D2d Ancillary Study Application

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Ancillary Study Number: AS13-04 Date Submitted (MM/DD/YYYY): 06/03/2013

Title of Proposal *(81 character limit)*: Vitamin D Supplementation to Reduce Vascular Risk Progression

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Other Key Persons (biosketch is not required)

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D2d Ancillary Study Subcommittee Use Only

Ancillary Studies Subcommittee Action: Approve Date: 06/10/2013
Steering Committee Action: Approve Date: 08/12/2013
DSMB Action: Please Select Date:

Approved Applications

Consent form required? Yes

D2d Ancillary Study Application

Part 1: Research Plan

1a. Anticipated Timeline and Enrollment

Planned Start Date	12/01/2013
Planned End Date	11/30/2017
Anticipated Enrollment	

1b. Hypothesis and Specific Aims

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

More than 79 million US adults have pre-diabetes, a condition which progresses to diabetes at a rate of approximately 10% per year (1,2). Lower blood vitamin D levels are associated with prevalent pre-diabetes, progression to overt diabetes, and excess cardiovascular disease (CVD) mortality (3-9). Thus, patients with pre-diabetes may be excellent candidates for early interventions with vitamin D supplementation to potentially reduce the development of type 2 diabetes (T2DM), atherosclerosis, and CVD events; however, such hypotheses have not been adequately tested in interventional studies. The proposed study, which is an ancillary study to D2d, will determine whether vitamin D3 supplementation reduces CVD progression, inflammation, and inflammatory markers in participants with pre-diabetes.

In prior short-term studies, we and others have shown that vitamin D treatment improves factors linked to vascular inflammation in pre-diabetes and T2DM: it improves insulin secretion and peripheral insulin action, suppresses the renin angiotensin system, decreases systemic inflammatory mediators, decreases macrophage cytokine release, and prevents monocyte adhesion and migration and macrophage cholesterol deposition by downregulation of endoplasmic reticulum (ER) stress (10-17). Thus, we hypothesize that long-term vitamin D3 supplementation will reduce vascular inflammation and atherosclerotic progression in pre-diabetes.

In the carotid atherosclerotic plaque, monocytes and macrophages exist in at least two distinctly differentiated phenotypes: classically activated M1 and alternatively activated M2 cells (18). We found that T2DM patients with higher 25(OH)D levels have decreased monocyte migration and adhesion to endothelial cells and reduction of both M2 and M1 membrane markers, suggesting that vitamin D suppresses both monocyte subsets present in the atherosclerotic plaque (16,17). In addition, active vitamin D supplementation reverses macrophage cholesterol deposition to generate cells with anti-atherogenic properties (15,16). We performed a small short-term pilot and feasibility trial in vitamin D-deficient patients with T2DM and found that vitamin D3 4000 international units (IU)/day decreases progression of carotid intima-media thickness (CIMT) and improves endothelial dysfunction [by brachial artery reactivity testing (BART)] compared to 400 IU/day. This evidence suggests that immune modulation by vitamin D may prevent CVD progression.

To further test our hypothesis, we propose an ancillary study (D2d-CVD) in 236 D2d participants, followed for the first two years of intervention. We expect the D2d-CVD study to accomplish the following specific aims:

1. To determine if vitamin D3 supplementation (4000 IU/day) improves non-invasive markers of subclinical CVD in patients with pre-diabetes. Participants will be evaluated for changes in a) vascular morphology (CIMT); b) systemic inflammation [hsCRP, matrix metalloproteinase 9 (MMP-9), tumor necrosis factor α (TNFα), interleukin (IL) 1]. Aim 1A: In mediation analyses, test whether changes in systemic inflammatory markers predict change in CIMT.

2. Determine if vitamin D3 supplementation (4000 IU/day) promotes an anti-atherogenic phenotype in monocytes derived from patients with pre-diabetes. At the same time points as in Aim 1, we will assess: membrane receptor phenotype, monocyte adhesion and migration and Aim 2A: in mediation analyses, test whether monocyte membrane receptor phenotype (M1 vs. M2 differentiated monocytes) or monocyte adhesion or migration predict change in CIMT.

1c. Significance and Brief Background

Please describe the scientific relevance (1 page limit).

Pre-diabetes has been called the “largest healthcare epidemic” in the United States. Pre-diabetes is the leading risk factor for T2DM and is present in a staggering 79 million adults in the U.S., more than 1 in 3 (2). While pre-diabetes progresses to T2DM at a rate of approximately 10% per year, it also constitutes an independent factor risk for CVD, with annual CVD mortality rates being nearly twice that of normoglycemic individuals (1). Hyperglycemia accounts for some of the increased risk of CV events in these patients. However, other risk factors such as hypertension and vascular inflammation are considered critical to the development of coronary disease (19). Therefore, it is imperative to elucidate mechanisms linking vascular inflammation to pre-diabetes and cardiovascular risk factors that may lead to novel therapies to treat these pervasive diseases.

