Official title: The acute effect of instant coffee in different forms on plasma metabolites

NCT number: XXX

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Study Protocol and Statistical Analysis Plan

The procedures are as follows:

1. Do not consume coffee, tea, chocolate, or other caffeinated foods and drinks for the whole day (i.e. 24hrs) before the study.
2. Take informed consent that explains what is in the capsule, what is done with the data, and the risks of a blood draw.
3. Venous fasting blood draw in the morning.
4. Ingestion of the capsule or a cup of coffee beverage after the blood draw.
5. Answer a short questionnaire (age, sex, race, education level, alcohol use, usual coffee and tea use [green, Oolong, black tea], intake of fruit, vegetables, legumes, whole grains, menopausal status, oral contraceptive use [affects caffeine metabolism])
6. Another venous blood draw after 1 hour.
7. Process and store the blood correctly.

Recruitment of study participants

Study participants (n=15) will be recruited via flyers (e.g., shopping malls, MRT stations, neighbourhood shops, community centres, food centres, tertiary institutions such as NUS), advertisements at on-campus notice boards, bus-stops and newspapers. Recruitment email within NUS will be sent by the respective administrative officers of the project team members. Recruitment will also be conducted through online platforms such as Telegram, Facebook and Instagram, as well as snowballing and convenience sampling through referrals. Participants will be screened for their eligibility over a phone call with a screening questionnaire before they are enrolled into the study.

A short screening questionnaire will be administered to each participant to obtain information on their basic demographics, lifestyle, and medical history. Inclusion criteria include individuals aged 21-64 years old and of Chinese ethnicity. Exclusion criteria include pregnancy, specific medication use including recent use of antibiotics, presence or history of diseases (e.g., cancer, cardiometabolic, endocrine), major surgical operations, depression, anxiety, caffeine intolerance, fear of needles and/or blood disorders. Written informed consent will be obtained from each participant prior to the

commencement of the study.

Briefing session and informed consent

A briefing session will take place before the day of the study visit through an arranged Zoom/phone call. Participants who opted for a zoom call will be sent a scheduled meeting link, where he/she will be briefed by a study team member and/or PI/ Co-I. Over the call, researchers will explain the project aims and intervention study, and answer questions that the participants may have. Participants will be given or emailed handouts on foods/drinks not allowed to be consumed one day (i.e. 24hrs) before the visit (if the visit is scheduled at 8am, participants should abstain from the stated food/drinks from the day before at 8am). Participants will also be reminded to bring their medication and dietary supplements along to visit 1 for recording purposes.

Written informed consent will be obtained from each participant on the day of the first study visit. This will be performed by a study team member and should be completed within half an hour prior to the start of the study. Only participants who consent and completed the study visit(s) will be compensated. Participants will be reimbursed $50 at the end of the second study visit. Participants who wish to withdraw after completing the first study visit will be reimbursed $20.

Pre-study Commencement:

Before commencing the study visits, participants will be requested to fast overnight (8-12h). Drinking plain unflavoured water is allowed. Participants will be told to avoid vigorous physical activity except for activities of daily living for at least 24 hours prior to the session.

The day (24 hrs) before the study visits, participants will also be requested to avoid certain foods and beverages that may confound the study design including coffee and tea, coffee or tea-flavoured foods, caffeine-containing beverages (i.e., Coca-Cola, energy drinks), supplements containing caffeine or of herbal/botanical origin, chocolate, and chocolate-containing foods. We will communicate this over a phone call/messaging app with the participants before commencing the study, and provide a pamphlet

containing pictures and a detailed list of “allowed” and “non-allowed” foods and beverages.

Study Visit and Blood Collection:

The 2 study visits will take place at Investigational Medicine Unit (IMU), NUS Yong Loo Lin School of Medicine, with random assignment to either coffee capsules first or coffee as a beverage first. Each participant should go through both capsule and beverage interventions. The number of participants randomised to start with the capsule will be similar to those starting with the beverage. During each visit, 12 mL of venous blood will be collected at 2 time points each – at fasting and 1 hour after the consumption of the coffee capsule/drink. During the first visit, we will also ask the participant to fill out a questionnaire about their personal characteristics (DOB, gender, education level, smoking habits, height, weight, usual [referring to the past year] consumption of coffee and tea [number of cups per day of caffeinated and decaffeinated], soft drinks with caffeine such as cola, energy drinks such as Red Bull, chocolate, and key foods in the diet such as fruits and vegetables, alcohol use, dietary supplement use, medication use, menopausal status (Annex E). The period between 2 study visits will range between 7 to 28 days.

