D2d Ancillary Study Application

Office Use
Ancillary Study Number: AS14-07 Date Submitted (MM/DD/YYYY): 03/03/2015

Title of Proposal *(81 character limit)*: Does vitamin D3 supplementation have an effect on markers of DNA damage?

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Other Key Persons (biosketch is not required)
Name: Gustavo G. Zarini Ancillary Study Role: Research Coordinator
Name: Ancillary Study Role:
Name: Ancillary Study Role:
Name: Ancillary Study Role:

D2d Ancillary Study Subcommittee Use Only

Ancillary Studies Subcommittee Action: Please Select Date
Steering Committee Action: Please Select Date
DSMB Action: Please Select Date

Approved Applications

Consent form required? Please Select

Part 1: Research Plan

1a. Anticipated Timeline and Enrollment

Planned Start Date	11/01/2015
Planned End Date	07/01/2020
Anticipated Enrollment	1,800

1b. Hypothesis and Specific Aims

Please describe the question(s) you are asking and what you expect to happen (3/4 page limit).

We expect vitamin D3 supplementation to increase serum vitamin D levels for the treatment group and the increase in serum vitamin D will be associated with a reduction in DNA damage. Our proposed mechanism is that increased serum vitamin D due to supplementation will lead to a corresponding increase in telomerase activity, which will slow the shortening rate of telomere length. We also expect vitamin D3 supplementation to reduce oxidative DNA damage as indicated by a decrease in 8-hydroxy-2"-deoxyguanosine (8OHdG). In addition, racial/ethnic differences in serum vitamin D may lead to supplementation working better for some race/ethnic groups to improve DNA damage. Blacks and Hispanics start with lower levels of serum vitamin D3 as compared to Whites (1). Several studies indicated that Blacks and Hispanic start with longer telomere length as compared to Whites (2-5) but their rate of attrition is greater than in Whites (4-6).

Primary Aim: To prospectively evaluate the effect of vitamin D supplementation on markers of DNA damage among multi-ethnic populations with prediabetes.
Hypothesis 1a: Vitamin D supplementation, as compared to placebo will slow the progression of DNA damage, as measured by leukocyte telomere length (LTL), in individuals from baseline to 36-month follow-up.
Hypothesis 1b: Individuals who receive vitamin D supplementation will have a greater increase in telomerase activity, as measured in peripheral blood mononuclear cells (PBMCs), than would individuals who receive placebo from baseline to 36-month follow-up.
Hypothesis 1c: Vitamin D supplementation, as compared to placebo will have a greater reduction in oxidative DNA damage, as measure by 8-hydroxy-2"-deoxyguanosine (8OHdG), in individuals from baseline to 36-month follow-up.

Secondary Aim: Race/ethnicity will modify the relationships between level of vitamin D supplementation (4000 IU/day versus placebo) and DNA damage (telomere length, telomerase activity and 8OHdG levels) from baseline to 36-month follow-up.
Hypothesis 2a: Race/ethnicity subgroups will modify the association between vitamin D supplementation level and leukocyte telomere length (LTL) over time.
Hypothesis 2b: Race/ethnicity subgroups will modify the association between vitamin D supplementation level and PBMC telomerase activity over time.
Hypothesis 2c: Race/ethnicity subgroups will modify the association between vitamin D supplementation level and 8OHdG levels over time.

Tertiary Aim: Serum vitamin D levels at 36-month for both groups combined (4000 IU and placebo) will be related to primary outcomes, with higher values of serum vitamin D resulting in higher values for leukocyte telomere length and telomerase activity and lower in 8OHdG levels.
Hypothesis 3a: Higher serum vitamin D levels will be associated with longer leukocyte length (LTL) at 36-month.
Hypothesis 3b: Higher serum vitamin D levels will be associated with higher PBMC telomerase activity at 36-month.
Hypothesis 3c: Higher serum vitamin D levels will be associated with lower 8OHdG levels at 36-month.

