STUDY PROTOCOL

PROTOCOL TITLE:
Cardio-vascular protective effects of wolfberry in middle-aged and older adults

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1. Background

An aging population is a global phenomenon (*1*) and likewise, Singapore is experiencing a rapid increase in the mean population age as well (*2*). The process of aging results in a progressive decline of numerous physiological processes, leading to an increased risk of health complications and cardio-metabolic diseases (*3*). Since aging significantly affects the heart and arterial systems (*4*, *5*), it has been associated with an increased risk of developing cardiovascular disease (CVD) (*3*).

CVD is one of the world's leading causes of death (*6*). In Singapore, 30 % of all total deaths or 16 deaths daily are attributed to CVD in 2016 (*7*). Many significant risk factors for CVD have been identified (*8*) and endothelial dysfunction due to oxidative stress and inflammation in the artery is considered as a major risk factor for CVD development (*9–11*). Mounting evidence emphasised the importance of measuring clinically relevant markers of the endothelial dysfunction when conducting CVD-related research beyond assessing the conventional blood lipid profiles.

Endothelial dysfunction-related clinical markers include endothelial progenitor cells (EPCs), brachial artery flow-mediated dilation (FMD) and carotid intima-media thickness (IMT). Circulating EPCs are bone marrow-derived mononuclear cells that have the capacity to migrate, proliferate, and differentiate into mature endothelial cells (*12*). EPCs also play crucial roles in the regeneration of the endothelial lining of blood vessels (*13*). Clinical studies documented a decreased number of circulating EPCs in CVD patients, suggesting that levels of circulating EPCs might be associated with vascular endothelial function and CVD risk (*14–16*). Brachial artery FMD and carotid IMT are well-known markers of early atherosclerosis, which significantly correlates with the development of CVD (*17–19*). Hence, an assessment of the circulating EPC, coupled with brachial artery FMD and carotid IMT can serve as surrogate markers of vascular endothelial function and indicate subclinical vascular pathological changes (*20*, *21*).

Furthermore, the global study of molecular lipids through the use of lipidomic technologies can provide a better understanding of the cardiovascular pathological states caused by alterations in lipid metabolism (*22*). Plasma lipid species and classes/subclasses have been found to be associated with CVD (*23*). More recently, plasma lipid species have also been associated with incidences of cardiovascular events, suggesting the usefulness of these biomarkers to predict

cardiovascular risk (*24*).

Nonetheless, there is a paucity of data from human clinical studies that observe the synergistic effects of wolfberry and healthy eating patterns on cardiovascular health. In addition, very limited studies apply novel analytical techniques to assess the complex cardiovascular pathological states in the nutrition field.

1.1 General introduction

The American Heart Association and other organizations recommended a healthy diet and lifestyle to combat CVD (*25*). Thus the development of a dietary strategy that improves endothelial function of the artery and regulates other CVD risk factors may reduce the risk of developing CVD. Although there is a growing of interest on evaluating both structural and functional arterial proprieties and assessing plasma lipidomic profiles when applying nutritional treatment, very limited data exist in the nutrition field.

The fruit of *Lycium barbarum*, also called wolfberry or goji berry, is a well-known traditional Chinese medicine plant and it has recently become increasingly popular in many countries around the world due to its health promoting properties (*26*, *27*). The biological activities and potential health benefit effects of wolfberries are generally explained by its chemical constituents including carotenoids, polysaccharides, and polyphenols (*28*) Among the various carotenoids, zeaxanthin, which has antioxidant and anti-inflammatory properties is predominant in wolfberry (*26*). In humans, zeaxanthin is located primarily in the macula and is believed to protect the retina from photo-oxidative damage by its antioxidant function (*28*). In addition, cardio-protective effects of zeaxanthin has been discussed and epidemiologic studies have shown strong associations between carotenoids, including zeaxanthin, and the risk of CVD and atherosclerosis (*29*). Although the effects of wolfberry extract or wolfberry supplementation on blood carotenoid concentrations and blood lipid and antioxidant status have been reported (*30–32*), the cardio-protective effect of consuming dried wolfberry as a whole food is limited.

