Effect of Vitamin D replacement on maternal and neonatal outcomes: a randomized controlled trial in pregnant women with hypovitaminosis D - Protocol.

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ABSTRACT

Introduction

The vitamin D recommended doses during pregnancy differ between societies. The WHO guidelines do not recommend routine prenatal supplementation, but they underscore the fact that women with the lowest levels may benefit most. The effects of routine supplementation during pregnancy on maternal and neonatal clinical outcomes have not been investigated in the Middle East, where hypovitaminosis D is prevalent. Our hypothesis is that in Middle Eastern pregnant women, a vitamin D dose of 3,000 IU/d is required to reach desirable maternal 25-hydroxyvitamin D [25(OH)D] level, and to positively impact infant bone mineral content (BMC).

Methods and analysis

This is a multicenter blinded randomized controlled trial. Pregnant women presenting to the Obstetrics and Gynecology clinics will be approached. Eligible women will be randomized to daily equivalent doses of cholecalciferol, 600IU or 3,000IU, from 15-18 weeks gestation until delivery. Maternal 25(OH)D and chemistries will be assessed at study entry, during the third trimester and at delivery. Neonatal anthropometric variables and 25(OH)D level will be measured at birth, and bone and fat mass assessment by DXA, at one month of age. 280 pregnant women allow to demonstrate a significant difference in the proportion of women reaching a 25(OH)D level ≥50 nmol/l at delivery, and a difference in infant BMC of 6(±10)g, for a 90% power and a 2.5% level of significance. The proportions of women achieving target 25(OH)D level will be compared between the 2 arms, using Chi-Square. Independent t-test will be used to compare mean infant BMC between the 2 arms. The primary analysis is an intention-to-treat analysis of unadjusted results.

Ethics and dissemination

The protocol has been approved by the Institutional Review Board at the American University of Beirut-Lebanon (IM.GEHF.22). The trial results will be published in peer-reviewed medical journals and presented at scientific conferences.

Trial registration number: NCT 02434380.

INTRODUCTION

Vitamin D physiology during pregnancy

Pregnancy is characterized by physiologic changes in mineral metabolism, to allow calcium accretion in the fetal skeleton [1-3]. These changes start in the first trimester, and culminate during the third trimester, a period during which fetal calcium requirements increase exponentially [2]. Indeed, it is in anticipation to such requirements that maternal calcitriol levels increase during pregnancy. While the total calcitriol levels double in the first trimester, free calcitriol levels do not increase until the third trimester, and remain so into lactation [2, 4]. Conversely, parathyroid hormone (PTH) levels decrease early on and increase back to mid-normal range by term [2]. Total calcium level decreases during pregnancy, due to hemodilution, while the ionized calcium level remains stable [2]. Vitamin D binding proteins also increase during pregnancy secondary to high estrogen levels [4, 5], but 25-hydroxyvitamin D [25(OH)D] level, the single best nutritional indicator of vitamin D status [6], remains stable [7]. The changes in calcitriol levels led to the description of pregnancy as a state of "absorptive hypercalciuria" [1, 2]. The above adaptive physiology is not only key to safety considerations when using vitamin D supplementation during pregnancy, but also to determining key biochemical and hormonal parameters to be monitored.

Maternal vitamin D status during pregnancy

Vitamin D deficiency during pregnancy is prevalent worldwide,

especially in developing countries [8]. In a systematic review of 18 studies conducted in Western countries during the first trimester, white Caucasian pregnant women were found to have a mean 25(OH)D level between 29 and 73 nmol/l [9]. Mean 25(OH)D levels were lower in non-Caucasian pregnant women, ranging between 15.2 and 43 nmol/l [9]. In addition to ethnicity, higher latitude was a significant predisposing factor for hypovitaminosis D [9]. Similarly, in non-Western countries, more than half of pregnant women who were beyond their first trimester had 25(OH)D levels below 75 nmol/l; these include countries such as India [10], Kuwait [11], Pakistan [12] and Turkey [13]. Even lower levels (< 25 nmol/l) have been reported at delivery in Saudi Arabia, Iran and United Arab Emirates [14]. Furthermore, immigrant women were at a particular risk [15, 16]. An observational study from Netherlands showed significantly lower 25(OH)D levels during the first trimester in immigrant pregnant women (Turkish, Moroccan and others), compared to western participants [17].

Association between maternal vitamin D status and maternal adverse outcomes

Vitamin D insufficiency during pregnancy is associated with adverse maternal outcomes such as increased risk of gestational diabetes mellitus (GDM), preeclampsia, Cesarean-section delivery and bacterial vaginosis [18]. In a recent meta-analysis of observational studies, the risk of GDM was found to be increased by 40-84 % in pregnant women with low 25(OH)D levels, defined as < 50 nmol/l or < 75 nmol/l, depending on the studies [19-21]. While preeclampsia risk was significantly increased in vitamin D insufficient women [22], C-section rates were inconsistently affected by vitamin D status [23]. However, these findings remain limited by the inherent biases of observational studies, inconsistent adjustment for confounders, in addition to the wide heterogeneity in vitamin D assays and vitamin D cutoffs definition.

