Medical University of South Carolina
Protocol

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Study Title: Preventing Health Disparities during Pregnancy through Vitamin D Supplementation

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A. **SPECIFIC AIMS**

This project, comprised of discrete studies, expert evaluation and translation for public health campaign, promotes racial health equity for pregnant women. A strong racial disparity in the US surrounds vitamin D status: African American women have 20-fold and Hispanic women 2.4-fold greater risk of deficiency than Caucasian women. In South Carolina, greater than 70% of African American, 33% of Hispanic and 12% of Caucasian pregnant women meet the Institute of Medicine's (IOM's) 2010 definition of vitamin D deficiency (1-3). Such disparity on the basis of race/ethnicity represents a serious public health issue. Yet, some—including the IOM—argue that vitamin D deficiency minimally affects maternal and fetal health and that no vitamin D supplementation study has shown improved pregnancy outcome (3, 4). Results of our two recently completed vitamin D trials involving 510 pregnant women throughout pregnancy suggest otherwise (5-7). In the NICHD trial, women supplemented with 400 IU vitamin D/day (the amount in most prenatal vitamins) vs. 2000 or 4000 IU/day not only had worse vitamin D status throughout pregnancy, but combined higher risk of comorbidities of pregnancy (gestational diabetes, hypertensive disorders, infection, preterm labor/birth and periodontal inflammation) (7). In trial 2, women at two South Carolina community health centers were supplemented with vitamin D and again, higher vitamin D status was associated with lower comorbidities of pregnancy risk (6, 8).

A novel finding from the NICHD trial was that serum levels of active, hormonal vitamin D ($1,25(OH)_2D$) during pregnancy were optimized at twice the level normally observed in non-pregnant women, levels that would be considered toxic in nonpregnant individuals. At no other time in life does this relationship exist. Is the hormone at such levels driving some vital process during pregnancy? If so, what is that process? There are hints as to why $1,25(OH)_2D$ is elevated in pregnant women. During pregnancy, the maternal immune system undergoes drastic changes for fetal protection. For example, if a solid organ composed of 50% mismatched cell markers (antigens) was transplanted into a new host, it would be rejected within hours; however, a fetus that is 50% mismatched with paternal antigens is protected from immune destruction through a process known as immune privilege. During pregnancy the body increases immune suppressive cells, reduces highly activated natural killer and cytotoxic T-cells with capacity to destroy the fetus and still has the capacity to fight off foreign pathogens. While the mechanisms that orchestrate this complex balance are unknown, we hypothesize that due to vitamin D's ability to regulate immune cells, vitamin D is responsible for many immune alterations associated with fetal immune privilege. We further hypothesize that correction of vitamin D deficiency during pregnancy will protect against immune-mediated disorders such as recurrent miscarriage, preterm birth, bacterial infections, periodontal inflammation and gestational diabetes. This premise,tested and confirmed by this project, will lead to public policy changes and equality in vitamin D status during pregnancy. To test the validity of these hypotheses, the goals of this project are as follows:

The central goal of this project is to realize racial equity for all pregnant women and their developing babies in the US. To achieve this goal, there are 3 specific objectives:

1) Determine how vitamin D deficiency leads to immune imbalance and subsequent disparities in pregnancy health outcomes;

2) Determine how vitamin D supplementation prevents such imbalance and disparities; and

3) Translate such findings into public health policy.

By comparing women of diverse racial/ethnic backgrounds who receive the current vitamin D IOM standard of 400 IU/day compared to 4000 IU/day [daily dose shown to achieve optimal production of active vitamin D hormone ($1,25(OH)_2D$)], the clear effects of vitamin D will be realized. Furthermore, these effects of sufficient vitamin D during pregnancy transcend the racial differences of the women.

B. **BACKGROUND AND SIGNIFICANCE**

Despite its discovery a hundred years ago, vitamin D has emerged as one of the most controversial nutrients and prohormones of the 21st century. Its role in calcium metabolism and bone health is undisputed but its role in immune function and long-term health is debated. There are clear indicators from in vitro and animal in vivo studies that point to vitamin D's indisputable role in both innate and adaptive immunity; however, the

translation of these findings to clinical practice, including the care of the pregnant woman, has not occurred. Until recently, there has been a paucity of data from randomized controlled trials to establish clear cut beneficial effects of vitamin D supplementation during pregnancy.

What is vitamin D? Vitamin D is a preprohormone that is made by most living plants and terrestrial animals. In the true sense of the word, vitamin D is not a "vitamin" because the main source of vitamin D is that which we synthesize ourselves—in our skin—with less than 10% coming from dietary sources. Vitamin D comes in two major forms—vitamin D_2 or ergocalciferol and vitamin D_3 or cholecalciferol. While certain plants are capable of making both forms of vitamin D, the major form made by plants is vitamin D_2 following ultraviolet B exposure of the provitamin D_2 ergosterol (9, 10). In comparison, humans can metabolize both vitamin D_2 and D_3, but can only synthesize *de novo* vitamin D_3. The *de novo* synthesis of vitamin D_3 in humans and other animals begins in the skin with the parent compound 7-dehydrocholesterol or provitamin D_3. Following exposure to ultraviolet B radiation in the range of 290-315 nm, 7-dehydrocholesterol becomes previtamin D_3. Through a subsequent thermal reaction in the skin, previtamin D_3 is isomerized into vitamin D_3. It is important to note that unlike other hormones in the body whose main substrate is cholesterol, vitamin D synthesis requires the 7-dehydrocholesterol precursor and sunlight at a specific wavelength and angle. Without this reaction, humans are dependent on dietary intake of vitamin D, which may be in the form of either vitamin D_2 or D_3. A western diet, however, provides less than 200 IU (5 μg) vitamin D/day, a point that was reestablished in our two recently completed vitamin D clinical trial during pregnancy (5).

Following its synthesis, vitamin D binds to vitamin D binding protein (VDBP) and finds its ways into the circulation. Dietary and endogenous vitamin D appear to act similarly with half-life between 12-24 hours, the length of time depending on how quickly the liver converts vitamin D to 25-hydroxy-vitamin D (also known as calcidiol). Vitamin D is measured in international units (IU) or micrograms with a known conversion of 40 IU equal to 1 microgram.

While there appears to be a differential conversion rate of the two forms of vitamin D to 25(OH)D (11), the conversion of either form is dependent on a functional liver and the activity of 25-hydroxylase. Thus, those with impaired liver function will have diminished conversion of vitamin D to 25(OH)D. Following its synthesis, 25(OH) then enters the circulation where it is tightly bound to VDBP. Only a small amount of 25(OH)D is unbound or "free." The half-life of 25(OH)D is 2-3 weeks, making it a much better indicator of the body's vitamin D status than vitamin D. Of note, 25(OH)D can be expressed as ng/mL or nmol/L. The conversion from ng/mL to nmol/L is 2.5; thus, a 25(OH)D concentration of 20 ng/mL equals 50 nmol/L.

Once 25(OH)D is formed in the liver, it enters the circulation. Best known is the processing of 25(OH)D by the kidney where 25(OH)D complexed with VDBP and megalin is taken up by the epithelial cells of the proximal tubules and converted to the active hormonal form of vitamin D—di-hydroxy-vitamin D ($1,25(OH)_2D$ or calcitriol)—by the action of the mitochondrial enzyme 1-α-hydroxylase.

$1,25(OH)_2D$'s endocrine effects include the following classic triad of action: (1) increase intestinal calcium (as Ca^{2+} ions) absorption through the actions of calbindin; (2) increase urinary calcium reabsorption; and (3) regulation of parathyroid hormone in a negative feedback loop that allows calcium to be absorbed from the gastrointestinal tract, reabsorbed from urine, and metabolized from bone in order to maintain calcium homeostasis within the body. Because calcium is essential to all tissues and organs, particularly the heart, skeletal muscle and brain, the body will scavenge calcium if necessary from the skeleton. Adequate vitamin D must be on hand to provide enough substrate to form 25(OH)D, which in turn, is converted to $1,25(OH)_2D$, whose half-life is 8 hours. In individuals with vitamin D deficiency, only trace amounts of vitamin D will be found in the body because whatever comes into the circulation is quickly converted to 25(OH)D and then to $1,25(OH)_2D$ to maintain calcium homeostasis.

For decades, it was thought that only the kidney has the capacity to metabolize 25(OH)D; however, extrarenal metabolism has been demonstrated in every organ system in the body (12-15). During pregnancy, the placenta is probably the most prominent site for extra-renal activation of vitamin D (16). It appears that the extrarenal function of vitamin D has more to do with immune function than with calcium metabolism and homeostasis. Support for this premise first came from the observations of Mellanby and others at the turn of the 20th century: during that time, Mellanby in his study of rachitic children and dogs noted an increased risk of respiratory infections in those afflicted (17, 18). Additional reports came from those working with tuberculosis

patients and the beneficial effect of being in sunlight and outdoors in the treatment of the condition (19). Weick (20) in 1967 and Rehman (21) in 1994 independently observed that children with rickets appeared ill, with decreased energy and activity, and were more susceptible to respiratory illnesses. Despite these observations, it was concluded that the condition of vitamin D deficiency led to weakness and malnutrition and was not a direct effect of vitamin D on the immune system. The mechanism of action of these processes and health derangements would not be understood until the advent of molecular biology.