Healthy eating patterns such as the Mediterranean diet, has also been linked to a reduced risk of CVD. Several mechanisms have been suggested to underlie the observed benefits including a decrease in inflammatory markers, an improvement in endothelial function, and reduced LDL atherogenicity (*33–35*). Only one recent study investigated the plasma lipidomic profiles with the

Mediterranean diet and there was an alteration of lipid metabolites with a longer acyl chain and higher number of double bonds after the Mediterranean diet (*35*). The Health Promotion Board (HPB) in Singapore recommends a healthy eating pattern "My Healthy Plate", to help Singaporeans practice healthy eating habits that can aid in weight control and chronic disease protection. To date, no study has assessed the impact of this healthy eating pattern against CVD risk in the Singapore population.

Thus collectively, consuming a wolfberry as part of a healthy eating pattern diet may improve endothelial function and alter lipid metabolites in Singapore's middle-aged and older adults who are at risk for CVD.

1.2 Rationale and justification for the study

CVD is a major disease burden which is constantly growing in Singapore. Moreover, with the rapid increase in the local older population, the cost and manpower for CVD care, particularly in older population, is becoming unsustainable. Due to this circumstance, it is very important to seek ways to resolve healthcare challenges by taking necessary preventative measures to improve CVD care

1.2.1 Rationale for the Study Purpose

Findings from the proposed research will provide the scientific evidence regarding the beneficial effects of consuming wolfberry with a healthy eating pattern diet on the 1) cardiovascular system, 2) lipidomic profiles, and 3) whole body anti-oxidant status in Singapore middle-aged and older individuals. Although EPC, brachial artery FMD, and carotid IMT are the significant clinical markers of endothelial function and cardiovascular health, they are rarely measured in the nutrition field particularly in middle-aged and older population. Beyond the conventional blood lipid profiles, the importance of assessing the structure and function of the complete set of lipids is emerging, however, the application of lipidomic technologies is very limited in the nutrition-related research projects as well.

Therefore, the outcomes of this project will support/validate the assessment of clinically relevant cardiovascular health-related indicators and the complete set of lipid metabolites in this population when applying nutritional treatment. Lastly, the results from the proposed research will assist a practical guidance of dietary behaviour changes providing cardiovascular health

promoting effects to a large proportion of the Singapore population and may result in reducing cost and manpower for CVD care.

1.2.2 Rationale for Doses Selected

Dose have been selected based on studies by Zanchet et al. 2017(14 g/day) (*32*) and Cheng et al. 2005 (15 g/day) (*31*) which likewise conducted clinical randomised controlled trials with whole dried wolfberry. Favourable outcomes on the cardiovascular health of the subjects post-intervention were observed with a similar dose of 15 g wolfberry/day.

1.2.3 Rationale for Study Population

The age demographics of the selected population will include only the middle-aged and older adults between 50 to 75 years old. The rationale for this selection is because of their lower threshold and higher susceptibility toward CVD manifestation since the structural and functional deterioration of the heart and arterial systems is associated with aging (*1*).

1.2.4 Rationale for Study Design

This is a 16-week parallel, single-blind (investigator), prospective randomised controlled trial (RCT). Specifically, the 16 week duration was selected to allow us to investigate the cardiovascular protective effects of our intervention diets.

2. Objectives and Hypothesis

2.1 Objectives

1. To assess the effects of consuming a wolfberry as part of a healthy eating pattern diet on endothelial function in middle-aged and older adults.
2. To assess the effects of consuming a wolfberry as part of a healthy eating pattern diet on lipidomic profiles in middle-aged and older adults.
3. To examine the effects of consuming a wolfberry as part of a healthy eating pattern diet on whole body carotenoids and anti-oxidant status in middle-aged and older adults.

2.2 Hypothesis

1. Consuming wolfberry with a healthy eating pattern diet will improve endothelial function when compared to a same diet without wolfberry.

2. Consuming wolfberry with a healthy eating pattern diet will improve the whole body carotenoid and oxidative stress status of subjects compared to those who consumed a similar diet without wolfberry.
3. Consuming wolfberry with a healthy eating pattern diet will improve blood and skin carotenoids status while decrease whole body oxidative stress status when compared to a same diet with no wolfberry.

2.3 Potential Risks and benefits

2.3.1 End Points – Efficacy

No benefit is guaranteed and you may not derive any benefit from participating. However, you will be able to contribute to knowledge with regards to the effects of a wolfberry enriched healthy diets on improvements in cardio-metabolic health in the middle-aged and older population.

2.3.2 End Points – Safety

There may be potential risks to human participants but they are minimal and the research group members and well-trained technicians will consistently pay close attention to ensure the participant's safety. More details are described below.