Data linking vitamin D and CVD comes primarily from observational studies, but a recent meta-analysis of 9 prospective cohort studies showed an association between low 25OHD levels and risk for CVD in only half of these studies (20). Randomized controlled trials in this area are scarce and do not demonstrate a significant effect on CV outcomes or mortality; however, major study limitations limit conclusions from the available trials (21-25). No randomized controlled trials have been published primarily examining the effects of vitamin D on CV outcomes or focusing on the use of vitamin D on markers of CVD progression in patients with pre-diabetes, and thus, the proposed study addresses an unmet clinical need.

Population-based and intervention studies have shown that CIMT is a valid and reliable surrogate measure of coronary atherosclerosis (26). Previous meta-analysis shows that patients with impaired glucose tolerance (IGT) have a CIMT 0.04 mm thicker than control subjects, the magnitude of which is about 1/3 of that observed in diabetics, supporting that pre-diabetes is associated with risk factors typical of T2DM (27,28). In T2DM patients, low levels of 25OHD are strongly and independently associated with increased CIMT (29); however, no studies have evaluated the effects of vitamin D supplementation on CIMT in pre-diabetics. Endothelial dysfunction reflected by impaired BART response is another subclinical marker for the development of CVD (30). The only interventional study of vitamin D on surrogate markers of CVD in diabetics showed that a single dose of 100,000 IU of vitamin D2 in those who are deficient significantly improves BART at 8 weeks after adjusting for blood pressure (BP) changes (31). Therefore, vitamin D treatment may slow the progression of vascular dysfunction in pre-diabetics when administered in early stage disease.

Inflammation of large arteries alters elasticity, increases stiffness, and contributes to the high prevalence of CVD in T2DM and pre-diabetes (32-34). Vitamin D influences multiple known mechanisms responsible for the increased vascular inflammation seen in patients with pre-diabetes. Long-term vitamin D3 supplementation significantly reduces plasma levels of CRP, MMP 1 and 9, and its inhibitor (TIMP-1) in vitamin D-deficient, non-diabetic individuals and has beneficial effects on the elastic properties of the common carotid artery in postmenopausal women (35,36). However, the effects of long-term vitamin D supplementation on systemic inflammatory markers of CVD in pre-diabetes are unknown. Monocytes and macrophages play a critical role in the development of atherosclerosis and exist in at least two distinctly differentiated phenotypes: classically activated M1 and alternatively activated M2 cells (37). Initial theories have suggested that M1 cells perpetuate inflammation associated with atherosclerosis, while the role of M2 cells is unclear (38). Our results from prior studies suggest that the presence of adequate vitamin D can shift this paradigm. We found that in macrophages from diabetic patients, 1,25(OH)2D3 supplementation switches vitamin D-deficient, M2-predominant proinflammatory macrophages into M1-predominant macrophages with reduced proinflammatory cytokine secretion, migration and adhesion to endothelial cells, and foam cell formation, all suggestive of an anti-atherogenic M1 phenotype (15-17). From these data, we devised the macrophage phenotype ratio (MPR) to determine the relative proportion of macrophage M1 versus M2 marker expression, finding a consistent correlation of higher MPR with higher 25(OH)D and suppressed atherogenic properties within diabetics (17). Therefore, the next logical step is to determine in vivo whether vitamin D supplementation in pre-diabetic subjects generates an anti-atherogenic monocyte phenotype that suppresses vascular inflammation to prevent atherosclerotic progression.

1d. Innovation & Impact

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

The expansion of the D2d study with this proposal represents a unique opportunity to determine the effects of a highly promising and novel intervention on cardiovascular outcomes. The Dd2-CVD will integrate the effects of vitamin D on progression of CVD and progression to overt diabetes and determine whether these effects are independent or interrelated. Furthermore, vitamin D deficiency is more prevalent in pre-diabetes, and current evidence demonstrate vitamin D as an immunomodulator and contributor to CVD risk factors. Thus, we are taking a mechanistically-directed approach to treat systemic inflammation for prevention of CVD with the potential to affect long-term outcomes. The D2d-CVD will be the first long-term randomized clinical trial studying the effects of vitamin D supplementation on CVD progression in pre-diabetes.