Association between maternal vitamin D level and neonatal adverse outcomes

Low maternal 25(OH)D levels were recently linked to fetal programming, and were found to be associated with adverse events, in neonates, resulting in small for gestational age (SGA) at birth [19, 23], and also later on during childhood, leading to reduced muscle and bone mass in off springs at 4 and 9 years of age [24, 25]. This may be explained by the fact that maternal vitamin D is essential for fetal musculoskeletal integrity, as it regulates neonatal bone accrual, possibly through specific proteins that are responsible for placental

calcium transport [26]. Recently, data from the Southampton Women's Survey (SWS) showed that maternal 25(OH)D level is significantly correlated with placental amino acid transporters expression, mediating various nutrient transport to the fetus [27]. Furthermore, maternal vitamin D may influence fetal muscle motor unit size, and consequently muscle mass and strength after birth [25]. Noteworthy that fetal bone development is one of the predictors of peak bone mass, adult bone mineral content and hip geometry, thus correlating with fracture risk later in life [26, 28].

Vitamin D replacement guidelines during pregnancy

The guidelines regarding vitamin D replacement or supplementation during pregnancy vary substantially.

The 2010 Institute Of Medicine (IOM) Report on Dietary Reference Intakes for Calcium and Vitamin D recommended 600 IU to pregnant women, as the recommended daily allowance (RDA); the RDA being the dose that is projected to allow to at least 97.5% of the pregnant women population to reach the desirable target 25(OH)D level ≥ 50 nmol/l [29]. This recommendation was based on observational studies, none of which were conducted in the Middle East [29]. Conversely, the Endocrine Society 2011 guidelines recommended that 1,500 to 2,000 IU daily of vitamin D are needed to reach a target 25(OH)D level ≥ 75 nmol/l (recommendation that was graded as weak, with moderate quality of evidence) [30]. The American College of Obstetricians and Gynecologists (ACOG) does not recommend screening for vitamin D level in pregnancy, and abides by the IOM recommendations [31]. Moreover, the WHO 2012 guidelines on vitamin D replacement during pregnancy did not recommend vitamin D supplementation as part of prenatal care [32]. This was based on a meta-analysis of vitamin D trials during pregnancy, that identified a limited number of high quality studies demonstrating a beneficial effect of supplementation on maternal and neonatal outcomes, and concluded that further randomized controlled trials (RCTs) are needed [33]. In the United Kingdom, however, pregnant women are considered at risk of vitamin D deficiency, and supplementation with 400 IU daily is required [34].

It is not clear whether any of the above recommended doses are applicable to non-western populations, with lower baseline vitamin D levels, such as in Lebanon and other Middle Eastern countries. Indeed, the WHO pregnancy guidelines clearly stated that *"Vitamin D supplementation will probably have the most benefit in populations of poor countries, those with darker skin color and in populations with a high prevalence of vitamin D deficiency. It is expected that this intervention would be acceptable to women who are not exposed to adequate amounts of sunshine*' [32]. This is particularly relevant to

our population that tends to avoid sunshine, wear concealed clothing or use sunblock, all resulting in the low 25(OH)D levels observed across the lifecycle.

Randomized controlled trials of vitamin D supplementation during pregnancy:

Two landmark randomized controlled trials have been conducted in the US [35] and UK [36]. Hollis et al showed that in US pregnant women, with a baseline 25(OH)D level around 60 nmol/l, a vitamin dose of 4,000 IU daily allowed to 82% of participants to reach a 25(OH)D level of 80 nmol/l, while only 70% and 50% reached this target in the intermediate (2,000 IU daily) and low dose (400 IU daily), respectively [35]. Cooper et al showed that vitamin D supplementation of 1,000 IU daily, compared to placebo, in UK pregnant women allowed a significant increase in Bone Mineral Content (BMC) of neonates, however, only when they were born in winter [36]. One study from India, comparing nonintervention to vitamin D supplementation groups, dose being dependent on 25(OH)D levels at 20 weeks gestation, showed that vitamin D supplementation resulted in a significant difference in the achieved 25(OH)D level at delivery (43.1(81.3) nmol/l in the former group versus 56.8(47.5) nmol/l in the latter group) [37]. Both Hollis et al. and Sablok et al. showed that vitamin D supplementation decreased the risk of preterm labor, gestational diabetes and hypertensive complications (all combined) [36, 37].

In the Middle East and North Africa region, there are few recent randomized controlled trials that attempted to determine the optimal regimen of vitamin D replacement in healthy pregnant women [38-41]. With the exception of Soheilykhah et al that assessed the effect of vitamin D supplementation on insulin resistance [40], the primary outcomes in these studies were mostly maternal and neonatal 25(OH)D levels (see Appendix 1). None of the other clinically important outcomes, such as neonatal size and other anthropometric measurements, neonatal bone mineral content, GDM and C-section rates, were evaluated, as primary outcomes, in any of these trials (Appendix 1).

We therefore compiled a registry of all ongoing vitamin D trials in pregnancy, as captured by their registration on clinicaltrial.gov [42] (Appendix 2). In these trials, different doses of vitamin D, reaching up to 7,000 IU daily are being administered, and the outcomes to be assessed include neonatal weight and length, childhood asthma, maternal bone mineral density, maternal adverse outcomes, including preeclampsia and preterm labor, and neonatal adverse outcomes, such as SGA. Only 2 of the ongoing trials are being conducted in the Middle

East, one in Iran and one in Israel, both start supplementation in the third trimester and use vitamin D doses of 7,000 IU and 2,000 IU daily, respectively. These latter studies aim at assessing the effect of vitamin D supplementation on offspring calcium status, maternal and infant vitamin D status, and bone status (by quantitative ultrasound) at one year of age. Neither addresses the applicability of IOM vitamin D dose recommendations in pregnant women in the Middle East. Three completed (unpublished) trials were identified (Appendix 2), 2 from the US and 1 from Pakistan. They assessed the effect of various doses of vitamin D on 25(OH)D level, immune function and periodontal disease.