1) Source of Materials: All data will be used solely for research purposes. The blood will be collected by an invasive procedure (intravenous cannulation) following a standard procedure provided by WHO (*36*) and collected blood samples will be used to measure selected clinical markers of health only. Other research materials will include: medical, diet logs, body composition, FMD and CIMT measurements. The original paper copies of all identifiable data will be kept in locked storage cabinets and rooms with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. Electronic copies of the data with identifiable participant information will be kept on a secure website or password-protected computers with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. All data will be de-identified prior to statistical analyses.

2) Blood Collections: Participants may experience soreness and bruising at the puncture site. Participants may also feel lightheaded and there is a slight risk of fainting. Direct pressure will be applied over the puncture site to minimize soreness and bruising. Participants will be seated in a cushioned chair with padded armrests to minimise the risk of falling if a participant becomes

lightheaded or faints during the blood draw. Dr. Kim's trained research staff will stay with participants at NUHS IMU and NUS OHC during the blood collection to assure the participant's safety. Participants will be asked not to donate blood for at least one month prior to, during, and for one month after the study due to potential alterations in the participant's blood profile that may affect study outcomes. In the unlikely event that the subject is injured while giving a blood sample, first aid will be provided and the subject will be directed to proper health treatment by the on-site study physicians.

3) Dietary Intervention: There is no known risk associated with the consumption of wolfberry or a healthy eating pattern diet according to earlier intervention studies of a similar nature (*30–32*) but there is a possibility of the stomach or bowels becoming upset if there is a change in usual customary dietary intakes. Subjects who experience these symptoms will be closely monitored and instructed to modify their wolfberry intake pattern by distributing the intake of wolfberry over 3 meals instead. If the symptoms persist, the Principal Investigator and/or the Co-investigator of this study may stop the subject's participation at any time if it is decided that it is in his/her best interests. If significant new findings develop during the course of this research that may relate to the subject's willingness to continue participation, they will be provided with this information.

4) Resonance Raman Spectroscopy: There is no known risk associated with non-invasive resonance Raman spectroscopic analysis which involves only the exposure of the palm to visible blue light.

5) Dual-energy X-ray absorptiometry (DXA): Body composition will be measured through non-invasive procedures. Specifically, body density will be determined using DXA. DXA involves some exposure to radiation; however, it is very small. The average absorbed dose of radiation, per scan, is equal to 1.424 mRem, totaling 4.272 mRem for all 3 scans. This amount of radiation exposure is less than or equal to one percent of the average exposure from a chest x-ray (5-20 mRem), and less than or equal to one-tenth of one percent of the average exposure from a full dental x-ray series (50-300 mRem).

6) High frequency ultrasonographic imaging: Ultrasonographic imaging has been used for over 20 years with excellent record. It is based on non-ionizing radiation and is generally considered safe when used prudently by appropriately trained healthcare providers (*37*).

3. STUDY POPULATION

3.1 Subject enrolment number

Recruitment will be done by an email which will be disseminated to the student and staff population within NUS. The recruitment notice will be sent out by the NUS Food Science and Technology administrative department via the NUS email and IVLE portal. In addition, validated posters will also be put up within the campus compounds on areas granted for poster pasting as well as on bulletin boards at neighbourhood void decks and community centres within the vicinity of NUH. A targeted fifty middle-aged and older men and women (aged 50 to 75 years, approximately half men and half women) will be recruited with the expectation that ≥ 40 subjects (≥ 20 subjects per group) will complete the study (≤ 20% dropout rate).

All participants who meet the inclusion criteria stipulated in *Section 3.3* may be enrolled for the study. There will be no restrictions based on gender or race but subjects will need to be adequately equipped with English literacy since the study material provided will be in printed in English only. Research material in other languages is unavailable due to the limitations in capability to translate all the documents effectively.

3.2 Criteria for Recruitment

Volunteers who responded will be interviewed over the phone by a qualified member of the research team. During the phone interview, the volunteer will be given a brief description of the study design and procedures to be followed. This will also include questions on the eligibility to participate in the research study based on a pre-determined set of criteria. If the volunteer is still interested and eligible, with their verbal approval, arrangements will be made for them come down for the screening visit. If the volunteer does not qualify for the study or declines to participate, these data will be destroyed following the completion of the phone conversation.