1c. Significance and Brief Background

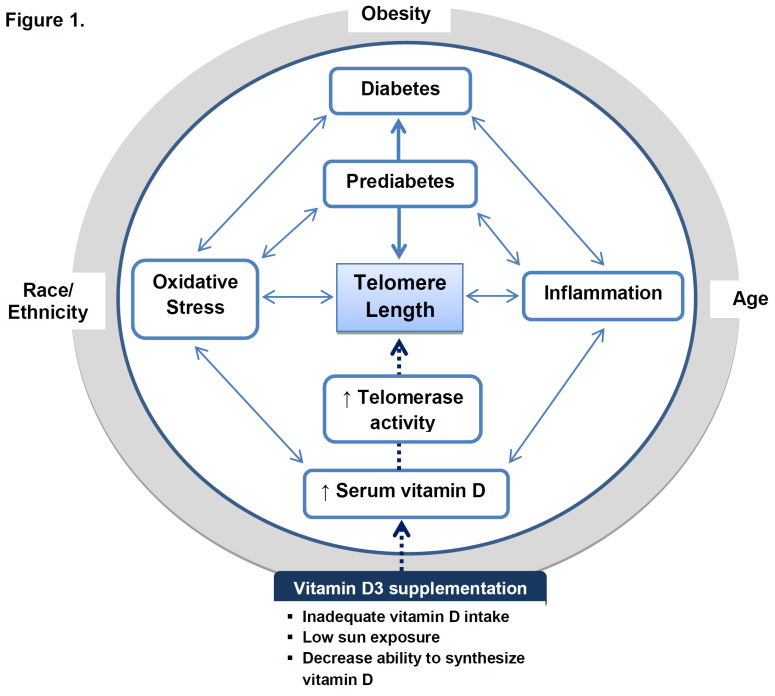
Please describe the scientific relevance (1 page limit).

Telomeres are structures of non-coding DNA that protect the erosion of coding chromosomes (7). Telomeres, the ends of eukaryotic chromosomes, undergo attrition with each replication (7, 8), and the replication potential of somatic cells correlates with telomere length (9). Telomerase, a cellular enzyme, adds the necessary telomeric DNA (T2AG3 repeats) onto the 3' ends of the telomeres by copying an RNA template sequence encoded by its telomerase RNA component (TERC) gene (10). Low levels of telomerase create short telomeres, which in turn signal exit from the cell cycle, cell senescence and apoptosis (11). Shorter telomere length and low telomerase activity have been linked to type 2 diabetes, oxidative stress, and mortality (2, 3, 12-17). Obesity, insulin resistance, hypertension and smoking, factors associated with diabetes and cardiovascular diseases, are also associated with shortened telomere length (18-23). Shorter telomeres have also been associated with oxidative stress and cardiovascular disease, independent of chronological age (2, 17). The erosion of telomere length is thought to be a consequence of oxidative stress (17). Oxidative stress increases the loss of telomere repeats per cell division (2). Leucocyte telomere length has been considered a biomarker of aging and overall oxidative stress (17). Epidemiological studies indicate that shortened telomere length has also been linked with oxidative stress, DNA damage (24-27) and inflammation (3, 27). Prediabetes and diabetes have been associated with oxidative stress and endothelial dysfunction (28). Moreover, inflammation and oxidative stress are precursors in the biology of aging (29, 30) and may have an individual or synergistic effect on telomere length. The positive impact of vitamin D on the actions of insulin secretion and inflammation has been based on observational studies (31). The biochemical mechanism for the association between vitamin D and inflammation, oxidative stress, and glycemic metabolism has not been established; however, the effect of these factors on telomere length has yet to be determined. Higher serum 25-hydroxy vitamin D [25(OH)D] levels in healthy women were positively associated with longer leucocyte telomere length (LTL) (32). Overweight African Americans without diabetes receiving vitamin D3 supplementation showed a significant increase in telomerase activity after 16 weeks in a randomized clinical trial (33). Vitamin D regulates the synthesis of estrogen hormones (34) indirectly influencing the expression of telomere length and telomerase activity. The presence of vitamin D receptors found in leukocytes further links vitamin D with telomere length (35). Additionally, vitamin D may also play a role in reducing oxidative stress; however, information is scarce regarding the relationship between vitamin D status and/or vitamin D3 supplementation with 8-hydroxy-2"-deoxyguanosine (8OHdG) an oxidative stress marker.

