PROTOCOL TITLE: **Optimization of 25(OH) vitamin D levels in African Americans**

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Optimization of blood 25-hydroxy-vitamin D levels in African Americans

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1.0 Objectives*

Two-thirds of the US population, particularly African Americans (AA), is at risk for inadequate or deficient 25-hydroxy-vitamin D (25(OH)VD). Epidemiological studies demonstrate an association between better health outcomes and higher blood levels of 25(OH)VD . Randomized controlled clinical trials have shown that, while supraphysiological high doses of VD are needed to achieve adequate blood levels of 25(OH)VD, not all subjects respond to them. Recent studies have also questioned the therapeutic effects of high-dose VD supplementation. This application presents our design for a randomized, double-blind, placebo-controlled clinical trial to test the hypothesis that supplementation with VD in combination with L-cysteine (LC) is more successful at optimizing the statuses of 25(OH)VD [biological signatures] and simultaneously decreasing TNF-α and IR [functional or clinical outcomes], suggesting a better therapeutic approach compared with supplementation with VD alone in AA subjects.

2.0 Background*

Previous human studies report that VD supplementation increases circulating 25(OH)VD status but has limited success in reducing insulin resistance (IR). Other human studies report that supplementation with LC lowers TNF-α and IR. This clinical trial will determine the impact of combined supplementation with VD+LC on increases in levels of 25(OH)VD as well as simultaneous reduction in TNF-α and IR biomarkers (mediators of clinical benefit) in the AA population. The mechanistic focus of this clinical trial will determine whether combined supplementation with VD and LC provides a more successful approach to increasing 25(OH)VD [biological signatures] as well as simultaneously decreasing TNF-α and IR [functional or clinical outcomes], compared with supplementation with VD alone. If the results are positive, future studies will investigate potential of this approach to reduce the inflammation, pain, and other health issues among AA in a full-scale efficacy trial.

3.0 Inclusion and Exclusion Criteria*

Informed written consent will be obtained from all subjects according to the IRB approved protocol. All subjects in this study will be adult AA volunteers and will meet the inclusion/exclusion criteria given below.

INCLUSION CRITERIA: (1) African-American adults aged 18-65 years willing to participate in 5 clinic visits where blood will be drawn; (2) participants who demonstrate an understanding of the risks and benefits of the protocol and sign the informed consent form; (3) participants willing to complete standard health history questionnaire before and during the study ***(Copy enclosed)***; (4) women with negative pregnancy tests (pregnancy test will be performed on all women participants)

EXCLUSION CRITERIA: (1) History of cardiovascular disease, sickle cell disease, or metabolic disorders including: diabetes, uncontrolled hypertension, hypothyroid, or hyperthyroid; (2) any sign of hepatic dysfunction or renal dysfunction, hypercalcemia, or nephrolithiasis; (3) those taking antiepileptic medications, glucocorticoids, or weight loss or other dietary supplements within the two months previous to enrollment or during the study; (4) those who visit tanning booths; (5) any subject who develops an infection and has to take antibiotics; and (6) women with positive pregnancy tests or those who are nursing. (7) adults unable to consent or prisoners. Any subject taking vitamin D or any other vitamin

or herbal supplement even advised by a doctor or over-the-counter will not be enrolled in the study.

4.0 Study-Wide Number of Subjects*

This is the only site for enrollment and will enroll 160 subjects.

5.0 Multi-Site Research* N/A (This is only site)

6.0 Study Timelines*

Potential subjects will be contacted over phone or potential subjects may call in response to advertisement and agrees to participate in this study will be asked to come to the clinic after fasting overnight. Fasting blood will be collected after written informed consent. Blood collected at V_{-1} will serve as the sample for the screening visit for the clinical trial. Subjects meeting the inclusion/ exclusion criteria will continue in the clinical trial. All subjects will fill out the complete standard health history before the study and at each visit during the study. This clinical trial will be registered at the ClinTrial.gov site.

Initially, all of the study subjects will be provided placebo supplementation as a placebo run-in period for one month before randomization. Visit 2 (V_0) is for obtaining baseline levels after the run-in period. AA-subjects will be block randomized into four groups. Members of the groups will ingest capsules containing placebo and supplements, orally, daily: (I) placebo (P); (II) LC (1000 mg); (III) VD (2000 IU); (IV) VD and LC. Each capsule will contain a placebo or cholecalciferol (2000 IU) or LC (1000 mg). Placebo group will take two placebo capsules a day in the morning; II LC group will receive two capsules of LC daily; (III) VD group will take two capsules and each capsule will contain 1000 IU VD daily; (IV) VD+LC group will take daily two capsule containing 1000 IU+500 mg LC. Supplements will be taken daily with food at breakfast for 6 months, while participants continue to carry on a normal lifestyle. During the placebo run-in period, all subjects will also take two placebo capsules daily. Based on previous experience, we expect a dropout rate of about 16% as a result of non-compliance, lost contact, taking another medicine, or a subject found to have another disorder. We expect that at least 30 subjects in each group will complete the study. Overnight fasting blood will be collected at each visit. Also, anthropomorphic data, body weight, and height (BMI), the record of any drug use, any adverse effects/complaints (related or unrelated), and Questionnaire will be collected at each visit by the nurse. It is likely to require ~90 minutes to collect the subject's history and other data in interviews with the nurse/dietician and investigators.

