Study Protocol Title:

Blackcurrant Modifies Gut Microbiota and Reduces the Risk of Postmenopausal Osteoporosis and Cardiovascular Disease: A Pilot Randomized Clinical Trial

NCT number: NCT04431960

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Study Protocol and Statistical Analysis Plan

1. Purpose/Introduction:

This study aimed to evaluate the dose-dependent effects of blackcurrant supplemen-tation on bone density in adult women and its relationship to bone metabolism. The fea-sibility of the pilot clinical study was also assessed. For this purpose, we conducted a pilot double-blind, randomized, placebo-controlled clinical study with blackcurrant supple-mentation for six months in peri- and early postmenopausal women aged 45–60. The primary endpoint was whole-body BMD. In addition, to delineate the underlying mecha-nisms of the action, changes in serum markers of bone metabolism following blackcurrant supplementation were also assessed.

2. Materials and Methods

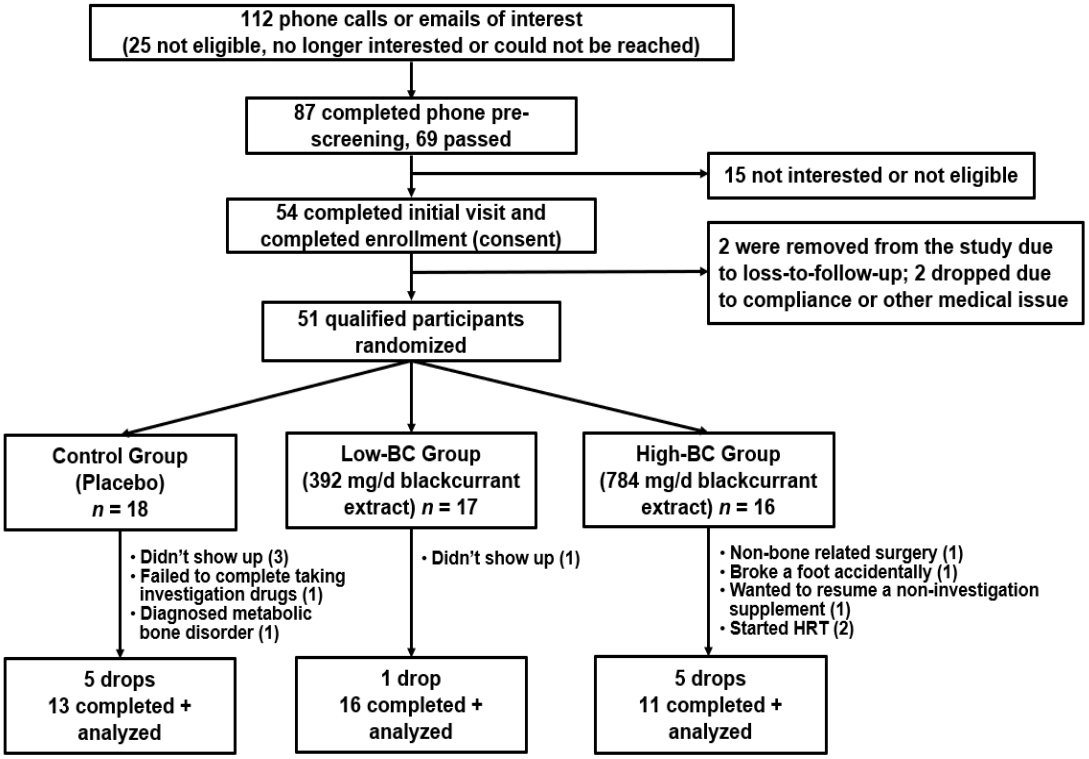
2.1. Study Participants

Forty peri- and postmenopausal women aged 45–60 were initially recruited from northeastern Connecticut through newspaper advertisements, flyers, and email. The inclusion criteria for this study were: (1) women aged 45–60 years old; (2) not on HRT for at least one year before the initiation of the study; (3) maintaining normal exercise level (<7 h/week) and willing to avoid exercise 24-h prior to blood and stool sampling and 12-h prior to bone measurements; (4) willing to ingest a dietary blackcurrant supplement or placebo as well as 400 mg calcium and 500 IU vitamin D daily; (5) willing to avoid other dietary supplements for the duration of the study, (6) willing to avoid intake of foods extremely rich in anthocyanins and fermented dairy products, (7) willing to have 3 blood draws and 2 bone scans and (8) willing to take a urine pregnancy test before each bone scan. Exclusion criteria included: (1) those with metabolic bone disease, renal disease, cancer, cardiovascular disease, diabetes mellitus, respiratory disease, gastrointestinal disease, liver disease, or other chronic diseases, (2) heavy smokers (>20 cigarettes/day), (3) perimenopausal women with any chance or plan of pregnancy, (4) taking prescription medications known to alter bone and Ca metabolism; (5) taking anabolic agents; (6) alcohol consumption exceeding 2 drinks/day. Subjects who met the recruitment criteria upon initial phone screening were immediately invited to the study laboratory at the University of Connecticut in Storrs, Storrs, CT, USA.

2.2. Study Design

Upon signing the consent form, an initial physical examination (body weight, height, waist circumference, and blood pressure) was conducted. The participants were also interviewed about their medical history and dietary behaviors by study personnel. Participants had a urine pregnancy test to confirm that they were not pregnant and thus eligible for the bone density measurement using dual-energy X-ray absorptiometry (DXA). All eligible participants completed an additional consent form for the DXA

assessment and scheduled their bone mass measures on or within ± 3 days of study visit 1 (month 0) and 3 (months 6) at the University of Connecticut Korey Stringer Institute Human Performance Laboratory. Participants were asked to refrain from taking the provided calcium supplements the day before the exam (24 h) to limit potential interference with the DXA measurements.