This proposal will combine clinical and basic research by characterizing the role of vitamin D in regulating monocyte differentiation and vascular inflammation, thus impacting the progression of atherosclerosis. The macrophage phenotype ratio (M1/M2) and monocyte adhesion and migration will be characterized over time as novel biomarkers for atherosclerotic disease progression. Based on our preliminary data showing that monocytes from vitamin D sufficient patients with T2DM have a predominantly M1 phenotype with less vascular adhesion and migration, we propose to examine in vivo whether vitamin D supplementation in pre-diabetes generates a similar monocyte phenotype and to validate whether this novel biomarker predicts CIMT response to vitamin D supplementation. Furthermore, the chronic inflammatory state in this population will allow us to determine whether vitamin D suppresses systemic inflammatory markers and if these changes correspond to changes in inflammatory monocytes and predict CIMT response to vitamin D.

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*

Study Type. We propose an ancillary study to assess markers of cardiovascular disease in a subset of the D2d study population for 2 years of follow-up. D2d-CVD will follow the D2d protocol; patients randomized in a double-blinded fashion to vitamin D3, 4000 IU/day versus placebo, will be asked to participate in the ancillary study. Aim 1 will determine the effects of vitamin D3 supplementation on early atherosclerosis assessed by CIMT (primary endpoint). Additionally, in Aim 1, we will investigate the effects of vitamin D3 on systemic inflammatory markers, and whether changes in inflammatory markers predict CIMT response. In Aim 2, we will assess whether vitamin D3 supplementation alters the monocyte receptor phenotype or monocyte adhesion and migration, and whether these properties predict change in CIMT. We expect to recruit all needed patients during the first 2 years of funding, with two years of follow-up and a year of data cleaning and analyses.

Population and Setting. Inclusion criteria are identical to those of the D2d protocol and include: 1) Pre-diabetes defined by meeting 2-out-of-3 of the following glycemic criteria: fasting plasma glucose 100-125 mg/dl, inclusive (impaired fasting glucose, IFG); 2 hour plasma glucose; 140-199 mg/dl, inclusive (impaired glucose tolerance, IGT); A1C 5.7-6.4%, inclusive; 2) Age ≥ 30 years with a Body Mass Index (BMI) 25 (23 for Asians) to 40 kg/m2. Exclusion criteria are also identical to those of the D2d protocol and are thus omitted here secondary to length.

Study design/schedule of assessments. All outcomes will be assessed at baseline, 1 year (M12), and 2 years (M24), and will include the following procedures: A) Carotid Artery Intima-Media Thickness (CIMT). A commercially-available ultrasound system will be used by a single, blinded vascular sonographer at each site for

Please continue Research Approach on next page.

CIMT. The distal 1 cm posterior wall of bilateral common carotid arteries will be measured using an automatic edge detection function, as previously described (39). Bilateral CIMT values will be averaged to obtain a single mean and maximal CIMT for each individual. For inflammatory markers, 0.5 ml of frozen serum will be obtained from the D2d Central Laboratory and stored at -80º C until analysis by the PI's laboratory. Samples will be analyzed by ELISA assays for systemic markers of inflammation associated with vascular disease (hsCRP, fibrinogen, TNFα, IL-1, MMP-9). B) Monocyte isolation. Forty ml of peripheral venous blood will be drawn from each individual, and monocytes will be isolated by standard Ficoll techniques, followed by CD14 marker-positive selection by magnetic beads. Monocytes will be stained with primary antibodies for markers of M1 differentiation (CCR7 and CD86) and M2 differentiation (CD163 and MR) and subjected to flow cytometry to determine the macrophage phenotype ratio. For adhesion assays, monocytes will be added to human umbilical vein endothelial cells (HUVEC) cells attached to culture plates, and adhered cells will be stained and quantified (17). For migration assays, Costar Transwells will be coated with fibronectin. Monocytes will be added to the upper chamber, and MCP1 will be added to the lower chamber as a stimulus for macrophage migration. Cells that migrate into the lower chambers will be counted (18).

Sample Size Calculations. Power computations are based on the proposed changes in CIMT over 2 years in the placebo group vs. the vitamin D group. Sample size calculations were based on two published placebo-controlled intervention studies of glucose-lowering agents in patients with pre-diabetes (40,41), in which the weighted annualized CIMT change was 0.010 mm in the placebo group vs. 0.0053 mm in the intervention group in 497 subjects. We assumed twice these changes in CIMT for both the control and vitamin D groups over 2 years, but for the standard deviations, conservatively utilized the higher values of the two studies and added an additional 25%, thus giving us estimated CIMT changes of 0.021 ± 0.22 mm for placebo and 0.010 ± 0.24 mm for intervention. Thus, 94 subjects per group will yield a power of 0.9 at the 0.05 level of significance. To account for a projected dropout rate of 10%/year, or 20% total, our goal will be to randomize 236 subjects.

