

Protocol

This trial protocol has been provided by the authors to give readers additional information about their work.

Protocol for: Roth DE, Morris SK, Zlotkin S, et al. Vitamin D supplementation in pregnancy and lactation and infant growth. *N Engl J Med* 2018;379:535-46. DOI: 10.1056/NEJMoa1800927

This supplement contains the following items:

1. Original protocol^a, final protocol, summary of changes
2. Original statistical analysis plan, final statistical analysis plan^b, summary of changes

^a 'Original protocol' was the version approved and current at the time that participant enrolment was launched in March 2014.

^b 'Final statistical analysis plan' includes definitions and analyses that were established prior to unmasking of treatment groups in May 2017. Unplanned/post-hoc analyses are described in the manuscript and supplemental methods.

**Randomized placebo-controlled trial of maternal vitamin D
supplementation during pregnancy and lactation to improve
infant linear growth in Dhaka, Bangladesh**

“Maternal Vitamin D for Infant Growth (MDIG) trial”

Table of Contents

Study Summary	4
1) Title	5
2) Trial registration	5
3) Protocol version	5
4) Funding	5
5) Roles and Responsibilities	5
a) Names, affiliations, and roles of protocol contributors	5
b) Name and contact information for the trial sponsor	6
c) Role of study sponsor and funders	6
d) Composition, roles, and responsibilities of governance/monitoring bodies	7
6) Introduction	7
a) Background and rationale	7
b) Explanation for choice of active agents and comparators	12
7) Objectives	15
a) Primary aims:	15
b) Secondary aims:	15
8) Trial design	15
9) Study setting	16
10) Participant eligibility criteria	17
a) Inclusion criteria:	17
b) Exclusion criteria:	17
11) Interventions	18
a) Intervention description	18
b) Criteria for discontinuing or modifying allocated interventions	19
c) Strategies to improve adherence to intervention protocols, and procedures for monitoring adherence	20
d) Relevant concomitant care and interventions that are permitted or prohibited during the trial	21
12) Outcomes	23
13) Participant timeline	26
14) Sample size	28
15) Recruitment	29
b) Provisional screening	30
c) Triage and tracking of provisionally eligible women	30
d) Detailed screening (of provisionally eligible women)	31
16) Allocation of intervention	32
a) Sequence generation	32
b) Allocation concealment mechanism	32
c) Implementation of allocation	32

17) Blinding (masking).....	32
a) Who will be blinded, and how.....	32
b) Conditions and procedures for unblinding	32
18) Data collection.....	33
a) Sequence and content of study visits	33
b) Verbal and clinical data collection methods (including point-of-care tests).....	36
c) Biological specimen collection and processing	39
d) Biomarker analyses	42
e) Epigenetic analyses	46
f) Data quality control.....	46
g) Safety monitoring.....	47
h) Methods to promote participant retention and complete follow-up	49
i) Definition and handling of protocol deviations and violations	49
19) Data management	50
20) Data analysis	51
a) Statistical methods (Aims #1, #2 and #3).....	51
b) Methods for subgroup or adjusted analyses	53
c) Definition of analysis population relating to protocol non-adherence	54
d) Analysis of the effect of vitamin D supplementation on infectious disease morbidity (Aim #4).....	54
e) Biomarker and epigenetic data analyses (Aims #5 and #6).....	54
21) Monitoring.....	54
a) Data monitoring.....	54
b) Interim analyses and stopping rules	55
22) Harms.....	55
23) Auditing	57
24) Research ethics approval	58
25) Protocol amendments.....	58
26) Consent or assent.....	58
27) Confidentiality	59
28) Declaration of interests.....	59
29) Access to data.....	59
30) Ancillary and post-trial care	59
31) Dissemination plans	59
a) Dissemination policy.....	59
b) Authorship eligibility guidelines and any intended use of professional writers	59
c) Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code.....	60
32) Informed consent materials.....	66
33) Biological specimens.....	67

Study Summary

Title	Randomized placebo-controlled trial of maternal vitamin D supplementation during pregnancy and lactation to improve infant linear growth in Dhaka, Bangladesh		
Short Title	Maternal vitamin D for infant growth (MDIG) trial		
Clinicaltrials.gov	NCT01924013		
Design	Randomized placebo-controlled dose-ranging trial		
Study Duration	4 years (2013 – 2017)		
Study site	Dhaka, Bangladesh		
Primary Objective	To determine whether maternal prenatal vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.		
Study Intervention		Group	Prenatal vitamin D3 supplement starting at 17-24 weeks gestation
		A	0 IU (placebo)
		B	4,200 IU/week
		C	16,800 IU/week
		D	28,000 IU/week
		E	28,000 IU/week
			Postpartum vitamin D3 for during period of lactation (to 6 months)
			0 IU (placebo)
			0 IU (placebo)
			0 IU (placebo)
			0 IU (placebo)
			28,000 IU/week
Primary Outcome	Infant length-for-age z-score (LAZ) at one year of age, based on WHO standards		
# of Subjects	1300		
Main Inclusion Criteria	<ul style="list-style-type: none"> • Age 18 years and above; • 17 to 24 completed weeks of gestation based on recalled last menstrual period (LMP) and/or 2nd trimester ultrasound; • Intends to permanently reside in the trial catchment area for at least 18 months 		
Main Exclusion Criteria	<ul style="list-style-type: none"> • History of medical conditions that may predispose the participant to vitamin D sensitivity, altered vitamin D metabolism and/or hypercalcemia, or history of renal calculi • Current high-risk pregnancy based on severe anemia, proteinuria, or hypertension • Multiple gestation, major congenital anomaly, or severe oligohydramnios based on maternal history and/or ultrasound • Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin with calcium and/or vitamin D. • Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency • Previous participation in the same study 		
Follow-up period	Prenatal: Enrolment (17-24 weeks gestation) until delivery Postnatal: Birth to 24 months of age		
Main Study Procedures	<ol style="list-style-type: none"> 1. Questionnaires 2. Anthropometry 3. Obstetric ultrasound 4. Specimen collection (Blood, urine, placenta, breast milk) 5. Morbidity surveillance 		

1) Title

Randomized placebo-controlled trial of maternal vitamin D supplementation during pregnancy and lactation to improve infant linear growth in Dhaka, Bangladesh.

Short title and acronym: **Maternal Vitamin D for Infant Growth (MDIG)** trial

Gates Foundation Project Name and #: Parathyroid-vitamin D axis dysregulation in early-onset infant stunting in resource-poor settings (OPP1066764)

2) Trial registration

Clinicaltrials.gov registration: NCT01924013

3) Protocol version

Version 1.0 – May 22nd, 2013

Version 1.1 – July 8th, 2013

Version 2 – September 20, 2013

4) Funding

Bill and Melinda Gates Foundation – Healthy Growth program

Program officer: Sindura Ganapathi

5) Roles and Responsibilities

a) Names, affiliations, and roles of protocol contributors

Role	Name	Institution
Principal Investigator	Daniel Roth	Hospital for Sick Children
Co-Investigator	Stanley Zlotkin	Hospital for Sick Children
Co-Investigator	Shaun Morris	Hospital for Sick Children
Co-Investigator	Rosanna Weksburg	Hospital for Sick Children
Co-Investigator / Statistician	Andy Willan	Hospital for Sick Children
Co-Investigator	Kellie Murphy	Mount Sinai Hospital
Co-Investigator	Prakesh Shah	Mount Sinai Hospital
Co-Investigator (Site PI)	Abdullah Al Mahmud	icddr,b
Co-Investigator	Tahmeed Ahmed	icddr,b
Co-Investigator	Munir Islam	icddr,b
Co-investigator	Alison Gernand	Pennsylvania State University
Co-investigator / consultant	Rashed Shah	Johns Hopkins University
Project manager	Brendon Pezzack	Hospital for Sick Children
Project manager	Elaine Gergolas	Hospital for Sick Children

b) Name and contact information for the trial sponsor

Principal Investigator: Daniel Roth

Address: 555 University Ave Toronto, ON M5G 1X8

Email: daniel.roth@sickkids.ca

Phone: 416-813-5795

Fax: 416-813-5663

Assistant (if applicable): Cindy Groff

Assistant email: cindy.groff@sickkids.ca

Project Manager: Brendon Pezzack

Address: 555 University Ave Toronto, ON M5G 1X8

Email: brendon.pezzack@sickkids.ca

Phone: 416-813-5793

Fax: 416-813-5663

Budget/Finance Contact: Florin Agarne

Address: 555 University Ave Toronto, ON M5G 1X8

Email: florin.argane@sickkids.ca

Phone: 416-813-8967

Fax: 416-813-5085

Bangladesh Site Principal Investigator: Abdullah Al MahmudAddress: Centre for Child and Adolescent Health, icddr,b
68, Shaheed Tajuddin Ahmed Sarani, Mohakhali
Dhaka 1212, Bangladesh

Email: mahmud@icddr.org

Phone: +880-2-9827001-10 Extension: 3807 OR 9827043

c) Role of study sponsor and funders

Study Sponsor/PI:

- overall responsibility for study design;
- collection, management, analysis, and interpretation of data;
- writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities

Study funder:

- Advisory role in study design.
- No role in collection, management, analysis, and interpretation of data;
- No role in writing of the report; and the decision to submit the report for publication,
- No authority over any of these activities

d) Composition, roles, and responsibilities of governance/monitoring bodies

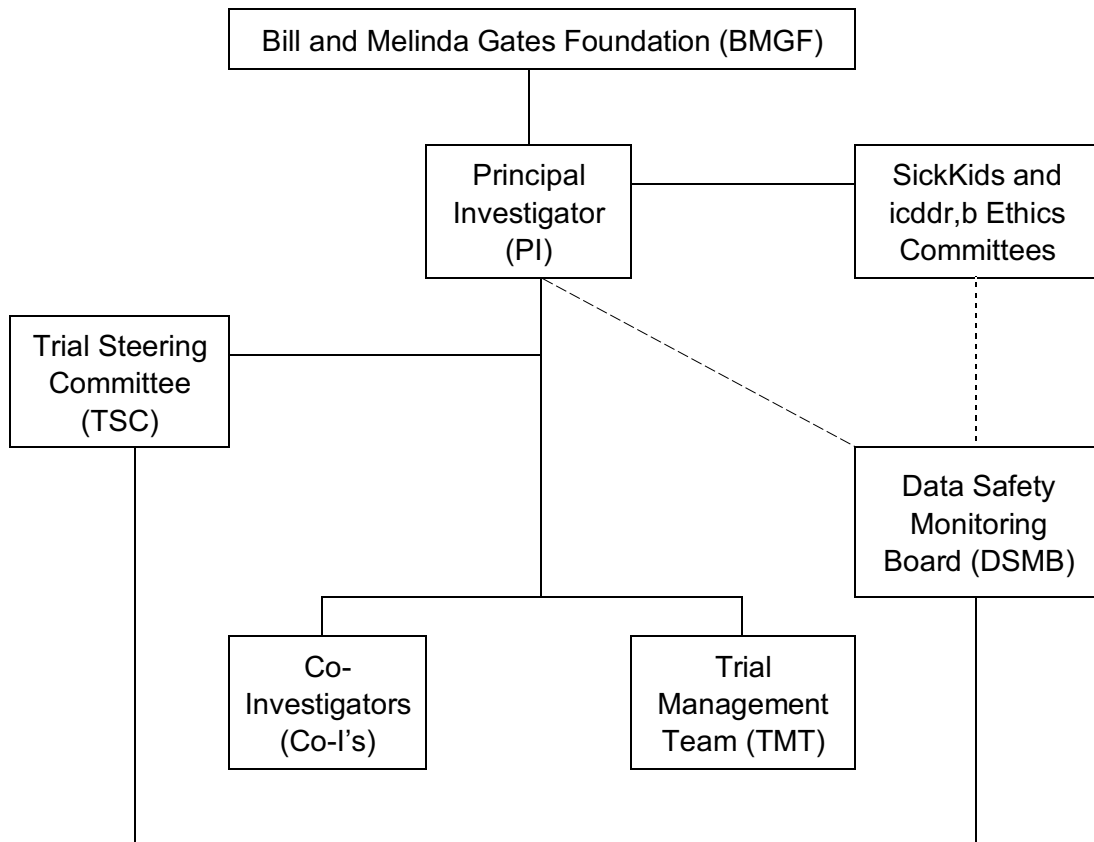


Figure 1: See Appendix for details of mandates and composition.

6) Introduction

a) Background and rationale

Worldwide, nearly 8 million children under the age of 5 die each year, predominantly in low-income countries in South Asia and sub-Saharan Africa¹. Although preventable child deaths are a persistent global health challenge, many countries in East and South Asia, including Bangladesh, have witnessed remarkable reductions in under-5 mortality rates over the past several decades; in fact, Bangladesh is on track to meet the United Nations Millennium Development Goal #4 (MDG-4) – a two-thirds reduction in child mortality between 1990 and 2015¹. Despite progress towards achieving the MDG-4 goal, fetal, infant and child rates of undernutrition in South Asia have been slower to decline^{2, 3}, suggesting that current child health programs are not adequate to influence the high rates of stunting (i.e., sex- and age-adjusted height or length less than 2 standard deviations below the median, as established by the World Health Organization growth standards).

Fetal and early childhood growth – particularly during the “first 1000 days” of pre- and postnatal development – has a profound effect on infectious disease susceptibility, mortality, and long-term functional and social outcomes⁴. In Bangladesh, it has been estimated that about one-third of term infants are low birth weight (LBW; defined as weight less than 2500 grams)⁵, a surrogate marker of intrauterine growth restriction (IUGR). Birth size strongly predicts postnatal growth, and growth faltering in low-income settings begins early (i.e., within the first 3 months of life⁶). In Bangladesh, stunting was estimated to affect nearly one-half of children younger than 5 years of age in 2005⁵. And, recent data suggest that the prevalence has remained virtually unchanged – 43% according to the Bangladesh demographic health survey (BDHS) 2007 and 41% according to BDHS 2011. The early onset of linear growth faltering (stunting) in low-income countries⁶, in spite of breast-feeding, strongly suggests that epigenetic prenatal events, nutritional and endocrine factors are the predominant causes of suboptimal growth in the first months of life. However, the causal pathways implicated in early childhood stunting in low-income settings remain poorly understood, limiting the ability of the public health community to design targeted interventions.

Vitamin D, parathyroid hormone (PTH), and parathyroid hormone-related peptide (PTHrP) are well-established endocrine modulators of bone mineral metabolism and skeletal development. However, there is surprisingly scant research addressing the role of the parathyroid-vitamin D axis in healthy infant growth. This trial will test our hypothesis that dysregulation of the parathyroid-vitamin D axis in the antenatal and early

postnatal period is an important and modifiable cause of linear growth faltering in resource-poor settings (**Figure 2**). ‘Dysregulation’ of the axis refers to: (1) a state of suboptimal vitamin D status (biochemical vitamin D deficiency, indicated by low circulating concentrations of 25-hydroxyvitamin D), (2) compensatory parathyroid gland hyperactivity marked by up-regulated PTH secretion, and possibly, (3) impaired PTHrP activity. In addition to the postulated direct effects of circulating maternal vitamin D metabolites on placental function⁷, maternal prenatal vitamin D status determines fetal/newborn vitamin D stores⁸ and maternal postpartum vitamin D intake determines breastfeeding infants’ vitamin D status⁹. Therefore, prenatal and postpartum maternal vitamin D supplementation is a feasible approach to influence vitamin D-dependent growth mechanisms in utero and during lactation.

There is a high prevalence of biochemical vitamin D deficiency among women and young infants in South Asia¹⁰. In Dhaka, we observed that 34% of pregnant women enrolled at 26 to 29 weeks

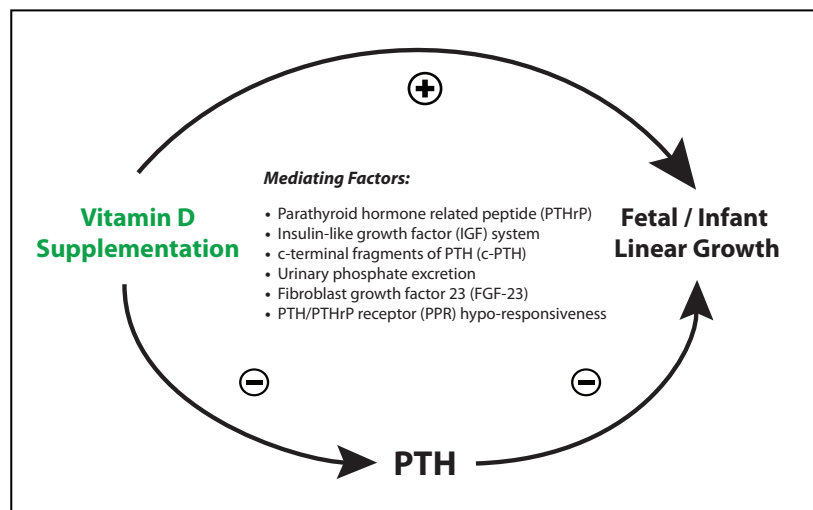


Figure 2: Conceptual framework showing the primary hypothesized relationships among vitamin D supplementation, parathyroid activity and fetal/infant growth, as well as factors postulated to mediate the effects of vitamin D and PTH on growth.

gestation (N=160) had serum 25-hydroxyvitamin D (25(OH)D) concentrations less than 30 nmol/L (a conventional definition of severe vitamin D deficiency), while 64% had 25(OH)D<50 nmol/L, the threshold for sufficiency recommended by the US Institute of Medicine (IOM). In the same study, 31% of newborns born to unsupplemented mothers had cord blood 25(OH)D<30 nmol/L and 81% had 25(OH)D<50 nmol/L¹¹. Nearly all women and newborns were deficient when a threshold of 25(OH)D<80 nmol/L was applied, which is a cut-off level proposed by some vitamin D researchers. In rural Sylhet, Bangladesh our research revealed that infants aged 1 to 6 months of age had a mean serum 25(OH)D concentration of 37 nmol/L (95% confidence interval, 30 to 43), and that the proportion of infants with 25(OH)D <25 nmol/L was 28% (95% confidence interval, 10 to 45)¹⁰. These findings suggest that vitamin D deficiency is not limited to urban areas.

Vitamin D and PTH are dominant endocrine regulators of bone mineral homeostasis through their direct and indirect actions on the kidney, intestine and bone¹². PTH is primarily secreted in response to a decrease in the serum calcium concentration, and acts to maintain the serum calcium concentration within a narrow physiological range by mobilizing calcium from bone, increasing intestinal calcium absorption, and decreasing renal calcium excretion. PTH and vitamin D are closely linked together through complex feedback loops. In particular, PTH regulates the renal conversion of the predominant circulating form of vitamin D (25-hydroxyvitamin D; 25(OH)D) to the active metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D), which is the primary hormonal mediator of intestinal calcium absorption. Yet, vitamin D also regulates PTH secretion, such that declines in serum 25(OH)D (the biomarker of vitamin D status) are associated with up-regulation of the parathyroid gland's release of PTH. Animal data support the hypothesis that maternal antenatal vitamin D status has important effects on fetal growth. Offspring of vitamin D-deficient guinea pigs have comparatively low birth weights, shorter lengths, reduced bone mineral content, and abnormal skeletal development at birth^{13, 14}. Offspring born to mice that lacked the gene for the vitamin D receptor (VDR-null mice) had low birth weights¹⁵ and impaired skeletal mineralization, primarily due to impaired maternal intestinal calcium absorption¹⁶. However, when the mouse fetuses were themselves VDR-knockouts, they were able to maintain normal serum fetal calcium concentrations, normal PTH concentrations, and normal growth in VDR-expressing mothers¹⁷. These findings suggest that adequate availability of the vitamin D metabolites in the maternal circulation may be more important for fetal growth than the direct effects of fetal vitamin D status.

The hypothesis that vitamin D deficiency and parathyroid hyperactivity adversely affect postnatal linear growth in humans has been most strongly suggested by data from studies of children with nutritional rickets; the classic metabolic bone disease of childhood caused by deficits of calcium and/or vitamin D. In rickets, secondary hyperparathyroidism has catabolic effects on the growing skeleton, leading to the bone demineralization that typifies the disease. However, stunting is also a common presenting feature among children with vitamin D-deficiency rickets^{18, 19}. Vitamin D treatment of rickets leads to an acceleration of linear growth, accompanied by a resolution of hyperparathyroidism^{18, 20, 21}.

Emerging data suggest that amelioration of infant vitamin D status may enhance linear growth even in the absence of clinically-overt vitamin D deficiency. A randomized vitamin D trial in India showed that postnatal infant vitamin D supplementation may reduce the risk of stunting among

at-risk infants²². In that trial, relatively low-dose postnatal infant vitamin D supplementation led to gradual increases in vitamin D status throughout early infancy. We speculate that earlier endowment with vitamin D stores (via prenatal maternal supplementation) may have resulted in a greater effect on stunting. However, the effects of maternal prenatal vitamin D supplementation on infant length have not been widely studied. A placebo-controlled trial of prenatal vitamin D supplementation conducted in the late 1970s in London, England showed minimal initial differences in birth length among infants born to supplemented versus unsupplemented mothers²³, but that the infants in the vitamin D group were significantly longer at one year of age²⁴. More recently, in a trial conducted in northern India, 299 women in the 2nd trimester of pregnancy were randomized to either a single 60,000 IU dose of vitamin D₃ or two 120,000 IU doses (whereby one dose was administered in each of the 2nd and 3rd trimesters). The trial also included an untreated control group of 43 women enrolled in the 3rd trimester who were already receiving standard care without vitamin D²⁵. The supplemented participants who were followed to delivery (n = 97; 32%) had infants that exhibited mean lengths at birth that were significantly higher than those in the untreated group²⁵. The largest prenatal vitamin D trial published to date was conducted in South Carolina, in which 479 women between 12 and 16 weeks gestation were randomized into treatment groups receiving 400 IU/day, 2000 IU/day or 4000 IU/day²⁶. Differences in mean birth weights were not found among the groups; however, birth lengths and postnatal outcomes were not reported.

We recently completed a randomized placebo-controlled double-blinded trial of maternal prenatal (3rd trimester) vitamin D supplementation in Dhaka, in which 160 women were randomized to receive either 35,000 IU/week (=5000 IU/day) or placebo until delivery. We observed that: maternal mean 25(OH)D was significantly higher at delivery after receiving vitamin D vs. placebo (134 vs. 39 nmol/L, P<0.001; N=133); and that cord 25(OH)D was significantly higher following vitamin D supplementation versus placebo (103 vs. 39 nmol/L; P<0.001; N=132). Vitamin D₃ at the studied dose did not cause maternal hypercalcemia or any other supplement-related serious adverse events; and, major adverse birth and neonatal outcomes were non-significantly less common in the vitamin D group, providing reassurances regarding short-term safety.

In preliminary analyses of the effect on infant length among 130 infants followed up to one year of age, we found that infants born to women in the vitamin D group had mean length-for-age z-scores (LAZ) that were significantly greater than infants in the placebo group throughout infancy (**Figure 3**). At 1 year of age, infants in the vitamin D group had LAZ that were, on average, 0.44 z-score units (95%CI 0.06, 0.82) higher than infants in the placebo group.

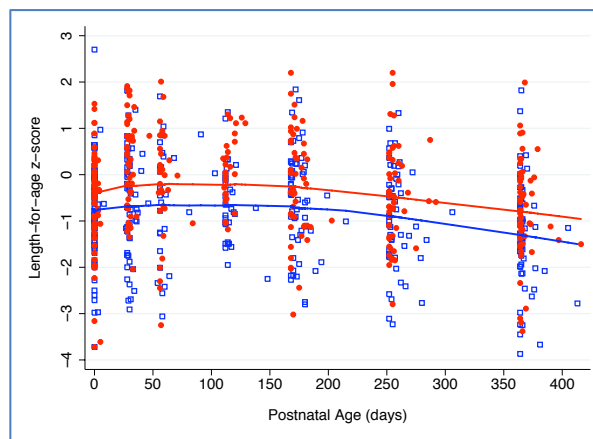


Figure 3: Length-for-age z-scores (LAZ) of infants born to mothers supplemented with vitamin D 35,000 IU/week in the third trimester (red filled circles; top fit line) versus placebo (blue hollow squares; bottom fit line). LAZ are calculated using WHO growth standards. Fit lines are LOWESS curves.

The specific mechanisms by which maternal vitamin D deficiency and parathyroid hyperactivity modulate fetal-infant linear growth are not well understood. PTH is critical for fetal and postnatal bone mineralization, yet its role in bone lengthening is unclear²⁷. In utero, PTH does not appear to directly influence transplacental calcium flux, a process more likely to be regulated by PTHrP²⁸. In the postnatal period, the direct effects of PTH on growth plate chondrocytes have not been convincingly demonstrated²⁹. In the context of the proposed trial, we will aim to study several nutritional, environmental and inflammatory mediating pathways by which the prevention of vitamin D deficiency and suppression of parathyroid hyperactivity may reduce the risk of early-onset stunting. These hypothesized pathways are summarized in Figure 4, and detailed further below (Section 18).

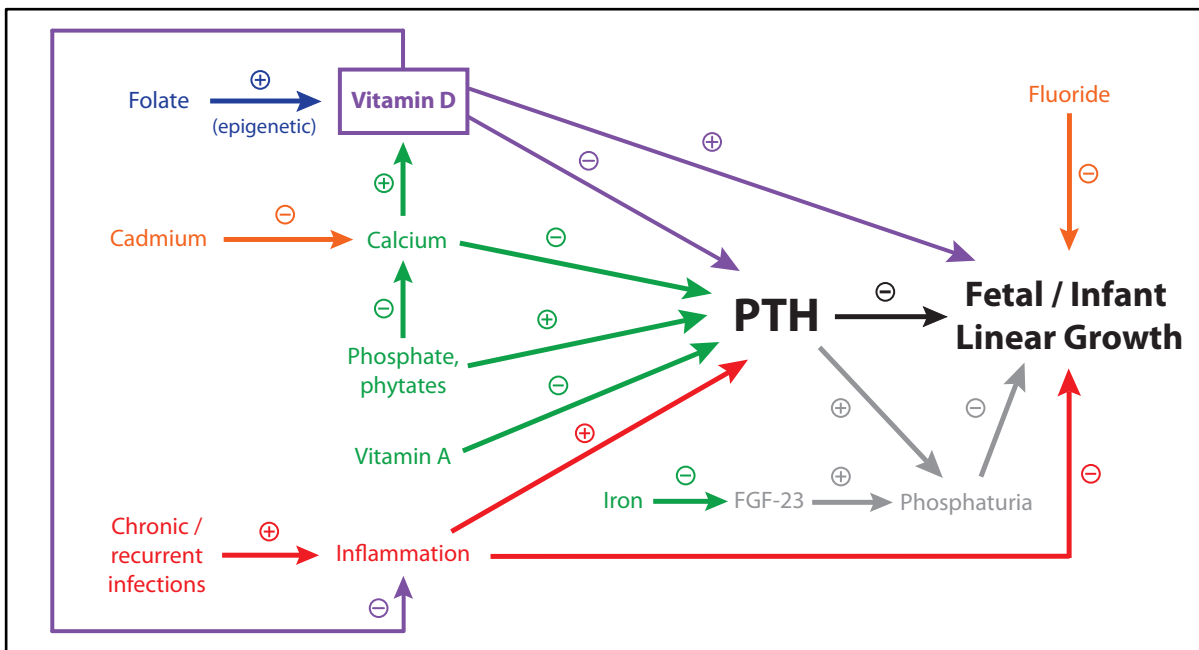


Figure 4. Hypothesized pathways by which nutrients, environmental contaminants, and infections interact with vitamin D and PTH effects on growth.

Epigenetic variation via methylation of genes involved in vitamin D metabolism may further explain inter-individual differences in the biochemical responses to vitamin D supplementation. We specifically hypothesize a pathway linking maternal folic acid intake, genes involved in perinatal vitamin D metabolism (CYP27B1 and CYP24A1), parathyroid activity, and birth size (Figure 5).

As a potent immunomodulator, vitamin D may mitigate episodic or chronic infection-related growth faltering. A range of studies have putatively linked vitamin D deficiency to an increased risk of infectious morbidity³⁰. In a case-control study in Sylhet, Bangladesh, we found that infants with acute lower respiratory infection had lower average 25(OH)D than community matched controls³¹, but the direction of causality is unclear (i.e., recurrent infections and general poor health may compromise vitamin D status, and/or vitamin D deficiency may raise the risk of infections). Therefore, by tracking episodes of illness and symptoms of infection (e.g., diarrhea) in pregnancy and early infancy, this trial will also enable us to investigate the effect of vitamin D supplementation on morbidity in infancy.

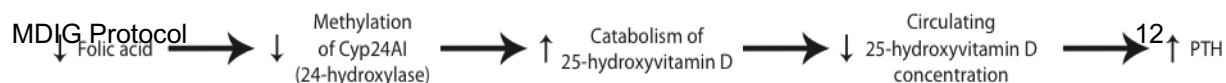


Figure 5. Hypothesized conceptual pathway linking folic acid intake, epigenetic regulation of vitamin D metabolic enzymes, and parathyroid hyperactivity in the prenatal period.

In summary, vitamin D deficiency and consequent parathyroid gland hyperactivity are postulated to increase the risk of fetal and early infant growth faltering in low-income settings, and in particular in South Asia. This trial of maternal pre- and postnatal vitamin D supplementation will directly test this hypothesis. In addition to this primary outcome, the trial will provide insight into other possible mediators and modifiers of the effect of vitamin D on growth through multiple sub-studies. These sub-studies will include 1) a pathway-wide assessment of biomarkers from the parathyroid-vitamin D axis and biomarkers associated with inflammation; 2) an analysis of epigenetic phenomena that affect vitamin D metabolism based on parental and cord blood as well as placental specimens (inclusion of the paternal blood specimen will enable the identification of allele-specific epigenetic changes); and 3) an analysis of the effect of supplementation on the incidence postnatal infant infectious disease.

b) Explanation for choice of active agents and comparators

Multiple vitamin D doses will be studied to enable the characterization of a dose-response effect on the primary outcome, while balancing considerations of sample size, safety and feasibility. Three prenatal vitamin D doses are proposed (4200 IU/week, 16,800 IU/week and 28,000 IU/week) based on published literature and our preliminary data. In addition, a postpartum phase will test the effect of continuing 4000 IU/d after delivery (**Figure 6**). The equivalent daily doses would be 600 IU/d, 2400 IU/d and 4000 IU/d respectively, based on known pharmacological principles and empiric data showing that weekly doses of 7X/week achieve similar 25(OH)D levels as doses of X IU per day³². For this reason, much of the cited literature below refers to daily doses.

Placebo: The current standard of antenatal care in Bangladesh and virtually all low-income countries does not include vitamin D supplementation. Moreover, a Cochrane Collaboration systematic review published in 2012 found a lack of evidence to support routine vitamin D supplementation during pregnancy³³. Our own data thus far do not suggest that there are discernible risks to individual mothers or infants in Dhaka who receive standard antenatal care excluding vitamin D supplementation (i.e., placebo). The hypothesized effect is a shift in the distribution of infant lengths rather than prevention or treatment of individual pathology. Even if shown to have significant effects on average linear growth, the vitamin D doses to be studied in this trial would require further investigation at a larger scale to justify their inclusion in a package of standard antenatal interventions. The inclusion of the placebo group in the proposed trial does not place participants at risk, will enable findings to have relevant policy implications, and will enable the study to test of effects of supplementation at the US/Canada RDA level. However, mothers or infants in the trial who are found to have clinical features of metabolic bone disease attributable to vitamin D deficiency during supplementation or follow-up will be promptly treated with vitamin D as required, outside of the study protocol.