Vitamin D appears to affect immune function in two ways: (1) upregulation of the innate immune system; and (2) downregulation of the adaptive immune system. Focusing on the innate immune system first, a major mechanism of action of vitamin D is via an endogenous antimicrobial peptide called cathelicidin (LL-37), which is generated in response to microbial invasion through activation of toll-2 receptors (TLR) on monocytes and macrophages (22-24). Not surprisingly, the vitamin D receptor (VDRE) is contained in the promoter region of the gene for LL-37. VDRE are found only in the LL-37 gene promoters of primates, suggesting that the ability of vitamin D to promote LL-37 antibacterial action is a relatively recent event in evolution. Both $1,25(OH)_2D$ and 25(OH)D have the ability to induce the expression of cathelicidin in monocyte/macrophage and epidermal lineage in cells that simultaneously have the 25(OH)D hydroxylase (25).

Significant support for the role of vitamin D in immune processes and function came in 2006 when Liu et al published their landmark study in *Science* (22). Serum samples taken from African American subjects with low 25(OH)D were inefficient in supporting cathelicidin mRNA induction; however, with the addition of 25(OH)D to those samples with low 25(OH)D levels this pattern was reversed. Thus, in this series of experiments, the addition of $25(OH)D_3$ restored the ability of sera from individuals with low 25(OH)D concentrations to support TLR2/1L-mediated induction of cathelicidin mRNA. A related study by Fabri, et al (26), showed that IFN-ɣ-mediated antimicrobial activity of human macrophages, especially important in HIV and tuberculosis patients, is dependent on vitamin D. Both study findings have implications for the pregnant woman and her developing fetus, but our understanding of such processes following maternal exposure to a pathogen or maternal infection remains scant. There is every reason to suggest that such processes are fully functional in the pregnant woman.

Vitamin D's role as a modulator of the immune system encompasses the adaptive immune system as well. $1,25(OH)_2D$ not only has the ability to affect processes within macrophages and monocytes, but also in T and B lymphocytes as well. The vitamin D receptor (VDR) is found on activated (but not resting) human T- and B-lymphocytes. Whereas $1,25(OH)_2D$ appears to activate the bacteriocidal process within macrophages and monocytes, it has different effects, that include suppression of T-cell proliferation and modulation of T-cell phenotype —with anti-inflammatory properties (27). By binding to the VDR on T cells, $1,25(OH)_2D$ acts to: (1) inhibit the proliferation of uncommitted T_H (helper) cells and (2) promote the proliferation of immunosuppressive regulatory T cells, or $T_{reg}S$, with notable accumulation of these cells at sites of inflammation (25). It appears that $1,25(OH)_2D$ suppresses certain B cell functions such as proliferation and immunoglobulin production and retards the differentiation of B-lymphocyte precursors to mature plasma cells *in vitro*. These *in vitro* findings explain the significant association between vitamin D deficiency and autoimmune diseases, such as systemic lupus erythematosus (28), multiple sclerosis (29-38), rheumatoid arthritis (34, 39, 40), diabetes—both types 1 (34, 41-45) and 2 (46-48), and certain cancers, such as colon (49-52), breast (53-58), and prostate (58-62). Additionally, the role of vitamin D in immune function intensifies the need to establish vitamin D sufficiency during pregnancy.

It is clear that vitamin D deficiency during pregnancy is common throughout the world yet what effect does deficiency have on the mother and her developing fetus? There is a strong relationship between maternal and fetal (cord blood) circulating 25(OH)D levels (63-66) such that maternal vitamin D deficiency is mirrored by neonatal vitamin D deficiency. With severe maternal vitamin D deficiency, the fetus rarely may develop rickets *in utero* with manifestation at birth (67). Such readily observable fetal and neonatal skeletal effects of profound vitamin D deficiency are easily understood in terms of cause and effect, but the more subtle effects of deficiency on the developing immune system, for example, and subsequent infection risk or immune dysfunction are more difficult to understand (22, 68-71).

Vitamin D status during pregnancy appears to play a role in fetal skeletal development, tooth enamel formation, and general fetal growth and development (72, 73). Mannion et al. (74), comparing growth parameters in newborn infants with the maternal intakes of milk and vitamin D during pregnancy, found an

association between vitamin D intake during pregnancy and birth weight. They reported with every additional 40 IU (1 µg) of maternal vitamin D intake, there was an associated 11-g increase in birth weight. Pawley and Bishop (75) in their study of 108 pregnant women and their offspring found a significant association between umbilical cord 25(OH)D concentrations and head circumference at 3 and 6 months' postnatal age that persisted after adjusting for confounding factors. Maghbooli et al. found significantly wider posterior fontanelle diameter in neonates of mothers with vitamin D deficiency (as defined by a 25(OH)D level <34.9 nmol/L or ~14 ng/mL) compared to neonates whose mothers were not deficient (76). Beyond growth, recent reports of neonates followed prospectively for acute viral infections and bronchiolitis from respiratory syncytial virus (RSV) support the premise that these states of deficiency do impact on the health of the young infant and suggest a greater role of vitamin D beyond bone health (77-79).

McGrath and others continue to investigate whether there are lasting effects of fetal and early infancy vitamin D deficiency on later adult disease processes such as anatomical changes of the brain, schizophrenia, multiple sclerosis, certain cancers, cardiovascular disease, and various other autoimmune diseases such as diabetes and lupus (80-95). Because vitamin D status has not been a consistent concern during pregnancy, long-term data are sparse. The few studies that have been conducted have focused more on discernible neonatal effects of vitamin D during pregnancy, rather than the long-latency and later health effects. Reports of neonatal seizures due to severe hypocalcemia or rickets *in utero* that is manifested at birth from severe maternal and thus fetal vitamin D deficiency are rare and do not further our understanding of potential epigenetic effects of vitamin D (96, 97). During pregnancy, supplementation with the current standard amount of vitamin D in prenatal vitamins—400 IU (10 µg) vitamin D/day—has minimal effect on circulating 25(OH)D concentrations in the mother and her infant at term (5, 98). It is also known that infants of women who were deficient throughout pregnancy will reach a state of deficiency more quickly and with greater severity than infants of women replete during pregnancy (67).

While there are numerous epidemiological studies that bear evidence of the association between vitamin D deficiency and altered health, definitive proof in terms of randomized controlled trials is often lacking. For example, higher circulating 25(OH)D levels have been linked with improved glucose handling and beta-cell function (99), and a reduction in risk for a growing list of long latency diseases that include cardiovascular disease (60, 100-104), multiple sclerosis (30, 35, 37), rheumatoid arthritis (39), systemic lupus erythematosus (28), type 1 and 2 diabetes (39), and various cancers (49, 55, 58, 105-110), but critics counter that while such findings are intriguing, they do not provide definitive evidence of causality or a mechanism of action that come from randomized controlled trials, and may lead to what is referred to as "circular epidemiology" (111). While we await the results of numerous clinical trials now underway to determine if there is a discernible effect of vitamin D in altering risk for various disease states to understand the role of vitamin D in health, it is important not to discount the mounting evidence from laboratory studies and prospective observation trials (77-79) that vitamin D—as a preprohormone—is essential in maintaining the immune system with profound implications (22, 112-114).

Significance: The central tenet of this project is enhanced understanding of the underlying mechanisms of action of vitamin D deficiency during pregnancy, which result in poor maternal and fetal health, and health disparities in pregnancy outcomes. By focusing on immune imbalance associated with vitamin D deficiency—first utilizing a well-developed mouse model of preterm birth and preeclampsia and then studying pregnant women themselves looking at adverse maternal and fetal health effects and markers of deficiency—we expect to show improvement in various pregnancy outcomes. During this project, we will complete the following activities:

1) Define more fully the health disparity during pregnancy between African American, Hispanic and Caucasian women on the basis of vitamin D status;

2) Demonstrate that vitamin D deficiency impairs placental function, leading to abnormal immune responses and adverse pregnancy outcomes;

(3) Demonstrate that vitamin D supplementation will enhance placental biology and protect against adverse pregnancy outcomes;

(4) Define the immune mechanisms by which vitamin D acts during pregnancy to enhance immune suppression and decrease inflammation for optimal health outcomes of mother and fetus through measurement and characterization of:

(a) immune T-cell modulatory changes during pregnancy (% change ratio of CD4+ and CD25+ over total lymphocyte population where there would be an increase in the ratio as a function of vitamin D repletion;

(b) inflammatory cytokine changes as a function of vitamin D status;

(c) tooth developmental changes as a function of vitamin D status;

(5) Validation of immune mechanisms of action of vitamin D through a mouse model developed by Dr. Martin Hewison at UCLA;

(6) Examine the metabolism and processing of vitamin D supplementation to better understand and prevent deficiency;

(7) Dissemination of findings at several levels through: a. the National Association of Community Health Centers; b. vitamin D think tank meetings with all involved partners; c. public health forum held regionally utilizing vitamin D grassroots organization/health center networks at community, state, and federal levels.