3.3 Inclusion Criteria

1. Ability to give an informed consent
2. Age 50 to 75 years
3. Willing to follow the study procedures

3.4 Exclusion Criteria

All subjects meeting any of the exclusion criteria at baseline will be excluded from participation

1. Significant change in weight (± 5% body weight) during the past 3 months
2. Allergy to wolfberry
3. Acute illness at the study baseline
4. Exercising vigorously over the past 3 months
5. Consistently following healthy eating pattern diet in the past 3 months
6. Smoking
7. Have an average weekly alcohol intake above 21 units per week (males) and 14 units per week (females): 1 unit = 360 mL of beer; 150 mL of wine; 45 mL of distilled spirits)
8. Pregnant, lactating, or planning pregnancy in the next 6 months
9. Taking dietary supplements which may impact the outcome of interests (e.g. vitamin supplements, antioxidant supplement etc.)
10. Prescribed and taking antihypertensive/cholesterol-lowering/type-2 diabetic medication/ traditional Chinese medicine which started less than 5 years prior to the intervention participation
11. Insufficient venous access to allow the blood collection
12. Exposed to radiation in the last 3 months or expected exposure to radiation in the coming 6 months

*Defined as having > 6 metabolic equivalents of exercise daily; approximately 20 mins of moderate intensity exercise (e.g. slow jogging) a day for older adults (*38*).

3.5 Withdrawal Criteria

Subjects are allowed to discontinue participation in the study at any point without giving reasons. Subjects' data will be able to be discarded if they choose to withdraw their participation unless otherwise agreed.

1. If subject is afflicted by an active infection requiring systemic antiviral or antimicrobial therapy that coincides to any of the 6 study visits.
2. Development of allergic reactions during the span of the study
3. Exercising vigorously during the span of the study
4. Smoking during the span of the study
5. Having an average weekly alcohol intake that above 21 units per week (males) and 14 units per week (females): 1 unit = 360 mL of beer; 150 mL of wine; 45 mL of distilled spirits) during the

span of the study

6. Pregnancy during the span of the study
7. Starts consuming dietary supplements which may impact the outcome of interest
8. Prescribed and taking antihypertensive/cholesterol-lowering/ type-2 diabetic medication
9. If subject is not following the prescribed diet
10. If a subject develops prolonged stomach or bowel upsets as a result of the intervention

3.6 Subject Replacement

Subject replacement will not be conducted.

4. TRIAL SCHEDULE

For this 16-week randomised, parallel-design prospective study, subjects are required to attend 6 visits including an initial screening visit. The duration for each visit is estimated to be approximately 5 hr for the pre- and post-intervention visits and approximately 2 hr for the screening and monthly visits in between. Following the initial screening visit, each visit would be separated by a 4-week interval. The trial schedule is depicted in the schematic attached in Appendix 1.

5. STUDY DESIGN

5.1 Summary of Study Design

For this 16-week randomised, parallel-design prospective study, subjects are required to attend 6 visits in total including an initial screening visit. The duration for each visit is estimated to be approximately 5 hr for the pre- and post-intervention visits and approximately 2 hr for the screening and three visits in between. Following the initial screening visit, each visit would be separated by a 4-week interval.

6. METHODS AND ASSESSMENTS

The following study procedure description will not involve the use of any audio or visual recording devices.

6.1 Randomisation and Blinding

Randomisation will be conducted using SAS 9.4 software which will assign the subjects either to

the intervention or control group. Single blinding (investigator) will be practiced.

6.2 Contraception and Pregnancy Testing

For pre-menopausal female subjects, a pregnancy test kit will be provided during each visit to determine for pregnancy during the study duration.

6.3 Study Visits and Procedures

6.3.1 Screening Visits and Procedures

Pre-screening: Volunteers who responded will be interviewed over the phone by a qualified member of the research team. During the phone interview, the volunteer will be given a brief description of the study design and procedures to be followed. This will also include questions on the eligibility to participate in the research study based on a pre-determined set of criteria. If the volunteer is still interested and eligible, with their verbal approval, arrangements will be made for them come down for the screening visit. If the volunteer does not qualify for the study or declines to participate, these data will be destroyed following the completion of the phone conversation.

Screening: Under the guidance of trained study personnel, the volunteers will first be briefed about the study and given ample time to carefully read through the informed consent form. They will also be provided an opportunity to question any doubts before acknowledging the consent form which will be kept by both the study personnel and participant. Participants who gave their acknowledgement will be further assessed for their study eligibility based on a pre-determined set of criteria including the completion of a healthy diet checklist. Following which, participants who fulfilled the previous criteria will be instructed to complete a medical history questionnaire. Based on the collected data, approval for participation will be finalised by either of the study investigators, Dr Khoo, Dr Chan or Dr Kim. If the participant does not qualify for the study or declines to participate, these data will be destroyed after the screening visit unless otherwise agreed.