The placebo run-in period is meant to stabilize subjects in the study and will prevent any effect due solely to inclusion in the study. Placebo and supplement capsules will be similar in appearance, taste, texture, and smell, and will be provided by the Dr. Shi/pharmacist, who will have the codes for which subjects are assigned to which supplement or placebo. Both subjects and investigators will be blinded to the supplement each subject will receive. Dr. Shi will randomize the subjects. Documentation about capsules/supplements to be used is enclosed on last page (page 9) of this protocol.

7.0 Study Endpoints*

Primary Endpoints: Blood levels of 25(OH)VD, TNF-α, and HOMA-IR.

Safety Endpoints: Serum calcium (Ca^{2+}), PTH, liver, and kidney function tests will be carried out at visits 0, 2, 4, and 6. Whole blood CBC and pregnancy tests will be done at all visits.
Secondary Endpoints: Blood levels of $1,25(OH)_2VD$, HbA_{1C}, and pro-inflammatory and anti-inflammatory cytokines (such as IL-2, IL-6, and IL-10).

We will also perform tests to monitor liver and kidney function tests at each visit and complete blood count analyses for any sign of any anemia or unexpected changes in white or red blood cell profiles.

8.0 Procedures Involved*

Fasting blood will be drawn after an overnight fast into precooled tubes kept in an ice bucket: 2 mL in a tube without anticoagulant (for the chemistry profile), 1 mL each EDTA-blood for HbA_{1c} and 1 ml for CBC, 8 mL for isolation of plasma, and 12 mL for PBMC isolation. Samples will be transported in an ice-bucket. EDTA-blood will be centrifuged, and the clear plasma saved in several aliquots for various assays. The plasma will be stored at -80°C. Information on age, height, body weight and records of any medication use will be collected from each patient at the time of enrollment. HbA_{1c}, CBC, and blood chemistry profile for glucose, Ca^{++}, liver, and kidney function tests, and pregnancy tests will be done at the clinical chemistry labs of LSUHSC-Shreveport. Frozen aliquots stored at -80°C will be used for all assays. Appropriate control and standards will be used, and analyses will be done in duplicate for each sample. This will eliminate inter-assay variability.

PBMC isolation and PCR studies: PBMC will be isolated using Histopaque (Sigma-Aldrich catalog # 10771) from EDTA -blood as described in our previous publications. We will assay GSH and VD metabolism genes in PBMC as described in our earlier studies.

25(OH)VD and $1,25(OH)_2VD$ assays: We have already standardized tests for simultaneous determinations of total 25(OH)VD and $1,25(OH)_2VD$ in human plasma using UPLC-MS/MS.

Whole blood total GSH, L-cysteine, will be determined using HPLC, and a whole blood Glucose-6-Phosphate Dehydrogenase (G6PD) Activity Assay Kit (Sigma, Cat # MAK015); TNF-α will be determined using an ELISA kit from R&D Systems (Minneapolis, MN). Insulin will be determined using ELISA kits from ALPCO Diagnostics (Salem, NH), and the HOMA insulin resistance index will be calculated (136). IL-2, IL-6, and IL-10 will be determined using ELISA kits from R&D Systems (Minneapolis, MN). All appropriate controls and standards as specified by the manufacturer's kits will be used; control samples will be analyzed each time to check the variation from plate to plate on different days to reduce the variability of assays. All of the analyses will be done in duplicate on each sample.

Total 24 ml blood will be collected (2 mL in a tube without anticoagulant (for the chemistry profile), 1 mL each EDTA-blood for HbA_{1c} and 1 ml for CBC, 8 mL for isolation of plasma, and 12 mL for PBMC isolation).

9.0 Data and Specimen Banking*

Plasma will be separated from blood. Aliquots of plasma stored at -80°C and will be used for all assays. Appropriate control and standards will be used, and analyses will be done in duplicate for each sample. Data on each subject will be stored in computer and will be protected by a password.