Hypothesis

The study hypothesis is that a high dose of vitamin D, equivalent to 3,000 IU/day, is needed to optimize maternal vitamin D level and neonatal musculoskeletal parameters, compared to a low dose of 600 IU/day.

Objectives

The two primary objectives of this trial, comparing the effect of a high dose versus a low dose vitamin D, are as follows:

- The proportion of women who will reach the IOM defined desirable 25(OH)D level ≥ 50 nmol/l at delivery.
- Infant bone mineral content (BMC) at one month of age.

The secondary objectives are to compare the effect of high dose versus low dose vitamin D on:

- Maternal outcomes:
  Mean maternal 25(OH)D level, at delivery.
  Mean maternal PTH level at delivery.
  Mean change in maternal urine calcium.
- Neonatal outcomes:
  Mean neonatal 25(OH)D level, at delivery.
  Mean neonatal PTH level, at delivery.
  Mean neonatal fat and lean mass, at one month of age.
  Mean neonatal knee to heel length at birth.

Exploratory outcomes include a composite outcome (incidence of GDM and C-section), maternal weight, blood pressure, ill days, fetal and neonatal anthropometric measures, including neonatal length and weight, rate of small for gestational age, APGAR score, placental weight, and 1α-hydroxylase activity, in addition to other placental and genetic studies, that characterize mineral and fuel metabolism.

METHODS AND ANALYSIS:

The protocol of this trial was developed based on the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT), see Appendices 3-6 for further details. This protocol is registered on clinicaltrial.gov (NCT 02434380, April 2015)

Study design

This study is a phase III, multicenter blinded randomized controlled, superiority trial, with 2 arms, conducted at the American University of Beirut - Medical Center (AUB-MC), Rafic Hariri University Hospital (RHUH), and Bahman Hospital.

Recruitment

Pregnant women in their first trimester will be recruited from the obstetric private clinics and out-patient departments of the three participating centers (AUB-MC, RHUH and Bahman Hospital). Information about the trial will be available as Arabic and English flyers in the Obstetrics and Gynecology Department, as well as the private clinics and out-patient departments of the three centers. The flow chart of participants and details of study visits are summarized in Figure 1.

Randomization

The allocation sequence will be computer-generated, permuted block randomization, stratified by study center, with a 1:1 allocation. The statistician will be responsible of sequence generation and treatment assignment. The senior pharmacist at AUB-MC will be responsible for treatment allocation.

Concealment and blinding

Vitamin D and placebo pills are manufactured to have a similar shape, color, size, smell and taste. The study medications will be stored at the AUB-MC pharmacy, and placebo and/or vitamin D pills will be dispensed in boxes. Boxes will be sequentially numbered as per the random allocation list by the pharmacist. The pharmacist keeps the list linking the randomization code to the participant identity/trial number and to the delivered box number. At each visit, the pharmacist allocates a box to every participant, containing enough pills until the next visit, with dates at which pills should be administered. The research assistant collects the boxes in sealed envelopes prior to

each participant's visit and delivers them at the end of the visit. The research assistants, the health care providers, the principal investigator, the co-investigators, and the biostatistician do not have access to code break, and are all blinded to the treatment allocation. The only personnel who will not be blinded will be the pharmacist.

Investigational Medicinal Product (IMP)

All participants receive once per week, 2 tablets that are similar in shape, color, size, smell and taste. Each tablet can be either a placebo or a 10,000 IU vitamin D (cholecalciferol), provided by Europharm.
The high dose group receives two tablets of 10,000 IU weekly (equivalent daily dose 2,857 IU).
The low dose group receives one tablet of 10,000 IU and one tablet of placebo alternating with two tablets of placebo on weekly basis (equivalent daily dose 714 IU).
Vitamin D supplementation present in prenatal multivitamins will be permitted up to 200 IU daily, which will raise the above mentioned treatment doses to approximately 3,050 IU and 900 IU daily, respectively.
The manufacturers had no role in the study design or implementation.

Study visits

a- Pre-screening visit
Trained research assistants will approach pregnant women who are in their first trimester during their routine prenatal visits to study sites. Eligible pregnant women willing to participate and to be compliant with the study protocol, and who provide written informed consent will be invited to a screening visit.

b- Screening visit
The screening visit will be scheduled to coincide with the nuchal translucency appointment date (between 11 and 13 weeks of gestation). During this visit, eligibility criteria will be verified and blood tests for 25(OH)D level, calcium, phosphate, magnesium, creatinine, and thyroid stimulating hormone (TSH) will be withdrawn. Urine calcium will be assessed in a fasting urine spot or 24-hour urine collection (Table 1). The level of 25(OH)D will be measured using the electrochemiluminescence immunoassay (ECLIA), at AUB-MC Clinical Chemistry laboratory. Reference ranges using this assay are defined as follows: Deficiency < 25 nmol/l, insufficiency: 25-62.4 nmol/l, desirable >62 nmol/l, toxic >374 nmol/l. AUB-MC Clinical Chemistry

laboratory partakes in the quality assurance, evaluation, and accreditation by the College of American Pathologists [43] and is a participant in the Vitamin D External Quality Assurance Surveillance (DEQAS) program [44].