Vitamin D 4,200 IU/week (to deliver 600 IU/d): This dose level reflects the North American recommended dietary allowance (RDA). Dietary guidelines released in November 2010 by the US Institute of Medicine (IOM) set the RDA for vitamin D for Canadian and American pregnant and lactating women at the same level as non-pregnant adults (15 mcg = 600 IU/day)³⁴. The RDA was set to promote bone health, and assumes inputs from a variety of sources in the setting of minimal sun exposure, rather than implying that 600 IU/day is a supplementation dose³⁵. The World Health Organization (WHO) does not recommend routine prenatal vitamin D supplementation, but existing WHO guidelines set a recommended daily intake (RDI) of vitamin D (from all sources) of 200 IU/day for most children and adults, including pregnant and lactating women. Typical maternal antenatal multiple micronutrient formulations that have been studied in low-income countries over the past 10-15 years have included 200 IU/day of vitamin D³⁶, a dose that would have only a small effect on serum 25(OH)D concentrations and thus would be unlikely lead to observable differences in growth outcomes compared to placebo.

Vitamin D 16,800 IU/week (to deliver 2400 IU/d): This dose level is expected to attain the proposed IOM threshold for sufficiency of serum 25(OH)D concentrations ≥ 50 nmol/L in most women in Dhaka. With respect to doses above the RDA, the tolerable upper intake level (UL) was increased by the IOM from the 1997 recommendations³⁷ to 4,000 IU/day in 2010³⁴, but there were few new data to support changes specific to pregnancy. Most antenatal vitamin D supplementation trials were conducted in the 1980s³⁸; in the past decade, only four additional pregnancy trials have been published^{25, 26, 39, 40}, only one of which (the Hollis trial⁴¹) included data from more than 100 participants. The Canadian Paediatric Society (CPS) has suggested consideration of maternal prenatal supplementation of 2,000 IU/day⁴². However, in 2011 the American College of Obstetricians and Gynecologists (ACOG) reiterated the lack of evidence to support routine high-dose prenatal vitamin D supplementation, but offered that “when vitamin D deficiency is identified during pregnancy, most experts agree that 1,000–2,000 IU per day of vitamin D is safe.”⁴³ The Endocrine Society (US) recently recommended that pregnant women at risk of vitamin D deficiency consume *at least* 1,400 IU/day, with an upper limit of 10,000 IU/day⁴⁴. Our pharmacokinetic findings among pregnant women in Dhaka have been consistent with published estimates of the vitamin D-25(OH)D dose-response relationship in non-pregnant adults (i.e., 0.7 nmol/L increase in 25(OH)D at steady-state for each 1 mcg/day of vitamin D^{345, 46}). To attain vitamin D sufficiency in the majority of women, according to the IOM standard of 50 nmol/L, we estimate that a dose of 2,000 to 2,500 IU/day will be required (by aiming for a group mean 25(OH)D of about 80 nmol/L). The 2,400 IU/day dose (administered as 16,800 IU/week) was selected as a multiple of the RDA of 600 IU/day to facilitate coherent dose-response comparisons between these doses. Although some authorities have recommended doses less than 2000 IU/day in Canada and the US (see above), such doses would be less likely to yield the 50 nmol/L steady-state in the majority of women in Dhaka (where baseline vitamin D status is lower than in typical women in North America).

Vitamin D 28,000 IU/week (to deliver 4000 IU/d): This dose is proposed to safely ensure suppression of PTH secretion in most participants. In a previous trial we observed that 35,000 IU/week (~5000 IU/day) potently suppressed maternal PTH production during pregnancy in Dhaka; however, earlier data suggested that 2,000 IU/day may have a weaker effect on PTH⁴⁷. From a mechanistic standpoint, it is important to study a dose that suppresses PTH; however,

based on the association between attained 25(OH)D and PTH at delivery in pregnant women in our preliminary trial, we expect that a dose of 4,000 IU/d (with an expected attained group mean 25(OH)D of ~110 nmol/L) will be sufficient for parathyroid suppression. The selection of 4000 IU/d balances desired physiological effects with safety considerations. Firstly, during our research, we found that 5000 IU/d (as 35,000 IU/week) did not provoke hypercalcemia and was not associated with any discernible adverse pregnancy outcomes. A reduction of that dose by 20% will provide a wide margin of safety for a larger study population that can be less intensely monitored for hypercalcemia. Secondly, 4000 IU/d will not exceed the IOM UL³⁴, a conservative margin of safe intake for the general population, even in the absence of clinical/biochemical monitoring.

Rationale for studying vitamin D 28,000 IU/week (~4000 IU/d) during lactation: Our preliminary findings in Dhaka revealed that infants born to women who received 3rd-trimester maternal vitamin D supplementation at 35,000 IU/week had higher LAZ during postnatal follow-up compared to infants born to women who received placebo. However, a critical unresolved issue is the mechanism through which prenatal supplementation impacts infant length. Prenatal supplementation may impact infant length through enhanced fetal skeletal growth, and/or latent effects on infant growth that result from the larger vitamin D stores provided by supplemented mothers during gestation. If the latter is a true phenomenon, as we speculate, then maintenance of the vitamin D steady-state during the period of lactation may accentuate the growth effects. There is a dose-response relationship linking maternal vitamin D status, breast milk vitamin D activity, and infant vitamin D status⁹. Vitamin D supplementation of lactating mothers with at least 2000 IU/day has been clearly linked to significant increases in the 25(OH)D of breast-fed infants⁴⁸. Maternal supplementation, rather than infant supplementation, is preferred in the context of this trial because of the possibility that the mechanism of effect of vitamin D on growth may involve the regulation of breast milk transfer of other endocrine factors (e.g., PTHrP), and may not only be related to the infant's 25(OH)D concentration. In addition, if shown to be beneficial, maternal supplementation may be more appealing in the public health context because it would support the optimality of exclusive breastfeeding, in that all nutrients would be delivered to the infant via breast milk without the need for infant nutrient supplementation. To maximize analytical efficiency (i.e., maintain sufficient numbers of participants in each intervention group) given resources/feasibility, we aim to test the postpartum effect only at the highest dose level (4000 IU/d). We will therefore assess the effect of maternal postpartum vitamin D3 supplementation at a dose of 28,000 IU/week (4000 IU/d) versus placebo on postnatal infant growth among infants born to women who received vitamin D 4000 IU/d during pregnancy.

Rationale for weekly doses instead of large single or infrequent intermittent 'bolus' doses: Experience with a large single dose (70,000 IU) in our initial pharmacokinetic studies in Dhaka revealed high inter-individual variability in 25(OH)D and the absence of a 25(OH)D steady-state⁴⁹, which were expected on the basis of published data⁵⁰. Large single doses may not be physiologically appropriate⁵¹, and have been associated with clinical adverse effects in adults⁵². Published pregnancy trials employing large single or intermittent vitamin D3 doses have not adequately reported pharmacokinetic and safety parameters^{25, 39}. Therefore, despite the potential practical advantages of infrequent dosing, it is currently more appropriate to replete

vitamin D stores using regular maintenance doses at short intervals (i.e., weekly or daily) that prevent large inter-dose fluctuations in 25(OH)D, and for which we have preliminary data.

7) Objectives

a) Primary aims:

1. To determine whether maternal prenatal oral vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week, administered as weekly doses) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh.
2. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.

b) Secondary aims:

3. Growth outcomes:

- i. To estimate the effect of prenatal +/- postpartum maternal vitamin D supplementation versus placebo on the prevalence of stunting at 1 year of age.
 - ii. To estimate the effects of maternal prenatal vitamin D supplementation on infant attained length at 2 years of age.
 - iii. To estimate the effect of maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo on infant attained length at 2 years of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.
4. To estimate the effect of maternal prenatal +/- postpartum vitamin D supplementation on the incidence of postnatal infant acute respiratory infections and acute diarrhea.
 5. To investigate the roles of specific hormones, nutrients, environmental contaminants, and inflammatory markers in the mediation or modification of the effect of vitamin D on infant stunting.
 6. To establish evidence that the vitamin D-parathyroid axis in pregnancy is influenced by epigenetic modification of genes involved in vitamin D metabolism.
 7. To estimate the effect of maternal prenatal vitamin D supplementation on the prevalence of low birth weight and small-for-gestational age.

8) Trial design

- Randomized
- Concealment of allocation and blinding throughout intervention and analysis
- Placebo-controlled
- Parallel-group
- Dose-ranging

- Superiority hypothesis testing framework
- 1:1 Allocation ratio across 5 groups

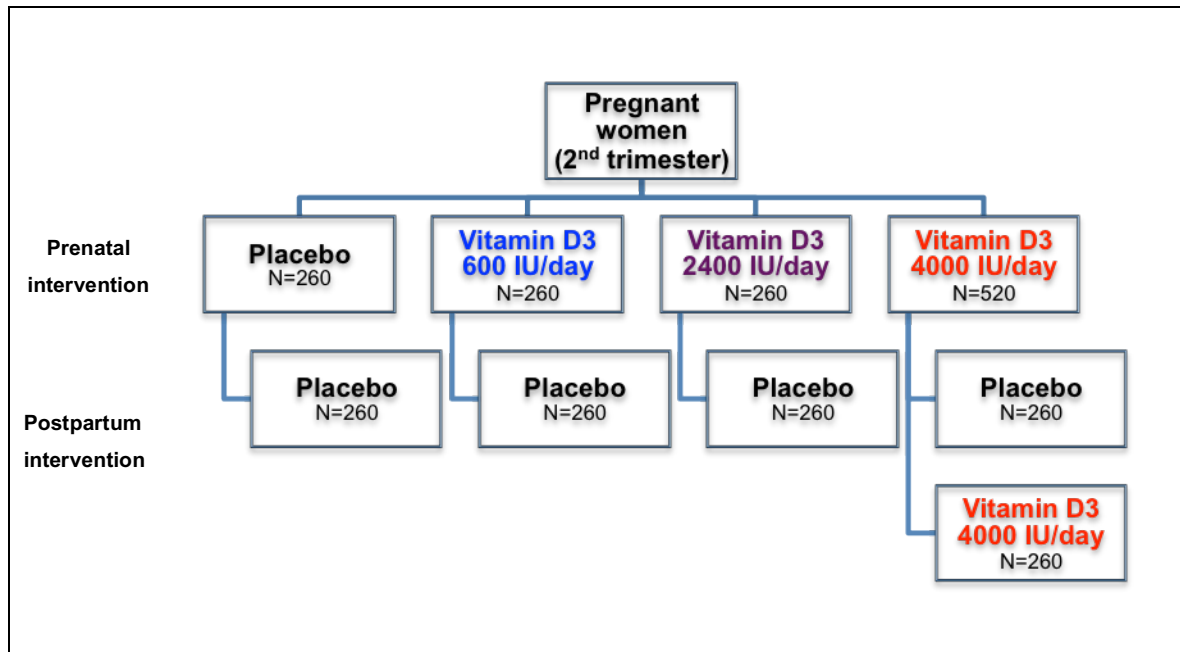


Figure 6: Trial Design

9) Study setting

The single-site trial will be conducted in **Dhaka, Bangladesh**. Enrolment and clinical activities will be based at the **Maternal and Child Health Training Institute (MCHTI), commonly known as Azimpur Maternity Center**, a government facility that provides low-cost health care to pregnant women and children in its referral area in central Dhaka, Bangladesh. MCHTI has outpatient clinics, an inpatient labour and delivery unit (152 beds), and inpatient paediatric services. There are approximately 30 women per day registered for antenatal care starting in the 2nd trimester. Low-income patients receive free care. Clinical staff includes obstetrician-gynecologists, pediatricians, anesthesiologists, medical house officers, nurses and paramedics. Basic laboratory and radiology services are available, including prenatal anatomical and dating ultrasound. Complicated patients (including newborns requiring respiratory support) are referred to nearby tertiary-care hospitals. The Dhaka wards/unions (char) near MCHTI that will be included in the trial catchment area include: Kamrangir char, Azimpur, Lalbag, and Hazaribag. We expect about three-quarters of the participants to be residents of Kamrangir char, a collection of urban slums on the Buriganga river, along the periphery of Dhaka city. The area is densely populated, with a total population of ~300,000, of which approximately 265,000 reside in slum settlements (National Institute of Population Research and Training, Dhaka, 2006). The literacy rate has been estimated at 29% (compared to the national average of 32%) and more than 30% of the residents have monthly incomes ≤ 5000 taka (~\$60 CAD). However, socioeconomic status varies greatly given the presence of some universities and government offices in the area. Income earners are mainly day labourers and many men work in local tanneries (the typical income of a tannery worker is about 6000 taka/month).

MCHTI (Azimpur maternity center) and its catchment area offer the operational advantages of efficient participant accrual, feasible perinatal specimen collection (cord blood and placenta), the cost efficiencies of facility-based enrolment, and the collaboration of MCHTI management: Dr. Sirajul Islam, Superintendent of Azimpur Maternity Centre, and Dr. Chinmoy Kanti Das, Coordinator of The Maternal and Child Health Training Institute (MCHTI).

Field and clinical operations will be managed by ICDDR,B with the collaboration of Shimantik, a partner implementing non-governmental organization that delivers maternal-child health services in Dhaka.

10) Participant eligibility criteria

a) Inclusion criteria:

- Age 18 years and above;
- 17 to 24 completed weeks of gestation (i.e., 17 weeks +0 days to 24 weeks + 0 days, inclusive) based on recalled last menstrual period (LMP) and/or ultrasound;

Rules for integrating information from recalled LMP and ultrasound:

- if there is a difference of >10 days between gestational age dated using the LMP and second trimester ultrasound, the estimated date of delivery will be adjusted as per the second trimester ultrasound (SOGC guidelines); otherwise (i.e., if the difference is =<10 days), the GA date based on LMP will be used.
- If there is more than one ultrasound, GA estimation should be based on the earliest of the ultrasounds for which a written report is available. If the earliest ultrasound was performed in the 1st trimester, and there is a difference of >5 days between gestational age dated using the LMP and 1st trimester ultrasound, the estimated date of delivery will be adjusted as per the 1st trimester ultrasound (SOGC guidelines); otherwise (i.e., if the difference is =<5 days), the GA date based on LMP will be used.
- Intends to reside in the trial catchment area (including Hazaribag, Azimpur, Lalbag, and Kamrangirchar) for at least 18 months;
- Provides written informed consent.

b) Exclusion criteria:

- History of any medical condition or medications that may predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia, including active tuberculosis or current therapy for tuberculosis, sarcoidosis, history of renal/ureteral stones, parathyroid disease, renal or liver failure, or current use of anti-convulsants.
- High-risk pregnancy based on one or more of the following findings by point-of-care testing:
 - Severe anemia: hemoglobin <70 g/L assessed by Hemocue
 - Moderate-severe proteinuria: ≥ 300 mg/dl (3+ or 4+) based on urine dipstick

- Hypertension: systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg
- High-risk pregnancy based on one or more of the following findings by maternal history and/or ultrasound:
 - Multiple gestation
 - Major congenital anomaly
 - Severe oligohydramnios
- Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin containing calcium and/or vitamin D.
- Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency.
- Previous enrolment in the trial during a previous pregnancy.

Note regarding the timing of supplement initiation: Starting supplementation in the mid-second trimester (17 to 24 weeks) balances the benefits of a prolonged period of supplementation with the practical consideration that pregnant women are not typically registered for antenatal care at Azimpur hospital prior to 20 weeks gestation.

11) Interventions

a) Intervention description

The experimental intervention is supplemental **oral vitamin D3 (cholecalciferol)**, a fat-soluble hormone precursor for which the natural sources are endogenous production in the skin upon ultraviolet B radiation exposure and some types of food (e.g., oily fish, fortified milk). The supplemental vitamin D will be provided in the form of small tablets (10 mm diameter) to be custom-manufactured by Toronto Institute for Pharmaceutical Technology (TIPT) in Toronto, Ontario, Canada (www.tipt.com). Each weekly dose will consist of a single tablet. Across trial groups, the tablets will only vary with respect to the vitamin D3 dose, per the chart below:

Group	Prenatal supplement	Postpartum supplement duration lactation (0 to 24 weeks postpartum)
A	0 IU (placebo)	0 IU (placebo)
B	4,200 IU/week (=600 IU/d)	0 IU (placebo)
C	16,800 IU/week (=2,400 IU/d)	0 IU (placebo)
D	28,000 IU/week (=4,000 IU/d)	0 IU (placebo)
E	28,000 IU/week (=4,000 IU/d)	28,000 IU/week (=4,000 IU/d)

See Appendix for Investigator brochure.

Tablet content will be verified by chemical analysis prior to the initiation of the trial, and at regular intervals throughout the trial (approximately every 6 months).

Supplementation will begin at enrolment and continue on a weekly basis throughout pregnancy and the postpartum period, until 24 weeks postpartum.

Mode and conditions of tablet consumption:

- Swallowed whole with any liquid (water, juice, tea, etc.) or, if necessary, chewed with or without a small amount of soft food such as yoghurt (if completely consumed).
- There is no specific time at which consumption must occur, as this is not relevant to long-term effect on vitamin D status.
- Tablet may be consumed with or without a meal, and the composition of meals is not specified, as this impacts negligibly on vitamin D absorption⁵³.

b) Criteria for discontinuing or modifying allocated interventions

i) Missed and late doses

A **missed dose** is considered to have occurred if a dose was not ingested on the scheduled day of administration, for a participant who remains on-study. Typical reasons may include:

- Participant not available in the home on a scheduled day of dose administration
- Participant refuses a scheduled dose due to nausea, etc.
- Scheduled visit cannot be completed due to political unrest, inclement weather, etc.

Although scheduled for specific 7-day intervals, a missed dose may be administered as a **late dose** on any day up to 7 days after the scheduled date of administration; thereafter, subsequent dosing continues as originally scheduled, even if this incurs an interval of less than 7 days between the late dose and the subsequent regularly scheduled dose.

- If a participant is not reached for one full week but then returns to follow-up on the subsequent scheduled visit day (i.e., 7 days following the missed visit), two doses may be administered simultaneously. A maximum of 2 doses may be administered together.
- If a participant returns to follow-up >7 days after a missed visit, the first missed dose will be considered to be a **completely missed dose**, but the second missed dose may be administered as a *late dose*. For example, if a participant is absent for a visit on day #14, and returns to follow-up 12 days later on day #26, the participant is considered to have *completely missed* the day #14 dose, but is administered the dose scheduled for day #21 as a *late dose*; had the participant returned to follow-up on day #21, she could have received the day #14 and day #21 doses simultaneously.

ii) Nausea/vomiting during or after dose administration

- If a participant complains of nausea or stomach upset on the scheduled day of dose administration, the dose may be deferred for up to 7 days (see above, per missed dose).
- If a participant vomits during or within 20 minutes of dose administration:
 - the dose may be repeated immediately; OR,
 - the dose may be considered 'missed' and deferred to another day (within 7 days), applying the same rules as for other missed doses.
- If vomiting occurs more than 20 minutes after a dose was swallowed, the dose will be considered to have been administered. In such cases, the dose will not be repeated, and dosing will continue as scheduled the following week.

iii) Refusal or deferral of a dose

- If a participant refuses a dose on the day of scheduled administration, the study worker will offer to return on a subsequent day (within the week) to deliver the dose (see above, per missed dose).
- However, if a participant refuses doses for 3 consecutive weeks, and on the 3rd week, expresses the intention to continue avoidance/refusal of the dose, no further supplementation will be offered on a weekly basis.
 - A participant with serial refusals of the supplement need not withdraw completely from the study, if she agrees to the follow-up procedures (e.g., sample collection, anthropometry, etc).
 - If a participant remains in follow-up, the study personnel will occasionally (at least monthly) discuss renewed supplement adherence with the participant.

iv) Adverse events that require supplementation discontinuation

- The only clinical events that will lead to study personnel withholding doses are those adverse events that are ascertained to be supplement-related (see below).
- However, individual doses may be considered missed, late or deferred due to intercurrent illnesses; approaches to missed doses are addressed as they would be for any other reason.
- The following specific events will lead to discontinuation of supplementation:
 - Confirmed hypercalcemia (see definition below): supplement administration will be stopped; however, observational follow-up (including biochemical assessment) will continue.
 - Symptomatic vitamin D deficiency: diagnosed by a study physician or consultant physician (e.g., osteomalacia) on the basis of clinical findings (e.g., persistent limb pain) and supported by biochemical evidence (i.e., hyperparathyroidism). Masked study supplement administration will be stopped and vitamin D supplementation will be instituted to correct severe vitamin D deficiency. Such participants will continue to be followed clinically. A low 25(OH)D concentration alone will not be grounds for cessation of the study supplement.
 - Fetal or infant death: Maternal supplementation will cease and participation will end.

c) Strategies to improve adherence to intervention protocols, and procedures for monitoring adherence

- Tablet ingestion will be directly observed by study personnel during home or clinic visits, except for certain contingencies (see below).
- Tablet supplies will be maintained/stored by study personnel, not in the homes of participants, except for certain short-term contingencies (see below).
- Study personnel will maintain a log of dose administration, including a record of all *missed doses*, *late doses* and *completely missed doses*.

- In situations where all of the following conditions are met, up to 4 tablets (one month's supply) may be given to a participant in advance, to be consumed without direct supervision:
 - Participant will be unavailable for study visit(s) due to travel
 - Participant expresses intent to self-administer dose(s)
 - Participant agrees to contact the study worker by phone or text message to confirm each weekly supplement self-administration in real-time.
 - Participant agrees to ensure the safe storage of the supplement tablets (e.g., out of reach of children). Participants will be given a child-proof container to hold the tablets.
 - Participant agrees not to give the supplements to any other person, even if she herself chooses not to consume them.

- d) Relevant concomitant care and interventions that are permitted or prohibited during the trial

The following co-interventions will be provided to all participants:

- Calcium 500 mg/day as calcium carbonate (Calbo, Square Pharmaceutical, Dhaka)
- Iron and folic acid supplementation: (66 mg elemental iron per day, and 350 mcg folic acid per day included in the standard formulation available in Bangladesh).
- Tetanus toxoid immunization: if not already received, two doses will be administered (4 weeks apart) prior to delivery according to current WHO recommendations.
- Antenatal monitoring and referral to physician as indicated for any complications.
- Counselling to promote optimal infant feeding and health maintenance (including exclusive breast-feeding for the first 6 months, with introduction of appropriate complementary foods starting at 6 months of age), care-seeking for illness, and routine immunizations.

Supplemental calcium or vitamin D not prescribed by the study protocol will be prohibited during the intervention phase of the trial (Phase 1). Participants will be questioned on a weekly basis as to whether they have consumed other nutrient supplements. A one-time warning will be given when non-study vitamin D or calcium supplementation is first reported; if the participant has not discontinued the non-study supplement at the time of the next weekly visit, the participant will be discontinued from further study supplementation. In such cases, participants may still continue clinical follow-up. There is no prohibition against supplemental calcium or vitamin D during the non-intervention phase 2 (after 6 months postpartum); however, no specific recommendations will be given.

Rationale for calcium co-intervention:

The provision of a calcium co-intervention in the context of the proposed study has two major advantages: (1) from a mechanistic standpoint, standard provision of calcium will act to mitigate any rate-limiting effects of serious maternal dietary calcium deficits on fetal growth or other outcomes, thereby isolating the effects of vitamin D; and (2) from a practical standpoint, the investigation of vitamin D supplementation in the context of routine calcium supplementation will facilitate the translation of our findings into anticipated future contexts in which supplemental

calcium is routinely provided. A factorial trial to discern the independent effects of calcium and vitamin D on growth was not feasible within the current funding/logistical constraints.

In the proposed trial, we will provide calcium supplementation (as calcium carbonate) at 500 mg/day elemental calcium to all participants. The World Health Organization (WHO) has recommended prenatal calcium supplementation at a dose of 1.5 to 2 g/day to reduce the risk of hypertensive diseases of pregnancy (HDPs, e.g., preeclampsia) in populations with low baseline dietary calcium intake and especially for women at high risk of HDPs (WHO 2011). In Bangladesh, typical daily calcium intake is low, estimated at 200-400 mg per day⁵⁴⁻⁵⁶. However, there are several reasons why a dose of 1.5 g was not selected for routine provision to participants in this trial:

- There are unresolved concerns about the safety of this high dose; trials have shown a significant increase in the risk of HELLP syndrome. A dissenting opinion in the WHO statement by Dr. Peter von Dadelszen (University of British Columbia) was based on speculation that calcium reduces the *diagnosis* of preeclampsia, but does not modify the underlying disease process (WHO, 2011).
- There is evidence that lower doses (< 1 g/day) may also reduce the risk of hypertensive disease of pregnancy (Hofmyer 2013, unpublished). Personal communications with Dr. Justus Hofmyer (University of the Witwatersrand, South Africa) have confirmed his viewpoint that a supplemental intake of 500 mg would be appropriate at present, on the basis of existing evidence.
- Currently, calcium supplementation is not standard of care in Bangladesh; in particular, 1.5 g/day is rarely prescribed in Bangladesh. Our experience is that if a woman has been prescribed calcium supplementation, it is at a dose of 500 mg per day.
- There are unresolved questions surrounding the feasibility of delivery mechanisms for daily calcium at high doses (1.5 to 2 g); in the WHO calcium supplementation trial, the regimen was one 500 mg tablet taken 3 times daily, for which adherence is likely to be low.
- There are potential adverse interactions between calcium and iron; at present, the WHO recommends that supplemental calcium be taken apart from iron, implying that supplementation would have to occur 4 times per day, likely further lowering adherence.
- There are no trials of combined high-dose vitamin D + calcium at the WHO-recommended doses; the combination poses theoretical risks of urolithiasis or other complications. These risks need to be studied separately before this combination can be widely implemented.
- In determining the appropriate calcium co-intervention dose for this study, we did not consider evidence suggesting a harmful or beneficial effect of calcium on long-term bone or cardiovascular outcomes. This was because the evidence is mixed, mostly drawn from elderly populations, and not felt to be relevant to relatively short-term supplementation during pregnancy.

In the trial setting, a daily dose of 500 mg of calcium is expected to raise the average participant intake above the estimated average requirement (EAR) established by the Institute of Medicine (800 mg) and to approach or attain the recommended dietary allowance (RDA) of 1000 mg/day for most women. A dose of 500 mg can be provided readily using an over-the-counter supplement available in Bangladesh, but higher doses would require multiple tablets.

Participants will be instructed and encouraged to take a single tablet of calcium per day in the morning, and to take the iron-folic acid supplement separately, in the evening, or with a meal.

With respect to the prevention of HDP, participants in this trial will undergo close clinical monitoring, including blood pressure measurement and urinalysis, that exceed the standard care which they would have otherwise experienced outside of the research context; therefore, some women may benefit from earlier recommendation and referral for treatment of signs of HDP.

12) Outcomes

Primary outcome measure: length-for-age z-score (LAZ) at one year of age.

Growth faltering in resource-poor settings primarily occurs in the first year of life⁶; thus any effects of the intervention are expected to be apparent by one year of age. Moreover, our preliminary trial data revealed a discernible effect of prenatal vitamin D supplementation on infant LAZ at one year. Since some infants might not be reached at exactly 1 year of age (52 weeks), measurements taken up to 60 weeks (i.e., up to 8 weeks past the scheduled time of ascertainment) will be included in the primary “one year” outcome analysis implying a range of 364 to 420 days (inclusive) for allowable timing of one-year measurements. The purpose of this range is to enable the inclusion of as many children as possible, even if they are not available for assessment during the 52nd week postnatal. LAZ based on WHO growth standards will be used to account for sex imbalances between groups and variance in exact age at the time of measurement.

Infant follow-up will continue to 2 years of age (with continued treatment masking) to establish the persistence of effects, to capture potential catch-up growth, and because stunting prevalence does not stabilize until 18-24 months of age⁶. To improve uniformity with respect to the timing of the 2-year visit, it will be scheduled at the time of the child’s 2nd birthday or up to 3 months afterwards (27 months after birth).

Other secondary growth outcomes will include weight, length, head circumference, limb length, mid-upper arm circumference and postnatal growth velocity. These secondary growth outcomes and additional safety outcomes are summarized below:

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
<u>Primary outcome:</u> Linear growth at one year of age	Efficacy	Length-for-age z-score (LAZ)	Value at 1 year of age	<ul style="list-style-type: none"> • Mean (primary analyses) • % below -2 SD 	Measurement obtained on or shortly after 52 weeks (up to 60 weeks)
Linear growth at two years of age	Efficacy	Length-for-age z-score (LAZ)	Value at 2 years of age	<ul style="list-style-type: none"> • Mean • % below -2 SD 	Measurement obtained on or just after the second-year birthdate (up to 27 months)

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
Weight and head circumference for age	Efficacy	Weight-for-age z-score (WAZ), weight-for-length (WFL), and head circumference for age z-score (HCAZ)	Values at one year or two years	<ul style="list-style-type: none"> • Mean • % below -2 SD 	Measurement obtained on or just after the one-year or two-year birthdates.
Weight, length, limb lengths, mid-upper arm circumference, head circumference	Efficacy	Raw weight, length, limb length, mid-upper arm circumference and head circumference measures	Values at specific timepoint	<ul style="list-style-type: none"> • Mean • Birth weight: % below 2500 g • % small-for-gestational age 	Measurement obtained closest to specified timepoint: <ul style="list-style-type: none"> • within 48 hours of birth. • Within 3 months of 1st and 2nd birthdays
Birth weight, length, head circumference z-scores	Efficacy	Birth weight, length, and head circumference z-scores	Value at birth	<ul style="list-style-type: none"> • Mean • % below -2 SD (WHO z-scores) 	Measurement obtained closest to specified timepoint: <ul style="list-style-type: none"> • within 48 hours of birth. • Within 3 months of 1st and 2nd birthdays
Linear growth velocity	Efficacy	Raw length measurement or LAZ	Change within a specified interval	<ul style="list-style-type: none"> • Mean 	Specified growth intervals: 1, 3, 6, 12, 18, and 24 months.
Gestational age at birth / preterm birth	Safety	Gestational age at birth (in days)	Value at birth, based on LMP +/- ultrasound	<ul style="list-style-type: none"> • Mean • % preterm (< 37 weeks) • % early preterm (< 34 weeks) 	<ul style="list-style-type: none"> • Delivery
Placental weight	Efficacy	Weight (grams)	Value	<ul style="list-style-type: none"> • Mean 	<ul style="list-style-type: none"> • Delivery
Vitamin D status	Efficacy	Serum 25(OH)D concentration	Values at specified timepoints, as well as change in concentration during pregnancy.	<ul style="list-style-type: none"> • Mean • % below/above specific thresholds 	<ul style="list-style-type: none"> • Delivery (maternal) • Cord blood • 3, 6 months postpartum
Maternal serum calcium	Safety	Serum calcium concentration	Value	<ul style="list-style-type: none"> • Mean • % above or below reference range 	Whenever measured
Uro-/nephrolithiasis	Safety	Presence on ultrasound	Ultrasound imaging	-	Delivery
Maternal referral for obstetric care	Safety	Any referral by study physicians for a suspected or diagnosed obstetric complication (even if referral not accepted)	-	-	Prenatal period, and up to 1 month postpartum.
Maternal hospitalization	Safety	Inpatient admission for any reason other than uncomplicated delivery.	-	-	Any time during trial
Child hospitalization	Safety	Inpatient admission for any reasons	-	-	Any time during trial

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
Infant morbidity	Efficacy/ Safety	Occurrence of episodes of skin infection, sepsis, diarrhea or acute respiratory infection (ARI).	-	Prevalence/ incidence	0 to 6 months of age.
Participant (maternal) death	Safety	Death from any cause at any time.	-	-	Any time during trial
Maternal death	Safety	Death while pregnant or within 42 days of termination of pregnancy, irrespective of the duration and site of the pregnancy, from any cause related to or aggravated by the pregnancy or its management but not from accidental or incidental causes.	-	-	Expected event count is 0.
Stillbirth	Safety	Intrauterine demise or delivery of a fetus that does not breathe or show any other evidence of life - e.g. beating of the heart, pulsation of the umbilical cord or definite movement of voluntary muscles	-	-	Cumulative over study; based on 2011 rate in Dhaka (2.6%)*, expected event count may be ~33.
Neonatal death	Safety	Death of live born infant within first 28 days of life	-	-	Cumulative over study; based on 2011 rate in Dhaka (3.6%)* event count may be ~42.
Post-neonatal infant death	Safety	Death of a child at any time during first year after 28 days.	-	-	Cumulative over study; based on 2011 rates (0.8%)* event count may be ~9.