The first 6 activities will generate supportive data for supplementing pregnant women with vitamin D at doses that enhance immune balance and regulation. With greater knowledge about vitamin D gained from this project, we will educate health care providers, policy makers, and the pregnant women themselves about why vitamin D is important and how to prevent deficiency (Activity 7). In Year 4, a "think tank"-type of vitamin D meeting of scientists, philosophers, politicians, health care providers, and educators will be hosted at MUSC. Also, a vitamin D public forum specifically about vitamin D during pregnancy will be launched. This will allow the creation of partners at the local, state and federal levels in changing the course of pregnancy outcomes in America. Such networking and interactions will allow utilization of existing infrastructure to sustain such change with the expectation that public health policy changes will be created that address the established need of pregnant women for vitamin D repletion.

SubObjective of Activity 7: Vitamin D Status and Health Implications for Women during Pregnancy: An MUSC Research-to-Practice Implementation and Assessment: Year 3-5 of Kellogg Foundation Grant

To accomplish Activity #7 (Objective #3), the following Objectives are to be met:

(1) Vitamin D status assessments by trimester of every woman presenting to MUSC for obstetrical care

(2) Determine the effect of vitamin D status on the pregnancy outcomes of women and their infants who receive health care through the MUSC health care system

(3) Assess the impact of real-time vitamin D status on the prescription of vitamin D supplementation during pregnancy by MUSC health care providers

C. PRELIMINARY STUDIES

Summary from two papers (one in press) (see appendix for manuscript):

Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2011;26(10):2341-57. Epub 2011/06/28. doi: 10.1002/jbmr.463. PubMed PMID: 21706518; PubMed Central PMCID: PMC3183324.

Wagner C, McNeil R, Hamilton S, Winkler J, Rodriguez Cook C, Warner G, et al. A Randomized Vitamin D Supplementation Trial in Two Community Health Center Networks in South Carolina. Am J Obstet Gynecol. 2013;in press.

In our recently completed NICHD vitamin D supplementation trial involving a diverse group of pregnant women less than 16 weeks of gestation, 4000 IU (100 µg) vitamin D_3/day was superior to 400- or 2000 IU/day in achieving circulating 25(OH)D of at least 40 ng/mL (100 nmol/L), the point at which $1,25(OH)_2D$ begins to be optimized (see Figure 2) (5). While 4000 IU/day was superior to 2000 IU/day in achieving the Institute of Medicine's threshold for sufficiency of a 25(OH)D concentration of ≥20 ng/mL (50 nmol/L), it was not statistically

significant—both 2000 and 4000 IU/day will achieve this level in pregnant women. If the goal, however, is to reach the point of $1,25(OH)_2D$ optimization of 40 ng/mL (100 nmol/L), then there is a clear advantage of taking 4000 IU/day. While there was restriction of randomization in the NICHD trial necessary for initial IRB approval, such a restriction was not in place for the second pregnancy Thrasher Research Fund Trial (described below). Even in the latter trial where women were randomized to either 2000 IU (50 μg) or 4000 IU (100 μg) vitamin D_3/day, there were no safety issues surrounding vitamin D supplementation and there was a trend where specific health complications of pregnancy such as preterm birth, preterm labor, hypertensive orders of pregnancy, gestational diabetes, and infection were higher in the 400 IU- compared with the 4000 IU-group, but did not reach statistical significance (115). When analyzed together as comorbidities of pregnancy and controlling for race, there were statistically significant differences between the 400 IU-, the 2000 IU-, and the 4000 IU groups with fewer events in the 4000 IU group (p=0.03) (115).

Consistent with the NICHD trial results, another recently completed study funded by the Thrasher Research Fund where women were randomized to either 2000 (50 μg)- or 4000 IU (100 μg) vitamin D_3/day showed that the while the mean change from maternal 25(OH)D baseline was +12.2±13.2 ng/mL in the 2000 IU group and +15.2±12.9 ng/mL in the 4000 IU group (p=0.16), the overall 25(OH)D rate of increase differed significantly between the two dose groups (p=0.036) with a higher rate in the 4000 IU group. Mean infant 25(OH)D level (ng/mL) was 22.1±10.3 in the 2000 IU group and 27.0±13.3 in the 4000 IU group (p=0.024). Secondary analysis indicated that preterm birth and labor were inversely related to pre-delivery vitamin D status (preterm birth OR=0.50 per 10 ng/mL, p=0.002; preterm labor or birth OR=0.72 per 10 ng/mL, p=0.012), an effect that persisted even after controlling for race. There were no adverse events associated with vitamin D supplementation (116).

Analysis of the two concurrent vitamin D pregnancy NICHD (n=350) and Thrasher Research Fund (n=157) trials was undertaken to assess the health characteristics and outcomes of this larger, combined group using a common data dictionary (8). As noted above, in the NICHD trial, women were randomized to 400 IU (10 μg), 2000 IU (50 μg) or 4000 IU (100 μg)/day, stratified by race. In Thrasher trial, participants were randomized to 2000 (50 μg) or 4000 IU (100 μg)/day, also stratified by race. All participants received study drugs from the same manufacturing lot. Studies administered identical questionnaires to produce comparable sociodemographic/clinical characteristics. Outcome measures included: (1) maternal baseline and delivery 25(OH)D; (2) neonatal 25(OH)D; (3) comorbidities of pregnancy: gestational diabetes, hypertensive disorders of pregnancy, infection—any, bacterial vaginosis (BV), and preterm birth without preeclampsia. In the combined cohort, there were 110 in the control group, 197 in 2000 IU group, and 189 in the 4000 IU group. The treatment groups differed on the basis of parity (p=0.022); insurance status (Medicaid status more common in the 2000 IU group p=0.006); and education (higher education level in the 400 IU group, p=0.012). No differences were noted between groups in baseline 25(OH)D (p=0.24), but differences between groups were noted in vitamin D status within one month of delivery (p<0.0001) and in cord blood (p=0.0001), with improved status in the 4000-IU group overall. No differences were noted between groups in terms of independent or combined comorbidities of pregnancy. There was a trend where preterm birth without preeclampsia was lower with increasing 25(OH)D concentration (Odds Ratio (OR) 0.83 (Confidence Interval (CI) 0.68-1.01); p=0.057).The risk of combined comorbidities of pregnancy adjusted for race/ethnicity and study was significantly lower with increasing 25(OH)D concentration (Odds Ratio per 10 ng/mL increase in 25(OH)D: 0.84; 95% CI 0.74-0.96; p=0.0095) (8).

The debate about what constitutes frank deficiency, insufficiency, and sufficiency continues (3, 117). The answer varies depending on the question—is the outcome bone integrity or immune function? There could be a different cut point for each category. Most would agree—including the Institute of Medicine in their most recent statement –that levels below 20 ng/mL (100 nmol/L) represent deficiency in the nonpregnant individual. There is less consensus with respect to pregnancy: based on our recent randomized controlled trial with pregnant women, it is clear that optimization of $1,25(OH)_2D$ does not occur until total circulating 25(OH)D levels have reached 40 ng/mL (100 nmol/L) (5).

The significance of these trials is that vitamin D status is improved with 4000 IU vitamin D_3 taken daily to achieve: (1) optimization of $1,25(OH)_2D$ production, and (2) improved cord blood 25(OH)D concentration. Having a higher 25(OH)D concentration was associated with improved health outcomes in both studies, but whether improved vitamin D status is a marker of some other parameter or a direct effect of vitamin D supplementation remains unclear at this time. Studies specifically designed and powered to answer the question of whether or

not vitamin D supplementation leads to improved health outcomes—lower risk of preterm birth, preeclampsia, and infection remain to be done. We can conservatively say that while we do not understand completely the role of $1,25(OH)_2D$ during pregnancy, achieving optimal production can be done safely with 4000 IU vitamin D_3/day, which appears to be the amount of vitamin D that would be conservatively generated by the body with adequate sunlight exposure over the duration of gestation.