Table 1: Trial events and outcomes measures

Trial event and outcomes measures	11-13 weeks (Screening visit)	15-18 weeks (Visit 1)	20 weeks	24-28 weeks	28-32 weeks (Visit 2)	37-42 weeks Delivery (Visit 3)	1mo post-partum (Visit 4)
Maternal height		*					
Maternal weight, blood pressure		*			*	*	
Maternal health and diet assessment		*			*	*	
Maternal 25(OH)D, Crea, Ca	×				×	×	
Maternal CBCD, ph, mg, alb, TSH, $1,25(OH)_2D$	×						
PTH	×					×	
Maternal glucose (1h)				*			
Maternal 24h (or spot) urine collection for Calcium and creatinine		×			×		
Maternal Vitamin D binding protein	×					×	
Maternal genetic pathways of vitamin D metabolism						×	
Fetal US: -crown -rump	*						
Fetal US: -Femur length -Abdominal circumference -Head circumference -Biparietal diameter			*				
Newborn weight, length, knee to heel length APGAR score						*	
Placental weight						×	
Placental studies						×	
Newborn 25(OH)D, Ca, PTH (cord blood)						×	
Newborn genetic pathways of vitamin D metabolism						×	
Infant bone/fat mass							×
Infant weight and length							*
Infant health and diet assessment							*

* test done for clinical purpose

× test done for research purpose

Eligibility criteria

Inclusion criteria:

- Pregnant women gestational age (GA) < 14 weeks at screening visit*.
- Middle Eastern origin; Middle East countries as defined by the World Bank (Bahrain, Egypt, Iran, Iraq, Palestine, Jordan, Kuwait, Lebanon, Oman, Qatar, Saudi Arabia, Syria, United Arab Emirates, Yemen) [45].
- 25(OH)D level between 25 and 75 nmol/l.
- Age > 18 years.
- Vitamin D supplementation ≤ 200 IU daily**.

*In the case where the pregnant woman presents after 13 weeks GA, she is still eligible for the screening visit provided that screening blood tests are done before 16.5 weeks of gestation and the first visit in the trial occurs before 18 weeks GA.
**If daily vitamin D supplementation is between 200 and 600 IU daily, at enrollment, the pregnant women will be advised to adjust prenatal multivitamin doses, in consultation with her primary obstetrician, to ensure that total vitamin D supplementation during the study does not exceed 1,400 IU per week, in consultation with the primary obstetrician.

Exclusion criteria:

- 25(OH)D level < 25 nmol/l, as it would be unethical to randomize pregnant women to the low dose of vitamin D, and 25(OH)D level > 75 nmol/l (30 ng/ml), as vitamin D supplementation with routine prenatal multivitamins would be sufficient.
- Known metabolic bone disease or chronic diseases associated with bone abnormalities (renal or liver diseases.
- Current medications likely to interfere with vitamin D metabolism (enzyme inducing anticonvulsants, anti-tuberculosis).
- Vitamin D supplementation > 600 IU daily*.
- Fetal physical anomalies on the initial ultrasound.
- Renal stones.
- Hyperparathyroidism.
- Uncontrolled thyroid dysfunction.
- Diagnosis of cancer in the last 10 years (other than basal cell carcinoma).
- Serum calcium >10 mg/dl.

- Diabetes mellitus type 1 or type 2.
- Previous GDM.
- Allergy to any component of vitamin D formulation.
  *If a pregnant woman is on a high dose of vitamin D supplementation, > 600 IU daily, vitamin D should be stopped at least one month prior to study entry, at the discretion of her primary physician.

c- First visit

During the first visit at 15-18 weeks GA, a questionnaire will be administered to collect maternal information on parity, demographics, smoking and alcohol history, exercise, previous medical problems, medications, dietary calcium and vitamin D intake, in addition to relevant paternal information. Pregnant women will be randomized early in their second trimester to one of two vitamin D doses as discussed above.

d- Second visit

This visit will take place at 28-32 weeks GA, during which maternal weight and blood pressure will be recorded, maternal health and diet assessed, in addition to assessment of adverse events, if any. We will check compliance to trial medication by pill counting. Blood and urine tests will be done (see Table 1).

e- Third visit

The third visit will coincide with the participant's delivery. When entering labor, the research team will be informed about each participant by the obstetrician, or by the participant, or her partner. The research assistant will visit the participant on the first day post-partum, and will record information on delivery mode, delivery course and complications, if any. In addition, neonatal measurements at birth such as length, weight and knee - heel length will be recorded in triplicates. Knee-heel length will be measured using hand-held vernier calipers [46]. Knee - heel length measurement is operator dependent; hence measurements will be done in triplicate, and only by pediatricians/neonatologists who are trained on how to use such instruments.
Neonatal 25(OH)D level will be obtained from cord blood whereas maternal blood tests will be withdrawn when the pregnant women presents in labor. In addition, blood tests at delivery include genetic studies such as vitamin D genes polymorphism and RNA expression of vitamin D polymorphisms. After delivery, placental sampling will be performed by a trained nurse and samples will be

preserved and stored at -80* C.

f- Fourth visit

This visit will occur when the infant is one month of age. He will undergo BMD assessment by DXA scan, Hologic machine, Horizon A, version 13.5.3.1, at AUBMC. Infant DXA assessment is performed by technicians certified by the International Society for Clinical Densitometry (ISCD). The technician positions the laser light so that it is centered about 2 cm below the iliac crest (or umbilicus/belly button) on the child, and observes the emerging image to ensure that the spine is centrally positioned and straight, and that the top of the iliac crests and all of L5 are visible.
In addition, during this visit, information about the infant health and feeding will be recorded using an interviewer-led questionnaire.