*Estimated event counts based on 2011 rates for Dhaka in Bangladesh Demographic Health Survey (BDHS).

Notes regarding growth outcome ascertainment based on anthropometric measurements and the application of World Health Organization (WHO) growth standards:

- *Measurement precision:* Measurement procedures will be conducted using an approach that minimizes errors at the time of data collection (see below, Data Collection).
- *Temporal consistencies:* A supervisor will conduct a same-day review of anthropometric measures in comparison to each child's previous set of measurements and will flag inconsistencies based on the following principles:
 - Length and head circumference (HC) will be assumed to be constantly increasing (even though there may be rare circumstances where they truly can decrease), such that any decline in a length or HC measurement will prompt a repeat length assessment at the earliest convenience (ideally, same week).

- Weight may vary, and can theoretically decline between closely spaced measurements. However, declines of greater than 10% will be flagged for repeat assessment at the earliest possible convenience (ideally, same week).
- *Pooled raw measures:* Paired/triplicate raw measures obtained on the same infant at the same visit will be averaged prior to analysis further improve precision.
- *Standardization for age and sex:* Weight-for-age (WAZ), length-for-age (LAZ), weight-for-length (WFL) and head circumference-for-age (HCAZ) will be calculated according to the sex-specific World Health Organization (WHO) growth standards^{57, 58}, using the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>).
- *Extreme outliers:* Extreme z-scores will be flagged based on the WHO Anthro software (< -6 SD or >6 SD for LAZ, >5 or <-6 for WAZ, >5 or <-5 for HCAZ, and <-5 or >5 for WFL). These extreme values will be manually reviewed to ensure they are not the result of data recording or entry errors. Real values that are extreme outlying z-scores are likely to be contributed by infants who are early preterm births and very low birth weight (VLBW); handling of these outliers is discussed below under Data Analysis.

13) Participant timeline

Enrolment and randomization will occur during the 2nd trimester (17 to 24 weeks gestation), supplementation with vitamin D and/or placebo will occur throughout the prenatal period and the first six months postpartum, the primary growth outcome will be ascertained at one year of age, and infants will continue follow-up until two years of age. The trial follow-up schedule is divided into phases. Phase 1 refers to the intervention period; and phase 2 refers to the observational follow-up period:

- **Phase 1a:** enrolment at 17-24 weeks gestation to birth (prenatal intervention).
- **Phase 1b:** birth to 6 months (including 6-month visit); postpartum intervention.
- **Phase 2a:** 6 to 12 months (including 12-month visit, may be extended to 15 months).
- **Phase 2b:** 12 to 24 months (including 24-month visit, may be extended to 27 months)

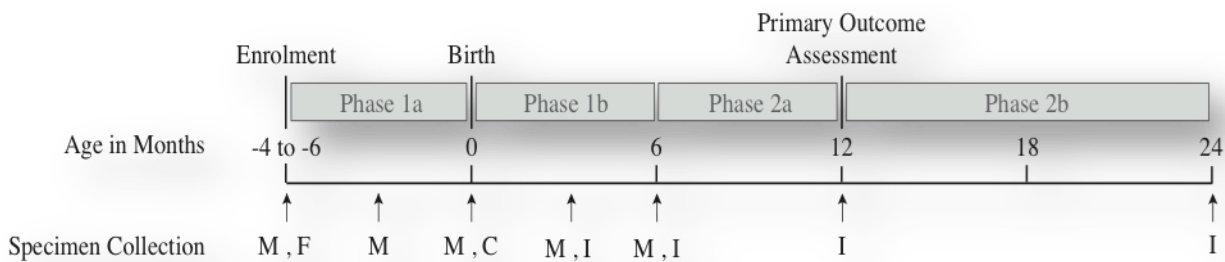


Figure 7: Scheduled specimen collection points for mothers (M), fathers (F), cord blood/placenta (C), and infants (I) are marked by vertical arrows.

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation				Close-out	
Timing (months, in relation to delivery)	-4 to -6	-4 to -6	-2.5	0	3	6	12	24
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
<i>Obstetric ultrasound</i>	X							
Allocation		X						
INTERVENTIONS:								
<i>Prenatal Supplementation</i>		◆—————◆						
<i>Postpartum Supplementation</i>				◆—————◆				
ASSESSMENTS:								
Visit frequency	Once	Weekly				Every 3 months		
<i>Maternal clinical assessment</i>	X		X	X	X	X		
<i>Maternal specimen collection</i>	X		X	X	X	X		
<i>Paternal specimen collection</i>	X							
<i>Birth outcome ascertainment</i>				X				
<i>Cord blood & placental specimen collection</i>				X				
<i>Infant Anthropometry</i>			X	X	X	X	X	X
<i>Infant morbidity assessments</i>			X	X	X	X	X	X
<i>Infant specimen collection</i>					X	X	X	X

* Paternal blood specimen collection will enable identification of allele-specific epigenetic patterns; however, paternal blood collection is not required for pregnant woman to join the trial, and therefore the consent process will be separate.

The prenatal timeline will be defined by gestational age (in weeks), based on the LMP and/or ultrasound results (see above). Postnatal follow-up visits will similarly be scheduled based on the infant's age in weeks during the first year, rather than 'anniversaries' of the birthdate, such

that 3 months = 12 weeks, 6 months = 24 weeks, 9 months = 36 weeks, and 12 months = 52 weeks. However, the visit at “24 months” will be based on the child’s 2nd birthdate.

All study activities (e.g., supplementation, visits, maternal and infant specimen collection) will be stopped for a mother-infant pair when any one of the following events occur:

- a) The 24-month infant visit is completed. This is the scheduled time at which participant discharge will routinely take place.
- b) Participant death.
- c) Consent for all types of follow-up is withdrawn.
- d) Participant moves location of residence and is lost to follow-up. Loss to follow-up will be considered to have occurred if study personnel receive information that the participant has moved away and will not return to follow-up at any time during pregnancy. However, a participant may be absent for a period of time (and thus miss supplement dosing and specimen collection), and yet return to follow-up without being excluded from the study.

14) Sample size

A total of **1,300 participants** will be randomized into 5 groups of 260 women each. The primary outcome analysis will be the between-groups comparison of mean length-for-age z-scores (LAZ) at 1 year of age, assuming up to 15% attrition from each group. To assess the effect of prenatal vitamin D, we plan to perform five primary between-group analyses – each vitamin D dose versus placebo (3 comparisons), and between adjacent doses (2 comparisons). A conservative approach to addressing multiple testing is to partition the alpha (risk of type 1 error) among the 5 comparisons (conventional overall 0.05 divided by 5); thus, each between-group comparison will be tested as a 2-sided test with an alpha of 0.01.

Assuming 90% power and a 1% risk of a type 1 error for each of 5 simultaneous 2-sided tests, the following figure shows the declining magnitude of the minimal detectable difference in LAZ that would be detectable with increasing sample size per group:

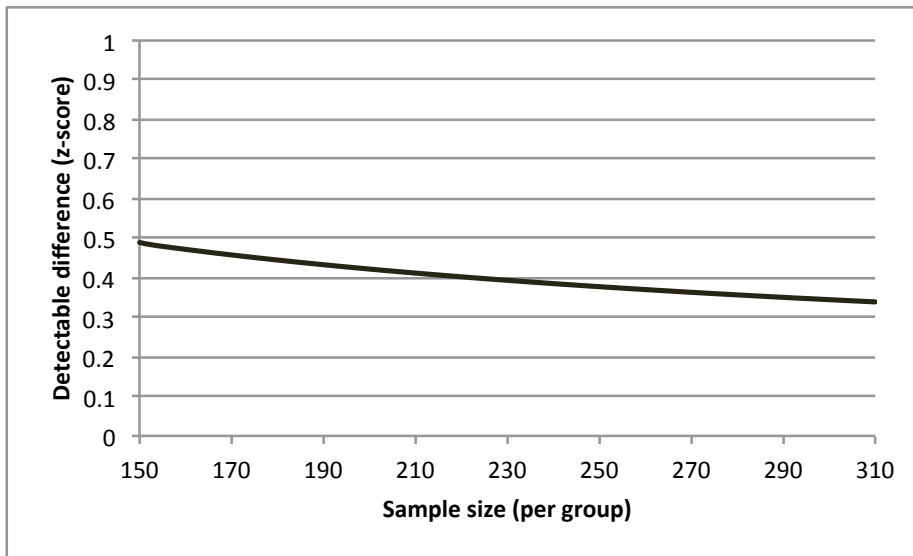


Figure 8: Sample size calculation

With 220 analyzable participants per group (~85% of enrolled), the minimum detectable difference in LAZ will be a z-score of 0.40, which equates to approximately 1.0 cm at 1 year of age based on WHO growth standards. In our completed preliminary trial, we observed an increase of 0.44 in LAZ (95% CI = 0.06 to 0.82) at one year, which corresponded to an increase of 1.1 cm (95% CI, 0.06 to 2.0), adjusted for gender (Roth et al. unpublished).

The secondary analysis at two years of age will enable detection of a ~1.2 cm difference between groups. For the 'postpartum effect' analysis of 4000 IU/day versus placebo postpartum among women who received 4000 IU/d antenatally, the comparison at one year of age will have a minimum detectable difference of 0.31 z-score units (assuming 90% power, a 5% risk of a type 1 error for a 2-sided test, and at least 220 participants per group). Based on WHO growth standards, this is approximately 0.77 cm at 1 year of age, and 0.96 cm at 2 years of age. Because the alpha is not subject to multiple testing, there is greater precision to detect a smaller difference for the postpartum effect analysis.

In order to enrol 1,300 women, we expect to provisionally screen up to 23,000 pregnant women for eligibility. The major reason for exclusion will be GA beyond 24 weeks.

15) Recruitment

Participants will be recruited through two mechanisms:

1. Routine antenatal care (ANC) visits at MCHTI.

All pregnant women who visit the outpatient or ultrasound departments of MCHTI for routine antenatal care/procedures will be approached by study personnel, informed about the study and asked if they would like to participate in an eligibility assessment.

2. Referral by government Family Welfare Visitors (FWVs) or non-governmental health promoters who encounter pregnant women in the community.

Where feasible, FWVs who work in the catchment area of MCHTI will be informed about the study and the basic eligibility criteria (mother's age and gestational age). If any of their clients meet the age and approximate gestational age criteria (i.e., before 3rd trimester), they will ask permission to provide the client's name and cell phone number (or address) to study personnel. If they consent to be contacted, study personnel will follow-up to initiate the screening process.

Eligibility screening is a two-step process:

- "*Provisional screening*" refers to the initial screening of MCHTI clients or women referred by FWVs on the basis of the eligibility criteria; age, known pregnancy, estimated first day of LMP, and Dhaka residence criteria; and,
- "*Detailed screening*" refers to the complete eligibility assessment supervised by a physician.

Women may be excluded at either stage due to non-eligibility and refusals (i.e., a woman who is provisionally eligible may refuse to undergo detailed screening; a woman who is fully eligible may refuse to enrol).

b) Provisional screening

Study personnel will assess the provisional eligibility of pregnant women presenting for antenatal care in the MCHTI outpatient department during regular clinic hours (typically, 9:00 am – 1 pm; 6 days per week; Saturday to Thursday). For women referred by FWVs, provisional screening may be conducted by phone, during a home visit, at MCHTI, or at the study field office located close to MCHTI (depending on what is most convenient for the prospective participant).

c) Triage and tracking of provisionally eligible women

- Provisionally eligible women who are between 17 and 24 weeks completed weeks will be referred immediately to the study physician for detailed screening (see below). Gestational age will be estimated based on maternal recall of the first day of the last menstrual period (LMP), with the help of a computer-based macro.
- Women beyond 24 weeks completed gestation (i.e., 24 weeks + 1 day or more) based on LMP will be excluded and will not be screened further.
- All pregnant women who meet the provisional criteria but have not yet reached 17 weeks gestation will be provided with an overview of the study purpose and procedures. They will be asked if they verbally consent to be contacted again about potential study participation. A list of these provisionally eligible women will consist only of first name, date of first day of LMP, and contact information (e.g., cell phone and/or address). The research staff will contact these women again shortly before they reach 17 weeks gestation to remind them to return to the study field office (or MCHTI) for detailed screening if they still meet the provisional screening requirements.

d) Detailed screening (of provisionally eligible women)

A study physician will supervise the detailed screening and informed consent process of all provisionally eligible women at 17 to 24 completed weeks gestation. Interviews with prospective participants will be conducted in a designated private area of the MCHTI outpatient department or field office (i.e., where a discussion can take place that other clients would be unlikely to overhear). The detailed screening and consent process will include the following items:

1. Confirmation of eligibility criteria pertaining to medical and obstetric factors.
2. With verbal consent to proceed, provisionally-eligible women will undergo assessment of the following clinical parameters:
 - **Blood pressure measurement** to exclude women with hypertension: Diastolic and systolic blood pressure will be measured using an automated digital blood pressure monitor. Two measurements will be taken, at least 1 minute apart; the highest of each of the systolic and diastolic values will be used for exclusion purposes (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg).
 - **Urine dipstick testing** to exclude women with moderate-severe proteinuria: Participants will be instructed to collect a random spot urine specimen (~30 mL) into a sterile dry container. Specimens will be screened immediately using a standard urine dipstick to measure urine protein.
 - **Finger-prick blood testing** to exclude women with severe anemia: A standard lancet will be used to prick the participant's finger and draw a drop of blood that will be used to measure hemoglobin concentration with a hand-held hemoglobinometer (Hemocue).
 - **Obstetric ultrasound** to confirm GA estimation and to exclude women with multiple gestation, major congenital anomalies, or severe oligohydramnios. *All participants will have an ultrasound at the time of enrolment to ensure all eligibility criteria are met.* However, as noted above, if a prior ultrasound report is available, GA estimation should be based on an earlier ultrasound, not the ultrasound conducted during enrolment.
3. Eligibility will be based on the 'best guess' GA estimate using an algorithm that integrates both LMP and ultrasound (see above, under eligibility criteria).
4. If a woman is found to be eligible, she will be offered the opportunity to participate and will be required to complete the detailed written consent process.
5. The consent process may occur over a period of more than 1 day, if necessary, to enable completion of the ultrasound and to provide the woman with adequate time to review the study information and decide whether or not to participate.
 - If the participant completes the written consent process more than 3 days after the start of the detailed screening process, the blood pressure measurement and urine dipstick testing must be repeated.
 - Hemoglobin measurement and the ultrasound need not be repeated again in these situations if completed within the 17-24 week gestational age window.
 - Enrolment must occur prior to 24 weeks completed gestation (i.e., last day of eligibility is 24 weeks + 0 days). Women who delay completing the written consent process and pass this time point cannot be included in the trial, even if they started the written consent process while still eligible.
6. If a woman is deemed ineligible to participate in the study based on her medical history, blood pressure, finger prick blood sample, urine dipstick test, or ultrasound,

or if or she chooses not to sign the consent form, she will be referred to the MCHTI antenatal care physician for standard care. Study staff will not assume further responsibility for the medical care of clinic patients who are not enrolled in the study.

(See below for details of the informed consent process that is integrated with detailed screening.)

16) Allocation of intervention

a) Sequence generation

The allocation sequence will be produced using a computer-generated random number sequence. We will use a simple randomization scheme; i.e., no stratification or blocking. The trial statistician will generate the allocation sequence.

b) Allocation concealment mechanism

The allocation sequence will not be viewed by the investigators or the field staff. This list will be provided to TIPT, the pharmaceutical company producing the supplements, which will package the supplements in individual participant supplement packs labelled with a unique study identifier.

c) Implementation of allocation

The pre-labelled supplement packets containing both phase 1a (prenatal) and phase 1b (postpartum) supplements will be provided to the field staff, and will be sequentially allocated to participants according to the order of enrolment. Using this method, there is no need for a treatment assignment to be concealed using envelopes, etc.

17) Blinding (masking)

a) Who will be blinded, and how

Participants, investigators, field personnel, study lab staff and data analysts will be blinded to vitamin D or placebo group allocation. The supplements will be identical in appearance and taste. Each supplement pack will only be labelled with the unique study identifier. The master list that links the unique identifier with group allocation will be maintained throughout the study at TIPT, and only released to the DSMB and/or investigators upon request.

b) Conditions and procedures for unblinding

The allocation scheme will be available to the DSMB and individual participants will be unmasked if they experience suspected supplement-related adverse events (i.e., hypercalcemia or clinical features suggestive of vitamin D toxicity). Because of the method of allocation concealment, unmasking of a single participant will not affect the masking of other participants.

Unmasking of individual participants' allocation is not planned, to enable ongoing blinded follow-up to 2 years postnatal, and beyond.

Methods: Data collection, management, and analysis

18) Data collection

a) Sequence and content of study visits

Follow-up will be conducted through pregnancy and up to two years postpartum/postnatal. Visits will routinely be conducted in the home, except when delivery occurs and when clinic visits are scheduled (i.e., typically when specimen collection is scheduled). Data will be collected using standardized data collection forms (DCF's). DCF's will be in a conventional paper format (although efforts will be made to identify opportunities for real-time electronic data capture).

1. Baseline visit (at enrolment)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	1A-B, 2, 3A-C

- i. Questionnaire (detailed): demographics, medical history, family and household factors, lifestyle factors related to vitamin D status
- ii. Maternal food frequency questionnaire
- iii. Blood pressure
- iv. Finger-prick blood test for severe anemia
- v. Urine dipstick testing for proteinuria and glycosuria
- vi. Maternal height and weight
- vii. Paternal height and weight (may be completed at a later time, as soon as possible after enrolment)
- viii. Obstetric ultrasonography
- ix. Maternal blood specimen collection
- x. Paternal blood specimen collection (may also be completed at a later time)
- xi. Supplement administration (first dose)

2. Prenatal routine weekly visits

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Home (or clinic)	Weekly (Phase 1a)	4A and 4B (once)

- i. Questionnaire (brief) to document on-going participation, health/vital status and medical events, and protocol deviations (e.g., non-study vitamin D supplement consumption).
- ii. Symptom checklist and morbidity surveillance (maternal)
- iii. Blood pressure measurement (only at 24 weeks, and then weekly from 36 weeks until delivery)
- iv. Supplement administration and adherence monitoring

3. 3rd-trimester clinical visit (30 weeks gestation)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	4A and 5

- i. All data collected during prenatal routine weekly visits (above)
- ii. Blood pressure
- iii. Maternal weight
- iv. Blood specimen collection
- v. Supplement administration (per routine weekly visit)

4. Labour and delivery

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Hospital	Once	6A

- i. All data collected during prenatal routine weekly visits (above)
- ii. Labour and delivery record (mode of delivery, complications, etc.)
- iii. Blood pressure and temperature
- iv. Maternal weight
- v. Maternal blood specimen collection
- vi. Cord blood specimen collection
- vii. Placental specimen collection

5. Neonatal assessment

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Hospital	Once	6B

- i. Questionnaire: vital status, complications and treatment, including referral to a neonatal care unit.
- ii. Neonatal anthropometry: weight, length, head circumference⁵⁹, upper-arm (i.e., humerus) length, and rump-to-knee (i.e., femur) length.
- iii. Neonatal clinical examination

6. Postnatal/postpartum routine weekly visits (0-6 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Home (or clinic)	Weekly (Phase 1b)	7

- i. Questionnaire (brief) to document on-going participation, health/vital status and medical events, infant feeding practices, supplement adherence, and protocol deviations (e.g., non-study vitamin D supplement consumption).
- ii. Symptom checklist (maternal)
- iii. Morbidity surveillance and brief clinical exam (infant)

- iv. Supplementation administration
- v. Health promotion (e.g., counselling regarding feeding and immunizations)
- vi. Infant length and weight (if not able to complete at scheduled clinic visit)
- vii. Maternal blood pressure (first postnatal visit only or if remains hypertensive)

7. Postnatal/postpartum clinic visits (0 to 6 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	3*	7 and 8 or 9A-B

- i. All data collected during postnatal routine weekly visits (above)
- ii. Maternal and infant food frequency questionnaires (at 6 months only)
- iii. Infant anthropometry: weight, length, and head circumference, mid-upper arm circumference, upper arm length, rump-to-knee length.
- iv. Maternal blood specimen collection (at 3 and 6 months)
- v. Maternal breast milk specimen collection (at 3 and 6 months)
- vi. Infant blood specimen collection (at 3 and 6 months), with test for anemia (at 6 months only)
- vii. Infant urine specimen collection (at 6 months only)

*All infants will have scheduled visits at 3 and 6 months postnatal. In addition, infants will have one additional set of anthropometric measurements (weight, length and head circumference) performed between birth and 8 weeks, but the specific timing of these additional visits will be randomly assigned at 2-week intervals. Random assignment of the supplementary anthropometry will be generated at the same time as the allocation sequence. Although dates of these visits will be scheduled in advance for each infant, the specific timing may be altered to accommodate participants' requests/schedules.

8. Postnatal/postpartum tri-monthly visits (9, 15, 18, and 21 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic (or home)	4	10

- i. Questionnaire (brief) to document on-going participation, health/vital status, infant/child feeding practices, and medical events.
- ii. Morbidity surveillance (infant)
- iii. Health promotion (e.g., counselling regarding feeding and immunizations)
- iv. Infant anthropometry: weight, length, and head circumference.

9. Postnatal/postpartum primary endpoint visit (age 52 weeks)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	11A,B

- i. Questionnaire (brief) to document on-going participation, health/vital status, infant/child feeding practices and medical events.
- ii. Maternal weight and height
- iii. Morbidity surveillance (infant)
- iv. Infant anthropometry: weight, length, head circumference, mid-upper arm circumference, upper-arm length, and rump-to-knee length.
- v. Infant blood specimen collection

10. Postnatal secondary endpoint visit (age 2 years)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	12

- i. Questionnaire (brief) to document on-going participation, health/vital status, infant/child feeding practices, and medical events.
- ii. Maternal weight and height
- iii. Morbidity surveillance (infant)
- iv. Infant anthropometry: weight, length, and head circumference, mid-upper arm circumference, upper-arm length, and rump-to-knee length.
- v. Infant blood specimen collection

The timing of visits will be scheduled in advance; however, the actual dates of visits may be postponed (changed to later dates) to accommodate participants. Additional unscheduled encounters may occur if the participant contacts study personnel to report new symptoms or clinical concerns, to ask questions regarding study participation, or to advise of an upcoming absence from the study area.

b) Verbal and clinical data collection methods (including point-of-care tests)

- 1. Questionnaires.** At every visit, questionnaires will guide study personnel in conducting structured, verbal, face-to-face interviews of the primary participant (mother). As a contingency, some data may be collected by phone (e.g., to confirm supplement consumption by participants who are temporarily outside of Dhaka). In some circumstances, other individuals may act as the respondents in the mother's absence (e.g., reporting a maternal or infant medical event).
- 2. Blood pressure measurement.** Maternal systolic and diastolic blood pressure (BP) will be measured using an automated digital blood pressure monitor (Microlife 3BTO-AP or equivalent). Two readings will be taken at least one minute apart and recorded; if either diastolic or systolic measurements differ between the paired readings by >10 mmHg, a third measurement will be performed. For analysis, paired readings will be

averaged; where a third reading was obtained, the single discordant reading will be excluded. BP will be measured at enrolment, 24 weeks, 30 weeks, and then weekly from 36 weeks until the first postpartum home visit; if hypertensive at postpartum measurement, will continue to be monitored weekly unless BP normalizes. BP may be measured in the home or clinic.

3. **Urine dipstick testing.** Urine dipsticks (Siemens Diagnostics Urinalysis Reagent Test Strips) will be used at enrolment and then after enrolment only if high blood pressure is detected (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg) or if there are symptoms of dysuria (for leukocyte esterase).
4. **Hemoglobin measurements.** At enrolment (as part of the eligibility screening process), maternal hemoglobin will be measured in a finger-prick blood sample using a handheld hemoglobinometer (Hb 201, Hemocue AB, Sweden). Infant haemoglobin will be measured at 6 months of age.
5. **Adult (maternal and paternal) height and weight.** Weight and height will be measured in the clinic using standard methods adapted from the CDC NHANES manual⁶⁰, and employing a digital floor scale (Tanita HD318, Tanita Corporation, Japan or equivalent) and a stadiometer (Leicester Height Measuring device, Chasmors Ltd., England or equivalent). Measurements will be performed in duplicate. Third measurements will be taken if the following discrepancies are noted between the paired measures: > 0.5 kg for weight; > 2 cm for height.
6. **Obstetric ultrasonography.** A second trimester ultrasonography will be performed at the time of enrolment by technicians at MCHTI using equipment available at the MCHTI (Just Vision 400, Toshiba, Japan; SONOACE X8, Medison, Korea). Technicians will be trained to collect specific data for study purposes, including number of fetuses, presentation, fetal heart rate, biparietal diameter, anterior-posterior thoracic diameter, crown-rump length, femoral length, abdominal circumference, placental position, amniotic fluid index, and presence/absence of major anomalies.
7. **Infant anthropometry.** Standardized procedures for infant anthropometry will be adapted from the Intergrowth-21 study manual (<http://www.intergrowth21.org.uk>). At each clinic visit, each infant will be measured independently by two study personnel, and the paired measurements will be compared; if they differ by more than the threshold values (7 mm for length; 5 mm for head circumference, upper arm length, knee-rump length, and mid-upper arm circumference; and, 50 g for weight), a second set of measurements will be performed and again compared. For all measurements except infant weight, if the second set differs by more than the threshold values, the procedure will be repeated a third time. Lengths and head circumferences will be recorded to the last completed unit (not to the nearest unit). The average (mean) of acceptable paired measures will be used in analysis.

- i. **Weight** will be measured in the clinic (or home) using a digital infant scale (Seca 334, Seca, Hamburg, Germany), to the nearest 5 g (up to 10 kg) and to the nearest 10 g (for > 10 kg).
 - ii. **Length** will be measured in the clinic using an infantometer (Seca 416, Seca, Hamburg, Germany), to the last completed 0.1 cm (1 mm). Measurement of length will be performed in the home using a portable infantometer (Seca 417, Seca, Hamburg, Germany).
 - iii. **Head circumference, mid-upper arm circumference (MUAC), upper-arm length, and rump-to-knee length** will be measured at scheduled clinic visits using a soft measuring tape or caliper, to the last completed 0.1 cm (1 mm).
8. **Dietary recall:** Estimation of usual dietary intake of vitamin D, calcium, phosphorus, and phytates among trial participants at enrolment (mothers) and 6 months postpartum, using a food frequency questionnaire.
9. **Neonatal examination:** As soon as possible after delivery, a physician will perform a standardized physical exam to document any congenital anomalies.
10. **Placental weight and dimensions:** After collection of cord blood and placental tissue specimens, the umbilical cord and membranes will be removed, blood drained, and then the placenta will be weighed to the nearest 0.1 g.
11. **Morbidity surveillance:** During routine weekly visits during Phase 1 (a & b), study personnel will review symptoms of possible infectious illness.
 - i. Maternal prenatal illness will be tracked using a symptom checklist at weekly prenatal visits. Temperature measurement and/or urine dip for leukocytes (indicator of urinary tract infection) will be performed in the home if clinical symptoms are suggestive of infection, based on standardized clinical algorithms.
 - ii. Infant episodes of diarrhea, acute respiratory infection (ARI) – subdivided as lower and upper tract subtypes, sepsis, or skin infections will be documented during the period from birth to 6 months of age. Conventional diarrhea and ARI case definitions will be based on maternal report of symptoms (e.g., cough, frequent stools, etc). When symptoms of ARI are reported (cough and/or difficulty breathing), the field worker will measure the infant's temperature and observe for rapid breathing, chest indrawing, or clinical danger signs (using the World Health Organization Integrated Management of Child Illness algorithms). Infants with suspected "pneumonia" (i.e., lower respiratory tract infection) or other serious infections will be referred to the hospital for assessment and treatment by a physician. The occurrence of other minor infections (e.g., skin infestations) and severe infections (e.g., meningitis) diagnosed during the follow-up period will be recorded.

c) Biological specimen collection and processing

Specimen Collection Schedule

Specimen	Phase 1a			Phase 1b		Phase 2a	Phase 2b
	Baseline	30 weeks	Delivery	3 months	6 months	12 months	24 months
Maternal urine	X		X		X		
Maternal venous blood	X	X	X	X	X		
Paternal venous blood	X						
Cord blood			X				
Placenta			X				
Infant venous blood				X	X	X	X
Infant urine					X		
Breast milk				X	X		

Trained phlebotomists will collect maternal venous blood, cord venous and arterial blood (after delivery of the placenta), and infant specimens according to standard operating procedures. The following general principles will apply:

- Specimens will be routinely collected in the clinic (or hospital) setting; however, in cases in which a home delivery occurs and study personnel are present during the delivery or immediately afterwards, placenta and cord blood specimens may be collected in the home. Venous blood specimen collection and urine collection will not be routinely performed in the home.
- Specimen collection will precede the weekly administration of the study supplement (in phase 1).
- To limit the extent to which biomarkers are influenced by acute inflammatory responses, mothers and infants experiencing an episode of acute diarrhea or acute respiratory illness at the time of scheduled sampling will have specimen collection postponed by 7 days. However, this will not apply to baseline specimens, or delivery specimens (maternal urine, maternal blood, cord blood and placental specimens).
- Maternal blood specimens at 30-weeks are primarily to document serum calcium in individual women, as a safety parameter. Baseline and delivery specimens will enable analyses related to the change in serum biomarkers from baseline to end of pregnancy (e.g., change in vitamin D status). Specimens collected during phase 1 (up to 6 months of age) will be used for planned/budgeted biomarker analyses (see below), and any remaining sample tissue/volume will be banked in Dhaka. Infant blood specimens in phase 2 (i.e., those collected at 12 and 24 months of age) will be bio-banked for additional analyses based on funding availability, or as part of future sub-studies.
- Processing of blood samples for plasma and serum separation:

Specimen type	Vacutainer tube	Centrifugation	Storage/transport for batched analyses	Storage/transport for same/next-day analyses
Plasma	EDTA tube (lavender top), or trace element EDTA	Immediately (within 30 minutes). Tube is kept on ice until	To be placed at -15 to -25°C immediately after centrifugation and aliquot preparation	Not applicable.