In a randomized, double-blind, placebo-controlled study, 8OHdG levels were reduced 25% after 6 months of treatment with vitamin D3 supplementation (36). Studies comparing race/ ethnicity and telomere length are inconclusive. It is reported that African Americans have longer LTL as compared to Whites (2-5, 37), but they have a faster age-related shortening of LTL (4-6), even after adjusting for gender, obesity and smoking (4). On the other hand, Blacks and Hispanics are at higher risk for vitamin D deficiency as compared to Whites (1). Further investigation to identify racial/ ethnic differences in markers of DNA damage and their relationship with vitamin D are needed.

Figure 1 shows conceptual framework of the proposed study, based on the literature and reported relationships of vitamin D.

Figure 1.



1d. Innovation & Impact

Please describe the research innovation and potential impact (1/2 page limit).

Major innovations of the proposed study are: 1) To investigate the causal relationship between vitamin D supplementation and markers of DNA damage, 2) To measure the effect of change in 25(OH)D levels in markers of DNA damage long term, 3) Inclusion of men and women from multi-ethnic populations, 4) The use of vitamin D3 supplementation at the Institute of Medicine (IOM) recommended upper limit (4000 IU/day) for 36 months duration.

Findings from the proposed study may indicate a potential role of vitamin D3 supplementation in slowing the process of aging and related diseases through telomerase re-activation, maintenance or slowed loss of telomere length and a reduction in oxidative stress. If serum vitamin D levels are associated with telomere length, telomerase activity and oxidative stress, then vitamin D3 supplementation could prove to be a cost effective way to slow the aging process, prevent age related chronic diseases. It is known that insufficient serum vitamin D could lead to morbidity and mortality (38, 39). Higher serum vitamin D had been associated with longer telomeres for women (32). No studies exist comparing race/ethnicity with duration of vitamin D3 supplementation and DNA damage. We expect to generate much needed data concerning the magnitude and direction of response of DNA damage with vitamin D3 supplementation duration by race/ethnicity. The results of this study is necessary to inform future research concerning recommendations or vitamin D3 supplementation for racial/ethnic groups with prediabetes.

1e. Research Approach

Please address the following: (1 and 1/2 page limit)

- *Study type (e.g. interventional or observational)*
- *Population and setting (inclusion/exclusion criteria)*
- *Study design (e.g. details of study procedures, outcome assessments, confounders, bias)*
- *Schedule of assessments*
- *Sample size (power) calculations*
- *Data analysis plan*

Population and setting: The proposed study will be conducted at the human nutrition laboratory at Florida International University. Our study will use blood samples of the first 1800 participants from the D2d multiethnic study. We will use all participants from baseline to 36 months and apply the following self-reported categories to assess race: White (62%) and Black (30%) and Other (8%). The specimens selected will be blood from participants who have the same inclusion/exclusion criteria of the parent study. Our recruitment of the samples will be based on receiving all baseline samples for placebo and intervention and as many completers as achieved in 36 months.

Study design: This secondary analysis of the parent D2d study is a longitudinal study (baseline, 12, 24, and 36 months) assessing the effect of dosage (treatment of 4000 IU/day of vitamin D3) versus placebo (control) by race/ethnicity on the progression or reduction of DNA damage. Outcome measurements are telomere length, telomerase activity, and 8-hydroxy-2”-deoxyguanosine (8OHdG) levels for oxidative damage.
Procedure: Samples will be delivered overnight with dry ice from the central processing center at Tufts University to our human nutrition laboratory at Florida International University. The samples will be inventoried as soon as they are received and placed in our freezer at -80° C. Analysis will begin once sufficient number of samples are received to process and continue until all samples are analyzed. We anticipate analyzing baseline and the 12-month time point within the first year and will continue to receive samples for the 24th and 36th month when available.