D. RESEARCH DESIGN AND METHODS (including data analysis)

Study Design: This is a randomized, placebo-controlled clinical trial of 450 pregnant women who will be enrolled between 10-14 weeks of gestation and followed until delivery. Following written, informed consent, each mother will be randomized to receive either placebo or 4000 IU/day vitamin D_3 plus the standard prenatal vitamin (containing 400 IU vitamin D_3). She will be followed monthly for a total of nine study visits. If, after 3 months of vitamin D supplementation, a mother is still frankly vitamin D deficient (less than 15 ng/ml), she will receive open label active vitamin D gummies for the remainder of her pregnancy. These visits will include the initial recruitment of mother at 10-14 weeks of pregnancy, monthly study visits, delivery study visit, and 18-month post-partum follow-up visit for mother and child. The primary outcome variable is maternal and neonatal health status as a function of maternal and infant vitamin D status. Secondary outcome variables to be analyzed, as a function of maternal vitamin D status during pregnancy will include: T-lymphocyte profile, immune function indicators, neonatal growth, inflammatory cytokine profile, methylation patterns of DNA of both mother and her neonate,

(1) Subject Selection Criteria and Recruitment: Any mother (18-45 years of age) who presents to her obstetrician or midwife at the Medical University of SC (MUSC), Charleston, SC obstetrical facilities within the first 14 weeks after her last menstrual period (LMP) with confirmation of a singleton pregnancy will be eligible for enrollment in the study. Mothers of diverse ethnic backgrounds (African-American, Asian, Caucasian and Hispanic) will be actively recruited. A database will be generated weekly of undelivered patients for the labor and delivery staff continue to alert the staff of impending admissions.

(2) Exclusion Criteria: Mothers with pre-existing calcium, uncontrolled thyroid disease, parathyroid conditions, or who require chronic diuretic or cardiac medication therapy including calcium channel blockers will not be eligible for enrollment into the study. Mothers with pre-existing sickle cell disease (not trait only), sarcoidosis, Crohn's disease, or ulcerative colitis may not participate in the study. In addition, because of the potentially confounding effect of multiple fetuses, mothers with multiple gestations will not be eligible for participation in the study. A sub-group of approximately 100 subjects with known diabetes, hypertension, HIV, or morbid obesity (body mass index > 49) will participate in the study.

(3) Subject Enrollment: The potential study subject's primary care provider will make first contact on behalf of the study and refer interested mothers to study personnel for further information and invitation. Upon enrollment into the study, expectant mothers at approximately 10-14 weeks' gestation will be randomized to receive one of two treatment regimens of vitamin D_3. They will be randomized to 1 of 2 groups: (1) Group A: 400 IU vitamin D_3/day—Standard dose treatment of placebo (0 IU vitamin D_3) plus prenatal vitamin (400 IU/day); or (2) Group B: 4,400 IU/day (4,000 IU/4 gummies/day + 400 IU/day in prenatal). To attain equal group numbers (n=150) and balanced numbers by racial/ethnic group (African-American, Caucasian and Hispanic, which represent the predominant groups delivering at MUSC), a stratified block randomization will be used.

(4) Race/Ethnicity Defined: Each mother will be asked to define the racial/ethnic group to which she belongs: (a) African-American, (b) Caucasian (including Asian and American Indian), and (c) Hispanic. Because few mothers of American Indian (0.08% of all deliveries) and Asian (1% of all deliveries) descent deliver at MUSC, there is insufficient power to include those groups individually in this study. In addition, the mother, by self-report, will be asked to define the race/ethnicity of the father of the baby.

(5) Study Site Population: **The Medical University of South Carolina** (MUSC) is an urban medical university in Charleston, SC that serves patients predominantly in the Charleston Tri-County area (80%) and 20% from surrounding regions in South Carolina. We perform 2000 deliveries each year. The ethnic composition of our obstetrical population is 57% African American, 36% Caucasian, and 6% Hispanic and 1% Asian. MUSC provides care to any woman presenting in labor who requires routine care or who is referred from outlying hospitals due

to high-risk pregnancy for which delivery at a Regional Perinatal Center (RPC) is appropriate, regardless of ability to pay.

(6) Enrollment and Follow-Up Subjects: The Study Coordinator will be responsible for assisting in screening, enrollment and follow-up. To maintain data quality, the Study Coordinator will review every form to check for basic problems such as missing data. Labor and delivery staff will alert the study coordinator or the Clinical PI's of the admission of any study subjects. Each month the Study Coordinators will generate a report on patient recruitment and retention to be reviewed jointly by the research team. A computer-generated calendar will serve as a reminder to contact recruited patients two days prior to their appointment. Each month the Study Coordinators will generate a report on patient recruitment and retention that will be reviewed by Dr. Wagner.

(7) Follow-up Infant Visit: The Study Coordinator will follow up by phone several times from birth to 18 months in order to verify subject's contact information. The infant (accompanied by mother) follow-up visit will take place at MUSC's CTRC Outpatient Clinic around 18 months postpartum. The infant will have blood drawn for vitamin D and will have a dental assessment of the primary teeth, including digital photographic images.

(8) Sample Size: With the assistance of Dr. Thomas Hulsey, epidemiologist and co-investigator of this application, sample size calculations were made. All outcome markers are ratio-scaled and normally distributed. Because of the possibility of premature delivery and patient attrition during this study, enrollment of additional subjects over that minimally needed, will provide a cost effective opportunity to conduct additional analyses examining changes in vitamin D associated with diet, seasonality, and maternal medical complications. As one of the major goals of this study is to explore maternal health disparities by ethnicity, the two supplemented groups (400 and 4,400 IU/day) will be balanced by ethnicity (equal numbers of Caucasian, African American, and Hispanic). This sampling distribution will be accomplished by stratified randomization. This sampling frame was selected to provide: (1) sufficient numbers to examine associations with covariates; (2) the volume of initial and follow up samples will be manageable by our laboratory; (3) over-samples to compensate for losses to follow up; and, (4) stabilizes the sophisticated statistical analysis techniques (repeated measures).

(9) Subject Sampling: Since this study will enroll equal numbers from each ethnic group, the actual enrollment will be approximately 150 in each group. The sampling procedure is designed to achieve an ethnically balanced study population with over-sampling to accommodate lost to follow-up and missed visits. The random assignment will be conducted as a stratified (by ethnicity) blocked (by supplementation group) design. The randomization schedule will be developed using ProcPlan in SAS statistical software. Dr. Hulsey has extensive experience in developing stratified blocked randomization allocation plans for clinical trials.

(10) Multi-Vitamin and Vitamin D Supplementation.

(a) **Preparation of Vitamin D Tablets**: The vitamin D_3 gummies will be manufactured by Vitafusion (Church and Dwight, Vancouver, Washington) and provided to the PI for this study at no cost in bottles with child-proof caps. Two different gummies will be produced: all will be identical in appearance due to the blinding of the study. Gummies will contain no vitamin D (placebo) or 1000 IU vitamin D_3. All subjects will be instructed to chew 4 gummies/day. All subjects will receive prenatal vitamins (pills or chewable, subject preference) once daily containing an additional 400 IU of vitamin D_3 (one/day). Thus, a total vitamin D intake by group will be 400 or 4400 IU/day. In a large study of this type, we believe that oral intake by gummy is a more reliable means to administer vitamin D to the subjects, many of whom are experiencing pregnancy nausea or dislike swallowing large capsules to achieve even the 400 IU vitamin D_3/day.

(b) **Maternal Supplementation**: At each visit, mothers enrolled in the Study will be given an additional 40-day supply of prenatal vitamins with 400 IU/vitamin D_3 per tablet and an additional 40-day supply of vitamin D_3 gummies (placebo or 1000 IU/gummy). The subjects and the investigators will not be aware to which vitamin D group the subjects have been assigned. Each mother will be instructed to take one prenatal vitamin along with four vitamin D_3 gummies daily. At the completion of each month at the time of the prenatal clinic visit, each mother enrolled in the study will receive another 40-day supply of the prenatal vitamins and the specified vitamin D_3 gummy strength to which she is assigned. Mother will be instructed to bring the vitamin D and prenatal containers with her to each follow-up visit. Pill counts will be performed monthly and compliance noted.

(11) Maternal Sociodemographic Questionnaire: Upon enrollment in the study, each mother will be asked to complete a sociodemographic questionnaire. This questionnaire will ascertain certain

sociodemographic information: maternal age, race of herself and her parents and grandparents, educational level, occupation of herself and the father of her infant, the number of people in her household including herself, and maternal insurance status. A Hollingshead index from 1-7 will be derived for each mother based on the educational status and occupation of the mother (and father if he is involved and living in the same residence as the mother).

(12) **Pregnancy Intake Survey and Monthly Maternal Health/Medication Questionnaire**: Upon enrollment, and then at each monthly obstetrical visit, the mother will be asked to complete a health assessment questionnaire to ascertain her use of medications (checklist) and over-the-counter preparations that may influence vitamin D/calcium homeostasis. She will be specifically asked questions regarding her use of cigarettes and alcohol, and overall health status. In addition, if mother has required hospitalization, this will be recorded and a copy of the hospital record obtained after she has signed a release of medical information form to obtain the medical information. In addition, e.g., if mother required tocolysis, antibiotics or IV rehydration, this will be recorded on the questionnaire.