Analytical Methods. All data analyses and cardiovascular imaging analyses will be performed at Washington University. Interpretation of CIMT studies will be performed by the Washington University Core Lab. For quality control of CIMT, 25 subjects (study or non-study) spread over all centers over the duration of the study will have CIMT performed twice each, one week apart. Studies will be reviewed to ensure consistency of ultrasound procedures. Quality control of interpretation of CIMT will consist of independent interpretation of 10% of studies by two additional cardiologists who specialize in cardiovascular imaging. Statistical analyses will be focused on comparing changes over time across groups in CIMT, inflammatory markers, macrophage phenotype ratio (MPR – the ratio of macrophage M1 markers to M2 markers), and macrophage adhesion and migration. Initially, t-tests will compare baseline values in the two groups. The longitudinal analytic strategy will be a mixed model repeated measures analysis of variance, comparing the change from baseline to 2 years in the control vs. vitamin D group. Subsequent to these analyses, we will adjust for covariates, including age, BP, body mass index, smoking status, changes in A1C, and baseline vitamin D level. Finally, the monocyte outcomes and inflammatory markers will be treated as covariates in mixed model repeated measures analyses that seek an understanding of their role in predicting the CIMT response to vitamin D therapy. Analyses will be intention to treat in that all subjects who provide any follow up data will be included.

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).

All new data acquisition can be achieved by prolongation of existing visits, with no need for additional visits. At each of 3 visits (Baseline, M12, and M24), 2 additional procedures will need to be performed. These procedures include the following (described in detail above under Research Approach):

1. Carotid intima-media thickness: Ultrasound of bilateral carotid arteries requires approximately 15-30 minutes to complete.

2. Blood samples: Collection of 4 x 10-ml green-top sodium heparin vaccutainer tubes can be performed at the same time as scheduled D2d blood samples and thus will only require an additional 5 minutes to obtain.

In total, the procedures for this proposed ancillary study would prolong 3 existing visits (at baseline, 12 months, and 24 months) by approximately 45 minutes to 1 hour . As an alternative to the baseline visit, the baseline measurements for D2d-CVD study may also be performed at the randomization visit to allow site and participant flexibility.

Each site will receive compensation for the additional required time for each ancillary study visit. Compensation for the site will consist of two global fees. The first takes into account effort for the ultrasonographer, CIMT procedures, and transfer of cardiovascular imaging data. The second takes into account effort for the site PI and research coordinator, to include additional blood draw, effort for packaging biospecimens (including supplies such as dry ice, biohazard bags, etc.), scheduling time, and IRB approvals. We will supply individual sites with blood collection tubes, styrofoam biospecimen transport boxes, and biospecimen shipment fees. Additional compensation will be provided to participants.

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
- ☐ 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 Cincinnati VA Medical Center
- ☑ 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).

Dr. Bernal-Mizrachi's lab space is located within the Washington University Medical Center and consists of 1,100 square feet. It has a hood for working with toxic chemicals and radioisotopes and is equipped for processing, storage, and analysis of blood and tissue samples. It houses 4 computers for data analysis, a Sorvall refrigerated centrifuge, a -20° non-frost freezer, and 3 -80° freezers. A separate room contains cell culture equipment, C02 incubator (Fisher 3530), a Biosafety Class II cabinet (Fisher Hamilton 54L92700), and a liquid nitrogen container (Thermo Electron 8030). We have access to a Nikon fluorescence microscope equipped with a CCD Camera and Magnafire image analysis tools and a 4°C cold room for sample preparation, temporary storage, and gamma counter. The Flow Cytometry and Fluorescence Activated Cell Sorting Core in the Department of Pathology and Immunology offers services for flow cytometry-based cell analysis and cell sorting, including 4 benchtop analyzers and 3 cell sorters with the sorting capacity of 7 traditional cell sorters.

Blood samples for monocytes will be collected at baseline, 1 year, and 2 years. Four 10-mL green top sodium heparin tubes will be obtained. These tubes will be placed in styrofoam boxes for transport in a sealed biohazard bag with adsorbent (towel) and gel packs for cooling. The package will be stored in the refrigerator (1-6°C) until pickup by FedEx for overnight delivery. For inflammatory markers, serum samples from the same time points as the monocytes will be requested from the D2d Central Laboratory and batch transported to the PI on dry ice.