Please continue Research Approach on next page.

Outcome assessments and confounder bias: We will analyze the following outcomes: 1) Telomere length will be measured by TeloTAGGG Telomere Length Assay kit (Roche Applied Science, IN, USA) on BioRad CFX96 real time PCR instrument (Hercules, CA, USA) with genomic DNA isolated from whole blood (QiaAmp DNA Mini Kit (Qiagen) CA, USA). For each sample, relative telomere to single copy number ratio (T/S) will be assayed by real-time PCR method described by Cawthon (40), and compared to a reference sample. PCR methods are suggested for larger sample sizes (hundreds to thousands) (41). There are two main PCR methods for single gene copy: Q-PCR or MMQ-PCR. The later method chosen to determine telomere length, by T/S ratio, was considered accurate (41). We investigated several DNA extraction kits for PCR including PureLink Genomic DNA Mini Kit (Life Technologies), QiaAmp DNA Mini Kit (Qiagen) and the Lahiri and Nurnberger (high salt) method and since they were shown to have some variation, we will use the same kit type as recommended (42). The three PCR extraction kits all showed acceptable reproducibility (42). 2) Telomerase activity will be measured by TeloTAGGG Telomerase PCR ELISA kit (Roche Applied Science, IN, USA) from serum following vendor's instructions. The absorbance will be measured at A450 nm using Biotek FLX 800 Microplate Spectrophotometer against reference wavelength A690 nm (BioTek Instruments, Inc., VT, USA). 3) 8-hydroxy-2"-deoxyguanosine (8OHdG) levels will be determined by Enzyme-Linked Immunosorbent method, the reagents manufactured by Cayman Chemicals (Ann Arbor, MI, USA). The analytical procedure will be followed precisely according to the manufacturer's instructions.
Major confounders for the assessment of vitamin D3 supplementation, race and DNA damage will be supplied as data by the D2d parent study. The following variables are available from time points: baseline, 12, 24 and 36 months: 1) age, 2) gender, 3) race and ethnicity, 4) 25(OH)D, 5) smoking status, 6) HbA1c, 7) FPG, 8) BMI, 9) waist circumference, 10) geographic location, 11) medications usage (all available), 12) calcium intake, 13) vitamin D intake, 14) nutritional supplements, 15) insulin, 16) systolic blood pressure, 17) diastolic blood pressure, 18) physical activity, 19) study pill adherence, and 20) study pill distribution. Potential biases that may be introduced by using the first 1800 out of the 2300 recruited include differences in sociodemographics composition and dropout rate. In order to reduce sample bias, we will use "intent to treat" analysis and will carry-over values for participants who dropped out.

Sample size: A sample size of 900 per dosage group (placebo and 4000 IU vitamin D supplementation) will provide at least 80% power to detect a small effect size, 0.17, for change over time, time*dosage interaction, and time*dosage*race interaction for F tests with 0.05 level of significance. It will also provide more than adequate power for the multiple linear regression. The power calculations were done in PASS 11.

Data analysis: All analyses will be done on an "intent to treat" basis. Additional analysis will be conducted to assess differences between completers and dropouts. For the primary and secondary aims, mixed model will be employed using telomere length, telomerase activity and 8OHdG levels as the dependent variable, respectively. The interaction between dosage and time will be included to evaluate the dosage effect on the change of the dependent variable over time. The three-way interaction among race, dosage and time will be included to evaluate the moderator effect of race on the relationship between dosage and the dependent variable over time. For the tertiary aim, multiple linear regression will be used to test the association between serum vitamin D level and three dependent variables at 36 months, respectively. Both models will include the adjustment variables that are major confounders of vitamin D and outcomes such as change in A1C, change in medications, and obesity indicators. Potential additional covariates will be tested by correlation with the dependent variable. These analyses will be done in SAS 9.3. A significance level of 0.05 will be used to decide the statistical significance. We do not expect the missing data to be large; however, we will assess the situation carefully and use an appropriate imputation method, if justified.