10.0 Data Management* and Confidentiality

Analyses of data will be performed by the biostatistician, Dr. Shi. A *p*-value of less than 0.05 for a statistical test will be considered significant. Data will be analyzed statistically to compare the 4 groups at baseline and various visits using ANOVA. The Wilcoxon signed-rank or paired *t*-test will be used to determine significant changes after supplementation for each treatment group, and the ANOVA test to compare the 4 treatment groups on changes. The results for the pairwise comparisons among the 4 time points will determine whether there is a significant change in the outcome variables over time. The effect of covariates or possible confounders (age, sex, BMI, initial levels of outcomes, etc.) will be controlled for and tested for their significant effects by including them in the repeated measures model used.

Based on animal studies, co-supplementation with LC+VD will be more successful and raise serum 25(OH)VD levels by nearly 30 ng/mL(17, 18). Our goal is to raise circulating 25(OH)VD levels by an average of 30 ng/mL, and reduce IR levels by 35% in all AA subjects receiving combined VD+LC supplementation. **T**o observe a significant response or change, a sample size of 7 for 25(OH)VD, and 27 for IR were considered adequate using a paired *t*-test conducted at 5% level of significance and 80% power. We chose a goal of 30 subjects in each group who complete the study in the VD+LC supplementation and other groups. Based on our experience from a previously completed clinical trial (H-09-073), enrollment of and consent from 160 AA volunteers who participate in blood collection for screening should give us more than the required sample size of 30 subjects in each group (120 in 4 groups) after factoring in a dropout rate of about 16% for screen failures, withdrawal before randomization, or those who did not complete all the visits. Our experience also suggests that nearly 190 subjects will need to be contacted to achieve our goal of 160 AA volunteers who complete the written consent form and are enrolled in this clinical trial. This takes into account the fact that only about 85% of those initially contacted will indeed show up for the screening visit and complete the written informed consent.

11.0 Provisions to Monitor the Data to Ensure the Safety of Subjects*

Vitamin D and L-cysteine is a common micronutrients and is widely present in many food items, such as milk, cheese, eggs and green vegetables. The dose used falls within normal range for a healthy person consuming a healthy diet. Thus, this research is considered and falls under minimal risk. The Data Safety Monitoring Plan committee will review clinical laboratory reports for any unanticipated observation. Names of members of DSMP committee are enclosed. DSMP will meet once in three months or as required in case any adverse side effect is reported by any subject or seen in clinical report.

12.0 Withdrawal of Subjects*

Subjects found to be consuming over the counter supplement, positive pregnancy tests or not able to follow the instructions will be withdrawn from the study. Any data collected on subject withdrawn from the study will not be used in any analyses. The investigator will ask a subject who is withdrawing whether the subject wishes to provide further data collection from routine medical care.

13.0 Risks to Subjects*

The blood collection for this study will require needle pricks to your vein, which may cause you minimal discomfort. You may experience some bruising and/or slight soreness at the blood collection site. There should not be any restriction on your normal activities due to participation

in this study. No other extra examinations, procedures or tests, such as, pulmonary functions tests, etc. are required for participation in this study. All participants will agree not to eat anything for about 8 hours before coming to the clinic for blood draw.

14.0 Potential Benefits to Subjects*

There is no direct benefit. You will be paid $50 for each visit during this study period to cover your transportation or other expenses. This is done because you will need to come to clinic for blood draw required for research investigations. Costs for all the tests done for research investigations will be paid by the investigators.

15.0 Vulnerable Populations*

15.1 Research does not involve Vulnerable population. Research involve only healthy adult African Americans.

16.0 Community-Based Participatory Research*

16.1 There is no involvement of the community in the design and conduct of the research.

17.0 Sharing of Results with Subjects*

17.1 Results will be shared by presentation of data in Scientific meetings. Data will not have any personal identifiers of any subject.

18.0 Setting

Blood will be drawn in the clinics located at the Margret palace in the clinical trial by the nurse coordinator. All the tests will be formed at the clinical laboratories of the LSUHS and the laboratory of Dr. Jain Room 5-314 of the School Building.