Ingestion of coffee capsule

A single dose will be provided to participants, in the form of two capsule or one cup of coffee beverage containing 1.7 grams a commonly used commercially available instant coffee (NESCAFE Taster's Choice House Blend Coffee Stick Packs consisting of 100% coffee beans and no other ingredients). 1.7 grams of instant coffee is the amount recommended for 1 cup of instant coffee by the manufacturer. Based on our previous measurements of caffeine in this coffee (Wedick, N.M., et al. Effects of caffeinated and decaffeinated coffee on biological risk factors for type 2 diabetes: a randomized controlled trial. Nutr J 2011;10, 93) the amount of caffeine for this amount of coffee is 59 mg which is substantially lower than the amount of coffee in a standard cup of brewed coffee which typically contains 92 mg of caffeine and much lower than the maximum amount of caffeine recommended per occasion which is 200 mg (EFSA Panel on Dietetic

Products, Nutrition and Allergies. Scientific Opinion on the safety of caffeine. EFSA J. 2015;13:4102.). The capsule used will be gelatin capsules that are purchased from NTUC FairPrice online. The brand is Now Foods. While the capsules are bovine sourced gelatin, it is not certified Halal or Kosher. The product empty capsules are produced under GMP (https://www.nowfoods.com/now/about-now/seals-certifications/gmp).

Sample processing and extraction

Blood will be collected in 4 Vacutainer® serum tubes (6mL each) and allowed to clot/stand at room temperature. After 30-40 min, serum will be separated from entire blood by centrifugation (2000g, 10 min, 24°C) and aliquoted into 1.5mL tubes. The remaining portion of samples that are left after serums have been separated from the samples will be discarded immediately after separation. All samples will be processed on the same day that they are collected. Samples will be stored at -80°C until all samples have been collected at the end of the intervention study and processed in a single batch. Briefly, thawed samples will be added with deuterated internal standards (for measure of control for extraction). Serum samples will be de-proteinised with acidified methanol, centrifuged (4000g, 10 min, 4°C) [15] and transferred into glass vials.

After collection of the blood samples at NUS, they will be transported to the NTU laboratory for measurements. Remaining samples may be stored NUS Saw Swee Hock School of Public Health Tissue Repository for up to 10 years for future measurements that are related to biomarkers of coffee intake and biomarkers related to its potential health effects, with the approval from the ethics board.

Targeted and untargeted metabolomic profiling

Serum samples will undergo targeted metabolomic profiling at the Singapore Phenome Centre located at NTU, which has state-of-the-art mass spectrometers and strong technical support. Targeted detection and quantification of a list of previously proposed coffee biomarkers (see metabolite assay list below) will be conducted on the serum samples using a Waters Acquity UHPLC-TQ-MS, and commercially available standards and deuterated standards. For atractyligenin, its lack of standard previously limited its detection but we have successfully synthesised this compound from its atractyloside

precursor, which can be used for level l identification and accurate quantification in this project. A quality control extract of pooled serum from all study participants will be injected at the beginning and end of each run, and in-between every 10 samples.

Data processing and Compound identification

Quantification of the biomarker compounds will be processed using MassLynx (Waters-specific software).

Metabolite assay

1.Atractyligenin

2.Trigonelline (HCl)

3.Caffeine

4.Theobromine

5.Theophylline (1,3-Dimethylxanthine)

6.Paraxanthine (1,7-Dimethylxanthine)

7.Caffeic acid

8.3,4-dihydroxyhydrocinnamic acid (dihydrocaffeic acid)

9.Quercetin

10.Cyclo(Pro-val)

11.Cyclo(Leu-Pro)

12.5-Acetylamino-6-formylamino-3-methyluracil

13.5-Acetylamino-6-amino-3-methyluracil hydrate

14.3-CQA (Chlororgenic acid)

15.1-methylxanthine

16.Kaempferol

17.1-methyluric acid

18.1,7-dimethyluric acid

Statistical analysis

We will calculate mean changes in serum concentrations of biomarkers between the fasting state and after ingestion of the capsule or coffee with corresponding 95% confidence intervals. Furthermore, we will test whether changes in concentrations differ between the coffee and the capsule intervention using paired t-tests.