Specimen type	Vacutainer tube	Centrifugation	Storage/transport for batched analyses	Storage/transport for same/next-day analyses
	tube (royal blue top)	centrifugation.	(0.25 mL aliquots); then, transported to	
Serum	Serum tube (red top)	In 30 to 60 minutes, at room temp.	icddr,b on dry ice, and transferred to a -80°C freezer.	2-8°C until transport in a cold box to icddr,b, where held at 2-8°C until analysis.
Whole blood	EDTA tube (lavender top), or trace element EDTA tube (royal blue top)	Removed before centrifugation.	2-8°C until transport in a cold box to icddr,b, where held at 2-8°C until analysis.	Not applicable.

- Whole blood aliquots (3 mL) will be drawn from EDTA tubes immediately after mixing (prior to centrifugation) for paternal blood, maternal delivery and cord blood samples.
 - Serum and EDTA tubes will be centrifuged at low speed for 15 minutes, and the supernatant transferred by plastic micropipette in 0.25 mL aliquots into labelled microfuge tubes.
1. **Maternal Urine:** Participants will be instructed to collect a spot urine specimen into a sterile dry container at baseline, delivery, and 6 months post-partum. One specimen aliquots (1.5 mL) will be held at 2 to 8°C until it is transported on ice to the laboratory for same or next-day analysis at icddr,b; other aliquots will be frozen at approximately -20°C until transfer to icddr,b on ice packs for long-term storage at -80°C..
 2. **Maternal venous blood:** A phlebotomist will collect 2 mL venous blood in a serum tube and 6 mL in an EDTA tube, at baseline, 30 weeks gestation, delivery, 3 months postpartum and 6 months postpartum. The delivery sample will be optimally collected during labour (2nd stage or later); however, if this is not feasible, a postpartum specimen will be obtained within 24 hours of delivery. Specimens will be considered to have been collected 'at delivery' if drawn within +/- 24 hours of delivery. At delivery, a trace element tube (royal blue-top; with EDTA; 6 mL) will be used in place of the regular EDTA tube.
 3. **Paternal venous blood:** A phlebotomist will collect 6 mL venous blood in an EDTA tube, at baseline. If the father is not able to contribute a blood sample at baseline, he can return at another time in the near future.
 4. **Cord blood:** The umbilical cord will be clamped and cut, the specific technique and timing for which will be determined by the attending physician or birthing attendant (i.e., cord clamping time is outside of the study protocol). Within 10 minutes of delivery (up to a maximum of 30 minutes after delivery of the placenta), a site on the umbilical cord attached to the placenta will be cleansed using dry cotton gauze to wipe away any maternal blood and then sterilized with an isopropyl alcohol swab. The umbilical vein will be cannulated and blood will be collected into the following

collection tubes (in this order): 1) one 5 ml serum (red-top) tube, 2) one 10 mL EDTA tube, 3) one 6 ml trace element tube (with EDTA), and 4) one 6 ml or 10 mL EDTA tube depending on the volume of blood collected. Attempts will be made to collect a total of about 20 to 40 mL of blood. If feasible, the umbilical artery will be cannulated and one 5 mL serum (red-top) tube will also be filled.

5. **Placental tissue:** Following delivery of the placenta, placental specimens from 4 quadrants of the placenta will be collected. Biopsies will be placed in RNALater and stored at 2 to 8°C until transfer to Toronto for epigenetic/gene expression studies.
6. **Infant venous blood collection:** During 4 clinical visits during the post-partum / infant follow-up phase, a phlebotomist will collect a single venous blood specimen by venipuncture of a superficial arm, hand, leg, or foot vein. Samples will be collected into an EDTA tube and a serum tube. At 3 months, the total blood volume will be 3 mL (2 mL in EDTA tube, 1 mL in serum tube); at 6 months, the total blood volume will be 6 ml (4ml in EDTA tube, 2 ml in serum tube); at 1 and 2 years of age, the total blood volume will be 5 mL per draw (4 mL in EDTA and 1 mL in a serum tube). The following measures will be taken to enhance infant comfort during the procedure (based on parental preference):
 - a. Swaddling
 - b. Breastfeeding, and/or
 - c. Oral dextrose/sucrose administration (2 mL of 12% dextrose or 24-25% sucrose solution applied to the tongue of infants within 2 minutes of the procedure).

For infant and maternal blood collection, a maximum of two attempts (where a single attempt is defined as having occurred if the skin is breached) will be made for each scheduled visit. If a sample cannot be obtained after two attempts, the study personnel will request that the participant return once more for another two attempts at the participant's convenience (typically within 1 week). If the two additional attempts are unsuccessful, no further attempts will be made until the next scheduled blood sample.

To directly benefit the infant, hemoglobin and ferritin at 6 months of age will be reported back to the study physician, and the results will be interpreted based on a standard algorithm. Infants with iron-deficiency anemia will be treated, and referred for specialist care if necessary.

7. **Infant urine specimen:** Infant urine specimens will be collected at 6 months of age. An adhesive plastic urine collection bag will be placed on the infant's perineum for a 2-hour urine collection period. Specimen aliquots (1.5 ml, each) will be held at 2 to 8°C until they are transported on ice to icddr,b for: 1) long-term storage at -80°C, or 2) sent to the laboratory for same or next-day analysis.
8. **Breast milk samples** will be collected at 3 and 6 months postpartum according to the SOP developed for the MAL-ED study. Breast milk sample collection will be

undertaken in a private setting, with appropriate sensitivity to the participant's comfort during the procedure. Specimen aliquots will be frozen at -20°C until transfer to icddr,b for long-term storage at -80°C.

Approaches to potential variations in the specimen collection schedule:

- Missed or late specimens: Participants may occasionally be absent on scheduled days of specimen collection, or events such as national holidays or general strikes may require minor changes to sampling schedules. The following principles will guide specimen collection in case of late or missed specimen collection:
 - In general, 'late' specimens will be collected at the earliest opportunity following the missed visit.
 - A specimen will only be considered 'missed' if the next scheduled time of collection of the same specimen type is reached before the missed specimen can be collected.
- Specimens unsuitable for analysis: A small proportion of scheduled urine or blood specimens may be found to be unsuitable for analysis, mislabelled, etc. In these situations, study personnel will request collection of a single replacement specimen from the participant. Individual participants will not have replacement specimens requested on more than one occasion during the trial.
- Unscheduled sampling to monitor potential adverse events: According to the standardized safety monitoring algorithm, study personnel will occasionally collect unscheduled specimens from participants to either follow-up on initial results suggestive of hypercalcemia or on the basis of other clinical concerns. If a participant refuses follow-up sampling that has been advised for safety reasons, such that a specimen cannot be obtained within 7 days of the scheduled date or the repeat sample cannot be obtained within a time period that is sufficient to enable a decision regarding the safety of the next scheduled supplement dose, then supplementation will stop (for safety reasons) but other forms of observational follow-up may proceed as per the protocol.

d) Biomarker analyses

Biomarkers of the vitamin D-parathyroid axis (vitamin D metabolites, PTH fragments, PTHrP, serum calcium, and FGF-23) will be serially quantified through the pre- and postnatal periods.

- *Vitamin D status*: Vitamin D status will be determined by the serum 25(OH)D concentration, which is a well-established biomarker⁶¹. Serum 25(OH)D will be assessed using state-of-the-art liquid chromatography tandem mass spectroscopy (LC-MS/MS) at the AFBM.
- *Serum calcium*: Maternal serum calcium concentration is the primary biochemical safety outcome, and will be measured at scheduled intervals during the intervention phase (baseline, 30 weeks of gestation, delivery, 3 months postpartum, and 6 months postpartum).
- *Parathyroid hormone (PTH)*: The PTH immunoassays currently used widely in clinical laboratories (so-called 2nd-generation assays) utilize antibodies specific to epitopes found

in the middle of the whole PTH protein (84-amino acid length). These immunoassays cross-react with PTH fragments that do not include the extreme N-terminal amino acid sequence responsible for PPR binding (see **Figure 9**); these assays are conventionally referred to as *intact PTH* (iPTH) assays. Newer assays utilizing epitopes specific to the bioactive N-terminus (first 3 amino acids) enable specific identification of ‘whole PTH’ (wPTH) molecules that include the full 84-amino acid protein (PTH(1-84)). wPTH is increasingly recognized as a more biologically relevant marker; in a recent epidemiologic study of adults on renal dialysis, wPTH correlated more precisely with adult mortality than iPTH⁶². Since wPTH may represent a relatively greater fraction of iPTH in the context of parathyroid hyperactivity, a linear conversion factor cannot be implemented⁶³.

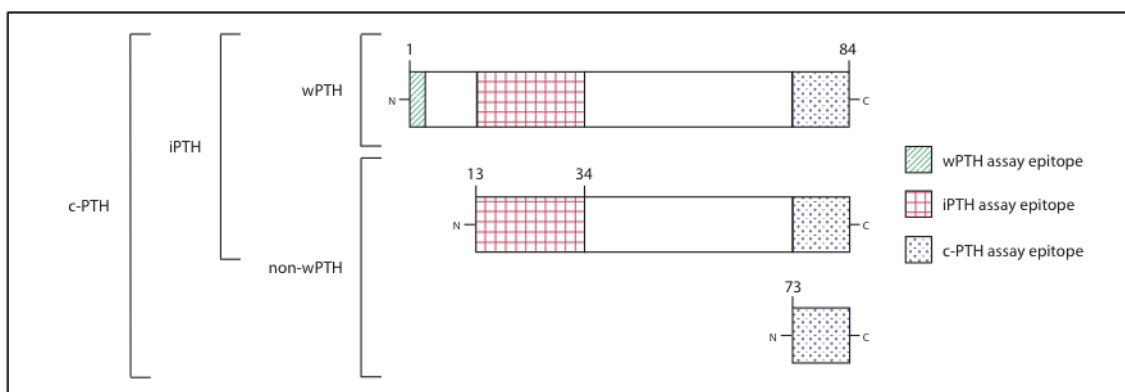


Figure 9: Schematic representation of the three types of PTH molecules to be assayed in the proposed study. Labeled brackets indicate the types of molecules that will be identified by each of 3 assays (c-PTH, iPTH, wPTH). The non-wPTH fraction will be calculated as c-PTH minus wPTH.

Commercially available assays now exist that utilize antibodies that bind truncated c-terminal fragments of PTH (c-PTH), thereby capturing virtually all PTH peptides, ranging from wPTH (i.e., PTH(1-84)) to peptides as short as the c-terminal 11 amino acids (i.e., PTH(73-84)). To capture the entire spectrum of vitamin D-parathyroid-growth associations, we will quantify multiple PTH fractions in the proposed study. wPTH, c-PTH, and iPTH will be measured by ELISAs (Immutopics) which employ the same biotinylated capture antibody to bind PTH at amino acids 39-84. We considered other available methods for the multi-target assessment of PTH; but the Immutopics assay was selected based on assay characteristics, epitope specificity, and specimen volume efficiencies. Evidence suggests that non-wPTH (i.e., PTH that lacks the bioactive N-terminal region) has biological effects that are generally opposite to those of wPTH⁶⁴. High circulating concentrations of c-PTH relative to wPTH have been associated with growth faltering in some studies of children with end-stage renal disease⁶⁵. We will thus investigate whether relative suppression of maternal and/or infant c-PTH secretion mediates the effect of vitamin D supplementation on fetal growth.

- *FGF-23* is a relatively recently discovered bone-derived protein, the expression of which may be triggered by 1,25(OH)₂D and which may act as a phosphaturic factor in the context of hyperparathyroidism⁶⁶. In many Gambian children with calcium-deficiency rickets and elevated PTH, FGF-23 concentrations were above the expected range⁶⁷. We hypothesize that FGF-23 may be a biomarker of parathyroid hyperactivity. FGF-23 will be

quantified in plasma using a 2nd-generation immunoassay that detects epitopes within the carboxyl-terminal (C) portion of the molecule (Immutopics).

- *Parathyroid hormone related peptide (PTHrP)*. Partially homologous to PTH, PTHrP is an established regulator of endochondral bone formation that is essential for long bone extension⁶⁸. In pregnancy, PTHrP is a critical regulator of maternal-fetal calcium flux²⁸. It is also speculated that PTHrP mediates postnatal infant growth since it is readily transferred from the mother to infant via breastmilk⁶⁹. In rats, antagonism of PTHrP during pregnancy causes fetal growth restriction⁷⁰ and, in a recent epidemiologic study, it was shown that fetal PTHrP concentrations were significantly lower in infants with IUGR compared to normal weight infants⁷¹. The systemic interplay between vitamin D, PTH and PTHrP in humans has not been well described, but animal studies suggest that elevations in PTH are associated with suppression of endochondral PTHrP expression⁷². We aim to test the hypothesis that maternal PTHrP production and its transfer via breast milk are mechanisms by which vitamin D supplementation enhances infant bone growth.
- *Urinary phosphate excretion*. PTH over-expression may increase urinary phosphate loss, as suggested by observations of children with hypophosphatemic rickets who manifest significant growth restriction and low serum phosphate concentrations⁷³. Infant urinary phosphate excretion will be measured and expressed as the phosphate-creatinine ratio and the renal maximum tubular reabsorption of phosphate per litre of glomerular filtration rate (TmP/GFR). It is also possible that severe parathyroid axis dysregulation may lead to end-organ PTH/PTHrP receptor (PPR) downregulation, which has been suspected in Indian toddlers with very low dietary calcium intake⁷⁴. Urinary cyclic AMP will be measured as a biomarker of PPR responsiveness.

Other biomarkers will be used to test mechanistic hypotheses during discrete developmental periods (see Figure 4 above):

- *Insulin-like growth factor (IGF) system*. In children with healing rickets, skeletal remineralization and the decline in PTH is accompanied by increases in circulating IGF-1 and IGF binding protein-3²¹. This observation is concordant with studies in animals in which secondary hyperparathyroidism is accompanied by diminished IGF-1 and IGFBP-3 secretion⁷⁵. IGFBP-3 appears to facilitate IGF-1 action, whereas binding of IGF-1 by IGFBP-1 may inhibit IGF-1 function. In epidemiologic studies, maternal IGFBP-1 concentrations were inversely associated birth weight (in Norway)⁷⁶, and elevated IGFBP-1 was found among small-for-gestational age newborns in Pakistan⁷⁷ as well as among stunted children in South Africa⁷⁸. We aim to explore whether suppression of fetal IGF-1 and IGFBP-3, as well as increased IGFBP-1 production, mediate the effect of maternal prenatal vitamin D supplementation on infant size. Thus, IGF system biomarkers will be studied in the perinatal period and early infancy via the cord blood sample and the first postnatal infant specimen.
- *Phosphates and phytates* may inhibit calcium absorption⁷⁹, and thus may interact with vitamin D and calcium intake. *Vitamin A* status may regulate PTH secretion⁸⁰. Also, retinols interact with vitamin D in the nucleus, via heterodimerization of the retinoic acid and vitamin D receptors. *Iron* status has been postulated to have a modifying effect on the vitamin D-parathyroid axis. Recently, in a study of Gambian children, hemoglobin

(interpreted by the authors as a surrogate marker of iron status) was shown to be inversely associated with FGF-23⁸¹, suggesting that iron may modify the effects of dietary calcium deficits or vitamin D deficiency on bone mineral metabolism. *Cadmium and fluoride* may be found in high concentrations in the groundwater in some regions of South Asia. Cadmium has been postulated to adversely affect calcium absorption in Bangladeshi women⁸², and fluoride excess has been linked to rickets in some parts of India⁸³. *Folic acid* is of unique interest because of its potential to act on maternal-fetal vitamin D status via epigenetic regulation of enzymes involved in vitamin D metabolism⁸⁴. In fact, folic acid supplementation may improve vitamin D status independently of vitamin D based on observations in pregnant Nepali women⁸⁵, and emerging evidence suggests that folic acid may influence the bioavailability of vitamin D during pregnancy by affecting the methylation pattern of 24-hydroxylase (cyp24A1), the enzyme that catabolizes 25(OH)D⁸⁴.

Maternal vitamin A status, folate, and cadmium exposure will be studied during the prenatal period; Serum retinol will be applied as a biomarker of maternal vitamin A status, and maternal folic acid intake will be considered to be reflected by the maternal serum folate concentration⁸⁶. Fluoride exposure will be studied among infants in the postnatal period.

- *Inflammatory cytokines*. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) may directly restrict linear growth through effects on osteoclast activity⁸⁷. Inflammation may also drive parathyroid hyperactivity⁸⁸; the pro-inflammatory chemokine interleukin-8 (IL-8) may specifically promote PTH secretion⁸⁹. On the other hand, vitamin D may dampen placental inflammation⁹⁰, thereby facilitating fetal development by optimizing transplacental nutrient transfer. To address the potentially key role of vitamin D in the regulation of immunity during the perinatal period, we will evaluate associations among vitamin D dose, parathyroid activity, and maternal peripartum or cord blood biomarkers of inflammation (TNFalpha, IL-6, IL-8, C-reactive protein).

A schedule of analytes is shown in Appendix C. Serum calcium will be measured routinely. However, as a cost-saving measure, additional analytes will be measured in a subset of specimens in the context of a nested case-control study.

Blood/urine/breastmilk biomarker analyses will be performed at three laboratories: the *Clinical Biochemistry Laboratory* (CBL) at ICDDR,B in Dhaka, the *Nutritional Biochemistry Laboratory* (NBL) at ICDDR,B in Dhaka, and the *Analytical Facility for Bioactive Molecules* (AFBM) at the Hospital for Sick Children (SickKids) in Toronto.

Maternal serum calcium and infant haemoglobin and ferritin will be reported back to the field in real-time and managed by study physicians according to specific protocols. All others assays will be performed as batched analyses, and not reported back to the field, as they will not have clearly-defined clinical implications.

e) Epigenetic analyses

In a subset of 200 mother-infant pairs, DNA and mRNA will be extracted from placenta specimens and blood samples using standard techniques as previously described⁹¹. Where possible, paternal whole blood specimens will be collected to enable the identification of allele-specific patterns. Genomic DNA will be subjected to bisulfite modification, which will convert unmethylated cytosine (C) to thymine (T) but leave methylated C intact. Amplified PCR products can be further used for quantitative pyrosequencing, in which a non-methylated C reads as T, and a methylated C reads as a C. Bisulfite modified DNA will be analyzed by pyrosequencing to assess DNA methylation (at single nucleotide (CpG) resolution) at several consecutive CpG sites within a ~150 bp region at the promoters of two genes involved in vitamin D metabolism (CYP27B1 and CYP24A1) in maternal and cord blood (and paternal blood, if feasible). Gene expression (mRNA) from placental specimens (fetal tissue) will be assessed by RT-PCR. Correlations between DNA methylation patterns and mRNA transcript levels will enable tissue-specific inferences regarding gene expression. Specimens will be banked, and if additional funding permits, microarray that can assess genome-wide methylation patterns will be pursued.

f) Data quality control

A range of quality assurance (QA) and quality control (QC) mechanisms will be established to ensure the highest feasible data quality.

Primary QA methods will include:

1. Development of detailed standard operating procedures (SOPs) for all field and laboratory components of the protocol.
2. Intensive training of study personnel, with competency assessments after initial training, and at least every 3 months thereafter.
3. Field-testing of data collection tools (e.g., questionnaires, anthropometry) prior to enrolment of trial participants.
4. Structured oversight of all activities by supervisory personnel, including review of all data forms.
5. Weekly clinical/field personnel meetings to review common errors, challenges, etc.
6. Weekly reports circulated to the PI and trial coordination team by the field team. The report will be manually generated by field staff and will contain trial metrics regarding accrual, withdrawal, protocol deviations/violations, and adverse events, QC metrics, and reports of equipment function and calibration. In addition, regular (bi-weekly) reports will be generated from the database itself (data queries); however, these may lag by ~2 weeks due to the time required for data entry.
7. Specific approaches to improve precision of the primary outcome data, including duplicate anthropometric measurements (see above).
8. Duplicate data entry and use of built-in range and consistency checks in the database.

Primary methods of QC will include:

1. Random spot observations of study procedures by supervising personnel, with the aim of providing real-time feedback to field personnel.
 2. Repetition of selected items from structured interviews from a sub-sample of visits/encounters.
 3. Random spot checks by supervisors to ensure home visits occurred as recorded.
 4. Random spot checks of individual participant tablet counts to ensure concordance with recorded number of tablets administered.
 5. Analysis of vitamin D3 content of tablets by high-performance liquid chromatography (HPLC) prior to trial initiation and at regular intervals throughout. In addition, masked sets of supplements will be sent to a 3rd party lab to verify that placebo and vitamin D tablets are labelled appropriately.
- g) Safety monitoring

Participant safety during the intervention phase will be monitored by study personnel based on:

1. Weekly follow-up visits that will include the use of a checklist of symptoms that may indicate vitamin D toxicity or other medical concerns. Monitoring for maternal and infant clinical events during clinic-based and household visits will follow a standard algorithm. In general, severe symptoms (e.g., severe headache) or persistence of mild-moderate complaints (e.g., persistent low-grade lower back pain) will prompt referral to the study physician.
2. Maternal serum calcium measured at scheduled intervals during the intervention phase (baseline, 30 weeks of gestation, delivery, 3 months postpartum, and 6 months postpartum).

Serum calcium is the best available biomarker of vitamin D toxicity⁹²; therefore, hypercalcemia will be the primary biochemical safety parameter in this trial. Nonetheless, based on our own experience with doses equivalent to up to 5000 IU/day (see above) and previously published trials conducted in the USA using doses up to 4000 IU/day during pregnancy⁴¹ and up to 6400 IU/day during lactation⁴⁸, we do not anticipate any episodes of hypercalcemia or other supplement-related adverse events.

- '*Possible hypercalcemia*' will be defined as a single serum calcium concentration >2.60 mmol/L. Serum calcium will be reported back to the study physician within 72 hours of specimen sampling (typically, reporting will occur within 48 hours). Any participant with possible hypercalcemia found through scheduled blood sampling will provide a second sample within 24 hours of the time that the study physician receives a report of an abnormal value. Abnormal serum calcium results will always be reported and confirmed before the time of the following week's dose (to enable a decision to be made about withholding supplementation). Severe derangements in serum calcium will be managed as urgently as possible by a non-study physician at a referral facility.
- '*Confirmed hypercalcemia*' will be defined as serum calcium concentration >2.60 mmol/L on two separate blood specimens, and will be considered the primary supplement-related

adverse event. The second, confirmatory specimen is required to prompt the cessation of study supplementation due to the possibility of a laboratory error, but clinical management need not await the second assay if the participant is symptomatic.

If the repeat serum calcium is normal, then supplementation will continue and a further repeat serum calcium will be measured one week after the first abnormal result, to confirm that it remains within the normal range. In the unlikely event that a participant has confirmed hypercalcemia, study personnel will follow the clinical course until normalization of serum calcium or one month post-partum (whichever occurs later), although medical care (including antenatal, perinatal and neonatal care) and additional non-study biochemical monitoring will be managed by non-study physicians. If medical referral for hypercalcemia is either not accepted or not adhered to by the participant, or non-study physicians do not request serial follow-up serum calcium measurements, then biochemical monitoring deemed appropriate by the study physician (in addition to routine study biochemistry) will be offered to the participant to document resolution of hypercalcemia or provide further justification for referral to a specialist. Participants with mild and asymptomatic hypercalcemia may not require referral or treatment beyond the cessation of supplementation, and will continue to participate in study follow-up. The study physician will be responsible for ensuring that any participant with confirmed or suspected moderate-severe hypercalcemia is assessed at a hospital by a clinician with expertise in the treatment of hypercalcemia, either immediately if indicated clinically or as soon as possible after the second serum calcium result is reported as abnormal (if the participant is otherwise asymptomatic). This assessment, and hospital admission if necessary, will be undertaken at a private clinic/hospital in Dhaka, since the availability of specialist care is inconsistent at public facilities. Costs of care will be covered by the study.

3. Maternal urine calcium:creatinine ratio at delivery.

In previous trials, we used regular monitoring of maternal urinary calcium:creatinine as a screening measure for vitamin D toxicity; however, this test is non-specific, and frequently led to the need for repeat testing, which was burdensome for participants and field staff. In no cases did an isolated calcium-creatinine ratio indicate vitamin D toxicity. Therefore, regular urine calcium:creatinine ratio will not be employed as a primary clinical safety monitoring tool in this study. However, we will measure urinary calcium:creatinine ratio among mothers at delivery, as a screening test for hypercalciuria, rather than overt vitamin D toxicity. Abnormal values (Ca:Crea>1 mmol/mmol) will prompt repeat testing, and either of the following criteria will be considered as a presumptive diagnosis of 'hypercalciuria'

- two consecutive urine samples with Ca:Crea>1 mmol/mmol, or
- one urine sample with Ca:Crea>1 mmol/mmol in the presence of persistent symptoms suggestive of possible uro/nephrolithiasis.

Participants with persistent symptoms of renal colic or hypercalciuria will be referred for renal ultrasound to assess for the presence of urolithiasis or nephrolithiasis (renal stones). Participants with uro- or nephrolithiasis will be referred to the DSMB for a decision regarding unblinding and possible discontinuation of the study supplement, to be decided on a case-by-case basis. Biochemical evidence of hypercalciuria alone will not trigger urgent DSMB review,

but will be reviewed at regular intervals. However, participants with hypercalciuria and an absence of stones will undergo repeat urine calcium:creatinine assessment one month later; if hypercalciuria is persistent, a repeat ultrasound will be undertaken.

h) Methods to promote participant retention and complete follow-up

Participant retention will be primarily promoted through frequent interaction between field-level study personnel and the participants (weekly during phase 1). Adherence to the schedule of clinic visits will be facilitated by compensation of participants for costs of transportation and time away from the home and/or work (equivalent of approximately \$5 USD per visit). The typical per-visit transportation cost to and from the clinic (e.g., by rickshaw) is expected to be 100 to 200 Taka (~\$1.25 to \$2.50). For comparison, the average household income in this low-income area of Dhaka is estimated to be about ~10,000 Taka per month (~\$125/month). A similar level of remuneration is currently being used without complications in ongoing studies in Dhaka. It is generally perceived to be high enough to adequately compensate participants for out-of-pocket expenses and time spent for visits (in transit and at the clinic), but low enough to avoid unduly influencing women to participate. Also, the payment in cash at the time of each visit prevents any undue influence to continue in the study that could occur if payment was delayed. Separate reimbursement may be made to directly compensate for transportation costs required when pregnant participants are in labour, to maximize the probability that pregnant participants will deliver at the Clinic. In many cases, this will not involve payment to participants, but rather direct payment to hired drivers on behalf of participants. Participants may decline some or all of the payments without affecting study participation.

Efforts will be made to obtain complete follow-up data from all enrolled participants. If a participant experiences a serious adverse event (e.g., hospitalization), follow-up and scheduled data collection will continue to the extent that is possible. Even in cases in which supplementation is stopped, data collection may continue, to the extent that the participant continues to consent to a modified form of participation.

i) Definition and handling of protocol deviations and violations

The terms *protocol deviation* and *protocol violation* refer to incorrect actions or omissions by study personnel or omissions of study procedures due to external factors, rather than participant actions, omissions or decisions. The terms are operationally defined as follows, for the purposes of monitoring and reporting of the trial:

- **Protocol deviations** will be considered to be all types of non-compliance with the written protocol by study personnel that do not materially increase the level of risk for individual participants, and do not sufficiently compromise data integrity to the extent to that a participant would need to have supplementation discontinued or be withdrawn from the study.
- **Protocol violations** will be considered to be acts of omission or errors by study personnel that materially increase the risk to one or more participants and/or require discontinuation of study supplementation or, if necessary, withdrawal of the affected

participant from the trial. The withdrawal from the study or discontinuation of supplementation may be justified by protocol non-compliance that seriously compromises the data integrity even if this did not present a serious risk to the individual (e.g., participant was enrolled who did not meet eligibility criteria).

For ease of operationalization, the terms 'deviation' and 'violation' will be considered mutually exclusive, rather than considering violations to be a subset of deviations. The distinction between deviations and violations is not inherently related to whether the act/omission was intentional or accidental. In some cases, a failure to follow the protocol correctly may occur because of events beyond the control of either the investigators, staff or participants (e.g., missed visit due to severe weather storm). Such instances will be referred to as protocol deviations or violations, as deemed otherwise appropriate based on the definitions above.

All protocol deviations and violations will be tracked closely by study personnel and supervisors, and will be recorded using specific data forms. Protocol deviations will, by definition, not require withdrawal of the affected participants, but will be carefully documented. Deviations will be reported in real-time to the ethics boards to the extent that is required by their regulations (e.g., if associated with a serious adverse event). In some cases, review of protocol deviations will result in application for protocol and/or study document amendments. Conversely, protocol violations typically will lead to discontinuation of supplementation (if occurring during Phase 1), and in some cases, may lead to withdrawal of the affected participant from the trial. Protocol violations will be reported to the DSMB and ethics boards to the extent required by the boards' regulations and requirements; an ethics board may only require immediate reporting of protocol violations that are associated with serious adverse events.

As noted above, protocol deviations and violations are errors/omissions by study personnel or those due to external factors. In contrast, participant actions or decisions that result in deviation from the protocol will be referred to as **protocol non-adherence**. This distinction is important because participants are free to voluntarily refuse procedures at any time. In general, protocol non-adherence will not result in withdrawal from the trial, but may in some cases result in discontinuation of study supplementation as a safety precaution; such instances are detailed above (e.g., refusal to discontinue the use of non-study vitamin D or calcium supplements).

19) Data management

Research personnel will record data on standardized data collection forms (paper-based). A site supervisor will review all forms for completeness and protocol deviations/violations before sending them to the data management center at icddr,b on a weekly basis.