Sample size calculation and justification

Sample size was calculated for the 2 primary outcomes: the proportion of pregnant women who will reach a 25(OH)D ≥ 50 nmol/l at delivery, and the infant bone mineral content (BMC) at one month of age; the largest number was considered the final sample size. Given that we have 2 primary outcomes, type I error was considered 2.5% [47]. Sample size calculation was done online [48].

Sample size calculation for the proportion of women who will reach a 25(OH)D ≥ 50 nmol/l at delivery:

Based on a retrospective lab study conducted at AUB-MC in 2014, the median 25(OH)D level in the Lebanese population was found to be 52 nmol/l (20.9 ng/ml). The low dose group will receive 10,000 IU vitamin D weekly, equivalent to 700 IU daily. The high dose group will receive 20,000 IU weekly vitamin D, equivalent to 2,850 IU daily. Considering that each 100 IU vitamin D supplementation increases the level by 1.7 nmol/l [49], the expected levels reached in the low dose and high dose arms would be 67 nmol/l and 106 nmol/l, respectively. This computation takes into consideration that all groups will be taking additional 200 IU vitamin D daily from their prenatal vitamin pills, thus increasing the final vitamin D intake, approximately, to 900 IU/ day in the low dose group and 3,050 IU/ day in the high dose group. The expected proportions of pregnant women who would reach a 25(OH)D level ≥ 50 nmol/l, using a standard deviation (SD) of 24.9 nmol/l, and assuming normality, would be 75 % and 98.4 %, in the low dose and high dose arms, respectively. To detect statistical significance between the 2 groups, for a 90% power and a type I error

of 2.5%, 50 participants per arm are needed.

Calculation was also done based on the results of a recently completed systematic review and meta-analysis of randomized controlled trials from the Middle East and North Africa (MENA), conducted by Chakhtoura et al, as part of a Master of Sciences in Health Research thesis project (available online from the Jafet Library at the American University of Beirut - Lebanon).
This meta-analysis showed that, in pregnant women from the MENA region, a vitamin D dose of 800-2,000 IU daily results in an increase in 25(OH)D level by 2.5 nmol/l, and a high dose of > 2,000 IU daily results in an increase in 25(OH)D level by 1.67 nmol/l. Starting from a baseline 25(OH)D level of 52 nmol/l, the 25(OH)D levels achieved would be 74.5 nmol/l and 103 nmol/l, in the low and high dose group, respectively. Accordingly, 83.6% and 98.3% would reach the target 25(OH)D level of 50 nmol/l, and 72 participants per arm are needed for an 80% power and a type I error of 2.5%. Noteworthy that the studies included in the aforementioned meta-analysis had a baseline 25(OH)D level of 20-27 nmol/l, lower than the expected levels in our participants.

Sample size calculation for infant BMC:

Estimations were based on the preliminary results of the MAVIDOS trial conducted by our collaborators at Southampton University, UK. They showed a significant difference of 6g (SD 10g) in neonatal mean BMC in the vitamin D supplemented group, compared to placebo, in winter season [36]. For a 90% power and a type I error 2.5%, considering an SD of BMC of 10g, to detect a 6 g difference in BMC between high dose and low dose groups, 69 participants per arm are needed. Taking into consideration that 25(OH)D levels in RHUH and Bahman hospital are lower compared to pregnant women presenting to AUB-MC and to pregnant women in UK, a significant improvement in BMC is expected throughout the year in the high dose group compared to low dose group.
The largest sample size of 69 participants per arm is our target. If we consider a 50% drop out rate, to be conservative, approximately 140 participants per arm should be recruited, for a total of 280 pregnant women for the whole study. If we consider that 50% of pregnant women presenting to clinics are eligible, approximately, a total of 560 pregnant women should be screened initially. If 30% of pregnant women accept to participate in clinical trials, approximately, 1,870 pregnant women should be approached initially.

Statistical analysis

Baseline demographic characteristics will be summarized using frequencies and percentages for categorical characteristics, and mean ±SD (or median and range) for continuous variables. Normality of all variables will be checked. Comparisons between dose groups will be performed using Chi-square tests for the categorical variables, and t-test for continuous variables, as appropriate.

Unadjusted analysis:

Two primary outcomes are considered:

a) The proportion of women who reach a 25(OH)D ≥ 50 nmol/l: binary outcome; The percent of women achieving 25(OH)D ≥ 50 nmol/l in the low dose will be compared to that in the high dose using Chi-Square, by constructing a 95% confidence interval for the difference and computing an unadjusted RR and its 95% confidence interval, along with the p-value. A number needed to treat (NNT) will also be computed.
b) The mean infant BMC at one month of age: continuous outcome; Independent t-test will be used to compare mean BMC between the 2 arms. 95% confidence interval for the difference will be calculated.