1f. References Cited
Please attach a list of references (PDF format document) with the application.

Part 2: Description of Data

2a. Required Sources of Data
Please select all that apply:

- ☑ Existing data collected as part of D2d study
  If yes, please complete ***D2d Ancillary Study Data Request Form***
- ☑ New data derived through use of stored biological specimens collected as part of D2d study
  If yes, please complete ***D2d Ancillary Study Specimen Request Form***
- ☐ New data derived through direct contact with D2d participants (e.g. procedure, survey, observation)
  If yes, please complete question *2b*
  If available, please include a copy of the proposed protocol with your application.

2b. New Data Acquisition, *if applicable*
Please describe the additional procedures, interventions or surveys required for new data acquisition and address the need for additional visits and/or the prolongation of existing visits. (1/2 page limit).

Not applicable.

Part 3: Facilities & Resources

3a. D2d Collaborating Clinical Sites

Please note: By marking the box next to a site(s), the ancillary study PI has secured the commitment of the site(s) to the proposed ancillary study to allow generation of new data by direct contact with D2d participants at the site.

Please select all D2d collaborating clinical sites that have agreed to participate in the proposed Ancillary Study.

- ☐ Atlanta VA Medical Center
- ☐ Baylor College of Medicine
- ☐ Beth Israel Medical Center
- ☐ Duke University Medical Center
- ☐ Florida Hospital Translational Research Institute
- ☐ HealthPartners Research Foundation
- ☐ Los Angeles Roybal
- ☐ Maine Medical Center Research Institute
- ☐ Medical University of South Carolina
- ☐ MedStar Health Research Institute
- ☐ NIDDK Phoenix
- ☐ Northwestern University
- ☐ Pennington Biomedical Research Center
- ☐ Stanford University Medical Center
- ☐ Tufts Medical Center
- ☐ Tulane University Health Sciences
- ☐ University of Kansas Medical Center
- ☐ University of Nebraska Medical Center
- ☐ University of Tennessee Health Science Center
- ☐ University of Texas Southwestern Medical Center

3b. Description of Clinical Laboratory Facilities, *if applicable*

Please describe clinical laboratory facilities and where and how bio-specimens will be handled (e.g. storage, shipping of biological material; laboratory staff experience).

The Laboratory directed by Dr. Huffman is exclusively available for the proposed research at FIU. The laboratory is comprised of a 1,700 square feet facility in the Academic Health Center (AHC-1) building with capabilities to determine biomarkers of cardiovascular disease risk factors, mitochondrial DNA, mitochondrial toxicity, genetic markers and oxidative stress. The equipment includes Westover Trinocular Microscope (1), Bio-Tek Universal Microplate Reader (1), Biotech Vertical ElectrophoresisSystem (1), iQ5 Bio-Rad RT PCR (1), Millipore Flow Cytometer (1), Bio-Rad Mycyler Thermal Cycler (1), Biotech Hortizontal Electrophoresis System (1), Beckman Coulter Spectrophotometer with Multi-cell capabilities (1), Perkin-Elmer Absorption Spectrophotometer (1), Fluorescence Spectrophotometer (1), Revco Ultra-Low Temperature Freezers (4), Beckman Centrifuge (1), Biological Safety Hood (2), Pelton and Crane Autoclave (1), CO2 Incubator (1), Micro-centrifuge (1), 4 Dell PCs and other basic laboratory equipment. There are devoted support activities provided by the institution, such as stockroom, autoclaving, dishwashing, hazardous waste disposal, etc. The laboratory meets all state/federal safety guidelines and is equipped with safety equipment such as eye wash stations, showers, flammable storage, fume and biological hoods, personal protection equipment, fire blankets and fire extinguishers. Additional core facilities are available in the physical vicinity that provide DNA sequencing, flow cytometry, microsatellite typing, as are additional cell culture facilities.