6.3.2 Study Visits and Procedures

Prior to each visit, subjects will be required to fast from the night before for approximately 10 to 12 hrs.

The following procedures will be conducted by trained research staff on the pre- and post-

intervention visits:

1. Sampling of fasting-state blood (60 mL) through intravenous cannulation from one of the subject's arms in a room at NUH IMU designated and approved for phlebotomy purposes.
2. Anthropometric measurements including height (pre-intervention visit only), weight and waist circumference at Food Science & Technology Programme, NUS.
3. Blood pressure measurement at Food Science & Technology Programme, NUS.
4. Skin carotenoid status analysis using resonance Raman spectroscopy at Food Science & Technology Programme, NUS.
5. Endothelial-dependent brachial artery FMD and carotid IMT using high-frequency ultrasonographic imaging at the National University Heart Centre
6. Body composition assay by DXA at the Orthopaedic Diagnostic Centre, NUHS
7. Questionnaires and assessment forms including a 3-day food record, Pittsburgh sleep quality index questionnaire, sleep evaluation questionnaire, perceived stress questionnaire, Montreal cognitive assessment, subjective appetite visual analogue scale and quantitative food frequency questionnaire (FFQ: pre-intervention visit only) at Food Science & Technology Programme, NUS.

The following procedures will be conducted by trained research staff on the three visits in between:

1. Sampling of fasting-state blood (20 mL) through intravenous cannulation from one of the subject's arms in a room at NUS OHC designated and approved for phlebotomy purposes.
2. Anthropometric measurements including weight and waist circumference at Food Science & Technology Programme, NUS.
3. Blood pressure measurement at Food Science & Technology Programme, NUS.
4. Skin carotenoid status analysis using resonance Raman Spectroscopy at Food Science & Technology Programme, NUS.
5. Questionnaires and assessment forms including a 3-day food record, Pittsburgh sleep quality index questionnaire and subjective appetite visual analogue scale at Food Science & Technology Programme, NUS.

The collected blood samples will be stored and analysed for the following parameters :

1. Blood lipid-lipoprotein concentrations: Total cholesterol, high-density lipoprotein

cholesterol, low-density lipoprotein cholesterol, and total triglyceride (every 4 weeks).

2. Blood carotenoid concentration: Fasting state lutein, zeaxanthin, lycopene, α-carotene, β-carotene, α-cryptoxanthin, and β-cryptoxanthin concentrations using high-performance liquid chromatography (HPLC; pre- and post-intervention only).

3. Oxidative stress and inflammatory-related parameters: Concentrations of plasma oxidative stress (malondialdehyde (MDA), glutathione peroxidase (GPX), and superoxide dismutase (SOD)) and inflammatory (tumour necrosis factor-α, interleukin-6, monocyte chemoattractant protein-1) biomarkers using commercially available ELISA assay kits (pre- and post-intervention only).

4. Concentrations of vasodilation and vasoconstriction-related biomarkers: nitric oxide, endothelial nitric oxide synthase, and endothelin-1 using commercially available ELISA assay kits (pre- and post-intervention only).

5. Endothelial progenitor cells will be analyzed and enumerated using flow cytometry.

6. Lipidomic profiles: The main classes of glycerophospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, including lysoglycerophospholipids) and sphingolipds (sphingomyelin and ceramides) will be analysed at pre- and post-intervention by high-performance liquid chromatography couple with targeted MRM analysis on triple-quadrupole mass spectrometers (pre- and post-intervention only).

7. Additional plasma will be stored for possible further analyses.

At the end of the subject's first visit, they will also be randomised into either the intervention or control arm using SAS 9.4 software although the intervention will only begin the following week.

One-to-one dietary counselling for each subject and an instruction sheet "My Healthy Plate dietary guide" will be provided by a research dietitian and trained research staff to achieve the healthy eating pattern diet while only subjects in the wolfberry intervention group will receive a provision of 500 g dried wolfberries every 4 weeks along with specific instructions to cook and consume 15 g/day wolfberry as part of a mixed-meal. Compliance with the diet interventions will be promoted by frequent online and in-person contact, 3-day food records (every 4 weeks) as well as photographs of the subject's meals which will be sent to the study personnel for regular checks on pre-determined dates.