Secondary and exploratory outcomes:
For secondary and exploratory outcomes, t-test will be used for continuous outcomes and Chi-square will be used for binary outcomes to compare means and proportions, respectively. Non-parametric tests including Wilcoxin sign rank test and Fisher exact test will be used, respectively, instead of t-test and Chi-square, when needed.
Relative Risk (RR) with corresponding confidence intervals will be calculated for dichotomous variables, and difference in means with their 95% confidence intervals will be used for additional analysis of continuous variables.

The primary analysis is an intention-to-treat analysis (ITT) of unadjusted results. ITT being defined as analysis of all participants as randomized, regardless of whether they respected the study protocol or not (effectiveness). P-values will be reported to four decimal places.
For the primary outcomes, p- values will be considered statistically significant if ≤ 0.025.
SPSS version 23 will be used to conduct statistical analysis.

In case of missing data, analysis restricted to results of individuals

with complete data will be done (with retrospective power calculation) and compared to analysis resulting from multiple imputations to try to test the robustness of results [50].

Additional analysis

-Subgroup analysis:

As discussed earlier, the IOM targets a 25(OH)D level of ≥ 50 nmol/l [29] and the Endocrine Society targets a level of ≥ 75 nmol/l [30]. Subgroup analysis based on 25(OH)D level at study entry (<50 nmol/l vs ≥ 50 nmol/l) will be done to explore whether the treatment effects persist across all 25(OH)D categories, whether below or above 50 nmol/l.

Sub-group analysis based on the season will be also performed, to check for interaction between vitamin D dose and the season of pregnant women enrollment.

-Sensitivity analysis:

Sensitivity analysis will be performed, including Per Protocol analysis and as treated analysis. In addition, an adjusted analysis will be done, including adjustment for variables that are not evenly distributed between the 2 arms, if any, and adjustment for variables that are clinically important (even if no imbalance in the baseline characteristics of the 2 groups); this includes baseline 25(OH)D level, pre-pregnancy BMI, season at enrollment, and smoking status.

Ethical considerations

We will restrict enrollment to pregnant women whose 25(OH)D levels range between 25 and 75 nmol/l. This is because it will be unethical to include women with levels <25 nmol/l in the trial, as there is a risk to randomly allocate them to the low dose arm. In addition, women with 25(OH)D level > 75 nmol/l will be excluded in order to prevent reaching supra-normal levels of 25(OH)D should they be allocated to the high vitamin D dose. Noteworthy that high dose of vitamin D (up to 4,000 IU daily) have been used in previous trials conducted during pregnancy with no reported adverse events (Appendix 1).
The infant radiation exposure resulting from the study procedure, BMD testing by DXA, is minimal. The radiation dose is estimated at 0.007 mSv for whole body DXA. This dose is equivalent to 20 hours of exposure to background radiation, based on Duke Radiation Safety online assessment and statement [51].

Safety considerations

Information on adverse events will be regularly collected soon after starting the trial intervention, and during each trial visit. In between visits, all participants will be called by the research team every 2 weeks to emphasize compliance with treatment regimens, and to inquire about adverse events. All information will be documented in case report forms, and discussed with the Trial Monitoring Committee (TMC) (see Appendix 3 for further details). The TMC will report any serious adverse event to IRB and Data Safety and Monitoring Board (DSMB) within 48 hours.

Dissemination

Trial results will be communicated to participants, to the public, to health care professionals at AUB-MC and in Lebanon. Results will be presented in scientific meetings conferences, and published in peer-reviewed medical journals, whether the results are in the expected direction or not.

DISCUSSION

Hypovitaminosis D is a well-recognized common public health problem in Lebanon and in most countries of the Middle East. Many observational studies suggest that maternal hypovitaminosis D is associated with adverse maternal and neonatal outcomes. Vitamin D RCTs in pregnancy are scarce, with small sample sizes, and their primary outcomes are mostly limited to measuring 25(OH)D levels in mothers and neonates. Furthermore, given the lack of evidence-based guidelines that define the optimal RDA for vitamin D supplementation during pregnancy in our population, and the limited number of randomized clinical trials completed so far in our region, this trial will fill an important knowledge gap. We will conduct this RCT to test the impact of 2 different doses of vitamin D replacement on clinically relevant maternal and neonatal outcomes in Middle Eastern women. The Lebanese and other Middle Eastern women are ideally suited for such trial, in view of the fact that the median 25(OH)D levels in this age group is relatively low, a level that is reflective of the median low levels registered in most countries from the Middle East, as well as those from Northern Africa [14]. The doses used will allow us to directly address the applicability of the IOM in our region. The findings of this trial will help guide public health policy maker regarding vitamin D supplementation in pregnant women and will allow a step

forward in evidence based recommendations, specific to the Middle East. Multiple outcomes that have never been targeted in any previous trial in pregnancy will be assessed as secondary or exploratory outcomes; indeed, the results will guide future research projects in this field.

Findings from our trial, and similar to results derived from nutrient RCTs, are prone to the confounding effect of several factors [52]. Indeed, the baseline 25(OH)D level, the dietary intake of vitamin D and other nutrients, such as calcium and proteins, sun exposure and others remain important predictors affecting the response to vitamin D supplementation, but very difficult to quantify accurately.

Trial status

The study was launched in July 27, 2015.