The flow of data is shown schematically in the diagram below:

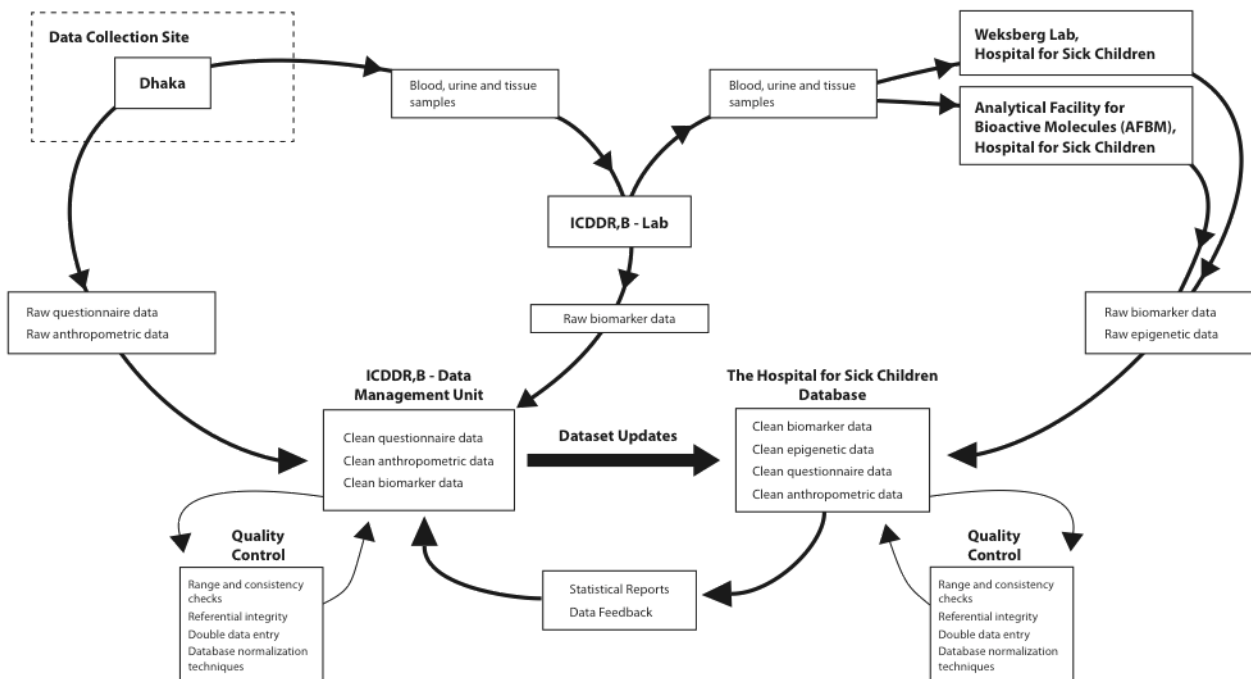


Figure 10: The flow of study data

The database will be designed using SQL Server (back-end) and entered using Visual Basic (front-end). A set of range and consistency checks will be built into the data capture system to provide immediate feedback to data entry personnel regarding errors or inconsistent data. Double data entry will be used to further reduce the rate of data entry errors. Data queries to establish the integrity of the database will be generated on a monthly basis and circulated for review to the investigators.

20) Data analysis

- a) Statistical methods (Aims #1, #2 and #3)

**A detailed data analysis plan is in development, and will be made available prior to the initiation of data analysis.*

The primary outcome measure – length-for-age z-score (LAZ) – will be derived from each length measure (cm) using the WHO child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Length at each visit will be based on the mean of paired measurements. LAZ at “one year” will be based on data from 52 to 60 weeks (364 to 420 days) postnatal (where day 0 is birth). Visit dates will be scheduled based on the number of weeks past the child’s birth date; therefore, the mode of ‘one year’ measurements should be 52 weeks (364 days) postnatal. If an infant has >1 length measurement during the period of 364 to

420 days, the measurement collected closest in time to day 364 will be used in the primary cross-sectional analyses; only one measurement per child will be used in the primary analysis.

For the analysis of the primary trial efficacy outcome (LAZ at one year of age), mean LAZ will be compared across groups using analysis of variance (ANOVA), without adjustment for covariates. To assess the effect of *prenatal* vitamin D on mean LAZ at one year of age, we plan to perform five primary between-group analyses – each vitamin D dose versus placebo (3 pairwise comparisons), as well as comparisons between all adjacent vitamin D doses (2 pairwise comparisons). Because the primary hypotheses relate to differences between two groups, each analysis will be akin to an independent samples t-test. The primary effect measure for each comparison will be expressed as a mean difference between groups with 95% confidence intervals (95% CI). However, an overall alpha for statistical significance for all 5 comparisons will be 0.05 (two-sided), and the Holm test will be used to account for multiple testing. Therefore, even if the 95% CI for a particular mean difference does not include 0, it is possible that it may not be considered statistically significant when all 5 comparisons are presented together.

The ‘postpartum effect’ will be assessed by the comparison of mean LAZ at about one year of age between 28,000 IU/week postpartum versus placebo among women who received 28,000 IU/week antenatally, using a similar statistical approach. The nominal alpha for statistical significance will be 0.05 (two-sided test) for this analysis. Because there is only one primary pairwise comparison related to the post-partum effect, no adjustment for the multiplicity of outcomes is planned.

In the primary analysis, all infants with LAZ at one year will be analysed ‘as randomized’ (i.e., an intent-to-treat approach), irrespective of supplementation adherence, and without imputation for missing data (i.e., infants for whom LAZ is unavailable at one year of age). The primary analyses will not be adjusted for baseline covariates; however, sensitivity analyses will involve adjustment for any covariates that substantially differ at baseline (enrolment), if any. In addition, a missingness analysis will be undertaken to understand the pattern of missing data (in particular to detect differential loss of data across groups), and multiple imputation methods will be used in sensitivity analyses to correct for these losses. Imputation will involve the regression of one-year LAZ on previous LAZ values earlier in infancy, in addition to other important covariates (e.g., gestational age at birth).

All longitudinal analysis of LAZ will employ generalized estimating equations (GEE) with robust variance estimation to account for within-subject correlation of repeated measures over time. Linear regression spline models, with knots at the major scheduled follow-up visit time points, will be used to analyse changes in LAZ over specific time intervals. Interaction terms for time and group allocation will be used to test between-group differences in the changes in LAZ during discrete time intervals.

In secondary analysis of the linear growth outcomes, participants will be classified as ‘stunted’ if $LAZ < -2$. Cross-sectional comparisons between groups with respect to the odds of stunting will be based on estimation of the unadjusted odds ratio, and use of a chi-square test to test the significance of a between-group difference in prevalence. Longitudinal analysis of the odds ratio for stunting will employ GEE, with a logit link and binomial distribution.

With respect to the additional anthropometric outcomes, weight-for-age, weight-for-length, head circumference-for-age, and growth velocity for age z-scores will be similarly derived based on WHO growth standards. For some analyses, birth weight will be adjusted for sex and gestational age using both a recently published country-specific fetal weight reference⁹³ and a US birth weight reference⁹⁴. Individually-customized fetal growth references have not yet been established to provide additional benefits beyond standardization for country/ethnicity⁹³.

Summary measures (e.g., means, frequencies, proportions, incidence rates) and effect estimates (i.e., regression coefficients) will be reported as point estimates and 95% confidence intervals. In general, odds ratios (OR) with corresponding confidence intervals will be used to compare dichotomous variables, and difference in means will be used for analysis of continuous variables. P-values will be reported to three decimal places with p-values less than 0.001 reported as <0.001. Statistical analyses will be performed using the Stata software package, with main analyses of primary outcomes performed in a blinded fashion.

b) Methods for subgroup or adjusted analyses

As noted above, extreme z-scores will be flagged based on the WHO Anthro software (< -6 SD or >6 SD for LAZ), and manually reviewed to ensure they are not the result of data recording or entry errors. Real values that are extreme outlying negative z-scores are expected to be predominantly contributed by infants who had early preterm births (<34 weeks gestation) and very low birth weight (VLBW, <1500 g). Therefore, the preferred approach to sensitivity analyses that address these outliers will be to stratify infants according to surrogate markers of the underlying biological processes leading to these outliers (i.e., early preterm versus term/ late preterm, or categories based on birth weight), rather than stratifying on the outcome itself (i.e., outlier LAZ or not). Although the primary analysis will *a priori* include all infants with one-year LAZ, on an intent-to-treat basis, the empiric distribution of LAZ and the influence of extreme outliers will be explored. Sensitivity analyses will be conducted that, a) exclude early preterm infants, b) include all infants but employ non-parametric methods (e.g., Mann-Whitney U test) or regression methods that are robust against outliers. Using age that is corrected based on gestational age at birth for the assignment of LAZ in preterm infants (rather than their chronological age) is not acceptable in the context of a randomized trial of a prenatal intervention because it induces a spurious temporal relationship between the period of supplementation and the timing of outcome ascertainment. In addition, it would result in substantial loss of early postnatal data.

In addition, sub-group analyses will be considered based on the following covariates:

- gestational age at birth, to isolate the effect of vitamin D on growth, relatively independent of effects on gestational duration.
- Sex, to understand potentially variable impact of the intervention on the growth of boys versus girls
- Maternal baseline vitamin D status
- Maternal body mass index (BMI)
- Supplement adherence (see below)

c) Definition of analysis population relating to protocol non-adherence

The primary analysis will be an intent-to-treat (as randomized) analysis. A per-protocol analysis of the effect of prenatal supplementation will be performed that is restricted to participants who meet the following criteria for acceptable adherence during the prenatal period:

- Consumed at least 90% of all scheduled prenatal doses
- Had no episodes of reported consumption of non-study vitamin D or calcium

d) Analysis of the effect of vitamin D supplementation on infectious disease morbidity (Aim #4)

We will estimate incidence rates of infant postnatal respiratory and diarrheal illness episodes from birth to 6 months of age. Incidence rate ratios will be reported to quantify between-group differences in illness rates, assuming an appropriate count distribution (i.e., Poisson or negative binomial), and accounting for repeated events within the same child using generalized estimating equations (GEE).

e) Biomarker and epigenetic data analyses (Aims #5 and #6)

Upon completion of phase 2a field activities (12-month follow-up completed for all participants), 300 cases (stunted infants) and 300 controls (non-stunted infants) will be randomly selected from among those mother-infants pairs with complete anthropometric datasets and appropriate sets of biological specimens for analyses of a range of biomarkers (outlined above). The control group will be frequency matched to cases based on gestational age at birth (+/- 4 days), sex, and season of birth. Structural equation modelling (path analysis) of biomarker data and newborn/infant anthropometry will be performed to obtain estimates of the epidemiological associations among mediators/modifiers of the vitamin D-parathyroid axis and fetal/infant growth.

For the epigenetic analyses, we will randomly select 50 mother-infant pairs from each prenatal intervention group (total 200) who have complete datasets, including maternal, paternal, cord, and placental specimens that are adequate for epigenetic analyses. Pyrosequencing results will be analyzed using PyroMark software (Qiagen). Statistical analyses will focus on between-group differences in methylation patterns at the CYP27B1 and CYP24A1 loci.

Detailed approaches to the statistical analyses to address aims #4, #5 and #6 are in development.

21) Monitoring

a) Data monitoring

A data and safety monitoring board (DSMB) will be constituted and authorized by the icddr, b Ethical Review Committee (ERC).

b) Interim analyses and stopping rules

There are no formal interim analyses of efficacy or stopping rules planned for this trial.

22) Harms

The primary clinical harm that can be caused by excessive vitamin D ingestion is hypercalcemia. Based on existing data in the study setting, the occurrence of hypercalcemia as a study-related adverse event is not anticipated in this trial. However, clinical and biochemical parameters will be monitored to detect this event. Maternal serum calcium will be monitored at regular intervals (see above), and the occurrence of the clinical features of hypercalcemia will be further monitored by asking participants about the following symptoms on a weekly basis:

- Decreased appetite
- Weight loss
- Vomiting
- Fever or chills
- Constipation
- Abdominal pain
- Excessive thirst
- More frequent urination
- Muscle weakness
- Back, arm, or leg pain
- Confusion
- Depression

Other potential discomforts or inconveniences in the trial include the following: 1) discomfort and the very low risk of bruising or infection from venous blood sampling; 2) the potential emotional effects of some questions we ask regarding health status and previous pregnancies, and 3) the time required for participation. All other procedures in this study, including the clinical assessment procedures, perinatal specimen collection, and neonatal examination present no more than a minimal risk.

With respect to the discomforts/risks related to blood drawing, we will take standard preventive measures, including universal infection control precautions, and we will employ trained phlebotomists or nurses to complete these tasks. The amount of blood to be drawn is low and not harmful.

With respect to the potentially sensitive nature of some of the questions (e.g., asking about prior pregnancy loss), we will ensure participants are able to answer these questions in a private setting (without partner/husband present) and have an opportunity to talk about concerns regarding these issues if they arise after the questioning. Research assistants will be specifically trained to check whether participants were bothered or upset by any of the questions and study staff will be able to arrange for appropriate mental health referrals if necessary.

Reporting of adverse events will employ the following definitions:

Adverse event (AE): Any untoward medical occurrence experienced by a participant. An adverse event does not necessarily have to have a causal relationship with the study intervention. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom or disease temporally associated with the application of an intervention, whether or not considered related to the intervention.

Serious adverse event (SAE): An adverse event qualifies as *serious* if it is accompanied by any of the following complications or outcomes:

- Death
- Life-threatening complication, such that death was averted by medical or surgical interventions (the term "life-threatening" in this context refers to an event in which the patient was at risk of death *at the time of the event*; it does not refer to an event, which hypothetically might have caused death, had it been more severe).
- Inpatient hospitalization or prolongation of an existing hospitalization (other than for uncomplicated delivery).
- Persistent or significant disability/incapacity following the resolution of the acute event. Disability means a substantial disruption of one's ability to carry out normal life functions.
- *In infants*: a diagnosis of cancer, neurological disorder (stroke, seizure disorder, encephalopathy, or structural brain abnormality), or major congenital anomaly whether or not these required inpatient hospitalization.

A study physician will be responsible for coordinating the management and documentation of all suspected or confirmed adverse events reported by field personnel, comprising the following actions:

1. Organizing the medical assessment and management of participants with AEs, in coordination with MCHTI medical personnel who may or may not already be involved in patient care (e.g., they would be necessarily involved if a complication occurs during delivery), including the transfer to a tertiary-care facility if necessary (i.e., all SAEs will require care at a medical facility). All medical care (including diagnostics and treatment), including consultation or admission to a tertiary level hospital if indicated, will be free of charge to the participant or family. For those patients treated at medical facilities, management will be guided by physicians not directly affiliated with the study and will be guided solely by the welfare of the patient.
2. Collection of urine and blood specimens as soon as possible for all participants with suspected or confirmed SAEs; these specimens will preferably be collected prior to medical interventions that would affect the interpretation of any biochemical tests (e.g., obtain a plasma calcium prior to initiation of diuretic therapy) if obtaining the specimen will not interfere in the indicated management of the patient. To the extent that is possible, environmental or genetic reasons for idiosyncratic reactions to vitamin D will be investigated through ancillary testing, but only upon receiving informed consent from the participant where appropriate.
3. Completion of necessary documentation for ethics boards.

4. Immediate (i.e., as soon as possible) reporting of all SAEs to the Dhaka-based principal investigator (or designate). Non-serious AEs will be discussed at least monthly with one designated co-investigator during the course of the study, to monitor patterns of multiple similar AEs not considered serious in individual.
5. Follow-up of the patient status in hospital, and forwarding relevant information to the study coordinator, until complete resolution of the AE.

The potential for a causal association between the study interventions and the SAE will be assessed according to the commonly-employed scheme below:

Determination of association between AE and supplementation

0	NO RELATIONSHIP	Inconsistent timely relationship (e.g., detection prior to participant starting supplementation) or evidence that a symptom, sign or laboratory finding is definitely related to an etiology other than the study supplements.
1	POSSIBLE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and a potential alternative etiology has not been investigated.
2	PROBABLE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and a potential alternative etiology is not apparent on the basis of limited investigations.
3	DEFINITE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and any possible alternative etiologies have been ruled out, or re-challenge with the suspected causative factor led to recurrence of the signs or symptoms.

Prompt reporting to the DSMB and ethics boards will be undertaken for *unanticipated* adverse events, the scope of which will include the following:

- An adverse event that is severe *and* considered to have a probable or very probable relationship to vitamin D supplementation (i.e., is considered a SAE at least probably caused by vitamin D) or another study procedure. SAEs that are explained by obstetric/perinatal complications seen in the study setting (e.g., obstructed labor) and not considered to be related to vitamin D supplementation or study procedures will not be considered 'unanticipated'.
- An atypical severe adverse event that is not readily explained by obstetric/perinatal or complications or other medical events unrelated to supplementation, even if the event would not otherwise be considered a manifestation of vitamin D toxicity or another study procedure.
- A pattern of any adverse events (not necessarily defined as SAEs) that collectively change the risk-benefit calculation for participation.

23) Auditing

An external monitoring plan is planned but details are pending confirmation of funding for these activities.

Ethics and dissemination

24) Research ethics approval

Research ethics committee/institutional review board approval will be obtained from the Hospital for Sick Children Research Ethics Board (REB) and the icddr,b ethical review committee (ERC) prior to initiation of participant contact.

25) Protocol amendments

Any amendments to the protocol will be reviewed and approved by the SickKids REB and icddr,b ERC prior to changes to study procedures. As deemed necessary by the trial steering committee and/or ethical review boards, changes to the protocol will be communicated to the trial participants.

26) Consent or assent

The consent process and all accompanying documents will be in Bangla, the national language of Bangladesh that is universally spoken and understood in Bangladesh. Literate women or their family members will be encouraged to read the form aloud, under the supervision of study personnel. Alternatively, because of variable levels of literacy, consent documents will be read out loud to prospective participants by study personnel if necessary. The project coordinator and study physicians will be required to have successfully completed an on-line ethics training course. All study personnel will be trained to ensure their understanding of the importance of promoting free and voluntary consent.

The participants will be screened for exclusion criteria before providing written consent to participate in the study. A study physician will be primarily responsible for the informed consent process, and in all cases will confirm consent and respond to any questions from participants prior to completion of the process. The initial components of the consent process overlap with detailed eligibility assessment, and thus will be overseen by the physician. However, other trained study workers (i.e., paramedics or research assistants) may assist in providing detailed explanations of study procedures, risks, and benefits to the prospective participants. The informed consent process will be conducted at screening and/or baseline visit in a designated room at the clinic. If a woman is interested in participating in the study, she will be given a consent form and asked to review it with her husband and/or family members. Prospective participants may take several days to consider participation, within the bounds of the gestational age inclusion criterion.

There will be three types of consent requested in this study

1. Primary study participation – this will encompass all maternal and infant activities.
2. Biorepository storage consent
3. Paternal consent – this will be limited to the involvement of the child's biological father, if available, for height/weight measurements and one blood specimen to be collected at or

near the time of enrollment. Fathers may choose to participate in one or both of anthropometry and/or specimen collection.

27) Confidentiality

All data will be collected in a manner that respects participants' privacy and confidentiality. We do not anticipate that any potentially sensitive information concerning health will be generated or disclosed in the context of this study.

28) Declaration of interests

The investigators do not have conflicts of interest to declare.

29) Access to data

The principal investigator and co-investigators will have access to the final trial dataset; there are no contractual agreements that limit such access for investigators.

30) Ancillary and post-trial care

Phase 1 (intervention phase): The study physician will assess all suspected adverse events or medical concerns. An obstetrician and pediatrician at MCHTI will be available for consultation. When medical issues cannot be resolved by MCHTI medical staff, or a situation requires urgent or critical care, referral will be made to a designated tertiary care hospital in Dhaka (Ad-Din Hospital). MCHTI physicians will be primarily responsible for arranging the referral, but study personnel will assist to ensure timely care. When possible, a study worker will accompany participants to the hospital, or meet the participant at the hospital (if the participant leaves directly from home rather than the clinic). In all cases, the study physician will follow and document the course of events, and obtain relevant information from the participant's medical records in order to document any adverse events fully. Costs of essential urgent/emergent medical care at the referral hospital will be covered by the study budget.

Phase 2 (observational phase): Medical care and referral during this period will only be facilitated by study physicians if a clinical issue is discovered at the time of a scheduled visit (e.g., severe malnutrition detected by anthropometry). Costs of initial referral for care will be covered by the study budget. However, medical care costs for problems that arise between scheduled visits will not be routinely covered, and will be considered on a case-by-case basis.

31) Dissemination plans

a) Dissemination policy

Results of the trial will be disseminated widely, without restriction, through scientific conferences and journal publications.

b) Authorship eligibility guidelines and any intended use of professional writers

Authorship eligibility will be based on the guidelines of the International Committee of Medical Journal Editors (http://www.icmje.org/ethical_1author.html).

- c) Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

The full protocol will be made publicly available following review and approval by the trial steering committee, DSMB, and ethical review boards in Canada and Bangladesh.

There are no specific plans at present regarding the mechanisms by which the public will be granted access to datasets and statistical code; however, in principle, public access is supported by the investigators. Requests for datasets and statistical code will be reviewed on a case-by-case basis.

REFERENCES

1. Rajaratnam JK, Marcus JR, Flaxman AD, Wang H, Levin-Rector A, Dwyer L, et al. Neonatal, postneonatal, childhood, and under-5 mortality for 187 countries, 1970-2010: a systematic analysis of progress towards Millennium Development Goal 4. *Lancet*. 2010; 375(9730):1988-2008.
2. George K, Prasad J, Singh D, Minz S, Albert DS, Muliylil J, et al. Perinatal outcomes in a South Asian setting with high rates of low birth weight. *BMC Pregnancy Childbirth*. 2009; 9:5.
3. de Onis M, Blossner M, Borghi E, Morris R, Frongillo EA. Methodology for estimating regional and global trends of child malnutrition. *Int J Epidemiol*. 2004; 33(6):1260-70.
4. Victora CG, Adair L, Fall C, Hallal PC, Martorell R, Richter L, et al. Maternal and child undernutrition: consequences for adult health and human capital. *Lancet*. 2008; 371(9609):340-57.
5. Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet*. 2008; 371(9608):243-60.
6. Victora CG, de Onis M, Hallal PC, Blossner M, Shrimpton R. Worldwide Timing of Growth Faltering: Revisiting Implications for Interventions. *Pediatrics*. 2010; 125(3):e473-80.
7. Evans KN, Bulmer JN, Kilby MD, Hewison M. Vitamin D and placental-decidual function. *J Soc Gynecol Investig*. 2004; 11(5):263-71.
8. Hollis BW, Pittard WB, 3rd. Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences. *J Clin Endocrinol Metab*. 1984; 59(4):652-7.
9. Taylor SN, Wagner CL, Hollis BW. Vitamin D supplementation during lactation to support infant and mother. *J Am Coll Nutr*. 2008; 27(6):690-701.
10. Roth DE, Shah MR, Black RE, Baqui AH. Vitamin D Status of Infants in Northeastern Rural Bangladesh: Preliminary Observations and a Review of Potential Determinants. *Journal of Health, Population and Nutrition*. 2010; 28(5):458-69.
11. Roth DE, Mahmud AA, Raqib R, Akhtar E, Perumal N, Pezzack B, et al. Randomized placebo-controlled trial of high-dose prenatal third-trimester vitamin D3 supplementation in Bangladesh: the AViDD trial. *Nutrition Journal*. 2013; 12:47.
12. Goltzman D. Emerging roles for calcium-regulating hormones beyond osteolysis. *Trends Endocrinol Metab*. 2010; 21(8):512-8.
13. Rummens K, van Bree R, Van Herck E, Zaman Z, Bouillon R, Van Assche FA, et al. Vitamin D deficiency in guinea pigs: exacerbation of bone phenotype during pregnancy and disturbed fetal mineralization, with recovery by 1,25(OH)2D3 infusion or dietary calcium-phosphate supplementation. *Calcif Tissue Int*. 2002; 71(4):364-75.
14. Finch SL, Rauch F, Weiler HA. Postnatal vitamin D supplementation following maternal dietary vitamin D deficiency does not affect bone mass in weanling guinea pigs. *J Nutr*. 2010; 140(9):1574-81.
15. Kovacs CS. Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies. *Am J Clin Nutr*. 2008; 88(2):520S-8S.
16. Rummens K, van Cromphaut SJ, Carmeliet G, van Herck E, van Bree R, Stockmans I, et al. Pregnancy in mice lacking the vitamin D receptor: normal maternal skeletal response, but fetal hypomineralization rescued by maternal calcium supplementation. *Pediatr Res*. 2003; 54(4):466-73.
17. Kovacs CS, Woodland ML, Fudge NJ, Friel JK. The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice. *Am J Physiol Endocrinol Metab*. 2005; 289(1):E133-44.
18. Soliman AT, Al Khalaf F, Alhemaidi N, Al Ali M, Al Zyoud M, Yakoot K. Linear growth in relation to the circulating concentrations of insulin-like growth factor I, parathyroid hormone, and 25-hydroxy vitamin D in children with nutritional rickets before and after treatment: endocrine adaptation to vitamin D deficiency. *Metabolism*. 2008; 57(1):95-102.

19. Weisberg P, Scanlon KS, Li R, Cogswell ME. Nutritional rickets among children in the United States: review of cases reported between 1986 and 2003. *Am J Clin Nutr.* 2004; 80(6 Suppl):1697S-705S.
20. Rajah J, Jubeh JA, Haq A, Shalash A, Parsons H. Nutritional rickets and z scores for height in the United Arab Emirates: to D or not to D? *Pediatr Int.* 2008; 50(4):424-8.
21. Bereket A, Cesur Y, Ozkan B, Adal E, Turan S, Onan SH, et al. Circulating insulin-like growth factor binding protein-4 (IGFBP-4) is not regulated by parathyroid hormone and vitamin D in vivo: evidence from children with rickets. *J Clin Res Pediatr Endocrinol.* 2010; 2(1):17-20.
22. Kumar GT, Sachdev HS, Chellani H, Rehman AM, Singh V, Arora H, et al. Effect of weekly vitamin D supplements on mortality, morbidity, and growth of low birthweight term infants in India up to age 6 months: randomised controlled trial. *BMJ.* 2011; 342:d2975.
23. Brooke OG, Brown IR, Bone CD, Carter ND, Cleeve HJ, Maxwell JD, et al. Vitamin D supplements in pregnant Asian women: effects on calcium status and fetal growth. *Br Med J.* 1980; 280(6216):751-4.
24. Brooke OG, Butters F, Wood C. Intrauterine vitamin D nutrition and postnatal growth in Asian infants. *Br Med J (Clin Res Ed).* 1981; 283(6298):1024.
25. Kalra P, Das V, Agarwal A, Kumar M, Ramesh V, Bhatia E, et al. Effect of vitamin D supplementation during pregnancy on neonatal mineral homeostasis and anthropometry of the newborn and infant. *The British journal of nutrition.* 2012; 108(6):1052-8.
26. 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.* 2011:n/a-n/a.
27. Kovacs CS. Bone development in the fetus and neonate: role of the calciotropic hormones. *Curr Osteoporos Rep.* 2011; 9(4):274-83.
28. Bond H, Dilworth MR, Baker B, Cowley E, Requena Jimenez A, Boyd RD, et al. Increased maternofetal calcium flux in parathyroid hormone-related protein-null mice. *J Physiol.* 2008; 586(7):2015-25.
29. Waller S. Parathyroid hormone and growth in chronic kidney disease. *Pediatr Nephrol.* 2011; 26(2):195-204.
30. Yamshchikov AV, Desai NS, Blumberg HM, Ziegler TR, Tangpricha V. Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists.* 2009; 15(5):438-49.
31. Roth DE, Shah R, Black RE, Baqui AH. Vitamin D status and acute lower respiratory infection in early childhood in Sylhet, Bangladesh. *Acta Paediatr.* 2010; 99(3):389-93.
32. Ish-Shalom S, Segal E, Salganik T, Raz B, Bromberg IL, Vieth R. Comparison of daily, weekly, and monthly vitamin D3 in ethanol dosing protocols for two months in elderly hip fracture patients. *J Clin Endocrinol Metab.* 2008; 93(9):3430-5.
33. De-Regil LM, Palacios C, Ansary A, Kulier R, Pena-Rosas JP. Vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev.* 2012; 2:CD008873.
34. Ross AC, Taylor CL, Yaktine AL, Del Valle HB. Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, Institute of Medicine. *Dietary Reference Intakes for Calcium and Vitamin D.* Washington, DC: The National Academies Press; 2010.
35. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab.* 2011; 96(1):53-8.
36. Shrimpton R, Huffman SL, Zehner ER, Darnton-Hill I, Dalmiya N. Multiple micronutrient supplementation during pregnancy in developing-country settings: policy and program implications of the results of a meta-analysis. *Food Nutr Bull.* 2009; 30(4 Suppl):S556-73.
37. Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. *DRI: Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and*

- Fluoride. Food and Nutrition Board IOM. Washington: National Academy Press; 1997. Available from: http://www.nap.edu/openbook.php?record_id=5776.
38. Roth DE. Vitamin D supplementation during pregnancy: safety considerations in the design and interpretation of clinical trials. *J Perinatol*. 2011; 31(7):449-59.
 39. Sahu M, Das V, Aggarwal A, Rawat V, Saxena P, Bhatia V. Vitamin D replacement in pregnant women in rural north India: a pilot study. *Eur J Clin Nutr*. 2009; 63(9):1157-9.
 40. Yu CK, Sykes L, Sethi M, Teoh TG, Robinson S. Vitamin D deficiency and supplementation during pregnancy. *Clin Endocrinol (Oxf)*. 2009; 70(5):685-90.
 41. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: Double blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res*. 2011; 26(10):2341-57.
 42. Godel JC, Irvine J, Onyett H, Saylor K, Schroter H, Young M, et al. Vitamin D supplementation: Recommendations for Canadian mothers and infants. *Paediatrics & Child Health*. 2007; 12(7):583-9.
 43. ACOG Committee Opinion No. 495: Vitamin D: Screening and supplementation during pregnancy. *Obstet Gynecol*. 2011; 118(1):197-8.
 44. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *The Journal of clinical endocrinology and metabolism*. 2011; 96(7):1911-30.
 45. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am J Clin Nutr*. 2003; 77(1):204-10.
 46. Aloia JF, Patel M, Dimaano R, Li-Ng M, Talwar SA, Mikhail M, et al. Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration. *Am J Clin Nutr*. 2008; 87(6):1952-8.
 47. Roth D, Mahmud A, Raqib R, Akhtar E, Black R, Baqui A. Pharmacokinetics of high-dose weekly oral vitamin D3 supplementation during the third trimester of pregnancy in Dhaka, Bangladesh. *Nutrients*. 2013.
 48. Wagner CL, Hulsey TC, Fanning D, Ebeling M, Hollis BW. High-dose vitamin D3 supplementation in a cohort of breastfeeding mothers and their infants: a 6-month follow-up pilot study. *Breastfeed Med*. 2006; 1(2):59-70.
 49. Roth DE, Mahmud A, Raqib R, Black RE, Baqui AH. Pharmacokinetics of a single oral dose of vitamin D3 (70,000 IU) in pregnant and non-pregnant women. *Nutr J*. 2012; 11(1):114.
 50. Ilahi M, Armas LA, Heaney RP. Pharmacokinetics of a single, large dose of cholecalciferol. *Am J Clin Nutr*. 2008; 87(3):688-91.
 51. Vieth R. How to optimize vitamin D supplementation to prevent cancer, based on cellular adaptation and hydroxylase enzymology. *Anticancer Res*. 2009; 29(9):3675-84.
 52. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA*. 2010; 303(18):1815-22.
 53. Wagner D, Sidhom G, Whiting SJ, Rousseau D, Vieth R. The bioavailability of vitamin D from fortified cheeses and supplements is equivalent in adults. *J Nutr*. 2008; 138(7):1365-71.
 54. Islam MZ, Lamberg-Allardt C, Karkkainen M, Ali SM. Dietary calcium intake in premenopausal Bangladeshi women: do socio-economic or physiological factors play a role? *Eur J Clin Nutr*. 2003; 57(5):674-80.
 55. Arsenault JE, Yakes EA, Islam MM, Hossain MB, Ahmed T, Hotz C, et al. Very Low Adequacy of Micronutrient Intakes by Young Children and Women in Rural Bangladesh Is Primarily Explained by Low Food Intake and Limited Diversity. *The Journal of Nutrition*. 2013; 143(2):197-203.