(13) **Maternal Dietary History**: Each mother will complete a standardized Food Frequency Questionnaire (FFQ) at the 2nd visit to ascertain her generalized eating pattern, with specific calculation of calcium and vitamin D intake. A Diet and Lifestyle Questionnaire will also be distributed. Dr. Sarah Rothenberg will use data obtained from the FFQ and Diet and Lifestyle Questionnaire to determine fish/shellfish consumption as it pertains to methylmercury intake and the interaction between vitamin D and methylmercury. This questionnaire is advantageous to collecting data over food diaries or 3-day food records for the following reasons: (a) The questionnaire does not rely on memory as much as other validated methods; (b) Its readability is designed for lower literacy populations; and (c) It accurately estimates individual and population-wide average daily intakes of all macro- and micronutrients. Individual and collective data can be gathered for statistical analysis to assess dietary intakes for this study population. This tool has been validated and reproduced against other dietary intake tools. Each completed FFQ form will be sent to the processing center (Berkeley, California) in batches of 50-100. Food models also will be provided to assist subjects in identifying most commonly consumed portion sizes rather than assuming each subject can accurately discern serving sizes on her own.

(14) **Maternal Physical Activity Assessment Questionnaire:** Each mother will be asked to complete a Physical Activity Assessment Questionnaire. This questionnaire utilizes a well-established physical activity instrument with known validity and reliability. Because physical activity can affect calcium metabolism and because a woman's activity level may change considerably during her pregnancy, it will be important to reassess maternal activity level throughout the entire period of gestation. Therefore, each mother will complete this questionnaire monthly.

(15) **Sunlight Exposure**: Two body sites will be scored at each monthly visit with a Smart Probe (measures skin color at 450 nm wavelength) to assess sunlight exposure.

(16) **Maternal Physical Examination**: As part of MUSC's OB clinic, each mother undergoes a physical examination at scheduled clinic visits, according to standard of care for the OB clinic. Some of these results of the physical exam will be recorded directly on the case report forms.

(17) **Maternal Dental Examination**: Women with 1 or more natural teeth and not having a health condition that requires antibiotic prophylaxis before periodontal probing are eligible for the periodontal examination.

(a) The commonly used full mouth periodontal examination (FMPE) may be done up to three times during the pregnancy (baseline, 28 weeks and 36 weeks). The FMPE protocol will be used for classification of periodontal disease. The major outcome measure of change in the periodontal measures will be correlated with change in serum circulating 25(OH)D concentration from baseline to 28 and 36 weeks. The FMPE will be done by trained personnel (Registered Dental Hygienist or Dentist) in a dental chair setting. Data are either recorded on paper or direct data entry will be done. The research instruments and questionnaires have all been previously used in the research environment.

(b) Gingival inflammation is measured using a salivary occult blood test (SOBT). The chairside SOBT is done monthly by rinsing the mouth with water and then spitting into a small paper cup. The outcome for SOBT will be analyzed with respect to the periodontal measures, and in particular, the percent of sites with BOP. Trained study personnel will perform the SOBT. Data will be recorded on visit questionnaire. This research instrument has been previously used in the research environment.

(c) Oral bacteria will be measured using a GenProbe for Chlamydia trachomatis at delivery or visit 7 (36 weeks). A swab is used to make the oral collection by gently swabbing the inside of the cheeks and oral cavity. The dichotomous outcome for Chlamydia (positive or negative) will be analyzed with respect to the periodontal measures, BOP, and birth outcomes. Trained study personnel will perform the oral bacterial test. Data will be recorded on the visit questionnaire. This research instrument has been previously used in the research environment.

(18) **Labor and Delivery Questionnaire**: Characteristics of each mother's labor and delivery will be recorded on a data form. Prior complications of pregnancy will be listed on the monthly pregnancy assessment questionnaire. Such complications at the time of delivery will be listed here according to ACOG definitions: e.g., preeclampsia; HELLP syndrome; gestational diabetes; hypertension; preterm labor; premature rupture of membranes (PROM); deep venous thrombosis or a coagulopathy; maternal infection such as varicella or herpes (primary or secondary); placental abnormalities such as previa or abruption. The following information about labor will be included: (a) length of labor, (b) use of pitocin augmentation, (c) time of rupture of membranes, and (d) complications of labor: fetal distress or decelerations, failure to progress, shoulder dystocia, breech presentation or abnormal fetal lie. Should the infant be delivered at an outlying hospital, mother will complete a release of medical information form so that medical records relating to the labor and delivery and the infant's health status/physical examination will be forwarded to the Clinical PI.

(19) **Maternal Body Mass Index (BMI) Measurement**: Pre-pregnancy height and weight of each mother will be recorded at the first outpatient visit to determine BMI (weight [kg]/height^2 [m^2]). During subsequent outpatient visits, only the mother's weight will be recorded.

(20) **Maternal Baseline and Monthly Calcium and C-Reactive Protein Serum Studies**: Whole blood (3.5 mL) will be obtained from each mother at the time of her scheduled obstetrical visit monthly as well as at delivery and placed in an SST tube. The blood will be sent to the Clinical Chemistry Laboratory. The following blood studies will be measured: total Ca and C-reactive protein. These results will be reported directly to Dr. Wagner, then entered into the Study's Database.

(21) **Maternal Baseline and Monthly Vitamin D and Calcium Homeostatic Parameters**: A 14 mL sample of whole blood will be drawn from the mother at the scheduled obstetrical visit monthly as well as at delivery and placed in an SST tube to be processed in the CTRC Clinical Laboratory for serum separation. The serum will be placed in a –80^{o}C freezer and sent to Dr. Bruce Hollis' laboratory on dry ice. Each serum sample will be analyzed for maternal circulating levels of total 25(OH)D, 1,25(OH)$_2$D, iPTH (5), batched for serum cytokine analysis, and batched for measurement of megalin and cubulin. 25(OH)D will be run on all subjects after 3 months supplementation. Mothers who are still frankly vitamin D deficient (less than 15 ng/ml) will be placed on open label vitamin D gummies for the remainder of their pregnancy.

(22) **Monthly Maternal Urine Sample**: A random urine sample will be obtained from the mother at each obstetrical visit to be sent to the Clinical Chemistry Laboratory for urinary Ca and creatinine measurement and derivation of the urinary Ca: creatinine ratio. This ratio will be used as an indicator of hypercalciuria that precedes hypercalcemia. Results of these urine studies will be reported directly to Dr. Wagner. An additional aliquot of the urine sample will be frozen for future cytokine analysis.

(23) **Cord Blood Vitamin D and Calcium Measurements**. At the time of delivery, 10-mL of whole blood will be drawn from the umbilical vein then sent to the CTRC Clinical Laboratory facility for processing. One red bullet (0.5 cc aliquot) will be sent to the Clinical Chemistry laboratory for measurement of total calcium and creatinine. The remaining blood will be processed in the CTRC Clinical Laboratory immediately for serum separation and placed in a –80^{o}C freezer then transported on dry ice to Dr. Hollis' laboratory for later determination of each neonate's circulating levels (at the time of delivery) of intact iPTH, total 25(OH)D, and 1,25(OH)$_2$D (5). Additional frozen serum will be batched for analysis of cytokines. Every effort will be made to collect cord blood at the time of delivery. An additional 15 mL plasma sample will be obtained from the cord for immune privilege flow cytometry studies.

(24) **Immune Privilege Flow Cytometry Studies:** Maternal plasma (14 mL) at first visit (baseline), fourth visit (24 weeks gestation) and seventh visit (36 weeks gestation), as well as 15 mL cord plasma will be analyzed by Dr. Jennifer Mulligan's laboratory for circulating immune cells by flow cytometric analysis. Plasma cytokines will be measured by ELISA per manufacturer's instructions. Expression of genes related to

vitamin D_3 activation and metabolism will be measured by custom PCR Array (SA Biosciences) for the following genes: VDR (vitamin D receptor), CYP2R1 (vitamin D 25-hydroxylase), CYP27B1 (1-alpha-hydroxylase), CYP24A1 (24-hydroxylase), and GC (vitamin D binding protein). The plasma immunoregulatory products to be examined include IL-10, TGF-beta, TNF-alpha, IFN-gamma, IL-2, IL-18 and IL-12.

(25) **Vaginal Self-Swabs:** At each visit, subjects will be asked to perform 3 vaginal self-swabs:

(a) First swab that the study coordinator will use to prepare a wet mount slide for identification of bacterial vaginosis and gram staining (these slides will be air dried and saved for batch analysis by the pathologists in Clinical Chemistry);

(b) Second swab will be a GenProbe swab for identification of Trichomonas vaginalis, Neisseria gonorrheae and Chlamydia trachomatis;

(c) Third swab will be a GenProbe swab for evaluation of vaginal cytokines.

When study subjects come into hospital for delivery, we will also obtain these three vaginal self-swabs. Vaginal cytokines that are of interest include: tumor necrosis factor-α, interleukin (IL)-1-α and 1-β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL13, IL-15, IL-17, IL-18, monocyte chemotactic protein (MCP), TGF-β, macrophage inflammatory protein (MIP), alpha-defensins, leukotriene B, s-tumor necrosis factor-R1, E-selectin, necrosis factor and cAMP element response binding. These cytokines can be batch-analyzed using a custom Luminex multiplex ELISA assay.