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Contributors

GEHF conceived the idea of the trial. MC, GEHF, AN designed and developed the study protocol. ZM provided advice on sample size, study design and randomization. CC and NH provided advice on trial design, endpoints, conduct and operating procedures. AA and MN provided advice on the logistic planning of the study. All authors made significant contributions to the protocol development. They all reviewed the draft versions and approved the final version of this manuscript.

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Competing interest

Nothing to declare

Ethics approval

This protocol has been approved by the Institutional review Board (IRB), at AUB-MC, RHUH and by the medical committee at Bahman Hospital (Protocol number IM.GEHF 22).

Provenance and peer review

Not commissioned; externally peer reviewed.

Abbreviations

25(OH)D: 25-Hydroxyvitamin D; DSMB: Data safety and monitoring board; GDM: gestational diabetes mellitus; IOM: Institute Of Medicine; IRB: Institutional Review Board; IU: International Unit; PTH: parathyroid hormone; RDA: Recommended Dietary Allowance; SD: Standard Deviation; TSC: Trial Steering

Committee; TSH: thyroid stimulating hormone; QUS: quantitative ultrasound; UK: United Kingdom

References:

1. Kovacs CS, El Hajj Fuleihan G. Calcium and bone disorders during pregnancy and lactation. *Endocrinol Metab Clin North Am* 2006;35(1):21-51.
2. Kovacs CS. Calcium and bone metabolism disorders during pregnancy and lactation. *Endocrinol Metab Clin North Am* 2011;40(4):795-826.
3. Parkes I, Schenker JG, Shufaro Y. Parathyroid and calcium metabolism disorders during pregnancy. *Gynecol Endocrinol* 2013;29(6):515-19.
4. Bouillon R, Van Assche FA, Van Baelen H, et al. Influence of the vitamin D-binding protein on the serum concentration of 1, 25-dihydroxyvitamin D3: significance of the free 1, 25-dihydroxyvitamin D3 concentration. *J Clin Invest* 1981;67(3):589.
5. Van Hoof H, De Sévaux R, Van Baelen H, et al. Relationship between free and total 1, 25-dihydroxyvitamin D in conditions of modified binding. *Eur J Endocrinol* 2001;144(4):391-96.
6. Zerwekh JE. Blood biomarkers of vitamin D status. *Am J Clin Nutr* 2008;87(4):1087S-91S.
7. Kovacs CS. Maternal vitamin D deficiency: fetal and neonatal implications. *Semin Fetal Neonatal Med* 2013;pii: S1744-165X(13)00006-1
8. Arabi A, El Rassi R, El Hajj Fuleihan G. Hypovitaminosis D in developing countries–prevalence, risk factors and outcomes. *Nat Rev Endocrinol* 2010; 6(10): 550-561.
9. Nassar N, Halligan GH, Roberts CL, et al. Systematic review of first-trimester vitamin D normative levels and outcomes of pregnancy. *Am J Obstet Gynecol* 2011;205(3):208.e1-08. e7.
10. Sahu M, Bhatia V, Aggarwal A, et al. Vitamin D deficiency in rural girls and pregnant women despite abundant sunshine in northern India. *Clin Endocrinol* 2009;70(5):680-84.
11. Molla AM, Al Badawi M, Hammoud MS, et al. Vitamin D status of mothers and their neonates in Kuwait. *Pediatr Int* 2005;47(6):649-52.
12. Hossain N, Khanani R, Hussain-Kanani F,et al. High prevalence of vitamin D deficiency in Pakistani mothers and their newborns. *Int J Gynaecol Obstet* 2011;112(3):229-33.
13. Ergür AT, Berberoğlu M, Atasay B, et al. Vitamin D deficiency in Turkish mothers and their neonates and in women of reproductive age. *J Clin Res Pediatr Endocrinol* 2009;1(6):266.
14. Bassil D, Rahme M, Hoteit M, et al. Hypovitaminosis D in the Middle East and North Africa: Prevalence, risk factors and impact on outcomes. *Dermatoendocrinol* 2013;5(2):274.