56. Hels O, Hassan N, Tetens I, Haraksingh Thilsted S. Food consumption, energy and nutrient intake and nutritional status in rural Bangladesh: changes from 1981-1982 to 1995-96. *Eur J Clin Nutr.* 2003; 57(4):586-94.
57. World Health Organization. WHO child growth standards : length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age : methods and development. Geneva: World Health Organization; 2006.
58. World Health Organization., Development. WHONfHa. WHO child growth standards : head circumference-for-age, arm circumference-for-age, triceps skinfold-for-age and subscapular skinfold-for-age : methods and development. Geneva: World Health Organization; 2007.
59. de Onis M, Onyango AW, Van den Broeck J, Chumlea WC, Martorell R. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. *Food Nutr Bull.* 2004; 25(1 Suppl):S27-36.
60. National Health and Nutrition Examination Survey. Anthropometry Procedures Manual. Center for Disease Control and Prevention; 2007. Available from: http://www.cdc.gov/nchs/data/nhanes/nhanes_07_08/manual_an.pdf.
61. Seamans KM, Cashman KD. Existing and potentially novel functional markers of vitamin D status: a systematic review. *Am J Clin Nutr.* 2009; 89(6):1997S-2008.
62. Melamed ML, Eustace JA, Plantinga LC, Jaar BG, Fink NE, Parekh RS, et al. Third-generation parathyroid hormone assays and all-cause mortality in incident dialysis patients: the CHOICE study. *Nephrol Dial Transplant.* 2008; 23(5):1650-8.
63. Kazama JJ, Omori K, Higuchi N, Takahashi N, Ito Y, Maruyama H, et al. Intact PTH assay overestimates true 1-84 PTH levels after maxacalcitol therapy in dialysis patients with secondary hyperparathyroidism. *Nephrol Dial Transplant.* 2004; 19(4):892-7.
64. Murray TM, Rao LG, Divieti P, Bringhurst FR. Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl-terminal ligands. *Endocr Rev.* 2005; 26(1):78-113.
65. Waller SC, Ridout D, Cantor T, Rees L. Parathyroid hormone and growth in children with chronic renal failure. 2005; 67(6):2338-45.
66. Martin A, David V, Quarles LD. Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev.* 2012; 92(1):131-55.
67. Braithwaite V, Jarjou LMA, Goldberg GR, Jones H, Pettifor JM, Prentice A. Follow-up study of Gambian children with rickets-like bone deformities and elevated plasma FGF23: Possible aetiological factors. *Bone.* 2012; 50(1):218-25.
68. Kronenberg HM. PTHrP and skeletal development. *Ann N Y Acad Sci.* 2006; 1068:1-13.
69. Budayr AA, Halloran BP, King JC, Diep D, Nissenson RA, Strewler GJ. High levels of a parathyroid hormone-like protein in milk. *Proc Natl Acad Sci U S A.* 1989; 86(18):7183-5.
70. Thota CS, Reed LC, Yallampalli C. Effects of Parathyroid Hormone Like Hormone (PTHrP) Antagonist, PTHrP(7-34), on Fetoplacental Development and Growth During Midgestation in Rats. *Biology of Reproduction.* 2005; 73(6):1191-8.
71. Briana DD, Boutsikou M, Baka S, Hassiakos D, Gourgiotis D, Marmarinos A, et al. N-terminal parathyroid hormone-related protein levels in human intrauterine growth restricted pregnancies. *Acta Obstetrica et Gynecologica Scandinavica.* 2007; 86(8):945-9.
72. Liu J, Lv F, Sun W, Tao C, Ding G, Karaplis A, et al. The Abnormal Phenotypes of Cartilage and Bone in Calcium-Sensing Receptor Deficient Mice Are Dependent on the Actions of Calcium, Phosphorus, and PTH. *PLoS Genet.* 2011; 7(9):e1002294.
73. Zivicnjak M, Schnabel D, Billing H, Staude H, Filler G, Querfeld U, et al. Age-related stature and linear body segments in children with X-linked hypophosphatemic rickets. *Pediatr Nephrol.* 2011; 26(2):223-31.
74. Khadilkar A, Mughal MZ, Hanumante N, Sayyad M, Sanwalka N, Naik S, et al. Oral calcium supplementation reverses the biochemical pattern of parathyroid hormone resistance in underprivileged Indian toddlers. *Arch Dis Child.* 2009; 94(12):932-7.

75. Sanchez CP, He YZ, Leiferman E, Wilsman NJ. Bone elongation in rats with renal failure and mild or advanced secondary hyperparathyroidism. *Kidney Int.* 2004; 65(5):1740-8.
76. Asvold BO, Eskild A, Jenum PA, Vatten LJ. Maternal Concentrations of Insulin-like Growth Factor I and Insulin-like Growth Factor Binding Protein 1 During Pregnancy and Birth Weight of Offspring. *American Journal of Epidemiology.* 2011; 174(2):129-35.
77. Akram SK, Carlsson-Skwirut C, Bhutta ZA, Söder O. Placental IGF-I, IGFBP-1, zinc, and iron, and maternal and infant anthropometry at birth. *Acta Paediatrica.* 2011; 100(11):1504-9.
78. Mamabolo RL, Alberts M, Levitt NS, Delemarre-van de Waal HA, Steyn NP. Association between insulin-like growth factor-1, insulin-like growth factor-binding protein-1 and leptin levels with nutritional status in 1-3-year-old children, residing in the central region of Limpopo Province, South Africa. *Br J Nutr.* 2007; 98(4):762-9.
79. Calvo MS, Kumar R, Heath H, 3rd. Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. *J Clin Endocrinol Metab.* 1988; 66(4):823-9.
80. Paik JM, Curhan GC, Forman JP, Taylor EN. Determinants of plasma parathyroid hormone levels in young women. *Calcif Tissue Int.* 2010; 87(3):211-7.
81. Braithwaite V, Jarjou LMA, Goldberg GR, Prentice A. Iron status and fibroblast growth factor-23 in Gambian children. *Bone.* (0).
82. Kippler M, Goessler W, Nermell B, Ekstrom EC, Lonnerdal B, El Arifeen S, et al. Factors influencing intestinal cadmium uptake in pregnant Bangladeshi women--a prospective cohort study. *Environ Res.* 2009; 109(7):914-21.
83. Khandare AL, Harikumar R, Sivakumar B. Severe bone deformities in young children from vitamin D deficiency and fluorosis in Bihar-India. *Calcif Tissue Int.* 2005; 76(6):412-8.
84. Novakovic B, Sibson M, Ng HK, Manuelpillai U, Rakyant V, Down T, et al. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem.* 2009; 284(22):14838-48.
85. Christian P, Jiang T, Khatry SK, LeClerq SC, Shrestha SR, West KP, Jr. Antenatal supplementation with micronutrients and biochemical indicators of status and subclinical infection in rural Nepal. *Am J Clin Nutr.* 2006; 83(4):788-94.
86. Green R. Indicators for assessing folate and vitamin B-12 status and for monitoring the efficacy of intervention strategies. *The American Journal of Clinical Nutrition.* 2011; 94(2):666S-72S.
87. Stephensen CB. Burden of infection on growth failure. *The Journal of Nutrition.* 1999; 129(2S Suppl):534S-8S.
88. Sainaghi PP, Bellan M, Antonini G, Bellomo G, Pirisi M. Unsuppressed parathyroid hormone in patients with autoimmune/inflammatory rheumatic diseases: implications for vitamin D supplementation. *Rheumatology (Oxford).* 2011.
89. Angeletti RH, D'Amico T, Ashok S, Russell J. The Chemokine Interleukin-8 Regulates Parathyroid Secretion. *Journal of Bone and Mineral Research.* 1998; 13(8):1232-7.
90. Liu NQ, Kaplan AT, Lagishetty V, Ouyang YB, Ouyang Y, Simmons CF, et al. Vitamin D and the regulation of placental inflammation. *J Immunol.* 2011; 186(10):5968-74.
91. Choufani S, Shapiro JS, Susiarjo M, Butcher DT, Grafodatskaya D, Lou Y, et al. A novel approach identifies new differentially methylated regions (DMRs) associated with imprinted genes. *Genome Res.* 2011; 21(3):465-76.
92. Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. *Am J Clin Nutr.* 2007; 85(1):6-18.
93. Mikolajczyk RT, Zhang J, Betran AP, Souza JP, Mori R, Gulmezoglu AM, et al. A global reference for fetal-weight and birthweight percentiles. *Lancet.* 2011; 377(9780):1855-61.
94. Oken E, Kleinman KP, Rich-Edwards J, Gillman MW. A nearly continuous measure of birth weight for gestational age using a United States national reference. *BMC pediatrics.* 2003; 3:6.

Appendices

32) Informed consent materials

See attached.

33) Biological specimens

		Phase 1a				Phase 1b			
		Maternal baseline	Maternal 30 weeks	Maternal delivery	Cord blood	Maternal 3 months	Infant 3 months	Maternal 6 months	Infant 6 months
Blood/ Serum/ Plasma	Calcium	X	X	X	X	X	X	X	X
	Phosphate								X
	Alkaline phosphatase								X
	Creatinine								X
	25(OH)D	X		X	X		X	X	X
	1,25(OH)2D	X		X	X			X	X
	Whole/bioactive PTH(1-84)	X		X	X			X	X
	C-terminal PTH(73-84)	X		X	X			X	X
	PTH(1-34) (intact PTH)	X		X	X			X	X
	PTHrP	X		X	X	X	X	X	X
	FGF-23	X		X	X			X	X
	IGF-1				X				X
	IGFBP-1 & 3				X				X
	Ferritin								X
	Retinol			X					
	Folate			X					
	Cadmium			X					
	C-reactive protein			X	X				
	IL-6, IL-8, TNF-alpha			X	X				
	<i>Biobank</i>	X	X	X	X	X	X	X	X
Urine	Creatinine			X					X
	Calcium			X					X
	Phosphate								X
	Fluoride								X
	Cyclic AMP								X
	<i>Biobank</i>	X		X					X
Breast milk	PTHrP					X		X	
	<i>Biobank</i>					X		X	

In addition, a single paternal blood specimen will be collected for epigenetic analyses.

**Randomized placebo-controlled trial of maternal vitamin D
supplementation during pregnancy and lactation to improve
infant linear growth in Dhaka, Bangladesh**

“Maternal Vitamin D for Infant Growth (MDIG) trial”

Table of Contents

Study Summary	4
1) Title	5
2) Trial registration	5
3) Protocol version	5
4) Funding	5
5) Roles and Responsibilities	5
a) Names, affiliations, and roles of protocol contributors	5
b) Name and contact information for the trial sponsor	6
c) Role of study sponsor and funders	6
d) Composition, roles, and responsibilities of governance/monitoring bodies	7
6) Introduction	7
a) Background and rationale	7
b) Explanation for choice of active agents and comparators	12
7) Objectives	15
a) Primary aims:	15
b) Secondary aims:	15
8) Trial design	16
9) Study setting	16
10) Participant eligibility criteria	17
a) Inclusion criteria:	17
b) Exclusion criteria:	18
11) Interventions	18
a) Intervention description	18
b) Criteria for discontinuing or modifying allocated interventions	19
c) Strategies to improve adherence to intervention protocols, and procedures for monitoring adherence	21
d) Relevant concomitant care and interventions that are permitted or prohibited during the trial	21
12) Outcomes	23
13) Participant timeline	27
14) Sample size	29
15) Recruitment	30
b) Provisional screening	30
c) Triage and tracking of provisionally eligible women	31
d) Detailed screening (of provisionally eligible women)	31
16) Allocation of intervention	32
a) Sequence generation	32
b) Allocation concealment mechanism	32
c) Implementation of allocation	33

17) Blinding (masking)	33
a) Who will be blinded, and how	33
b) Conditions and procedures for unblinding	33
18) Data collection	33
a) Sequence and content of study visits	33
b) Verbal and clinical data collection methods (including point-of-care tests).....	37
c) Biological specimen collection and processing	40
d) Biomarker analyses	45
e) Epigenetic analyses	49
f) Data quality control.....	49
g) Safety monitoring.....	50
h) Methods to promote participant retention and complete follow-up.....	52
i) Definition and handling of protocol deviations and violations.....	53
19) Data management	54
20) Data analysis	55
a) Statistical methods (Aims #1, #2 and #3).....	55
b) Methods for subgroup or adjusted analyses	56
c) Definition of analysis population relating to protocol non-adherence	57
d) Analysis of the effect of vitamin D supplementation on infectious disease morbidity (Aim #4).....	57
e) Biomarker and epigenetic data analyses (Aims #5 and #6)	57
21) Monitoring.....	58
a) Data monitoring.....	58
b) Interim analyses and stopping rules.....	58
22) Harms	58
23) Auditing.....	61
24) Research ethics approval.....	61
25) Protocol amendments.....	61
26) Consent or assent.....	61
27) Confidentiality	62
28) Declaration of interests	62
29) Access to data	62
30) Ancillary and post-trial care	63
31) Dissemination plans	63
a) Dissemination policy	63
b) Authorship eligibility guidelines and any intended use of professional writers	63
c) Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	63
32) Informed consent materials	69
33) Biological specimens.....	70

1) Study Summary

Title	Randomized placebo-controlled trial of maternal vitamin D supplementation during pregnancy and lactation to improve infant linear growth in Dhaka, Bangladesh		
Short Title	Maternal vitamin D for infant growth (MDIG) trial		
Clinicaltrials.gov	NCT01924013		
Design	Randomized placebo-controlled dose-ranging trial		
Study Duration	4 years (2014 – 2018)		
Study site	Dhaka, Bangladesh		
Primary Objective	To determine whether maternal prenatal vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.		
Study Intervention	Group	Prenatal vitamin D3 supplement starting at 17-24 weeks gestation	Postpartum vitamin D3 for during period of lactation (to 6 months)
	A	0 IU (placebo)	0 IU (placebo)
	B	4,200 IU/week	0 IU (placebo)
	C	16,800 IU/week	0 IU (placebo)
	D	28,000 IU/week	0 IU (placebo)
	E	28,000 IU/week	28,000 IU/week
Primary Outcome	Infant length-for-age z-score (LAZ) at one year of age, based on WHO standards		
# of Subjects	1300		
Main Inclusion Criteria	<ul style="list-style-type: none"> • Age 18 years and above; • 17 to 24 completed weeks of gestation based on recalled last menstrual period (LMP) and/or 2nd trimester ultrasound; • Intends to permanently reside in the trial catchment area for at least 18 months 		
Main Exclusion Criteria	<ul style="list-style-type: none"> • History of medical conditions that may predispose the participant to vitamin D sensitivity, altered vitamin D metabolism and/or hypercalcemia, or history of renal calculi • Current high-risk pregnancy based on severe anemia, proteinuria, or hypertension • Multiple gestation, major congenital anomaly, or severe oligohydramnios based on maternal history and/or ultrasound • Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin with calcium and/or vitamin D. • Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency • Previous participation in the same study 		
Follow-up period	Prenatal: Enrolment (17-24 weeks gestation) until delivery Postnatal: Birth to 24 months of age		
Main Study Procedures	<ol style="list-style-type: none"> 1. Questionnaires 2. Anthropometry 3. Obstetric ultrasound 4. Specimen collection (Blood, urine, placenta, umbilical cord, breast milk, nasal swabs) 5. Morbidity surveillance 		

2) Title

Randomized placebo-controlled trial of maternal vitamin D supplementation during pregnancy and lactation to improve infant linear growth in Dhaka, Bangladesh.

Short title and acronym: **Maternal Vitamin D for Infant Growth (MDIG)** trial

Gates Foundation Project Name and #: Parathyroid-vitamin D axis dysregulation in early-onset infant stunting in resource-poor settings (OPP1066764)

3) Trial registration

Clinicaltrials.gov registration: NCT01924013

4) Protocol version

Version 1.0 – May 22nd, 2013

Version 1.1 – July 8th, 2013

Version 2.0 – September 20th, 2013

Version 3.0 – July 4th, 2014

Version 3.1 – April 21, 2015

Version 3.2 – November 12, 2015

Version 3.3-June 28, 2016

Version 3.4- March 5, 2017

5) Funding

Bill and Melinda Gates Foundation – Healthy Growth program

Program officer: Jeffrey Murray

6) Roles and Responsibilities

a) Names, affiliations, and roles of protocol contributors

Role	Name	Institution
Principal Investigator	Daniel Roth	Hospital for Sick Children
Co-Investigator	Stanley Zlotkin	Hospital for Sick Children
Co-Investigator	Shaun Morris	Hospital for Sick Children
Co-Investigator	Rosanna Weksberg	Hospital for Sick Children
Co-Investigator / Statistician	Andy Willan	Hospital for Sick Children
Co-Investigator	Kellie Murphy	Mount Sinai Hospital
Co-Investigator	Prakesh Shah	Mount Sinai Hospital
Co-Investigator (Site PI)	Abdullah Al Mahmud	icddr,b
Co-Investigator	Tahmeed Ahmed	icddr,b
Co-Investigator	Munir Islam	icddr,b
Co-investigator	Alison Gernand	Pennsylvania State University
Co-investigator / consultant	Rashed Shah	Save the Children, USA
Project Manager	Eszter Papp	Hospital for Sick Children

b) Name and contact information for the trial sponsor**Principal Investigator: Daniel Roth**

Address: 686 Bay Street Toronto, ON M5G 0A4
Email: daniel.roth@sickkids.ca
Phone: 416-813-7654 X328807
Fax: 416-813-6520

Project Manager: Eszter Papp

Address: 686 Bay Street Toronto, ON M5G 0A4
Email: eszter.papp@sickkids.ca
Phone: 416-813-5793 X302025
Fax: 416-813-6520

Budget/Finance Contact: Jamie La

Address: 686 Bay Street Toronto, ON M5G 0A4
Email: jamie.la@sickkids.ca
Phone: 416-813-7654 X301724
Fax: 416-813-5085

Bangladesh Site Principal Investigator: Abdullah Al Mahmud

Address: Centre for Child and Adolescent Health, icddr,b
68, Shaheed Tajuddin Ahmed Sarani, Mohakhali
Dhaka 1212, Bangladesh
Email: mahmud@icddrb.org
Phone: +880-2-9827001-10 Extension: 3807 OR 9827043

c) Role of study sponsor and funders*Study Sponsor/PI:*

- overall responsibility for study design;
- collection, management, analysis, and interpretation of data;
- writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities

Study funder:

- Advisory role in study design.
- No role in collection, management, analysis, and interpretation of data;
- No role in writing of the report; and the decision to submit the report for publication,
- No authority over any of these activities

d) Composition, roles, and responsibilities of governance/monitoring bodies

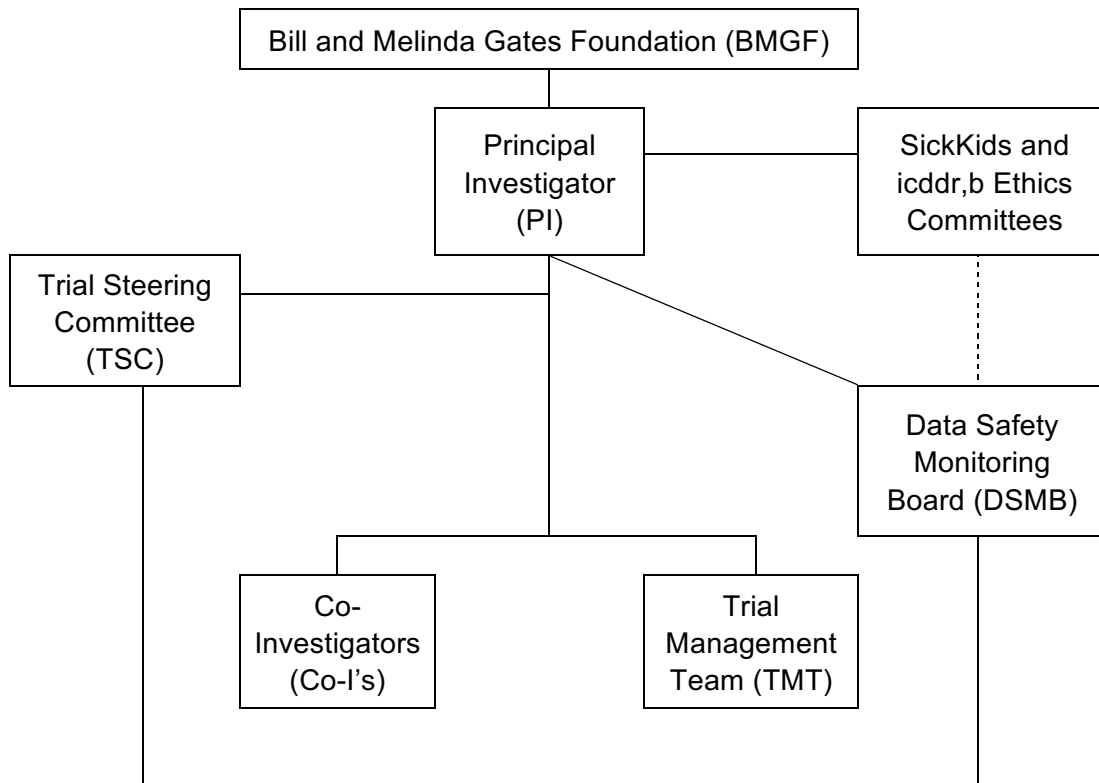


Figure 1: See Appendix for details of mandates and composition.

7) Introduction

a) Background and rationale

Worldwide, nearly 8 million children under the age of 5 die each year, predominantly in low-income countries in South Asia and sub-Saharan Africa¹. Although preventable child deaths are a persistent global health challenge, many countries in East and South Asia, including Bangladesh, have witnessed remarkable reductions in under-5 mortality rates over the past several decades; in fact, Bangladesh is on track to meet the United Nations Millennium Development Goal #4 (MDG-4) – a two-thirds reduction in child mortality between 1990 and 2015¹. Despite progress towards achieving the MDG-4 goal, fetal, infant and child rates of undernutrition in South Asia have been slower to decline^{2,3}, suggesting that current child health programs are not adequate to influence the high rates of stunting (i.e., sex- and age-adjusted height or length less than 2 standard deviations below the median, as established by the World Health Organization growth standards).

Fetal and early childhood growth – particularly during the “first 1000 days” of pre- and postnatal development – has a profound effect on infectious disease susceptibility, mortality, and long-term functional and social outcomes⁴. In Bangladesh, it has been estimated that about one-third

of term infants are low birth weight (LBW; defined as weight less than 2500 grams)⁵, a surrogate marker of intrauterine growth restriction (IUGR). Birth size strongly predicts postnatal growth, and growth faltering in low-income settings begins early (i.e., within the first 3 months of life⁶). In Bangladesh, stunting was estimated to affect nearly one-half of children younger than 5 years of age in 2005⁵. And, recent data suggest that the prevalence has remained virtually unchanged – 43% according to the Bangladesh demographic health survey (BDHS) 2007 and 41% according to BDHS 2011. The early onset of linear growth faltering (stunting) in low-income countries⁶, in spite of breast-feeding, strongly suggests that epigenetic prenatal events, nutritional and endocrine factors are the predominant causes of suboptimal growth in the first months of life. However, the causal pathways implicated in early childhood stunting in low-income settings remain poorly understood, limiting the ability of the public health community to design targeted interventions.

Vitamin D, parathyroid hormone (PTH), and parathyroid hormone-related peptide (PTHrP) are well-established endocrine modulators of bone mineral metabolism and skeletal development. However, there is surprisingly scant research addressing the role of the parathyroid-vitamin D axis in healthy infant growth. This trial will test our hypothesis that dysregulation of the parathyroid-vitamin D axis in the antenatal and early

postnatal period is an important and modifiable cause of linear growth faltering in resource-poor settings (**Figure 2**). ‘Dysregulation’ of the axis refers to: (1) a state of suboptimal vitamin D status (biochemical vitamin D deficiency, indicated by low circulating concentrations of 25-hydroxyvitamin D), (2) compensatory parathyroid gland hyperactivity marked by up-regulated PTH secretion, and possibly, (3) impaired PTHrP activity. In addition to the postulated direct effects of circulating maternal vitamin D metabolites on placental function⁷, maternal prenatal vitamin D status determines fetal/newborn vitamin D stores⁸ and maternal postpartum vitamin D intake determines breastfeeding infants’ vitamin D status⁹. Therefore, prenatal and postpartum maternal vitamin D supplementation is a feasible approach to influence vitamin D-dependent growth mechanisms in utero and during lactation.

There is a high prevalence of biochemical vitamin D deficiency among women and young infants in South Asia¹⁰. In Dhaka, we observed that 34% of pregnant women enrolled at 26 to 29 weeks gestation (N=160) had serum 25-hydroxyvitamin D (25(OH)D) concentrations less than 30 nmol/L (a conventional definition of severe vitamin D deficiency), while 64% had 25(OH)D<50 nmol/L, the threshold for sufficiency recommended by the US Institute of Medicine (IOM). In the

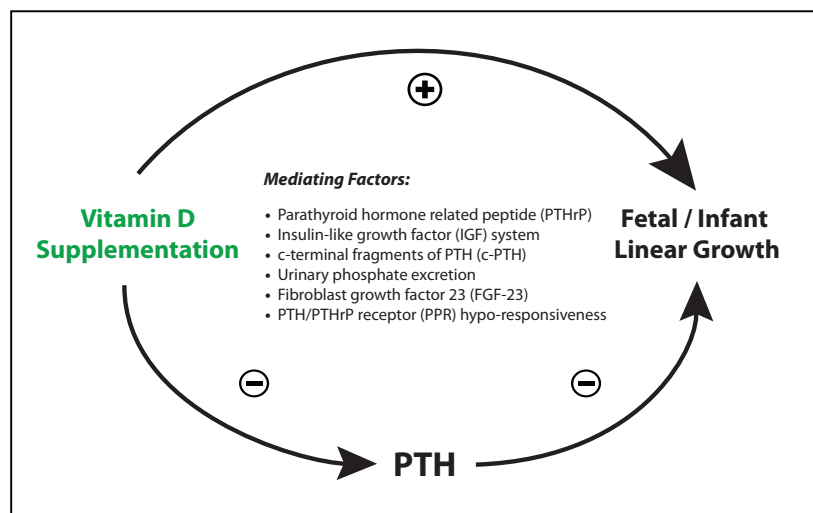


Figure 2: Conceptual framework showing the primary hypothesized relationships among vitamin D supplementation, parathyroid activity and fetal/infant growth, as well as factors postulated to mediate the effects of vitamin D and PTH on growth.

same study, 31% of newborns born to unsupplemented mothers had cord blood 25(OH)D<30 nmol/L and 81% had 25(OH)D<50 nmol/L¹¹. Nearly all women and newborns were deficient when a threshold of 25(OH)D<80 nmol/L was applied, which is a cut-off level proposed by some vitamin D researchers. In rural Sylhet, Bangladesh our research revealed that infants aged 1 to 6 months of age had a mean serum 25(OH)D concentration of 37 nmol/L (95% confidence interval, 30 to 43), and that the proportion of infants with 25(OH)D <25 nmol/L was 28% (95% confidence interval, 10 to 45)¹⁰. These findings suggest that vitamin D deficiency is not limited to urban areas.

Vitamin D and PTH are dominant endocrine regulators of bone mineral homeostasis through their direct and indirect actions on the kidney, intestine and bone¹². PTH is primarily secreted in response to a decrease in the serum calcium concentration, and acts to maintain the serum calcium concentration within a narrow physiological range by mobilizing calcium from bone, increasing intestinal calcium absorption, and decreasing renal calcium excretion. PTH and vitamin D are closely linked together through complex feedback loops. In particular, PTH regulates the renal conversion of the predominant circulating form of vitamin D (25-hydroxyvitamin D; 25(OH)D) to the active metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D), which is the primary hormonal mediator of intestinal calcium absorption. Yet, vitamin D also regulates PTH secretion, such that declines in serum 25(OH)D (the biomarker of vitamin D status) are associated with up-regulation of the parathyroid gland's release of PTH. Animal data support the hypothesis that maternal antenatal vitamin D status has important effects on fetal growth. Offspring of vitamin D-deficient guinea pigs have comparatively low birth weights, shorter lengths, reduced bone mineral content, and abnormal skeletal development at birth^{13,14}. Offspring born to mice that lacked the gene for the vitamin D receptor (VDR-null mice) had low birth weights¹⁵ and impaired skeletal mineralization, primarily due to impaired maternal intestinal calcium absorption¹⁶. However, when the mouse fetuses were themselves VDR-knockouts, they were able to maintain normal serum fetal calcium concentrations, normal PTH concentrations, and normal growth in VDR-expressing mothers¹⁷. These findings suggest that adequate availability of the vitamin D metabolites in the maternal circulation may be more important for fetal growth than the direct effects of fetal vitamin D status.

The hypothesis that vitamin D deficiency and parathyroid hyperactivity adversely affect postnatal linear growth in humans has been most strongly suggested by data from studies of children with nutritional rickets; the classic metabolic bone disease of childhood caused by deficits of calcium and/or vitamin D. In rickets, secondary hyperparathyroidism has catabolic effects on the growing skeleton, leading to the bone demineralization that typifies the disease. However, stunting is also a common presenting feature among children with vitamin D-deficiency rickets^{18,19}. Vitamin D treatment of rickets leads to an acceleration of linear growth, accompanied by a resolution of hyperparathyroidism^{18,20,21}.

Emerging data suggest that amelioration of infant vitamin D status may enhance linear growth even in the absence of clinically-overt vitamin D deficiency. A randomized vitamin D trial in India showed that postnatal infant vitamin D supplementation may reduce the risk of stunting among at-risk infants²². In that trial, relatively low-dose postnatal infant vitamin D supplementation led to gradual increases in vitamin D status throughout early infancy. We speculate that earlier endowment with vitamin D stores (via prenatal maternal supplementation) may have resulted in

a greater effect on stunting. However, the effects of maternal prenatal vitamin D supplementation on infant length have not been widely studied. A placebo-controlled trial of prenatal vitamin D supplementation conducted in the late 1970s in London, England showed minimal initial differences in birth length among infants born to supplemented versus unsupplemented mothers²³, but that the infants in the vitamin D group were significantly longer at one year of age²⁴. More recently, in a trial conducted in northern India, 299 women in the 2nd trimester of pregnancy were randomized to either a single 60,000 IU dose of vitamin D3 or two 120,000 IU doses (whereby one dose was administered in each of the 2nd and 3rd trimesters). The trial also included an untreated control group of 43 women enrolled in the 3rd trimester who were already receiving standard care without vitamin D²⁵. The supplemented participants who were followed to delivery (n = 97; 32%) had infants that exhibited mean lengths at birth that were significantly higher than those in the untreated group²⁵. The largest prenatal vitamin D trial published to date was conducted in South Carolina, in which 479 women between 12 and 16 weeks gestation were randomized into treatment groups receiving 400 IU/day, 2000 IU/day or 4000 IU/day²⁶. Differences in mean birth weights were not found among the groups; however, birth lengths and postnatal outcomes were not reported.

We recently completed a randomized placebo-controlled double-blinded trial of maternal prenatal (3rd trimester) vitamin D supplementation in Dhaka, in which 160 women were randomized to receive either 35,000 IU/week (=5000 IU/day) or placebo until delivery. We observed that: maternal mean 25(OH)D was significantly higher at delivery after receiving vitamin D vs. placebo (134 vs. 39 nmol/L, $P < 0.001$; $N = 133$); and that cord 25(OH)D was significantly higher following vitamin D supplementation versus placebo (103 vs. 39 nmol/L; $P < 0.001$; $N = 132$). Vitamin D3 at the studied dose did not cause maternal hypercalcemia or any other supplement-related serious adverse events; and, major adverse birth and neonatal outcomes were non-significantly less common in the vitamin D group, providing reassurances regarding short-term safety.