(26) **Placental Pathology** Placentas will be obtained at time of delivery for research purposes. Each placenta will be placed in the Labor and Delivery refrigerator until transport to Pathology, Dr. Michael Caplan's lab refrigerator. Dr. Caplan will record gross placental findings, membranes, fetal surface, maternal surface, and placental parenchyma, using methodology already in place in Dr. Caplan's pathology laboratory.

(27) **DNA and mRNA Expression:** At visit 2, each study subject will have an additional 6 mL of blood drawn in a plasma tube for analysis of DNA and RNA by Dr. Inderjit Singh's lab. Specifically, vitamin D binding protein DNA and vitamin D receptor will be analyzed per subject as per established protocol. mRNA expression of CYP24A1, CYP27B1, IL-4, IFNg, IL-6, IL-17A, IL-23, FoxP3, and 24,25(OH)2D also will be measured using methodology already in place in Dr. Singh's laboratory and previously reported (118).

(28) **Methylation**: Cord blood, buccal mucosal swabs, and placental tissue samples will be used to determine the variation in infant DNA methylation as a function of maternal vitamin D status, maternal pregnancy complications, and infant and maternal characteristics. The methylation will be probed at both the whole genome level and at the specific locus level. To evaluate the methylation status of the entire genome we will utilize a liquid chromatography-mass spectroscopy based method, which determines the proportion of methylated cytosine to total cytosine in the entire genome. We will use locus-specific methylation probes to determine the methylation of the promoter regions for cortisol receptor, vitamin D receptor, and other steroid receptor sites. Locus-specific methylation will be determined by bisulfite sequencing to determine the methylation of individual cytosine base pairs at the nucleotide level.

(29) **Metabolism and Processing of Vitamin D:** Dr. Sarah Rothenberg will examine the effect of dietary methylmercury intake on the metabolism and processing of vitamin D supplementation during pregnancy. A recent publication reported the ability of methylmercury to inhibit the bioactive form of vitamin D to upregulate the vitamin D receptor, thereby potentially negatively altering the innate immune system (119). To examine this pathway in this cohort, at visits 2, 7 and delivery, each study subject will have an additional 6 mL (1.2 teaspoons) of whole blood drawn in a royal blue vacutainer. Additionally, data obtained from both the FFQ and Diet and Lifestyle Questionnaire will determine fish/shellfish consumption as it pertains to methylmercury intake. The methylmercury levels will be determined and compared with the vitamin D status and vitamin D receptor gene expression of the subjects.

(30) **Child Dental Visit** At 18 months of age, the dentist or hygienist will ask routine dental utilization questions, examine and make digital images (take photographs) of the child's teeth. Likewise, an oral bacterial collection may be done by a gentle swabbing of the mouth.

Activity #7 (Objective #3): Through the joint efforts of this Kellogg-sponsored project, the Medical University of South Carolina and GrassrootsHealth.net, activity #7 represents a significant public health breakthrough. GrassrootsHealth.net is a nonprofit organization located in San Diego and affiliated with the

University of California in San Diego, whose sole purpose is to improve the health of individuals throughout the world through education about vitamin D and the adverse effects on health during times of deficiency. Through the assistance of GrassrootsHealth.net and the University of California's San Diego's CME office, we developed educational media and CME credits for physicians and other health care professionals to teach them about vitamin D, its role in immune function and overall health, with the latest scientific information (GrassrootsHealth.net received funding for this during year 4 of the Kellogg project).

We launched "Protect Our Children Now" campaign on April 7, 2015. Protect Our Children NOW! is a community outreach and clinical implementation project to reduce the incidence of preterm births quickly, easily, and safely by addressing the vitamin D deficiency epidemic through the engagement of pregnant women in a value-changing program of Good Health vs. 'Treating Illness.' The purpose is to implement vitamin D testing and supplementation of pregnant women as standard of care. Specifically, it is to help them achieve a serum level of at least 40 ng/mL to aid in the prevention of many comorbidities of pregnancy.

The Protect Our Children NOW! project involves educating prenatal care practitioners by providing free CME opportunities about vitamin D, and the importance of testing serum levels. These CME courses were and are offered to MUSC physicians sponsored by the University of California San Diego and GrassrootsHealth. In addition to the CME credits, each physician and health care provider receives 'D*certification' for this project.

Once again, these goals can only be reached when the Obstetrical community embraces the importance of vitamin D supplementation during pregnancy as an important factor in affecting the outcome of the health and well-being of the mother and the developing fetus during pregnancy. To this end, MUSC has partnered with GrassrootsHealth to implement universal vitamin D screening of all pregnant women who present to MUSC for their obstetrical care.

The Obstetrics Department at MUSC embraced the mounting evidence that vitamin D deficiency is not beneficial to our pregnant women and began universal screening in September 2015 of all pregnant women presenting for obstetrical care at MUSC. Specifically, all pregnant women who present for obstetrical care within the MUSC system have a baseline 25(OH)D concentration measured with appropriate counseling and supplementation to achieve a total circulating 25(OH)D concentration of at least 40 ng/mL by second trimester with repeat measure now enacted at the 24-28 weeks' gestation obstetrical visit to coincide with the planned glucose tolerance test. This change in practice began on September 1, 2015, after the Obstetrics Department at MUSC unanimously agreed to implement this change. Without surveillance data, however, there is no way to assess the effectiveness of this program and its impact on pregnancy outcomes at MUSC. To address this deficit, we have implemented the following:

Methods for Activity #7 (Objective #3):
Using the MUSC Perinatal Database and the associated laboratory data, the vitamin D status of all women who present for obstetrical care at MUSC and who deliver their infants at MUSC will be assessed. We will request a waiver of consent and HIPAA as it is not practical to consent these women and their babies after they have been discharged, and the data will be queried in a manner that will reduce the risk of loss of confidentiality by utilizing deidentified data after the laboratory data are merged with the PINS database by Myla Ebeling. The data to be abstracted from PINS includes complications of the pregnancy, complications of the delivery and abnormal diagnoses of the offspring child. The data will be used to address specific research questions as noted above. Grassroots Health will analyze the deidentified data as an independent entity that is already involved in the Kellogg Vitamin D Pregnancy Project.

E. PROTECTION OF HUMAN SUBJECTS

1. RISKS TO THE SUBJECTS

This is a randomized, double-blind, placebo-controlled trial of vitamin D supplementation during pregnancy, funded by the Kellogg Foundation. Dr. Carol Wagner is the PI of the study. For the parent protocol, 300 women must complete the study (through delivery) for the necessary statistical power (100 Hispanic, 100 African-American, and 100 Caucasian); half of each ethnicity will be randomized to 400 IU vitamin D/day and half to 4400 IU vitamin D/day. These women will be between 18-45 years of age, in good general health; exclusion criteria will be: mothers with pre-existing parathyroid disease or uncontrolled thyroid disease, mothers with multiple fetuses, and mothers with sickle cell disease, sarcoidosis, Crohn's disease, or ulcerative colitis. There will be a subgroup of pregnant women (projected 100 total) of all ethnicities who will have known diseases such as diabetes, hypertension, HIV or a body mass index > 49; these women will also be randomized to either 400 IU vitamin D/day or 4400 IU vitamin D/day, but in a separate randomization scheme, not dependent on completed subjects yielding a statistical power difference.

The targeted/planned enrollment table allows for a 30% dropout rate; information for our two previous large randomized clinical trials of vitamin D and pregnancy supports this rate. The targeted/planned enrollment table does NOT include the subgroup of pregnant women of all ethnicities who will have known diseases.

Targeted/Planned Enrollment Table

Total Planned Enrollment 450

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	150	0	150
Not Hispanic or Latino	300	0	300
Ethnic Category: Total of All Subjects*	450		
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	5	0	5
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	150	0	150
White	295	0	295
Racial Categories: Total of All Subjects*	450	0	450

**The "Ethnic Category: Total of All Subjects" must be equal to the"Racial Categories: Total of All Subjects".*

Pregnant women, 18-45 years of age will be recruited, of Hispanic, African-American and Caucasian ethnicities (may have a few Asians; will not exclude American Indian and Native Hawaiian if they are interested). All subjects will be receiving their prenatal care in the Charleston, SC area. All women will be less than 14 weeks of gestation at enrollment. This is a vitamin D supplementation study in pregnant women; so therefore, all women must be pregnant. The research question to be addressed is relevant only to women (pregnancy). A blood sample from the child of the pregnant women will be collected at birth, and the child will have a visit (with mother) at around 18 months of age to examine the first 4 baby teeth that have erupted and also to obtain a sample of the child's blood at that age for measurement of vitamin D.