6.3.3 Post Study Follow-up and Procedures

A monetary stipend of $ 600 will be issued after the subject's completion of the intervention. However, the monetary stipend will be pro-rated as shown in the table below depending on the number of visits completed if a subject decides to withdraw during the course of study. Volunteers who are deemed ineligible after screening will also be reimbursed $ 10 for their participation in the screening procedure. Specifics of the amount of reimbursement provided after each visited are described in the following table.

Visit	**Financial payment/ incentive**
Screening	$ 10
Visit 1	$ 150
Visits 2 to 4	$ 50 each
Visit 5	$ 300
Total	**$ 610**

6.3.4 Discontinuation Visit and Procedures

Subjects who voluntary withdraw during the 16-week intervention will not be replaced and the monetary stipend will be pro-rated according to the point in which they decided to withdraw during the study. No follow-up evaluations are required for subjects who withdraw from the study. However, if withdrawal is attributed to an illness or injury afflicted due to the subject's involvement in the study, NUS will be responsible for all compensations.

7. TRIAL MATERIALS

Dried whole wolfberry (500 g) would be issued to the subjects in the intervention group free of charge every 4 weeks and will be replenished during each visit. Subjects will be advised to consume these wolfberries as a part of a whole meal i.e. by cooking it with rice (wholegrain), a dietary staple in Singapore.

7.1 Trial Product

The fruit of *Lycium barbarum*, also called wolfberry or goji berry, is a well-known traditional Chinese medicine plant and it has recently become increasingly popular in many countries around the world due to its health promoting properties (*26*, *27*).

7.2 Storage and Drug Accountability

Dried wolfberries will be stored at 4 ^{o}C in a conventional refrigerator at all times to maintain product freshness and consistency.

8. TREATMENT

8.1 Rationale for Selection of Dose

Based on studies by Zanchet et al. 2017(14 g/day) and Cheng et al. 2005 (15 g/day) which likewise conducted human clinical interventions with dried wolfberry, favourable outcomes on the cardiovascular health of the subjects post-intervention were observed. Thus, a similar dose of 15 g wolfberry/day would be used.

8.2 Specific Restrictions / Requirements and Concomitant therapy

Dietary supplements which may impact the outcome of interests (e.g. vitamin supplements, antioxidant supplement, fish oil supplements etc.) should not be consumed by the subjects. In addition, if subjects have been prescribed and are taking antihypertensive, cholesterol-lowering or type-2 diabetic medication within the last 5 years prior to the intervention, they would be excluded from the study as well.

Records of all medications (prescription and over the counter), vitamin and mineral supplements, taken by the participant will be obtained from the medical history questionnaire. Eligibility for the study participation will be will be finalised by either of the study investigators, Dr Khoo, Dr Chan or Dr Kim.

8.3 Blinding

Study personnel, investigators and outcome assessors will be blinded from the subject's assignment. Unblinding of the study personnel will only be done upon completion of the intervention and analytical procedures.

9. SAFETY MEASUREMENTS

The study poses no more than minimal risk to research participants and the research group members will consistently pay attention to assure the participant's safety.

9.1 Definitions

Definitions of UPIRTSO and serious adverse events as well as other safety management procedures will be further elaborated in Sections 9.2 – 9.4.

9.2 Collecting, Recording and Reporting of UPIRTSO events to the NHG Domain Specific Review Boards (DSRB) and Serious Adverse Events (SAEs) to the Health Science Authority (HSA)

Safety analysis for adverse events will be performed after weekly by the study investigators and personnel involved. All problems involving local deaths related to the study or not, will be reported within 24 hours after first knowledge to the NHG DSRB. Any other SAEs will be reported not later than 7 calendar days after first knowledge to the NHG DSRB. Follow-up information will be actively sought and submitted as it becomes available. A complete follow-up report will be submitted within 8 additional days.

Medical and scientific judgements regarding the relatedness and severity of the occurrence will performed by Principal Investigator Dr. Khoo.