In preliminary analyses of the effect on infant length among 130 infants followed up to one year of age, we found that infants born to women in the vitamin D group had mean length-for-age z-scores (LAZ) that were significantly greater than infants in the placebo group throughout infancy (**Figure 3**). At 1 year of age, infants in the vitamin D group had LAZ that were, on average, 0.44 z-score units (95%CI 0.06, 0.82) higher than infants in the placebo group.

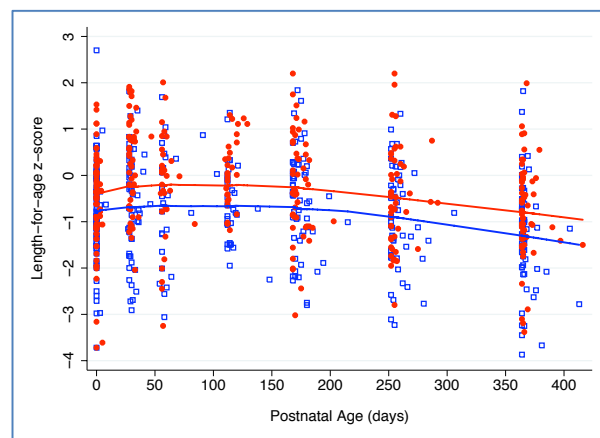


Figure 3: Length-for-age z-scores (LAZ) of infants born to mothers supplemented with vitamin D 35,000 IU/week in the third trimester (red filled circles; top fit line) versus placebo (blue hollow squares; bottom fit line). LAZ are calculated using WHO growth standards. Fit lines are LOWESS curves.

The specific mechanisms by which maternal vitamin D deficiency and parathyroid hyperactivity modulate fetal-infant linear growth are not well understood. PTH is critical for fetal and postnatal bone mineralization, yet its role in bone lengthening is unclear²⁷. In utero, PTH does not appear

to directly influence transplacental calcium flux, a process more likely to be regulated by PTHrP²⁸. In the postnatal period, the direct effects of PTH on growth plate chondrocytes have not been convincingly demonstrated²⁹. In the context of the proposed trial, we will aim to study several nutritional, environmental and inflammatory mediating pathways by which the prevention of vitamin D deficiency and suppression of parathyroid hyperactivity may reduce the risk of early-onset stunting. These hypothesized pathways are summarized in Figure 4, and detailed further below (Section 18).

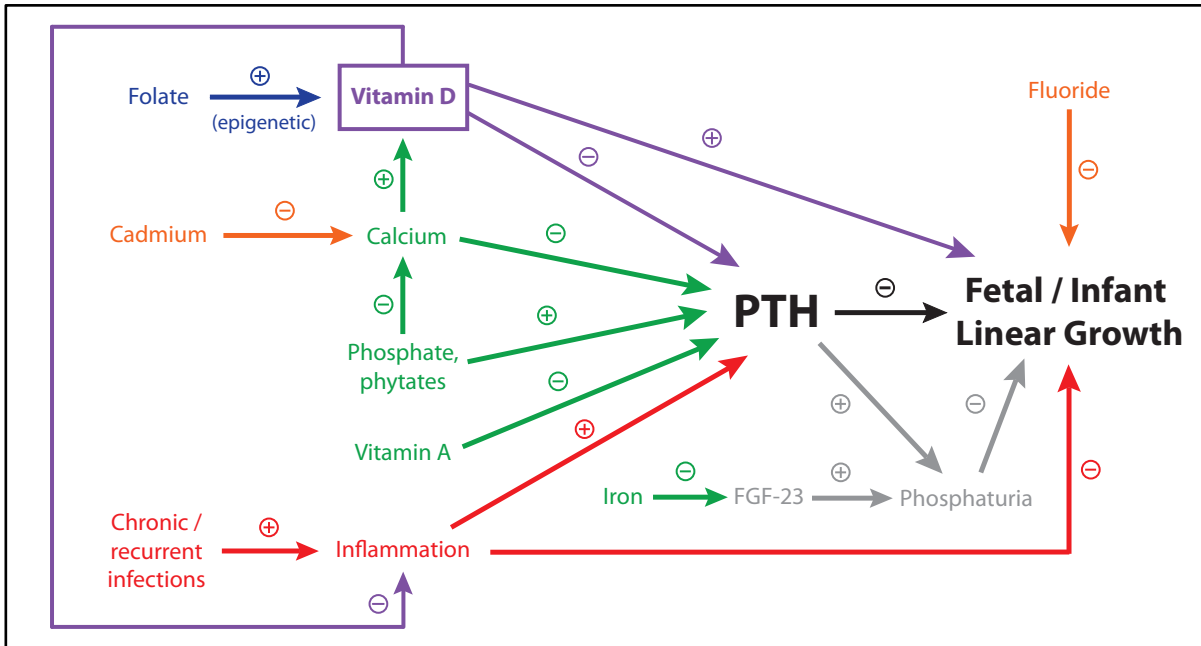


Figure 4. Hypothesized pathways by which nutrients, environmental contaminants, and infections interact with vitamin D and PTH effects on growth.

Epigenetic variation via methylation of genes involved in vitamin D metabolism may further explain inter-individual differences in the biochemical responses to vitamin D supplementation. We specifically hypothesize a pathway linking maternal folic acid intake, genes involved in perinatal vitamin D metabolism (CYP27B1 and CYP24A1), parathyroid activity, and birth size (Figure 5).

As a potent immunomodulator, vitamin D may mitigate episodic or chronic infection-related growth faltering. A range of studies have putatively linked vitamin D deficiency to an increased risk of infectious morbidity³⁰. In a case-control study in Sylhet, Bangladesh, we found that infants with acute lower respiratory infection had lower average 25(OH)D than community matched controls³¹, but the direction of causality is unclear (i.e., recurrent infections and general poor health may compromise vitamin D status, and/or vitamin D deficiency may raise the risk of

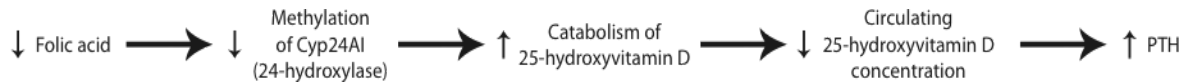


Figure 5. Hypothesized conceptual pathway linking folic acid intake, epigenetic regulation of vitamin D metabolic enzymes, and parathyroid hyperactivity in the prenatal period.

infections). Therefore, by tracking episodes of illness and symptoms of infection (e.g., diarrhea) in pregnancy and early infancy, this trial will also enable us to investigate the effect of vitamin D supplementation on morbidity in infancy.

In summary, vitamin D deficiency and consequent parathyroid gland hyperactivity are postulated to increase the risk of fetal and early infant growth faltering in low-income settings, and in particular in South Asia. This trial of maternal pre- and postnatal vitamin D supplementation will directly test this hypothesis. In addition to this primary outcome, the trial will provide insight into other possible mediators and modifiers of the effect of vitamin D on growth through multiple sub-studies. These sub-studies will include 1) a pathway-wide assessment of biomarkers from the parathyroid-vitamin D axis and biomarkers associated with inflammation; 2) an analysis of epigenetic phenomena that affect vitamin D metabolism based on parental and cord blood as well as placental specimens (inclusion of the paternal blood specimen will enable the identification of allele-specific epigenetic changes); and 3) an analysis of the effect of supplementation on the incidence postnatal infant infectious disease.

b) Explanation for choice of active agents and comparators

Multiple vitamin D doses will be studied to enable the characterization of a dose-response effect on the primary outcome, while balancing considerations of sample size, safety and feasibility. Three prenatal vitamin D doses are proposed (4200 IU/week, 16,800 IU/week and 28,000 IU/week) based on published literature and our preliminary data. In addition, a postpartum phase will test the effect of continuing 4000 IU/d after delivery (**Figure 6**). The equivalent daily doses would be 600 IU/d, 2400 IU/d and 4000 IU/d respectively, based on known pharmacological principles and empiric data showing that weekly doses of 7X/week achieve similar 25(OH)D levels as doses of X IU per day³². For this reason, much of the cited literature below refers to daily doses.

Placebo: The current standard of antenatal care in Bangladesh and virtually all low-income countries does not include vitamin D supplementation. Moreover, a Cochrane Collaboration systematic review published in 2012 found a lack of evidence to support routine vitamin D supplementation during pregnancy³³. Our own data thus far do not suggest that there are discernible risks to individual mothers or infants in Dhaka who receive standard antenatal care

excluding vitamin D supplementation (i.e., placebo). The hypothesized effect is a shift in the distribution of infant lengths rather than prevention or treatment of individual pathology. Even if shown to have significant effects on average linear growth, the vitamin D doses to be studied in this trial would require further investigation at a larger scale to justify their inclusion in a package of standard antenatal interventions. The inclusion of the placebo group in the proposed trial does not place participants at risk, will enable findings to have relevant policy implications, and will enable the study to test of effects of supplementation at the US/Canada RDA level. However, mothers or infants in the trial who are found to have clinical features of metabolic bone disease attributable to vitamin D deficiency during supplementation or follow-up will be promptly treated with vitamin D as required, outside of the study protocol.

Vitamin D 4,200 IU/week (to deliver 600 IU/d): This dose level reflects the North American recommended dietary allowance (RDA). Dietary guidelines released in November 2010 by the US Institute of Medicine (IOM) set the RDA for vitamin D for Canadian and American pregnant and lactating women at the same level as non-pregnant adults (15 mcg = 600 IU/day)³⁴. The RDA was set to promote bone health, and assumes inputs from a variety of sources in the setting of minimal sun exposure, rather than implying that 600 IU/day is a supplementation dose³⁵. The World Health Organization (WHO) does not recommend routine prenatal vitamin D supplementation, but existing WHO guidelines set a recommended daily intake (RDI) of vitamin D (from all sources) of 200 IU/day for most children and adults, including pregnant and lactating women. Typical maternal antenatal multiple micronutrient formulations that have been studied in low-income countries over the past 10-15 years have included 200 IU/day of vitamin D³⁶, a dose that would have only a small effect on serum 25(OH)D concentrations and thus would be unlikely lead to observable differences in growth outcomes compared to placebo.

Vitamin D 16,800 IU/week (to deliver 2400 IU/d): This dose level is expected to attain the proposed IOM threshold for sufficiency of serum 25(OH)D concentrations ≥ 50 nmol/L in most women in Dhaka. With respect to doses above the RDA, the tolerable upper intake level (UL) was increased by the IOM from the 1997 recommendations³⁷ to 4,000 IU/day in 2010³⁴, but there were few new data to support changes specific to pregnancy. Most antenatal vitamin D supplementation trials were conducted in the 1980s³⁸; in the past decade, only four additional pregnancy trials have been published^{25,26,39,40}, only one of which (the Hollis trial⁴¹) included data from more than 100 participants. The Canadian Paediatric Society (CPS) has suggested consideration of maternal prenatal supplementation of 2,000 IU/day⁴². However, in 2011 the American College of Obstetricians and Gynecologists (ACOG) reiterated the lack of evidence to support routine high-dose prenatal vitamin D supplementation, but offered that “when vitamin D deficiency is identified during pregnancy, most experts agree that 1,000–2,000 IU per day of vitamin D is safe.”⁴³ The Endocrine Society (US) recently recommended that pregnant women at risk of vitamin D deficiency consume *at least* 1,400 IU/day, with an upper limit of 10,000 IU/day⁴⁴. Our pharmacokinetic findings among pregnant women in Dhaka have been consistent with published estimates of the vitamin D-25(OH)D dose-response relationship in non-pregnant adults (i.e., 0.7 nmol/L increase in 25(OH)D at steady-state for each 1 mcg/day of vitamin D₃^{45,46}). To attain vitamin D sufficiency in the majority of women, according to the IOM standard of 50 nmol/L, we estimate that a dose of 2,000 to 2,500 IU/day will be required (by aiming for a group mean 25(OH)D of about 80 nmol/L). The 2,400 IU/day dose (administered as 16,800 IU/week) was selected as a multiple of the RDA of 600 IU/day to facilitate coherent dose-

response comparisons between these doses. Although some authorities have recommended doses less than 2000 IU/day in Canada and the US (see above), such doses would be less likely to yield the 50 nmol/L steady-state in the majority of women in Dhaka (where baseline vitamin D status is lower than in typical women in North America).

Vitamin D 28,000 IU/week (to deliver 4000 IU/d): This dose is proposed to safely ensure suppression of PTH secretion in most participants. In a previous trial we observed that 35,000 IU/week (~5000 IU/day) potentially suppressed maternal PTH production during pregnancy in Dhaka; however, earlier data suggested that 2,000 IU/day may have a weaker effect on PTH⁴⁷. From a mechanistic standpoint, it is important to study a dose that suppresses PTH; however, based on the association between attained 25(OH)D and PTH at delivery in pregnant women in our preliminary trial, we expect that a dose of 4,000 IU/d (with an expected attained group mean 25(OH)D of ~110 nmol/L) will be sufficient for parathyroid suppression. The selection of 4000 IU/d balances desired physiological effects with safety considerations. Firstly, during our research, we found that 5000 IU/d (as 35,000 IU/week) did not provoke hypercalcemia and was not associated with any discernible adverse pregnancy outcomes. A reduction of that dose by 20% will provide a wide margin of safety for a larger study population that can be less intensely monitored for hypercalcemia. Secondly, 4000 IU/d will not exceed the IOM UL³⁴, a conservative margin of safe intake for the general population, even in the absence of clinical/biochemical monitoring.

Rationale for studying vitamin D 28,000 IU/week (~4000 IU/d) during lactation: Our preliminary findings in Dhaka revealed that infants born to women who received 3rd-trimester maternal vitamin D supplementation at 35,000 IU/week had higher LAZ during postnatal follow-up compared to infants born to women who received placebo. However, a critical unresolved issue is the mechanism through which prenatal supplementation impacts infant length. Prenatal supplementation may impact infant length through enhanced fetal skeletal growth, and/or latent effects on infant growth that result from the larger vitamin D stores provided by supplemented mothers during gestation. If the latter is a true phenomenon, as we speculate, then maintenance of the vitamin D steady-state during the period of lactation may accentuate the growth effects. There is a dose-response relationship linking maternal vitamin D status, breast milk vitamin D activity, and infant vitamin D status⁹. Vitamin D supplementation of lactating mothers with at least 2000 IU/day has been clearly linked to significant increases in the 25(OH)D of breast-fed infants⁴⁸. Maternal supplementation, rather than infant supplementation, is preferred in the context of this trial because of the possibility that the mechanism of effect of vitamin D on growth may involve the regulation of breast milk transfer of other endocrine factors (e.g., PTHrP), and may not only be related to the infant's 25(OH)D concentration. In addition, if shown to be beneficial, maternal supplementation may be more appealing in the public health context because it would support the optimality of exclusive breastfeeding, in that all nutrients would be delivered to the infant via breast milk without the need for infant nutrient supplementation. To maximize analytical efficiency (i.e., maintain sufficient numbers of participants in each intervention group) given resources/feasibility, we aim to test the postpartum effect only at the highest dose level (4000 IU/d). We will therefore assess the effect of maternal postpartum vitamin D3 supplementation at a dose of 28,000 IU/week (4000 IU/d) versus placebo on postnatal infant growth among infants born to women who received vitamin D 4000 IU/d during pregnancy.

Rationale for weekly doses instead of large single or infrequent intermittent 'bolus' doses:

Experience with a large single dose (70,000 IU) in our initial pharmacokinetic studies in Dhaka revealed high inter-individual variability in 25(OH)D and the absence of a 25(OH)D steady-state⁴⁹, which were expected on the basis of published data⁵⁰. Large single doses may not be physiologically appropriate⁵¹, and have been associated with clinical adverse effects in adults⁵². Published pregnancy trials employing large single or intermittent vitamin D3 doses have not adequately reported pharmacokinetic and safety parameters^{25,39}. Therefore, despite the potential practical advantages of infrequent dosing, it is currently more appropriate to replete vitamin D stores using regular maintenance doses at short intervals (i.e., weekly or daily) that prevent large inter-dose fluctuations in 25(OH)D, and for which we have preliminary data.

8)

9) Objectives

a) Primary aims:

1. To determine whether maternal prenatal oral vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week, administered as weekly doses) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh.
2. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.

b) Secondary aims:

3. Growth outcomes:
 - i. To estimate the effect of prenatal +/- postpartum maternal vitamin D supplementation versus placebo on the prevalence of stunting at 1 year of age.
 - ii. To estimate the effects of maternal prenatal vitamin D supplementation on infant attained length at 2 years of age.
 - iii. To estimate the effect of maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo on infant attained length at 2 years of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.
4. To estimate the effect of maternal prenatal +/- postpartum vitamin D supplementation on the incidence of postnatal infant acute respiratory infections and acute diarrhea. [*For further details, see Appendix for sub-study protocol, "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh"*]
5. To investigate the roles of specific hormones, nutrients, environmental contaminants, and inflammatory markers in the mediation or modification of the effect of vitamin D on infant stunting.
6. To establish evidence that the vitamin D-parathyroid axis in pregnancy is influenced by epigenetic modification of genes involved in vitamin D metabolism.
7. To estimate the effect of maternal prenatal vitamin D supplementation on the prevalence of low birth weight and small-for-gestational age.

10) Trial design

- Randomized
- Concealment of allocation and blinding throughout intervention and analysis
- Placebo-controlled
- Parallel-group
- Dose-ranging
- Superiority hypothesis testing framework
- 1:1 Allocation ratio across 5 groups

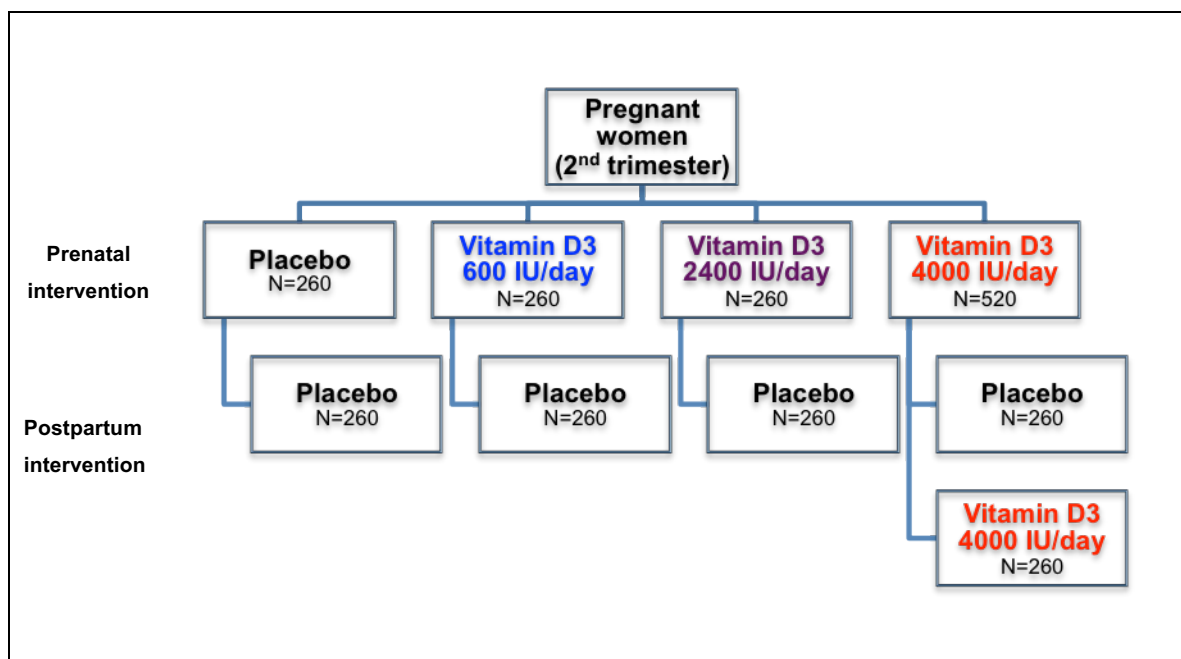


Figure 6: Trial Design

11) Study setting

The single-site trial will be conducted in **Dhaka, Bangladesh**. Enrolment and clinical activities will be based at the **Maternal and Child Health Training Institute (MCHTI), commonly known as Azimpur Maternity Center**, a government facility that provides low-cost health care to pregnant women and children in its referral area in central Dhaka, Bangladesh. MCHTI has outpatient clinics, an inpatient labour and delivery unit (152 beds), and inpatient paediatric services. There are approximately 30 women per day registered for antenatal care starting in the 2nd trimester. Low-income patients receive free care. Clinical staff includes obstetrician-gynecologists, pediatricians, anesthesiologists, medical house officers, nurses and paramedics. Basic laboratory and radiology services are available, including prenatal anatomical and dating ultrasound. Complicated patients (including newborns requiring respiratory support) are referred to nearby tertiary-care hospitals. The Dhaka wards/unions (char) near MCHTI that will be

included in the trial catchment area include: Kamrangir char, Azimpur, Lalbag, and Hazaribag. We expect about three-quarters of the participants to be residents of Kamrangir char, a collection of urban slums on the Buriganga river, along the periphery of Dhaka city. The area is densely populated, with a total population of ~300,000, of which approximately 265,000 reside in slum settlements (National Institute of Population Research and Training, Dhaka, 2006). The literacy rate has been estimated at 29% (compared to the national average of 32%) and more than 30% of the residents have monthly incomes \leq 5000 taka (~\$60 CAD). However, socioeconomic status varies greatly given the presence of some universities and government offices in the area. Income earners are mainly day labourers and many men work in local tanneries (the typical income of a tannery worker is about 6000 taka/month).

MCHTI (Azimpur maternity center) and its catchment area offer the operational advantages of efficient participant accrual, feasible perinatal specimen collection (cord blood and placenta), the cost efficiencies of facility-based enrolment, and the collaboration of MCHTI management: Dr. Sirajul Islam, Superintendent of Azimpur Maternity Centre, and Dr. Chinmoy Kanti Das, Coordinator of The Maternal and Child Health Training Institute (MCHTI).

Field and clinical operations will be managed by ICDDR,B with the collaboration of Shimantik, a partner implementing non-governmental organization that delivers maternal-child health services in Dhaka.

12) Participant eligibility criteria

a) Inclusion criteria:

- Age 18 years and above;
- 17 to 24 completed weeks of gestation (i.e., 17 weeks +0 days to 24 weeks + 0 days, inclusive) based on recalled last menstrual period (LMP) and/or ultrasound;

Rules for integrating information from recalled LMP and ultrasound:

- if there is a difference of >10 days between gestational age dated using the LMP and second trimester ultrasound, the estimated date of delivery will be adjusted as per the second trimester ultrasound (SOGC guidelines); otherwise (i.e., if the difference is \leq 10 days), the GA date based on LMP will be used.
 - If there is more than one ultrasound, GA estimation should be based on the earliest of the ultrasounds for which a written report is available. If the earliest ultrasound was performed in the 1st trimester, and there is a difference of >5 days between gestational age dated using the LMP and 1st trimester ultrasound, the estimated date of delivery will be adjusted as per the 1st trimester ultrasound (SOGC guidelines); otherwise (i.e., if the difference is \leq 5 days), the GA date based on LMP will be used.
- Intends to reside in the trial catchment area (including Hazaribag, Azimpur, Lalbag, and Kamrangirchar) for at least 18 months;
 - Provides written informed consent.

b) Exclusion criteria:

- History of any medical condition or medications that may predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia, including active tuberculosis or current therapy for tuberculosis, sarcoidosis, history of renal/ureteral stones, parathyroid disease, renal or liver failure, or current use of anti-convulsants.
- High-risk pregnancy based on one or more of the following findings by point-of-care testing:
 - Severe anemia: hemoglobin <70 g/L assessed by Hemocue
 - Moderate-severe proteinuria: ≥ 300 mg/dl (3+ or 4+) based on urine dipstick
 - Hypertension: systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg
- High-risk pregnancy based on one or more of the following findings by maternal history and/or ultrasound:
 - Multiple gestation
 - Major congenital anomaly
 - Severe oligohydramnios
- Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin containing calcium and/or vitamin D.
- Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency.
- Previous enrolment in the trial during a previous pregnancy.

Note regarding the timing of supplement initiation: Starting supplementation in the mid-second trimester (17 to 24 weeks) balances the benefits of a prolonged period of supplementation with the practical consideration that pregnant women are not typically registered for antenatal care at Azimpur hospital prior to 20 weeks gestation.

13) Interventions

a) Intervention description

The experimental intervention is supplemental **oral vitamin D3 (cholecalciferol)**, a fat-soluble hormone precursor for which the natural sources are endogenous production in the skin upon ultraviolet B radiation exposure and some types of food (e.g., oily fish, fortified milk). The supplemental vitamin D will be provided in the form of small tablets (10 mm diameter) to be custom-manufactured by Toronto Institute for Pharmaceutical Technology (TIPT) in Toronto, Ontario, Canada (www.tipt.com). Each weekly dose will consist of a single tablet. Across trial groups, the tablets will only vary with respect to the vitamin D3 dose, per the chart below:

Group	Prenatal supplement	Postpartum supplement duration lactation (0 to 24 weeks postpartum)
A	0 IU (placebo)	0 IU (placebo)
B	4,200 IU/week (=600 IU/d)	0 IU (placebo)
C	16,800 IU/week (=2,400 IU/d)	0 IU (placebo)
D	28,000 IU/week (=4,000 IU/d)	0 IU (placebo)
E	28,000 IU/week (=4,000 IU/d)	28,000 IU/week (=4,000 IU/d)

See Appendix for Investigator brochure.

Tablet content will be verified by chemical analysis prior to the initiation of the trial, and at regular intervals throughout the trial (approximately every 6 months).

Supplementation will begin at enrolment and continue on a weekly basis throughout pregnancy and the postpartum period, until 24 weeks postpartum.

Mode and conditions of tablet consumption:

- Swallowed whole with any liquid (water, juice, tea, etc.) or, if necessary, chewed with or without a small amount of soft food such as yoghurt (if completely consumed).
- There is no specific time at which consumption must occur, as this is not relevant to long-term effect on vitamin D status.
- Tablet may be consumed with or without a meal, and the composition of meals is not specified, as this impacts negligibly on vitamin D absorption⁵³.

b) **Criteria** for discontinuing or modifying allocated interventions

i) Missed and late doses

A **missed dose** is considered to have occurred if a dose was not ingested on the scheduled day of administration, for a participant who remains on-study. Typical reasons may include:

- Participant not available in the home on a scheduled day of dose administration
- Participant refuses a scheduled dose due to nausea, etc.
- Scheduled visit cannot be completed due to political unrest, inclement weather, etc.

Although scheduled for specific 7-day intervals, a missed dose may be administered as a **late dose** on any day up to 7 days after the scheduled date of administration; thereafter, subsequent dosing continues as originally scheduled, even if this incurs an interval of less than 7 days between the late dose and the subsequent regularly scheduled dose.

- If a participant is not reached for one full week but then returns to follow-up on the subsequent scheduled visit day (i.e., 7 days following the missed visit), two doses may be administered simultaneously. A maximum of 2 doses may be administered together.
- If a participant returns to follow-up >7 days after a missed visit, the first missed dose will be considered to be a **completely missed dose**, but the second missed dose may be administered as a *late dose*. For example, if a participant is absent for a visit on day #14, and returns to follow-up 12 days later on day #26, the participant is considered to have *completely missed* the day #14 dose, but is administered the dose scheduled for day #21 as a *late dose*; had the participant returned to follow-up on day #21, she could have received the day #14 and day #21 doses simultaneously.
- If a participant does not successfully ingest a weekly scheduled dose (due to refusal or nausea/vomiting) only one additional attempt will be made to ingest the dose again (during the same visit or during a visit up to 7 days after the scheduled date).

ii) Nausea/vomiting during or after dose administration

- If a participant complains of nausea or stomach upset on the scheduled day of dose administration, the dose may be deferred for up to 7 days (see above, per missed dose).
- If a participant vomits during or within 20 minutes of dose administration:
 - the dose may be repeated immediately; OR,
 - the dose may be considered 'missed' and deferred to another day (within 7 days), applying the same rules as for other missed doses.
- If vomiting occurs more than 20 minutes after a dose was swallowed, the dose will be considered to have been administered. In such cases, the dose will not be repeated, and dosing will continue as scheduled the following week.

iii) Refusal or deferral of a dose

- If a participant refuses a dose on the day of scheduled administration, the study worker will offer to return on a subsequent day (within the week) to deliver the dose (see above, per missed dose).
- However, if a participant refuses doses for 3 consecutive weeks, and on the 3rd week, expresses the intention to continue avoidance/refusal of the dose, no further supplementation will be offered on a weekly basis.
 - A participant with serial refusals of the supplement need not withdraw completely from the study, if she agrees to the follow-up procedures (e.g., sample collection, anthropometry, etc).
 - If a participant remains in follow-up, the study personnel will occasionally (at least monthly) discuss renewed supplement adherence with the participant.

iv) Adverse events that require supplementation discontinuation

- The only clinical events that will lead to study personnel withholding doses are those adverse events that are ascertained to be supplement-related or the occurrence of a medical condition that potentially or theoretically increases sensitivity to vitamin D supplementation (see below).
- However, individual doses may be considered missed, late or deferred due to intercurrent illnesses; approaches to missed doses are addressed as they would be for any other reason.
- Following clinical review by the Trial Steering Committee (TSC), the following specific events will lead to discontinuation of supplementation:
 - Confirmed hypercalcemia (see definition below): supplement administration will be stopped; however, observational follow-up (including biochemical assessment) will continue.
 - Symptomatic vitamin D deficiency: diagnosed by a study physician or consultant physician (e.g., osteomalacia) on the basis of clinical findings (e.g., persistent limb pain) and supported by biochemical evidence (i.e., hyperparathyroidism). Masked study supplement administration will be stopped and vitamin D supplementation will be instituted to correct severe vitamin D deficiency. Such

participants will continue to be followed clinically. A low 25(OH)D concentration alone will not be grounds for cessation of the study supplement.

- Fetal or infant death: Maternal supplementation will cease and participation will end.
- Onset of a medical condition or initiation of a medication following enrolment that the TSC concludes may reasonably predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia (e.g., tuberculosis or therapy for tuberculosis, sarcoidosis, renal/ureteral stones, parathyroid disease, renal or liver failure, or use of anti-convulsants).

c) Strategies to improve adherence to intervention protocols, and procedures for monitoring adherence

- Tablet ingestion will be directly observed by study personnel during home or clinic visits, except for certain contingencies (see below).
- Tablet supplies will be maintained/stored by study personnel, not in the homes of participants, except for certain short-term contingencies (see below).
- Study personnel will maintain a log of dose administration, including a record of all *missed doses*, *late doses* and *completely missed doses*.
- In situations where all of the following conditions are met, up to 4 tablets (one month's supply) may be given to a participant in advance, to be consumed without direct supervision:
 - Participant will be unavailable for study visit(s) due to travel
 - Participant expresses intent to self-administer dose(s)
 - Participant agrees to contact the study worker by phone or text message to confirm each weekly supplement self-administration in real-time.
 - Participant agrees to ensure the safe storage of the supplement tablets (e.g., out of reach of children). Participants will be given a child-proof container to hold the tablets.
 - Participant agrees not to give the supplements to any other person, even if she herself chooses not to consume them.

d) Relevant concomitant care and interventions that are permitted or prohibited during the trial

The following co-interventions will be provided to all participants:

- Calcium 500 mg/day as calcium carbonate (Calbo, Square Pharmaceutical, Dhaka)
- Iron and folic acid supplementation: (66 mg elemental iron per day, and 350 mcg folic acid per day included in the standard formulation available in Bangladesh).
- Tetanus toxoid immunization: if not already received, two doses will be administered (4 weeks apart) prior to delivery according to current WHO recommendations.
- Antenatal monitoring and referral to physician as indicated for any complications.
- Counselling to promote optimal infant feeding and health maintenance (including exclusive breast-feeding for the first 6 months, with introduction of appropriate complementary foods starting at 6 months of age), care-seeking for illness, and routine immunizations.