Our laboratory staff has 7 years of experience handling blood samples, performing human monocyte isolation, monocyte staining for flow cytometry, monocyte migration and adhesion to HUVEC, and analysis of macrophage cytokine production. Also, our laboratory is experienced in performing multiplex assessments using the Affymetrix platform for systemic markers of vascular inflammation (TNFα, IL-1, hsCRP, MMP-9, fibrinogen).

The Cardiovascular Imaging and Clinical Research Core Laboratory at WU (WU Core Lab) operates within 350 square feet for clinical research studies at the university. State-of-the art equipment is available for the performance and analysis of CV studies (e.g. echocardiograms, vascular ultrasound and endothelial function studies) and ambulatory BP and telemetry. Available equipment includes two large ultrasound machines and two portable systems equipped with cardiac and vascular transducers. Equipment is also available for oxygen consumption studies (VO2 max) and ambulatory BP monitoring. There are computers and a server/workstation for data storage and statistical analysis, and a digital ultrasound review and reading station (ProSolv).

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

The primary participant burden is time. Overall, the time commitment is reasonable (extension of 3 visits by 1 hour) and can be minimized by efficiency of procedures (D2d-CVD blood draws can be performed at the time of D2d blood draws). Additionally, participants will be reimbursed for the extra time required for the ancillary study. The burden of additional safety risk is low as the only additional procedures will include additional blood collection (without a second venipuncture) and CIMT, as described above. CIMT requires ultrasonography at frequencies for which there are no known risks to humans.

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.).

See safety risks above. To maintain confidentiality, all data requested from the D2d Coordinating Center will be de-identified. Additionally, all data and specimens required for new data acquisition will be de-identified prior to their transport from the ancillary study sites, the D2d Coordinating Center, or the D2d Central Laboratory to Washington University. In addition, any hard-copy research records will be housed within the PI's research area in locked cabinets and/or locked rooms within locked suites located on the Washington University (WU) campus in accordance with HIPAA guidelines. Electronic data will be maintained on WU network servers, which are HIPAA-compliant with 2-key security and monitored access. Cardiovascular imaging data will be sent directly through a web-based, password-protected image transfer protocol and database maintained by the WU Core Laboratory. Major characteristics of the database include: 1) Secure (accessible only to those who are authorized and HIPAA-compliant) with accessibility depending on the individual role on the project; 2) Data transfer encrypted via SSL; 3) Firewall protected; 4) Expandable (able to support thousands of records and link subjects across studies and projects); 5) Allows imported data from external sources; 6) Allows export of data in multiple formats, including Microsoft excel, SAS, or in a comma or tab delimited format.

4c. Burden on Collaborating Clinical Sites

The CIMT studies require a commercially available ultrasound system equipped with high-frequency transducers (8-15MHz) for each site and an ultrasonographer qualified to perform the studies. Prior to study initiation, any collaborating staff who require it will come to WU for a 1-day training program for CIMT at the WU Core Lab that will include review of study background, imaging protocol, instrumentation, image acquisition and submission. There will also be a hands-on session under supervision by medical and technical staff. Each site must be certified in CIMT studies, achieved by submitting a sample study to the WU Core Lab for review that must be 100% adherent to protocol. For transfer of CV studies, sites must have the ability to store studies in a HIPAA-compliant fashion electronically for the duration of the study. For transfer of biospecimens, sites must have an individual trained in shipment of dangerous goods so samples can be packaged appropriately, as well as a standard refrigerator for storage of 4°C samples pending shipment by FedEx.

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.

Informed consent will occur at each clinical site by its respective investigators. IRB approval with an additional consent form will thus be necessary at each clinical site. We will provide copies of the detailed protocol including all risks and benefits to each site in order to facilitate preparation for submission to their IRB. We will also be available for any assistance needed by individual sites with IRB submission. In addition, we will also submit an IRB proposal to the Washington University Human Research Protection Office for data analysis.

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:

- [x] Data extraction and transfer
- [x] 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.

We plan to apply to NIH RFA-HL-14-004, for Ancillary Studies in Clinical Trials. Given that this is an RFA, we see this as a unique opportunity to be competitive for securing funding.

6b. Planned Date of Submission to Funding Agency 09/24/2013

Part 7: NIH Biosketch

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

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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)* Carlos Bernal-Mizrachi Digitally signed by Carlos Bernal-Mizrachi DN: cn=Carlos Bernal-Mizrachi, o, ou, email=cbernal@dom.wustl.edu, c=US Date: 2013.06.02 14:01:09 -05'00' Date 04/28/2013

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