Part 4: Potential Burden on the D2d study

*Please describe the potential burden of the ancillary study **on the D2d study** in relation to the following and provide ways to minimize the burden:*

4a. Participant Burden and Potential for Compromising Participant Retention

There will be no additional procedures and or risk for the participants other than those experienced in parent study.

4b. Participant Safety and Confidentiality

Please describe measures taken to ensure participant safety and confidentiality and address plan for data management (secure storage, monitoring etc.).

The ancillary study will use unidentifiable data from the D2d parent study. Data provided by the parent study will be coded to not identify individuals. Confidentiality will be maintained at the parent study sites.

4c. Burden on Collaborating Clinical Sites

There is no burden on the collaborating clinical sites. All data will be shipped from the central site.

4d. Regulatory Requirements

Please describe how informed consent will be obtained and describe plan for local IRB approval. If available, please attach a copy of the informed consent form.

This study protocol will be submitted to the Institutional Review Board (IRB) at Florida International University (FIU) for use of residual blood, which is an exempt classification since we will be using blood samples that are coded with no identifiable markings. Data confidentiality will be strictly maintained. The data previously collected will not contain names, addresses, phone numbers, date of birth, social security numbers and/or medical records of the participants. The new data for blood analysis will be identified with participants' ID only and will be stored on a password-protected computer in a locked laboratory with limited access. The automated databases will be accessible through a password protected security system and backups (USB drives) are kept in a locked cabinet in the PI's locked office. All data will be subjected to double entry and data cleaning.
The consent forms will be obtained at the main study site.

Part 5: D2d Support

Please note: The costs associated with the use of resources (D2d or other) must be included in the plans for funding the ancillary study. All negotiated services must be documented in a letter of commitment from the provider of such services.

Please select resources at the D2d Coordinating Center or D2d Central Laboratory that will be required. There will be a fee associated with these services:

- ☑ Data extraction and transfer
- ☑ Specimen selection and transfer
- ☑ Data analyses *(depending upon staff availability, proposed analyses may be conducted by the analytical team at the D2d Coordinating Center*)
- ☐ Other study-specific services for studies that will collect new data in real time

Part 6: Funding

6a. Funding Source(s)

Please describe ancillary study funding source(s) or plans to apply for funding.

National Institutes of Health (NIH) and National Institute of Diabetes and Digestive and Kidney (NIDDK).

6b. Planned Date of Submission to Funding Agency 06/05/2015

Part 7: NIH Biosketch

*Please attach NIH Biosketches **for PI only** with your application upon submission.*

D2d Ancillary Study Application

Part 8: Acknowledgement of D2d Ancillary Studies Policies & Procedures

I have read and agree to abide by the policies and procedures for D2d Ancillary Studies as described in the document titled: *D2d Ancillary Studies Policies and Procedures & Instructions for Submission of Proposals*, and specifically regarding the presentation and publication of ancillary study results and data sharing policies.

Principal Investigator Signature *(e-signature accepted)* Fatma Huffman Digitally signed by Fatma Huffman DN: cn=Fatma Huffman, o=FIU, ou=Dietetics and Nutrition, email=huffmanf@fiu.edu, c=US Date: 2015.03.02 15:52:36 -05'00' Date 3-2-2015

Part 9: Attachments

Please indicate all documents that are included with your application:

- ☑ NIH Biosketches for PI only ***(required for all applications)***
- ☑ References Cited ***(required for all applications)***
- ☑ D2d Ancillary Study Data Request Form
- ☑ D2d Ancillary Study Specimen Request Form

Please note: The following forms are not required with application, but are required *prior to study initiation:*

- ☐ Ancillary Study Proposal
- ☐ Ancillary Study Manual of Procedures
- ☐ Informed Consent Form *(required for studies involving generation of new data via direct contact with D2d participants)*
- ☐ IRB Approval Letter

Final steps to submission:
Save a copy of this form to your computer.
Click on the "Submit" button, which will open an email and automatically attach application. In that email, attach additional documents checked above to complete application then send.

Submit

Thank you for your interest in the D2d Study