9.3 Safety Monitoring Plan

1) All data will be used solely for research purposes. The blood will be collected by an invasive procedure (intravenous cannulation) following a standard procedure provided by WHO (*36*) and collected blood samples will be used to measure selected clinical markers of health only. Other research materials will include: medical, diet logs, body composition, FMD and CIMT measurements. The original paper copies of all identifiable data will be kept in locked storage cabinets and rooms with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. Electronic copies of the data with identifiable participant information will be kept on a secure website or password-protected computer with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. All data will be de-identified prior to statistical analyses

2) Blood Collections: Participants may experience soreness and bruising at the puncture site. Participants may also feel lightheaded and there is a slight risk of fainting. Direct pressure will be applied over the puncture site to minimize soreness and bruising. Participants will be seated in a cushioned chair with padded armrests to minimise the risk of falling if a participant becomes lightheaded or faints during the blood draw. Dr. Kim's trained research staff will stay with participants at NUHS IMU and NUS OHC during the blood collection to assure the participant's safety. Participants will be asked not to donate blood for at least one month prior to, during, and for one month after the study due to potential alterations in the participant's blood profile that may affect study outcomes. In the unlikely event that the subject is injured while giving a blood sample, first aid will be provided and the subject will be directed to proper health treatment by the on-site study physicians.

3) Dietary Intervention: There is no known risk associated with the consumption of wolfberry or a healthy eating pattern diet according to earlier intervention studies of a similar nature (*30–32*) but there is a possibility of the stomach or bowels becoming upset if there is a change in usual customary dietary intakes. Subjects who experience these symptoms will be closely monitored and instructed to modify their wolfberry intake pattern by distributing the intake of wolfberry over 3 meals instead. If the symptoms persist, the Principal Investigator and/or the Co-investigator of this study may stop the subject's participation at any time if it is decided that it is in his/her best interests. If significant new findings develop during the course of this research that may relate to the subject's willingness to continue participation, they will be provided with this information.

4) Resonance Raman Spectroscopy: There is no known risk associated with non-invasive resonance Raman spectroscopic analysis which involves only the exposure of the palm to visible blue light.

5) Dual-energy X-ray absorptiometry (DXA): Body composition will be measured through non-invasive procedures. Specifically, body density will be determined using DXA. DXA involves some exposure to radiation; however, it is very small. The average absorbed dose of radiation, per scan, is equal to 1.424 mRem, totaling 4.272 mRem for all 3 scans. This amount of radiation exposure is less than or equal to one percent of the average exposure from a chest x-ray (5-20 mRem), and less than or equal to one-tenth of one percent of the average exposure from a full dental x-ray series (50-300 mRem)

6) High frequency ultrasonographic imaging: Ultrasonographic imaging has been used for over 20 years with excellent record. It is based on non-ionizing radiation and is generally considered safe when used prudently by appropriately trained healthcare providers (*37*).

9.4 Complaint Handling

On-site complaints will be handled by the study investigators, Dr Khoo, Dr Chan, Dr Kim, and her research staff professionally. If a complaint raised cannot be adequately managed by the available personnel, a consensus will be reached in a meeting held at the end of the test day with other involved members to manage the issue as soon as possible.

A complete follow-up report will be prepared within 8 additional calendar days to be disseminated to all members of the research project. Electronic copies of the report with identifiable participant information will be kept on a secure website or password-protected computer with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff.

10. DATA ANALYSIS

10.1 Data Quality Assurance

To ensure a reliable execution of the study protocol, compliance with the diet interventions will be promoted by frequent online and in-person contact, food records as well as photographs of the subject's meals which will be sent to the study personnel for regular checks on pre-determined dates. Any biases will also be minimised by blinding of the study investigator and outcome assessors.

Analytical accuracy and validity will be maintained by ensuring that all involved personnel are properly briefed and trained prior to commencing any analysis. Complete data entry will occur within one week of data collection. A random 10% of all observations will be double entered weekly. A data entry error rate exceeding 1% will prompt a review of data entry and coding procedures and double entry of all data since the last data review. The distributional characteristics of each dependent variable will be assessed and the data transformed as needed if the assumption of normality is violated.

10.2 Data Entry and Storage

Subjects will be assigned a subject ID, with the link between their subject ID and name kept separate from the data. Personal identifiers to be collected include the participant's name, email address and contact number. The linkage between subjects' personal identifiers and data will be stored both on paper and electronically on password-protected computers belonging to the Co-Investigator, Dr Kim. Data stored on paper and the computers will be kept in Dr Kim's office, with access code required for entry.

In summary, data from on-site health assessments including anthropometric, blood pressure and skin carotenoid status as well as medical and diet logs will first be recorded on paper. The original paper copies of all identifiable data will be kept in locked storage cabinets and rooms with access limited to Dr. Khoo, Dr Chan, Dr. Kim her research staff. Subsequently, all research entries will be collated for storage electronically. Electronic copies of the data with identifiable participant information will be kept on a secure website with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. All data will be de-identified prior to statistical analyses. There will be scheduled changes to passwords. There will be encryption of all email or protection by password of all data with personal identifiers.

