a 12-week randomized double-blinded placebo-controlled trial, Osteoporos Int 29(4) (2018) 881-891.
[47] C. Christodoulides, C. Lagathu, J.K. Sethi, A. Vidal-Puig, Adipogenesis and WNT signalling, Trends Endocrinol Metab 20(1) (2009) 16-24.
[48] S. Heinonen, J. Buzkova, M. Muniandy, R. Kaksonen, M. Ollikainen, K. Ismail, A. Hakkarainen, J. Lundbom, N. Lundbom, K. Vuolteenaho, E. Moilanen, J. Kaprio, A. Rissanen, A. Suomalainen, K.H. Pietilainen, Impaired Mitochondrial Biogenesis in Adipose Tissue in Acquired Obesity, Diabetes 64(9) (2015) 3135-45.
[49] J.P. Walhin, J.D. Richardson, J.A. Betts, D. Thompson, Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men, J Physiol 591(24) (2013) 6231-43.
[50] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25(4) (2001) 402-8.
[51] B.A. Watkins, Endocannabinoids, exercise, pain, and a path to health with aging, Mol Aspects Med 64 (2018) 68-78.
[52] M.A.I. Al-Obaide, R. Singh, P. Datta, K.A. Rewers-Felkins, M.V. Salguero, I. Al-Obaidi, K.R. Kottapalli, T.L. Vasylyeva, Gut Microbiota-Dependent Trimethylamine-N-oxide and Serum Biomarkers in Patients with T2DM and Advanced CKD, J Clin Med 6(9) (2017).
[53] C.K. Enders, Applied missing data analysis, Guilford Publications, New York, 2010.

[54] D. Capitanio, M. Vasso, S. De Palma, C. Fania, E. Torretta, F.P. Cammarata, V. Magnaghi, P. Procacci, C. Gelfi, Specific protein changes contribute to the differential muscle mass loss during ageing, Proteomics 16(4) (2016) 645-56.
[55] G.R. Adams, B.M. Hather, K.M. Baldwin, G.A. Dudley, Skeletal muscle myosin heavy chain composition and resistance training, J Appl Physiol (1985) 74(2) (1993) 911-5.
[56] R. Wehrens, Chemometrics with R Multivariate Data Analysis in the Natural Sciences and Life Sciences, (2011).
[57] B.-H. Mevik, R. Wehrens, The pls Package: Principal Component and Partial Least Squares Regression in R, 2007 18(2) (2007) 23.

Table 1. Timetable of activities planned during the course of the study directly related to participants

	Weeks										
Activity	-4	-2	0	3	6	9	12	15	18	21	24
Enrollment	x										
Screening (liver function, TSH)		x									
Randomization			x								
Intervention			x	x	x	x	x	x	x	x	x
Body composition			x								x
Fat biopsy (optional)			x								x
Blood for outcomes			x				x				x
Liver function assessment			x				x				x
Pill counts			x				x				x
Food intake assessment			x								x
Physical activity survey			x								x
Self-report adverse event			x	x	x	x	x	x	x	x	x

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number of manuscript
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	1
	2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	3	Date and version identifier	n/a
Funding	4	Sources and types of financial, material, and other support	17
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1, 17
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	17
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	n/a
Introduction			

Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3-5
	6b	Explanation for choice of comparators	5
Objectives	7	Specific objectives or hypotheses	5
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5-6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6-7
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8-9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	9-10
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n/a
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	10-14
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	14

Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	6
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	9
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	9
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	13-15
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	n/a
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	16
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	14-15
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	14
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14-15

Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	17
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n/a
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	10
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	16
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	6
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	16
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	6
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	16-17
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	17
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n/a
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	17

	31b	Authorship eligibility guidelines and any intended use of professional writers	n/a
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	n/a
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	n/a

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.