Activity #7 (Objective #3) will not consent any additional subjects, because we are requesting a waiver of consent and HIPAA for deidentified record review of women who delivered babies at MUSC since September 2015.

b. Sources of Materials

The sources of research material will be maternal interview, history and physicals at clinic visits, the questionnaires, the lab reports for blood and urine analyses, the dental periodontal exam results, the dental salivary occult blood results, the vaginal swab results, the placental pathology report, the medical records of each mother and her infant following delivery, and the photographic teeth results on the infants.

The material/data that is routinely found in a maternal health record during pregnancy (including information on past pregnancies and disease history) will be utilized in the study. There are additional materials and data from questionnaires, blood, urine, saliva, infant teeth, placental tissue and vaginal samples that will be obtained for research purposes only.

A master list will be stored with study files accessible for clinical intervention only. The PI and study staff will have access to the master list. All archived samples and samples provided to collaborating laboratories will be identified with study subject numbers only. The DNA samples collected will have no recontact.

c. Potential Risks

Potential Risks of Phlebotomy and Blood Removal: The total amount of blood to be drawn at each maternal visit is as follows: visits 1 and 4, 31.5 mL; visits 2 and 8 (delivery), 22.5 mL; visits 3, 5, and 6, 17.5 mL; and visit 7, 37.5 mL. The total amount of blood drawn over the study will not exceed 200 mL. The risks of maternal phlebotomy include a slight pain when the needle is inserted into the vein. Momentary discomfort may occur. Pressure will be applied to the arm following the procedure to minimize the risk of bruising. There is a slight chance of inflammation of the vein and/or blood clot formation, but this is extremely rare. Fainting could occur, but this is rare. The same risks apply to the blood draw for the child. Every attempt will be made to minimize discomfort, including application of a topical anesthetic (EMLA cream) to the child's arm prior to venipuncture. These procedures were in place for our two previous vitamin D supplementation trials during pregnancy (HR #10727 and HR #16476).

Potential Risks of Dental Procedures: The risks of the dental procedures include temporary discomfort associated with periodontal probing and temporary discomfort of a dry mouth after providing a saliva specimen. Because of the transient bacteremia with periodontal probing, the few women who are usually premedicated with antibiotics before routine dental treatments will be excluded from the periodontal probing.

Potential Risk of Obtaining Umbilical Cord Blood: The total amount of blood to be collected from the umbilical cord is 30 mL. Since this procedure will be performed after delivery, there is no risk to either the mother or the infant during this procedure.

Potential Risks of Vitamin D Supplementation: There is a possibility that the treatment (dose of vitamin D) that each subject receives may prove to be less effective or to have more side-effects than the other study treatment (other dose of vitamin D) or other available treatments. The risks of maternal high dose vitamin D supplementation are hypercalciuria and hypercalcemia. There is a small risk that the woman's blood calcium level would increase in response to higher dose vitamin D supplementation. In the more than 540 women in the two prior vitamin D pregnancy supplementation trials (5, 6) and in the more than 340 mothers and infants who participated in the recently completed vitamin D lactation supplementation trial conducted at MUSC and the University of Rochester, this has not happened previously at this dose. The dose of vitamin D (4400 IU/day) used in this study is just over the limits established by the Institute of Medicine in 2010 (3). The PI and co-investigator do have an IND from the FDA (IND 66,346) for the administration of this dose of vitamin D and a higher dose in pregnant and lactating women; this protocol has been added to the IND. If, after 3 months on vitamin D supplementation, a woman is still frankly vitamin D deficient (less than 15 ng/ml), she will receive open label vitamin D gummies as requested by the FDA (IND 66,346). In two previous large randomized controlled trials of vitamin D supplementation during pregnancy and one large randomized controlled trial of vitamin D

supplementation during lactation, there was no difference in serum calcium or urinary calcium/creatinine ratios between the treatment groups (please see attached papers).

Potential Risks to the Fetus: During pregnancy, the developing fetus' vitamin D levels are about 70% of maternal serum vitamin D levels (5, 6). In the more than 540 women who participated in our two previous vitamin D supplementation studies, there was no identified increased risk to the developing fetus as per our two Data Safety and Monitoring Committees. Yet, some unknown risk of high dose maternal vitamin D supplementation on the fetus could be possible. By monthly monitoring of maternal serum vitamin D levels, we will ensure that the fetal levels remain in the normal range.

Potential Risks of Vaginal Swabs: Vaginal swabs obtained by the subjects do not carry a risk greater than insertion of a tampon.

Potential Risk of Breach of Confidentiality: As with any research study, there is a small risk that medical information, laboratory reports and data could become available to individuals outside the study. Every effort will be made to ensure that this does not happen.

Potential of Unknown Risks: The experimental trial may have unknown side effects that previously have not been reported (unexpected and unanticipated adverse events or effects).

The only alternative to this treatment regimen with vitamin D is the standard prenatal vitamin prescription (with 400 IU vitamin D_3 per tablet) taken daily throughout pregnancy. This regimen may not be advantageous if a woman is deficient in vitamin D.

2. ADEQUACY OF PROTECTION AGAINST RISKS

a. Recruitment and Informed Consent

The study subject's primary care provider(s) will make first contact on behalf of the study so there is no appearance of a breach in confidentiality. Once a subject has expressed interest in this research study, to obtain informed consent, one of the study coordinators will explain to each subject in lay language the purpose, benefits and risks of the investigation. Consent will be given following an informative discussion period and either by reading the Informed Consent to the subject or by allowing the subject to read the consent, then reviewing it with the subject. All study coordinators have taken the Research Coordinator course and have passed their Miami CITI. Every attempt will be made to conduct this study in full compliance for the protection of the subjects. For those women who are not fluent in English and whose first language is Spanish, a Spanish version of the consent form will be used (translated by BiLingo from IRB approved English consent and submitted as an amendment to IRB). Following the subject's verbal agreement to participate in the study, the subject will sign the informed consent; the study coordinator also will sign the consent. A copy of the signed, written informed consent will be given to the study subject. The original informed consents will be placed in locked research file in study coordinators' office, 20 Ehrhardt St, #4. A copy of the informed consent also will be placed in the subject's study binder (located in locked cabinets at same location). A study coordinator note will be placed in Epic identifying an MUSC woman as participating in a vitamin D and pregnancy RCT.

b. Protection against Risk

All of the investigators have conducted several clinical trials; they are well versed in the issues of patient/subject confidentiality and have completed the Miami CITI course. They are nationally and internationally known in their fields. All study coordinators have completed the Miami CITI and the Research Coordinator course at MUSC. The endeavors of the PI, co-investigators and study coordinators to maintain subject confidentiality will be ensured.

All maternal blood, urine, and vaginal samples as well as infant blood and placental tissue will be labeled with the patient's name and medical record number to ensure proper tracking. Each study subject (and infant) then will be assigned a specific study number without any reference to the woman or infant's name, which will be used to enter all data into the web database. We will follow all HIPAA guidelines to maintain protection of patient/subject information. With these precautions in place, there is a remote risk that the samples and data could be linked to the woman and her infant; however, the study number and the name will be kept in a separate file in the locked offices of the study coordinators. The data will be entered in a secured database with only the study number entered, thus ensuring that the number and data will be kept separately from the woman and infant's names. All this information will be kept confidential, and when reported in scientific journals, the information will not have any identifying information regarding study subjects.

Should any untoward reaction occur, subjects would receive treatment at the Medical University of South Carolina. The study will cover the costs of laboratory tests; however, additional costs of treatment will be the responsibility of the subject as described in the informed consent.

See main section #5 Data Safety and Monitoring Plan that follows for full details; this is a clinical trial. This study meets the guidelines of clinicaltrials.gov as a clinical trial requiring a Data Safety and Monitoring Plan and a Data and Safety Monitoring Committee. Two of the members of the Data and Safety and Monitoring committee are scientists external to MUSC well known in the field of vitamin D metabolism and calcium metabolism. Two of the members are physician scientists external to MUSC, who are also well known in the field of calcium and vitamin D metabolism. A fifth member will be Dr. Tom Hulsey, epidemiologist at MUSC with considerable experience serving on the DSMC of other clinical trials. Dr. Hulsey was the chair of the DSMC of the two prior vitamin D supplementation trials conducted by this study team. He will maintain the database of the study, follow HIPAA guidelines, and conduct ongoing interim analyses to ensure that the risk: benefit analyses remain in favor of benefit to the subjects.

3. POTENTIAL BENEFITS OF THE PROPOSED RESEARCH TO THE SUBJECTS AND OTHERS

Pregnant women enrolled in the study may avoid vitamin D deficiency during their pregnancy. The developing fetus may have more normal calcium and vitamin D levels, better bone mineralization, and improved immune function and brain development. Another potential benefit of this study is that data will be generated that will translate into recommendations for optimal vitamin D status during pregnancy. Increasing numbers of Americans are becoming aware of the risks of hypovitaminosis D, and have begun taking supplemental vitamin D. Without monitoring, the potential for overdosing—although small, exists. There needs to be additional controlled trials that will serve as a guide for practitioners and health care policy makers when recommending the DRI for vitamin D during pregnancy in all ethnicities.