11. SAMPLE SIZE AND STATISTICAL METHODS

11.1 Determination of Sample Size

Outcomes of interests in this research include the difference in plasma zeaxanthin and MDA concentrations. Previous research reported that the plasma zeaxanthin concentration was significantly increased after consuming 15 g/day of wolfberry for 28 days (0.058 ± 0.011 μmol/L, mean ± SEM) compared to not consuming wolfberry (0.006 ± 0.003 μmol/L, mean ± SEM) (*31*). For the current study, presuming the proposed experiment yields similar results as previously, ≥ 12 participants per group will provide ≥ 95% power at $\alpha = 0.05$ (two-tailed) to statistically confirm a similar difference. Also, recent research reported that the plasma MDA concentration was decreased after consuming 14 g/day of wolfberry with meal for 45 days (-3 ± 1 μmol/mg of protein, mean ± SEM) compared to not consuming wolfberry (3 ± 1 μmol/mg of protein, mean ± SEM) (*32*). For the current study, presuming the proposed experiment yields similar results as previously, ≥ 20 participants per group will provide ≥ 95% power at $\alpha = 0.05$ (two-tailed) to statistically confirm a similar difference.

Although the primary outcome of this research is the difference of endothelial function after consuming a healthy eating pattern diet with wolfberry versus a same diet without wolfberry, power calculations based on endothelial function-related parameters were not done due to the lack of preliminary data. As such, the endothelial function-related responses in the current study will be used to calculate effect sizes for future research.

11.2 Statistical and Analytical Plans

The main effects of meal, time, and their interactions on the dependent variables will be determined by repeated-measures analysis of variance (ANOVA). All analyses will be performed in SAS 9.4 (SAS Institute Inc., Cary, NC) and results will be expressed as mean ± SE, otherwise noted, and statistical significance will be accepted at $p < 0.05$ (two-tailed).

12. ETHICAL CONSIDERATIONS

12.1 Informed Consent

The consent process will take place on the screening visit for potential research participants. Under the guidance of trained study personnel, the volunteers will first be briefed about the study and given ample time to carefully read through the informed consent form. They will also be provided an opportunity to question any doubts before acknowledging the consent form which will be kept by both the study personnel and participant. Participants who gave their acknowledgement will be further assessed for their study eligibility based on a pre-determined set of criteria including the completion of a healthy diet checklist. Following which, participants who fulfilled the previous criteria will be instructed to complete a medical history questionnaire. Based on the collected data, approval for participation will be finalised by either of the study investigators, Dr Khoo, Dr Chan or Dr Kim. If the participant does not qualify for the study or declines to participate, these data will be destroyed after the screening visit unless otherwise agreed.

Special provisions to cater to non-English speakers or English illiterate subjects are unavailable for this study due to the limitations in capacity and resource constraints to accommodate for these additional needs.

12.2 IRB review

This study does not involve any restricted or prohibited human biomedical research as defined in the Third and Fourth Schedules of HBRA. Each participating institution has been provided with and has approved this protocol and the associated informed consent documents.

12.3 Confidentiality of Data and Patient Records

All data will be used solely for research purposes. The blood will be collected by an invasive procedure (intravenous cannulation) following a standard procedure provided by WHO (*36*) and collected blood samples will be used to measure selected clinical markers of health only. Other research materials will include: medical, diet logs, body composition, FMD and carotid IMT measurements. The original paper copies of all identifiable data will be kept in locked storage cabinets and rooms with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. Electronic copies of the data with identifiable participant information will be kept on a secure website or password-protected computer with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff. All data will be de-identified prior to statistical analyses.

13. PUBLICATIONS

Data for publication will be retained by the PI and/or Co-Investigator for a minimum of 10 years for use in this research study and if agreeable, future nutritional research in Singapore by NUS and/or NUS's collaborators for as long as they are necessary.

14. RETENTION OF TRIAL DOCUMENTS

The original paper copies of all identifiable data will be kept in locked storage cabinets and rooms with access limited to Dr. Khoo, Dr Chan, Dr Kim and her research staff. Electronic copies of the data with identifiable participant information will be kept on a secure website with access limited to Dr. Khoo, Dr Chan, Dr. Kim and her research staff.

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List of Attachments

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