19.0 Resources Available

Dr. Jain and Dr. Levine have conducted and published the results of many clinical studies carried out in African Americans. This team has previously conducted three clinical trials and has a track record of successfully enrolling African Americans volunteers following IRB guidelines and a commitment to maintaining high ethical standards. Dr. Jain will spend 60% of his time and Dr. Levine will spend 10% of his time. Dr. Jain have been the PI of three clinical trials carried out to translate from basic to clinical research and investigations of the efficacy of micronutrients in type 1 and type 2 diabetic patients. Dr. Jain has a demonstrated track record of past collaborations, conducting clinical trials and many years of co-publications with co-investigator Dr. Levine. As a result of these previous experiences, I am very much aware of the importance of frequent communication among all of the investigators, compliance, regular research meetings, the construction of a realistic time line, and the need for high level of ethics and high quality clinical research protocols. Dr. Jain has published over 169 articles in peer-reviewed journals with over 14000 citations. A qualified nurse will be hired to draw blood. Nurse coordinator and Dr. Jain will also be responsible for enrolling volunteers, obtaining informed written consent, making telephone calls and compliance with visits and supplementation. Dr. Jain and nurse coordinator will also be responsible for coordination with IRB and obtaining any required modifications. 50% salary support is requested for the nurse coordinator. Dr. Jain and nurse coordinator will coordinate with the Research pharmacy and Dr. Shi to ensure random subject assignment of placebo, VD, LC, VD+LC for each group of subjects. Dr. Shi is a biostatistician and will do data management and statistical analyses of data.

20.0 Prior Approvals

20.1 We have already approval from the Biosafety committee. We also have IND waiver from the FDA. Copy of FDA letter enclosed with this protocol.

21.0 Recruitment Methods

IRB approved poster will be displayed in the hospital and Medical School building asking for volunteers to participate in this clinical trial. PI and nurse will also visit the local health fairs, community gathering such as churches, to educate all the African American community members about the clinical trial and ask for willingness to participate in this study. Investigators will also ask other faculty, nurses, residents and fellows about the clinical trial and help in contacting healthy adults to participate in this study. Volunteers will be informed in layman language about this study, time needed to participate in this study, any reimbursement participant will receive and how many visits are required to successfully complete the enrollment and study. Similarly, we will also advertise in the local newspaper for AA volunteers to participate in our clinical trial. All volunteers will be reimbursed $50 for each visit for the time and travel. Check will be mailed to the address provided by the department office.

22.0 Local Number of Subjects

We plan to enroll in 160 subjects.

23.0 Provisions to Protect the Privacy Interests of Subjects

No body other than research team is permitted to access any sources of information about the subjects.

24.0 Compensation for Research-Related Injury

This research involves Minimal Risk to subjects. You will not be given any other monetary compensation for taking part in this study. The costs for tests done because you are in the research study will be at no charge to you and will be paid for by the study sponsor. However, you or your insurance company must pay for all tests and procedures that are not part of the study but that your doctors feel are needed to help you.

25.0 Economic Burden to Subjects

There is no cost to research participant for any medical or laboratory tests performed by the Nurse/investigators.

26.0 Consent Process

We will obtain written informed consent from all the participants using IRB approved consent form. Each consent form will be signed by a witness. Dr. Jain and Dr. Levine have conducted and published the results of many clinical studies carried out in African Americans. This team has previously conducted three clinical trials and has a track record of successfully enrolling African Americans volunteers following IRB guidelines and a commitment to maintaining high ethical standards. Our research will not include whole genome sequencing (i.e., sequencing of a human germline or somatic specimen with the intent to generate the genome or exome sequence of that specimen).

27.0 Process to Document Consent in Writing

This research presents no more than minimal risk of harm to subjects and involves written documentation of consent will be required. A copy of the signed and dated consent document will be given to the person signing the document.

28.0 Drugs or Devices

This research involves dispensing micronutrient supplements vitamin D and L-cysteine. These are also sold over the counter in any discount store. These nutrients are normal part of our food consumed by the public, such as milk, cheese, eggs and green vegetables. The supplement capsules will be provided by the investigators free of any cost to the participants.

Capsules details.

1. The study subjects will ingest capsules containing placebo and supplements, orally, daily: (I) placebo (P); (II) L-cysteine (LC) (1000 mg); (III) Vitamin D (VD) (2000 IU); (IV) VD+LC groups

2. To start with each group/subject will have placebo run-in period for one month and all subjects will take two placebo capsules daily.

3. After one month of Placebo run-in period, all subjects will be randomized into four groups. Each capsule will contain a placebo or cholecalciferol (2000 IU) or LC (1000 mg) or VD+LC. Supplements will be taken daily with food at breakfast for 6 months.

4. Placebo group will take two placebo capsules a day in the morning; II LC group will receive two capsule of LC (500 mg each) daily; (III) VD group will take two capsule of VD (1000 IU) daily; (IV) VD+LC group will take daily two capsule of combination (VD 1000 IU VD+ 500 mg LC) daily.

5. Supplements will be taken daily with food at breakfast for 6 months.

6. We are working with Company named: Superior Supplement Manufacturing, Fountain Valley California, to get these capsules made. These capsules will look similar and will be of similar size.