The risks to the subjects in participating in this study (phlebotomy, hypercalciuria, hypercalcemia, confidentiality issues) are reasonable for the following reasons: The issue of adequate vitamin D levels in pregnant women and in women in general is a recent public health problem that reflects the vitamin D-poor content of modern diets and markedly decreased sunlight exposure (a relatively new phenomenon in our evolution) that follows the recommendation of the American Cancer Society and the American Dermatological Society. Thus, the issue of maternal supplementation with vitamin D becomes relevant for thousands of women and their developing fetuses. Hypovitaminosis D during pregnancy is a preventable disorder whose occurrence is widely pervasive in the African American community (affects >75% of reproductive age African Americans) with the majority of their neonates being hypovitaminotic *in utero* and at birth. Hypovitaminosis D is also prevalent in the Hispanics (affects approximately 60% of women of reproductive age). Secondly, the maximal dose that women will take for 10-40 weeks gestation is 5-fold lower than the per kg dose given to preterm and term infants routinely in total parenteral nutrition and formulas. Compared to the Masaii tribe in East Africa, who receive 5-9 hours of

sunlight per day and achieve vitamin D levels well above that achieved taking 4000 IU vitamin D_3, day, this supplementation regimen is aimed at reversing the vitamin D deficiency epidemic in the U.S. and other areas of the world. We now know that vitamin D deficiency has far more clinical implications than integrity of the skeleton, including impaired immunity and increased risk of various disease states, including diabetes, neoplasia, and multiple sclerosis. Through vitamin D supplementation during pregnancy, we believe that participation will beneficially improve overall health status of mother and her developing fetus. Because of the close monitoring of the pregnant women and their developing fetuses through pregnancy, we will have minimized the risks of participation in this study. Should a woman develop hypervitaminosis D, hypercalciuria or hypercalcemia, there are appropriate safeguards built into the study to minimize the risk to the mother and her infant.

4. IMPORTANCE OF THE KNOWLEDGE TO BE GAINED

The issue of maternal supplementation with vitamin D becomes relevant for thousands of women and their unborn fetuses due to recent public health information showing that the majority of pregnant women have inadequate vitamin D levels and decreased sunlight exposure. The potential co-morbidities of pregnancy caused by vitamin D insufficiency or deficiency are increasing and occur with more frequency in African-American and Hispanic ethnicities. As shown in our previous vitamin D supplementation pregnancy trials, hypovitaminosis D is a preventable disorder with adequate supplementation; we strongly believe that the DRI for pregnant women, especially darkly pigmented individuals is woefully inadequate. Optimal vitamin D supplementation becomes an issue for the majority of pregnant women in this country and is necessary to decrease health disparities due to ethnicity.

The results of our previous vitamin D supplementation clinical trials in pregnancy did not show any detrimental effects (hypercalcemia or hypercalciuria) of daily intake of 4000 IU of vitamin D (5, 6). While in the NICHD vitamin D supplementation trial, there was a trend toward reduction in co-morbidities of pregnancy (5), in the Thrasher Research Fund trial involving community-based women living at or near the poverty level, there was evidence of reduced preterm labor, preterm birth, and infection as a function of maternal total circulating 25(OH)D levels, effects that persisted even after controlling for race (6). Yet, in both studies where the health status of the mother was not the focus of the trial nor was immune function, these are post-hoc analyses and can only suggest correlation. In the present RCT, we hope to show statistical differences in the co-morbidities, and show improved immune function with improved vitamin D status.

Based on the data of vitamin D supplementation in normal adult men and women of diverse ethnic backgrounds, our previous vitamin D pregnancy and lactation trials, and the current practice of safely supplementing with 5-8 fold higher IU/kg doses in infants and young children, there is little risk of hypervitaminosis D occurring in pregnant subjects enrolled in this study. Rather, it is quite likely that the woman and her fetus (later, infant) will achieve more satisfactory vitamin D and calcium homeostasis that will yield improved immune function and reduction in co-morbidities of pregnancy. Should a woman develop hypervitaminosis D, hypercalciuria or hypercalcemia, there are appropriate safeguards built into the study to minimize the risk to mother and her infant. Thus, the risks to the subjects are reasonable in relation to the importance that reasonably may be expected to result.

5. SUBJECT SAFETY AND MINIMIZING RISKS (Data and Safety Monitoring Plan)

DATA AND SAFETY MONITORING PLAN: The operation of the DSMB is crucial to the safety of the subjects who will be enrolled in the study. Following the recommendations of the DSMB will be an essential and fundamental part of this Research Protocol.

A. **Creation of a Data and Safety Monitoring Board (DSMB)**: This grant application meets NIH policy and Guidelines on the inclusion of a Data and Safety Monitoring Plan for clinical trials mL and

http://grants.nih.gov/grants/guide/notice-files/not98-084.html). MUSC as the Institute and Center (IC) of this grant will have a Data and Safety Monitoring Committee (DMSC) in place as outlined in this grant. Two of the members of the DSMC are scientists external to MUSC well known in the field of vitamin D and calcium metabolism. Two of the members are physician scientists external to MUSC who also are well known in the field of calcium and vitamin D metabolism. A fifth member will be Dr. Tom Hulsey, epidemiologist at MUSC with considerable experience serving on the DSMC of other clinical trials (current and completed). He will maintain the database of the study, follow HIPAA guidelines, and conduct ongoing interim analyses to ensure that the risk: benefit analyses remain in favor of benefit to the subjects.

B. Conduct ongoing monitoring of interventional trial by those who have appropriate expertise to accomplish the trial's mission: The MUSC clinical lab is required to notify the PI of any critical lab values. The Study Coordinators will be responsible for checking laboratories of the women within 72 hours of their reporting and then entering the laboratory data into the computer. They will be provided with a set of normative laboratory values as a reference. The Clinical PI will review values during the weekly study meeting or will be notified if a value falls outside of the referent value range. All data will be verified independently by the Data Processing Center under the direction of Dr. Hulsey. The DSMC will be notified via telephone and fax with source documents and adverse report sheets if a subject's value falls outside the referent value range. In addition, all adverse events will be reviewed semiannually by the DSMC, whose report will be forwarded to the Kellogg Foundation on a quarterly basis and the IRB yearly. The Investigators will report all Serious Adverse Events by telephone to the IRB and the DSMC; in addition, the IRB, the DSMC, and Kellogg Foundation will receive a written report within 10 days of the Clinical Investigators' knowledge of the Event. In addition, the investigators will generate a quarterly report to the DSMC regarding subject enrollment, subject completion, adverse events and serious adverse events. The DSMC will review the report and a summary letter with their findings sent to the IRB.

C. Interim Data Analyses & Monitoring: Interim analyses for the evaluation of safety and efficacy will be conducted based on the recommendations of the Data Safety & Monitoring Board. The DSMC will serve to monitor for safety and efficacy. DSMB reports produced by Dr. Hulsey's team will include summary statistics: on mother recruitment (expected vs. actual), data form quality (completion and timeliness of forms); tracking of data editing; demographics of the randomized mothers; aggregate safety; aggregate efficacy; and related information. The DSMB also will monitor the trial from the standpoint of futility using the techniques of Lan and Wittes (119*).*

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G. CONSULTANTS

Where applicable, attach electronic versions of appropriate letters from all individuals confirming their roles in the project. Go to the application under "additional uploads" to attach this information.

n/a

H. FACILITES AVAILABLE

Describe the facilities available for this project including laboratories, clinical resources, etc.

Study subjects will be seen in obstetrical clinics at MUSC; interested pregnant women not being followed at any of these clinics can consent for the study and be seen in the CTRC at MUSC. Research laboratories will include several in the Children's Research Institute – Dr. Bruce Hollis/Dr. Carol Wagner, Dr. Jennifer Mulligan and Dr. Inderjit Singh as well as several in Obstetrics and Gynecology – Dr. Louis Guillette and Dr. David Soper/Dr. Gweneth Lazenby, and laboratories of Dr. Michael Caplan and Dr. Scott Argraves at MUSC. The laboratory of Dr. Sarah Rothenberg is located in the Department of Environmental Health Sciences, University of South Carolina.

I. INVESTIGATOR BROCHURE

If applicable, attach the electronic version of the investigator brochure. Go to the application under "additional uploads" to attach this information.

n/a

J. APPENDIX

Attach any additional information pertinent to the application, such as surveys or questionnaires, diaries or logs, etc. Go to the application under "additional uploads" to attach this information.

Relevant Papers:

Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2011;26(10):2341-57. Epub 2011/06/28. doi: 10.1002/jbmr.463. PubMed PMID: 21706518; PubMed Central PMCID: PMC3183324.

Wagner C, McNeil R, Hamilton S, Winkler J, Rodriguez Cook C, Warner G, et al. A Randomized Vitamin D Supplementation Trial in Two Community Health Center Networks in South Carolina. Am J Obstet Gynecol. 2013;in press.