The participants were taught how to complete 3-day food records (FR) and physical activity records and asked to complete them one week prior to each study visit at months 0, 3, and 6. Subjects who were taking any dietary supplements that are known to affect bone metabolism were asked to stop taking them. After the initial screening visit, subjects underwent a 2-week equilibration period, followed by a 6-month clinical trial period. To avoid potential bone deterioration related to calcium and vitamin D deficiency, all participants took a calcium citrate caplet daily that includes 400 mg calcium and 500 IU vitamin D (Bayer AG, Leverkusen, Germany) beginning 2 weeks before the study and lasting for the duration of the study. After the 2-week equilibration period, study participants were randomly assigned to three groups and asked to consume: (1) one capsule containing 392 mg of blackcurrant powder (low BC group); (2) two capsules containing 392 mg blackcurrant powder per capsule, total 784 mg of blackcurrant powder (high BC group); or (3) one placebo capsule (control group) daily for 6 months. Each 392 mg blackcurrant extract contained 176 mg anthocyanins (min. 40% delphinidin-3-rutinoside, 35% cyanidin-3-rutinoside, 10% delphinidin-3-glucoside and 7% cyanidin-3-glucoside). One capsule was equivalent to about 142 fresh blackcurrants. The blackcurrant powder and composition information was provided by Just the Berries, New Zealand (Just the Berries PD Corporation, Los Angeles, CA, USA). The placebo was

an identical-looking capsule that contained rice powder (supplied by Beehive Botanicals Hayward, WI, USA). The extract and placebo were encapsulated in vegetarian capsules and packaged into coded containers for daily dosing to participants (Beehive Botanicals Hayward, WI). Distribution of the supplements was performed blindly at the study center at month 0 (study visit 1: after the 2-week equilibration period) and month 3 (study visit 2).

Aside from the exclusion of dietary supplements, foods extremely rich in anthocyanins (all berries, grapes, red wine, and berry juices), and fermented dairy products containing viable *Bifidobacteria* or *Lactobacilli*, all participants were instructed to keep their usual dietary habits for the duration of the study. Subjects provided a 12-h fasting blood sample and stool specimen at each study visit at months 0, 3, and 6 for biochemical and metagenome sequencing analysis, and a physical examination (height, weight, waist circumference, blood pressure) was conducted at the initial visit (month 0), month 3, and the end of the treatment period (month 6). This clinical trial was registered at ClinicalTrials.gov (NCT04431960). The proposed project and its procedures were reviewed and approved by the University of Connecticut Institutional Review Board (HR20-0035) prior to the initiation of the project.

2.3. Dietary Intake and Physical Activity Assessment

During the trial, participants were asked to complete 3-day FR and physical activity records at months 0, 3, and 6. The FR data were analyzed using the Nutrition Data System for Research (NDSR, University of Minnesota Nutrition Coordinating Center, Minneapolis, MN, USA). The 3-d FR data included all foods and beverages consumed during two non-consecutive weekdays plus a weekend day of the week following the test visits. The physical activity records were used to estimate the metabolic equivalent of task (MET) scores to confirm participants maintained normal exercise levels (<7 h/week) during the entire intervention period and avoided exercise 24-h prior to study visits.

2.4. Bone Density Assessments

BMD analyses were conducted at the University of Connecticut Korey Stringer Institute by a licensed radiologic technician at baseline and 6 months using DXA (GE Healthcare Lunar) equipped with appropriate software for whole-body, head, arms, legs, trunk, ribs, spine, and pelvis BMD. Specific positioning and analysis guidelines were followed according to the operator's manual. Calibration, maintenance, and quality control of the equipment were routinely conducted following the maintenance instructions in the manual.

2.5. Blood Collection and Pretreatments

Fasted blood samples were collected at months 0, 3, and 6. The blood samples (80 mL) from subjects who fasted for 12 h were used to determine biomarkers for bone metabolism. Whole blood samples were collected in serum tubes (BD Vacutainer,

Mississauga ON, Canada), centrifuged at 3500× *g* for 15 min at 4 °C, divided into aliquots, and stored at −80°C until analyzed.

2.6. Measurements of Serum Biomarkers of Bone Metabolism

Changes in the amino-terminal propeptide of type 1 procollagen (P1NP), bone-specific alkaline phosphatase (BALP), osteocalcin (OC), carboxy-terminal crosslinked telopeptide of type 1 collagen (CTX1), and sclerostin at baseline, 3 months, and 6 months were measured in serum using commercially available ELISA kits.

3. Statistical Analysis

Data are reported as mean ± standard deviation (SD) unless specified otherwise. Differences with two-sided $p < 0.05$ were considered significant. Data were analyzed using analysis of variance methods with PROC MIXED in SAS (Version 9.4, SAS Institute, Cary, NC, USA) to determine the main and interaction effects of the two factors, treatment (0, 392, or 784 mg/day blackcurrants), and time (baseline, 3 months, and 6 months). The significance of differences in baseline characteristics was tested by ANOVA or Chi-square/Fisher's Exact test. The significance of changes in all outcome variables was tested by repeated-measures ANOVA with treatment as levels of between-subjects factors and time as levels of within-subjects factors. Percent changes in BMD and bone biomarkers between control and low BC groups and between control and high BC groups were evaluated using *t*-tests.