15. Lips P. Worldwide status of vitamin D nutrition. *J Steroid Biochem Mol Biol* 2010;121(1):297-300.
16. Van der Meer I, Middelkoop B, Boeke A, et al. Prevalence of vitamin D deficiency among Turkish, Moroccan, Indian and sub-Sahara African populations in Europe and their countries of origin: an overview. *Osteoporos Int* 2011;22(4):1009-21.
17. Van Der Meer IM, Karamali NS, Boeke AJP, et al. High prevalence of vitamin D deficiency in pregnant non-Western women in The Hague, Netherlands. *Am J Clin Nutr* 2006;84(2):350-53.
18. Hollis BW, Wagner CL. Vitamin D and pregnancy: skeletal effects, nonskeletal effects, and birth outcomes. *Calcif Tissue Int* 2013;92(2):128-39.
19. Aghajafari F, Nagulesapillai T, Ronksley PE, et al. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. *Br Med J* 2013;346.
20. Poel Y, Hummel P, Lips P, et al. Vitamin D and gestational diabetes: a systematic review and meta-analysis. *Eur J Intern Med* 2012;23(5):465-69.
21. Wei S-Q, Qi H-P, Luo Z-C, et al. Maternal vitamin D status and adverse pregnancy outcomes: a systematic review and meta-analysis. *J Matern Fetal Neonatal Med* 2013;26(9):889-99.
22. Tabesh M, Salehi-Abargouei A, Tabesh M, et al. Maternal vitamin D status and risk of pre-eclampsia: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2013;98(8):3165-73.
23. Thorne-Lyman A, Fawzi WW. Vitamin D during pregnancy and maternal, neonatal and infant health outcomes: a systematic review and meta-analysis. *Paediatr Perinat Epidemiol* 2012;26 Suppl 1:75-90.
24. Javaid M, Crozier S, Harvey N, et al. Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study. *The Lancet* 2006;367(9504):36-43.
25. Harvey NC, Moon RJ, Sayer AA, et al. Maternal Antenatal Vitamin D Status and Offspring Muscle Development: Findings From the Southampton Women's Survey. *J Clin Endocrinol Metab* 2013;99(1):330-37.
26. Martin R, Harvey NC, Crozier SR, et al. Placental calcium transporter (PMCA3) gene expression predicts intrauterine bone mineral accrual. *Bone* 2007;40(5):1203-8.
27. Cleal J, Day P, Simner C, et al. Placental amino acid transport may be regulated by maternal vitamin D and vitamin D-binding protein: results from the Southampton Women's Survey. *Br J Nutr* 2015:1-8.
28. Javaid MK, Lekamwasam S, Clark J, t al. Infant growth influences proximal femoral geometry in adulthood. *J Bone Miner Res* 2006;21(4):508-12.
29. Ross AC. The 2011 report on dietary reference intakes for calcium and vitamin D. *Public Health Nutr* 2011;14(05):938-39.
30. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011;96(7):1911-30.

31. Practice ACOG. ACOG Committee Opinion No. 495: Vitamin D: Screening and supplementation during pregnancy. *Obstet Gynecol* 2011;118(1):197.
32. WHO Guideline: Vitamin D supplementation in pregnant women 2012. Available from http://apps.who.int/iris/bitstream/10665/85313/1/9789241504935_eng.pdf?ua=1(accessed in November 2015).
33. De-Regil LM, Palacios C, Ansary A, et al. Vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev* 2012;2.CD008873.
34. NICE Guidelines 2008. Avialable from http://www.nice.org.uk/guidance/PH11(accessed in November 2015).
35. Hollis BW, Johnson D, Hulsey TC, et al. Vitamin D supplementation during pregnancy: Double*blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res* 2011;26(10):2341-57.
36. Cooper C, Harvey N, Bishop N et al. MAVIDOS Study Group. Maternal Gestational Vitamin D Supplementation and Offspring Bone Mass: A Multicentre Randomised, Double-Blind, Placebo-Controlled Trial (MAVIDOS). *Abstract presented at the American Society for Bone and Mineral Research (ASBMR) Annual Meeting; Oct. 9-12, 2015; Seattle* 2015.
37. Sablok A, Batra A, Thariani K, et al. Supplementation of vitamin D in pregnancy and its correlation with feto-maternal outcome. *Clin Endocrinol (Oxf)* 2015 Oct;83(4):536-41.
38. Dawodu A, Saadi HF, Bekdache G, et al. Randomized controlled trial (RCT) of vitamin D supplementation in pregnancy in a population with endemic vitamin d deficiency. *J Clin Endocrinol Metab* 2013;98(6):2337-46.
39. Sabet Z, Ghazi A, Tohidi M, et al. Vitamin D supplementation in pregnant Iranian women: effects on maternal and neonatal vitamin D and parathyroid hormone status. *Acta Endocrinol* 2012;8(1):59-66.
40. Soheilykhah S, Mojibian M, Moghadam MJ, et al. The effect of different doses of vitamin D supplementation on insulin resistance during pregnancy. *Gynecol Endocrinol* 2013;29(4):396-99.
41. Shakiba M, Iranmanesh MR. Vitamin D requirement in pregnancy to prevent deficiency in neonates: a randomised trial. *Singapore Med J* 2013;54(5):285-88.
42. ClnicalTrials.Gov. Available from: https://clinicaltrials.gov/(accessed in November 2015).
43. College of American Pathologists. Available from: www.cap.org (accessed in November 2015).
44. DEQAS. Available from: www.deqas.org (accessed in November 2015).
45. World Bank definition of Middle East countries. Available from http://data.worldbank.org/about/country-and-lending-groups (accessed in November 2015).
46. Skinner A, Cieslak Z, MacWilliam L, et al. The measurement of knee *heel length in newborn infants using a simple vernier calipers. *Acta Paediatr* 1997;86(5):512-17.
47. Bender R, Lange S. Adjusting for multiple testing–when and how? *J*

Clin Epidemiol 2001;54(4):343-49.
48. Online sample size calculation. Avaialble from: http://www.stat.ubc.ca/~rollin/stats/ssize/ (accessed in November 2015).
49. Heaney RP, Davies KM, Chen TC, et al. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am J Clin Nutr* 2003;77(1):204-10.
50. Little RJ, D'Agostino R, Cohen ML, et al. The prevention and treatment of missing data in clinical trials. *N Engl J Med* 2012;367(14):1355-60.
51. Duke Radition Safety. Available from: http://www.safety.duke.edu/Radsafety/consents/default.asp. (accessed in November 2015).
52. Heaney RP. Guidelines for optimizing design and analysis of clinical studies of nutrient effects. *Nutr Rev*. 2014;72(1):48-54