Supplemental calcium or vitamin D not prescribed by the study protocol will be prohibited during the intervention phase of the trial (Phase 1). Participants will be questioned on a weekly basis as to whether they have consumed other nutrient supplements. A one-time warning will be given when non-study vitamin D or calcium supplementation is first reported; if the participant has not discontinued the non-study supplement at the time of the next weekly visit, the participant will be discontinued from further study supplementation until she discontinues the non-study supplement. In such cases, participants may still continue clinical follow-up. There is no prohibition against supplemental calcium or vitamin D during the non-intervention phase 2 (after 6 months postpartum); however, no specific recommendations will be given.

Rationale for calcium co-intervention:

The provision of a calcium co-intervention in the context of the proposed study has two major advantages: (1) from a mechanistic standpoint, standard provision of calcium will act to mitigate any rate-limiting effects of serious maternal dietary calcium deficits on fetal growth or other outcomes, thereby isolating the effects of vitamin D; and (2) from a practical standpoint, the investigation of vitamin D supplementation in the context of routine calcium supplementation will facilitate the translation of our findings into anticipated future contexts in which supplemental calcium is routinely provided. A factorial trial to discern the independent effects of calcium and vitamin D on growth was not feasible within the current funding/logistical constraints.

In the proposed trial, we will provide calcium supplementation (as calcium carbonate) at 500 mg/day elemental calcium to all participants. The World Health Organization (WHO) has recommended prenatal calcium supplementation at a dose of 1.5 to 2 g/day to reduce the risk of hypertensive diseases of pregnancy (HDPs, e.g., preeclampsia) in populations with low baseline dietary calcium intake and especially for women at high risk of HDPs (WHO 2011). In Bangladesh, typical daily calcium intake is low, estimated at 200-400 mg per day⁵⁴⁻⁵⁶. However, there are several reasons why a dose of 1.5 g was not selected for routine provision to participants in this trial:

- There are unresolved concerns about the safety of this high dose; trials have shown a significant increase in the risk of HELLP syndrome. A dissenting opinion in the WHO statement by Dr. Peter von Dadelszen (University of British Columbia) was based on speculation that calcium reduces the *diagnosis* of preeclampsia, but does not modify the underlying disease process (WHO, 2011).
- There is evidence that lower doses (< 1 g/day) may also reduce the risk of hypertensive disease of pregnancy (Hofmyer 2013, unpublished). Personal communications with Dr. Justus Hofmyer (University of the Witwatersrand, South Africa) have confirmed his viewpoint that a supplemental intake of 500 mg would be appropriate at present, on the basis of existing evidence.
- Currently, calcium supplementation is not standard of care in Bangladesh; in particular, 1.5 g/day is rarely prescribed in Bangladesh. Our experience is that if a woman has been prescribed calcium supplementation, it is at a dose of 500 mg per day.
- There are unresolved questions surrounding the feasibility of delivery mechanisms for daily calcium at high doses (1.5 to 2 g); in the WHO calcium supplementation trial, the regimen was one 500 mg tablet taken 3 times daily, for which adherence is likely to be low.

- There are potential adverse interactions between calcium and iron; at present, the WHO recommends that supplemental calcium be taken apart from iron, implying that supplementation would have to occur 4 times per day, likely further lowering adherence.
- There are no trials of combined high-dose vitamin D + calcium at the WHO-recommended doses; the combination poses theoretical risks of urolithiasis or other complications. These risks need to be studied separately before this combination can be widely implemented.
- In determining the appropriate calcium co-intervention dose for this study, we did not consider evidence suggesting a harmful or beneficial effect of calcium on long-term bone or cardiovascular outcomes. This was because the evidence is mixed, mostly drawn from elderly populations, and not felt to be relevant to relatively short-term supplementation during pregnancy.

In the trial setting, a daily dose of 500 mg of calcium is expected to raise the average participant intake above the estimated average requirement (EAR) established by the Institute of Medicine (800 mg) and to approach or attain the recommended dietary allowance (RDA) of 1000 mg/day for most women. A dose of 500 mg can be provided readily using an over-the-counter supplement available in Bangladesh, but higher doses would require multiple tablets. Participants will be instructed and encouraged to take a single tablet of calcium per day in the morning, and to take the iron-folic acid supplement separately, in the evening, or with a meal.

With respect to the prevention of HDP, participants in this trial will undergo close clinical monitoring, including blood pressure measurement and urinalysis, that exceed the standard care which they would have otherwise experienced outside of the research context; therefore, some women may benefit from earlier recommendation and referral for treatment of signs of HDP.

14) Outcomes

Primary outcome measure: length-for-age z-score (LAZ) at one year of age.

Growth faltering in resource-poor settings primarily occurs in the first year of life⁶; thus any effects of the intervention are expected to be apparent by one year of age. Moreover, our preliminary trial data revealed a discernible effect of prenatal vitamin D supplementation on infant LAZ at one year. Since some infants might not be reached at exactly 1 year of age (52 weeks), measurements taken up to 60 weeks (i.e., up to 8 weeks past the scheduled time of ascertainment) will be included in the primary “one year” outcome analysis implying a range of 364 to 420 days (inclusive) for allowable timing of one-year measurements. The purpose of this range is to enable the inclusion of as many children as possible, even if they are not available for assessment during the 52nd week postnatal. LAZ based on WHO growth standards will be used to account for sex imbalances between groups and variance in exact age at the time of measurement.

Infant follow-up will continue to 2 years of age (with continued treatment masking) to establish the persistence of effects, to capture potential catch-up growth, and because stunting prevalence does not stabilize until 18-24 months of age⁶. To improve uniformity with respect to

the timing of the 2-year visit, it will be scheduled at 24 months of age (104 weeks) or up to 3 months afterwards (27 months after birth).

Other secondary growth outcomes will include weight, length, head circumference, limb length, mid-upper arm circumference and postnatal growth velocity. These secondary growth outcomes and additional safety outcomes are summarized below.

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
Primary outcome: Linear growth at one year of age	Efficacy	Length-for-age z-score (LAZ)	Value at 1 year of age	<ul style="list-style-type: none"> • Mean (primary analyses) • % below -2 SD (WHO z-scores) 	Measurement obtained on or shortly after 52 weeks (up to 60 weeks)
Linear growth at two years of age	Efficacy	Length-for-age z-score (LAZ)	Value at 2 years of age	<ul style="list-style-type: none"> • Mean • % below -2 SD (WHO z-scores) 	Measurement obtained on or just after the second-year birthdate (up to 27 months)
Weight and head circumference for age; weight for length	Efficacy	Weight-for-age z-score (WAZ), weight-for-length (WFL), and head circumference for age z-score (HCAZ)	Values at 1 year or 2 years of age	<ul style="list-style-type: none"> • Mean • % below -2 SD (WHO z-scores) 	Measurement obtained on or just after the one-year or two-year birthdates.
Weight, length, head circumference z-scores	Efficacy	Weight, length, and head circumference z-scores	Values at birth	<ul style="list-style-type: none"> • Mean • % below -2 SD (WHO z-scores) 	Measurement obtained within 48 hours of birth.
Weight, length, limb lengths, mid-upper arm circumference, head circumference	Efficacy	Raw weight, length, limb length, mid-upper arm circumference and head circumference measures	Values at specific timepoints	<ul style="list-style-type: none"> • Mean • Birth weight: % below 2500 g • % small-for-gestational age 	Measurement obtained closest to specified timepoint: birth, < 2, 3, 6, 9, 12, 15, 18, 21 and 24 months.
Linear growth velocity	Efficacy	Raw length measurement or LAZ	Change within a specified interval	<ul style="list-style-type: none"> • Mean 	Specified growth intervals between: birth, < 2, 3, 6, 9, 12, 15, 18, 21 and 24 months.
Gestational age at birth / preterm birth	Safety	Gestational age at birth (in days)	Value at birth, based on LMP +/- ultrasound	<ul style="list-style-type: none"> • Mean • % preterm (< 37 weeks) • % early preterm (< 34 weeks) 	<ul style="list-style-type: none"> • Delivery
Placental weight	Efficacy	Weight (grams)	Value	<ul style="list-style-type: none"> • Mean 	<ul style="list-style-type: none"> • Delivery

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
Vitamin D status	Efficacy	Serum 25(OH)D concentration	Values at specified time-points, as well as change in concentration during pregnancy.	<ul style="list-style-type: none"> • Mean • % below/above specific thresholds 	<ul style="list-style-type: none"> • Delivery (maternal) • Cord venous blood • 3 months postpartum (infant), 6 months postpartum (maternal and infant)
Maternal serum calcium	Safety	Serum calcium concentration	Value	<ul style="list-style-type: none"> • Mean • % above or below reference range 	Whenever measured
Uro-/nephrolithiasis	Safety	Presence on ultrasound	Ultrasound imaging	-	Delivery
Maternal referral for obstetric care	Safety	Any referral by study physicians for a suspected or diagnosed obstetric complication (even if referral not accepted)	-	-	Prenatal period, and up to 1 month postpartum.
Maternal hospitalization	Safety	Inpatient admission for any reason other than uncomplicated delivery.	-	-	Any time during trial
Child hospitalization	Safety	Inpatient admission for any reasons	-	-	Any time during trial
Infant morbidity	Efficacy/ Safety	Occurrence of episodes of skin infection, sepsis, diarrhea or acute respiratory infection (ARI).	-	Prevalence/ incidence	0 to 6 months of age.
Participant (maternal) death	Safety	Death from any cause at any time.	-	-	Any time during trial
Maternal death	Safety	Death while pregnant or within 42 days of termination of pregnancy, irrespective of the duration and site of the pregnancy, from any cause related to or aggravated by the pregnancy or its management but not from accidental or incidental causes.			Expected event count is 0.

Outcome	Type	Measurement variable / definition	Analysis metric	Method of aggregation	Timing
Stillbirth	Safety	Intrauterine demise or delivery of a fetus that does not breathe or show any other evidence of life - e.g. beating of the heart, pulsation of the umbilical cord or definite movement of voluntary muscles	-	-	Cumulative over study; based on 2011 rate in Dhaka (2.6%)*, expected event count may be ~33.
Neonatal death	Safety	Death of live born infant within first 28 days of life	-	-	Cumulative over study; based on 2011 rate in Dhaka (3.6%)* event count may be ~42.
Post-neonatal infant death	Safety	Death of a child at any time during first year after 28 days.	-	-	Cumulative over study; based on 2011 rates (0.8%)* event count may be ~9.

*Estimated event counts based on 2011 rates for Dhaka in Bangladesh Demographic Health Survey (BDHS).

Notes regarding growth outcome ascertainment based on anthropometric measurements and the application of World Health Organization (WHO) growth standards:

- *Measurement precision:* Measurement procedures will be conducted using an approach that minimizes errors at the time of data collection (see below, Data Collection).
- *Temporal consistencies:* A supervisor will conduct a same-day review of anthropometric measures in comparison to each child's previous set of measurements and will flag inconsistencies based on the following principles:
 - Length and head circumference (HC) will be assumed to be constantly increasing (even though there may be rare circumstances where they truly can decrease), such that any decline in a length or HC measurement will prompt a repeat length assessment at the earliest convenience (ideally, same week).
 - Weight may vary, and can theoretically decline between closely spaced measurements. However, declines of greater than or equal to 10% will be flagged for repeat assessment at the earliest possible convenience (ideally, same week).
- *Pooled raw measures:* Paired raw measures obtained on the same infant at the same visit will be averaged prior to analysis further improve precision.
- *Standardization for age and sex:* Weight-for-age (WAZ), length-for-age (LAZ), weight-for-length (WFL) and head circumference-for-age (HCAZ) will be calculated according to the sex-specific World Health Organization (WHO) growth standards^{57,58}, using the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>).
- *Extreme outliers:* Extreme z-scores will be flagged based on the WHO Anthro software (< -6 SD or >6 SD for LAZ, >5 or <-6 for WAZ, >5 or <-5 for HCAZ, and <-5 or >5 for WFL). These extreme values will be manually reviewed to ensure they are not the result of data recording or entry errors. Real values that are extreme outlying z-scores are likely to be contributed by infants who are early preterm births and very low birth weight (VLBW); handling of these outliers is discussed below under Data Analysis.

15) Participant timeline

Enrolment and randomization will occur during the 2nd trimester (17 to 24 weeks gestation), supplementation with vitamin D and/or placebo will occur throughout the prenatal period and the first six months postpartum, the primary growth outcome will be ascertained at one year of age, and infants will continue follow-up until two years of age. The trial follow-up schedule is divided into phases. Phase 1 refers to the intervention period; and phase 2 refers to the observational follow-up period:

- **Phase 1a:** enrolment at 17-24 weeks gestation to birth (prenatal intervention).
- **Phase 1b:** birth to 6 months (including 6-month visit); postpartum intervention.
- **Phase 2a:** 6 to 12 months (including 12-month visit, may be extended to 15 months).
- **Phase 2b:** 12 to 24 months (including 24-month visit, may be extended to 27 months)

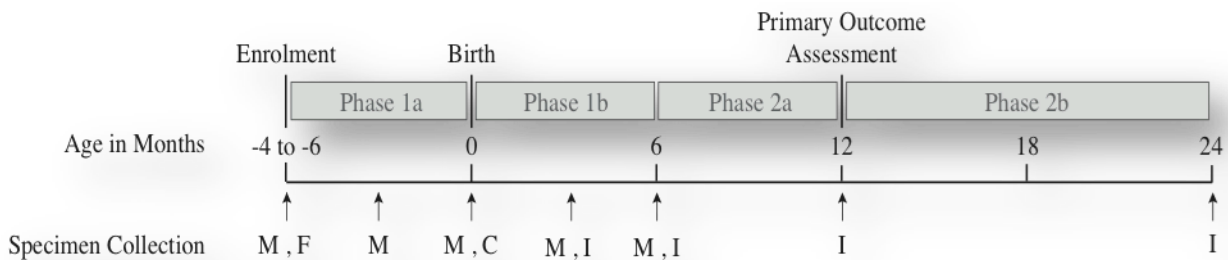


Figure 7: Scheduled specimen collection points for mothers (M), fathers (F), cord blood / cord tissue / placenta (C), and infants (I) are marked by vertical arrows.

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
Timing (months, in relation to delivery)	-4 to -6	-4 to -6	-2.5	0	3	6	12	24
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
Obstetric ultrasound	X							
Allocation		X						
INTERVENTIONS:								
Prenatal								
Supplementation								
Postpartum								

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
Timing (months, in relation to delivery)	-4 to -6	-4 to -6	-2.5	0	3	6	12	24
<i>Supplementation</i>								
ASSESSMENTS:								
Visit frequency	Once	Weekly					Every 3 months	
<i>Maternal clinical assessment</i>	X		X	X	X	X		
<i>Maternal specimen collection</i>	X		X	X	X	X		
<i>Paternal specimen collection</i>	X							
<i>Birth outcome ascertainment</i>				X				
<i>Cord blood/tissue & placental specimen collection</i>				X				
<i>Infant Anthropometry</i>				X	X	X	X	X
<i>Infant morbidity assessments</i>				X	X	X	X	X
<i>Infant specimen collection</i>					X	X	X	X

* Paternal blood specimen collection will enable identification of allele-specific epigenetic patterns; however, paternal blood collection is not required for pregnant woman to join the trial, and therefore the consent process will be separate.

The prenatal timeline will be defined by gestational age (in weeks), based on the LMP and/or ultrasound results (see above). Postnatal follow-up visits will similarly be scheduled based on the infant's age in weeks during the first year, rather than 'anniversaries' of the birthdate, such that 3 months = 12 weeks, 6 months = 24 weeks, 9 months = 36 weeks, 12 months ("1 year") = 52 weeks, and 24 months ("2 years") = 104 weeks.

All study activities (e.g., supplementation, visits, maternal and infant specimen collection) will be stopped for a mother-infant pair when any one of the following events occur:

- The 24-month infant visit is completed. This is the scheduled time at which participant discharge will routinely take place.
- Participant (maternal) death prior to delivery.
- Fetal or infant death at any time.
- Consent for all types of follow-up is withdrawn.
- Participant is lost to follow-up. Loss to follow-up will be considered to have occurred if: 1) study staff determine conclusively that the participant cannot be contacted for the

purposes of data collection for the duration of the period of scheduled follow-up (e.g., they have been informed of the participant's emigration from Bangladesh), OR 2) three months have passed since the scheduled but missed 24-month postnatal visit. A participant may be absent for a period of time (and thus miss supplement dosing, specimen collection, and other data collection), and yet return to follow-up without being excluded from the study or considered 'lost to follow-up'.

16) Sample size

A total of **1,300 participants** will be randomized into 5 groups of 260 women each. The primary outcome analysis will be the between-groups comparison of mean length-for-age z-scores (LAZ) at 1 year of age, assuming up to 15% attrition from each group. To assess the effect of prenatal vitamin D, we plan to perform five primary between-group analyses – each vitamin D dose versus placebo (3 comparisons), and between adjacent doses (2 comparisons). A conservative approach to addressing multiple testing is to partition the alpha (risk of type 1 error) among the 5 comparisons (conventional overall 0.05 divided by 5); thus, each between-group comparison will be tested as a 2-sided test with an alpha of 0.01.

Assuming 90% power and a 1% risk of a type 1 error for each of 5 simultaneous 2-sided tests, the following figure shows the declining magnitude of the minimal detectable difference in LAZ that would be detectable with increasing sample size per group:

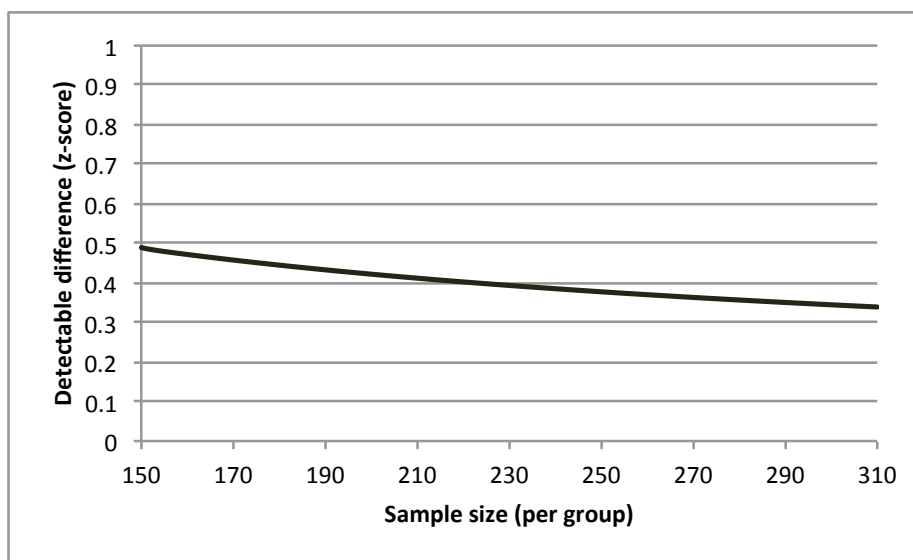


Figure 8: Sample size calculation

With 220 analyzable participants per group (~85% of enrolled), the minimum detectable difference in LAZ will be a z-score of 0.40, which equates to approximately 1.0 cm at 1 year of age based on WHO growth standards. In our completed preliminary trial, we observed an increase of 0.44 in LAZ (95% CI = 0.06 to 0.82) at one year, which corresponded to an increase of 1.1 cm (95% CI, 0.06 to 2.0), adjusted for gender (Roth et al. unpublished).

The secondary analysis at two years of age will enable detection of a ~1.2 cm difference between groups. For the 'postpartum effect' analysis of 4000 IU/day versus placebo postpartum among women who received 4000 IU/d antenatally, the comparison at one year of age will have a minimum detectable difference of 0.31 z-score units (assuming 90% power, a 5% risk of a type 1 error for a 2-sided test, and at least 220 participants per group). Based on WHO growth standards, this is approximately 0.77 cm at 1 year of age, and 0.96 cm at 2 years of age. Because the alpha is not subject to multiple testing, there is greater precision to detect a smaller difference for the postpartum effect analysis.

In order to enrol 1,300 women, we expect to provisionally screen up to 23,000 pregnant women for eligibility. The major reason for exclusion will be GA beyond 24 weeks.

17) Recruitment

Participants will be recruited through two mechanisms:

1. Routine antenatal care (ANC) visits at MCHTI.

All pregnant women who visit the outpatient or ultrasound departments of MCHTI for routine antenatal care/procedures will be approached by study personnel, informed about the study and asked if they would like to participate in an eligibility assessment.

2. Referral by government Family Welfare Visitors (FWVs) or non-governmental health promoters who encounter pregnant women in the community.

Where feasible, FWVs who work in the catchment area of MCHTI will be informed about the study and the basic eligibility criteria (mother's age and gestational age). If any of their clients meet the age and approximate gestational age criteria (i.e., before 3rd trimester), they will ask permission to provide the client's name and cell phone number (or address) to study personnel. If they consent to be contacted, study personnel will follow-up to initiate the screening process.

Eligibility screening is a two-step process:

- "*Provisional screening*" refers to the initial screening of MCHTI clients or women referred by FWVs on the basis of the eligibility criteria; age, known pregnancy, estimated first day of LMP, and Dhaka residence criteria; and,
- "*Detailed screening*" refers to the complete eligibility assessment supervised by a study physician.

Women may be excluded at either stage due to non-eligibility and refusals (i.e., a woman who is provisionally eligible may refuse to undergo detailed screening; a woman who is fully eligible may refuse to enrol).

b) Provisional screening

Study personnel will assess the provisional eligibility of pregnant women presenting for antenatal

care in the MCHTI outpatient department during regular clinic hours (typically, 9:00 am – 1 pm; 6 days per week; Saturday to Thursday). For women referred by FWVs, provisional screening may be conducted by phone, during a home visit, at MCHTI, or at the study field office located close to MCHTI (depending on what is most convenient for the prospective participant).

c) Triage and tracking of provisionally eligible women

- Provisionally eligible women who are between 17 and 24 weeks completed weeks will be referred immediately to the study physician for detailed screening (see below). Gestational age will be estimated based on maternal recall of the first day of the last menstrual period (LMP), with the help of a computer-based macro.
- Women beyond 24 weeks completed gestation (i.e., 24 weeks + 1 day or more) based on LMP will be excluded and will not be screened further.
- All pregnant women who meet the provisional criteria but have not yet reached 17 weeks gestation will be provided with an overview of the study purpose and procedures. They will be asked if they verbally consent to be contacted again about potential study participation. A list of these provisionally eligible women will consist only of first name, date of first day of LMP, and contact information (e.g., cell phone and/or address). The research staff will contact these women again shortly before they reach 17 weeks gestation to remind them to return to the study field office (or MCHTI) for detailed screening if they still meet the provisional screening requirements.

d) Detailed screening (of provisionally eligible women)

A study physician will supervise the detailed screening and informed consent process of all provisionally eligible women at 17 to 24 completed weeks gestation. Interviews with prospective participants will be conducted in a designated private area of the MCHTI outpatient department or field office (i.e., where a discussion can take place that other clients would be unlikely to overhear). The detailed screening and consent process will include the following items:

1. Confirmation of eligibility criteria pertaining to medical and obstetric factors.
2. With verbal consent to proceed, provisionally-eligible women will undergo assessment of the following clinical parameters:
 - **Blood pressure measurement** to exclude women with hypertension: Diastolic and systolic blood pressure will be measured using an automated digital blood pressure monitor. Two measurements will be taken, at least 1 minute apart; the highest of each of the systolic and diastolic values will be used for exclusion purposes (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg).
 - **Urine dipstick testing** to exclude women with moderate-severe proteinuria: Participants will be instructed to collect a random spot urine specimen (~30 mL) into a sterile dry container. Specimens will be screened immediately using a standard urine dipstick to measure urine protein. A positive test will require a second dipstick test, and a second positive result will lead to exclusion from the trial.
 - **Finger-prick blood testing** to exclude women with severe anemia: A standard lancet will be used to prick the participant's finger and draw a drop of blood that

will be used to measure hemoglobin concentration with a hand-held hemoglobinometer (Hemocue).

- **Obstetric ultrasound** to confirm GA estimation and to exclude women with multiple gestation, major congenital anomalies, or severe oligohydramnios. *All participants will have an ultrasound at the time of enrolment to ensure all eligibility criteria are met.* However, as noted above, if a prior ultrasound report is available, GA estimation should be based on an earlier ultrasound, not the ultrasound conducted during enrolment.
3. Eligibility will be based on the 'best guess' GA estimate using an algorithm that integrates both LMP and ultrasound (see above, under eligibility criteria).
 4. If a woman is found to be eligible, she will be offered the opportunity to participate and will be required to complete the detailed written consent process.
 5. The consent process may occur over a period of more than 1 day, if necessary, to enable completion of the ultrasound and to provide the woman with adequate time to review the study information and decide whether or not to participate.
 - If the participant completes the written consent process more than 3 days after the start of the detailed screening process, the blood pressure measurement and urine dipstick testing must be repeated.
 - Hemoglobin measurement and the ultrasound need not be repeated again in these situations if completed within the 17-24 week gestational age window.
 - Enrolment must occur prior to 24 weeks completed gestation (i.e., last day of eligibility is 24 weeks + 0 days). Women who delay completing the written consent process and pass this time point cannot be included in the trial, even if they started the written consent process while still eligible.
 6. If a woman is deemed ineligible to participate in the study based on her medical history, blood pressure, finger prick blood sample, urine dipstick test, or ultrasound, or if or she chooses not to sign the consent form, she will be referred to the MCHTI antenatal care physician for standard care. Study staff will not assume further responsibility for the medical care of clinic patients who are not enrolled in the study.

(See below for details of the informed consent process that is integrated with detailed screening.)

18) Allocation of intervention

a) Sequence generation

The allocation sequence will be produced using a computer-generated random number sequence. We will use a simple randomization scheme; i.e., no stratification or blocking. The trial statistician will generate the allocation sequence.

b) Allocation concealment mechanism

The allocation sequence will not be viewed by the investigators or the field staff. This list will be provided to TIPT, the pharmaceutical company producing the supplements, which will package

the supplements in individual participant supplement packs labelled with a unique study identifier.

c) Implementation of allocation

The pre-labelled supplement packets containing both phase 1a (prenatal) and phase 1b (postpartum) supplements (in separate packets) will be provided to the field staff, and will be sequentially allocated to participants according to the order of enrolment. Using this method, there is no need for a treatment assignment to be concealed using envelopes, etc.

19) Blinding (masking)

a) Who will be blinded, and how

Participants, investigators, field personnel, study lab staff and data analysts will be blinded to vitamin D or placebo group allocation. The supplements will be identical in appearance and taste. Each supplement pack will only be labelled with the unique study identifier and the phase (prenatal or postnatal). The master list that links the unique identifier with group allocation will be maintained throughout the study at TIPT, and will only be released to the DSMB and/or investigators upon request.

b) Conditions and procedures for unblinding

The allocation scheme will be available to the DSMB and individual participants will be unmasked if they experience suspected supplement-related adverse events (i.e., hypercalcemia or clinical features suggestive of vitamin D toxicity). Because of the method of allocation concealment, unmasking of a single participant will not affect the masking of other participants. Unmasking of individual participants' allocation is not planned, to enable ongoing blinded follow-up to 2 years postnatal, and beyond.

Methods: Data collection, management, and analysis

20) Data collection

a) Sequence and content of study visits

Follow-up will be conducted through pregnancy and up to two years postpartum/postnatal. Visits will routinely be conducted in the home, except when delivery occurs and when clinic visits are scheduled (i.e., typically when specimen collection is scheduled). Data will be collected using standardized data collection forms (DCFs). DCFs will be in a conventional paper format (although efforts will be made to identify opportunities for real-time electronic data capture).

1. Baseline visit (at enrolment)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	1A-B, 2, 3A-D

- i. Questionnaire (detailed): demographics, medical history, family and household factors, lifestyle factors related to vitamin D status
- ii. Maternal food frequency questionnaire
- iii. Blood pressure
- iv. Finger-prick blood test for severe anemia
- v. Urine dipstick testing for proteinuria
- vi. Maternal height and weight
- vii. Paternal height and weight (may be completed at a later time, as soon as possible after enrolment)
- viii. Obstetric ultrasonography
- ix. Maternal blood and urine specimen collection
- x. Paternal blood specimen collection (may also be completed at a later time)
- xi. Supplement administration (first dose)

2. Prenatal routine weekly visits

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Home (or clinic)	Weekly (Phase 1a)	4A and 4B (first weekly visit)

- i. Questionnaire (brief) to document on-going participation, health/vital status and medical events, and protocol deviations (e.g., non-study vitamin D supplement consumption)
- ii. Questionnaire (brief) to document household socioeconomic status and family characteristics, only during the first weekly visit.
- iii. Symptom checklist and morbidity surveillance (maternal)
- iv. Blood pressure measurement (only at 24 weeks, and then weekly from 36 weeks until delivery)
- v. Supplement administration and adherence monitoring

3. 3rd-trimester clinical visit (30 weeks gestation)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	4A and 5

- i. All data collected during prenatal routine weekly visits (above)
- ii. Blood pressure
- iii. Maternal weight
- iv. Blood specimen collection
- v. Supplement administration (per routine weekly visit)

4. Labour and delivery

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Hospital	Once	6A

- i. All data collected during prenatal routine weekly visits (above)
- ii. Labour and delivery record (timing of events, mode of delivery, complications, etc.)
- iii. Blood pressure and temperature
- iv. Maternal weight
- v. Maternal blood and urine specimen collection
- vi. Cord blood (venous and arterial) specimen collection
- vii. Placental weight and umbilical cord insertion location recorded
- viii. Placental and umbilical cord specimen collection

5. Neonatal assessment

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Hospital	Once	6B

- i. Questionnaire: vital status, complications and treatment, including referral to a neonatal care unit.
- ii. Neonatal anthropometry: weight, length, head circumference⁵⁹, upper-arm (i.e., humerus) length, and rump-to-knee (i.e., femur) length.
- iii. Neonatal clinical examination by physician

6. Postnatal/postpartum routine weekly visits (0-6 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Home (or clinic)	Weekly (Phase 1b)	7

- i. Questionnaire (brief) to document on-going participation, health/vital status and medical events, infant feeding practices, supplement adherence, and protocol deviations (e.g., non-study vitamin D supplement consumption).
- ii. Symptom checklist (maternal)
- iii. Morbidity surveillance and brief clinical exam (infant)
- iv. Supplementation administration
- v. Health promotion (e.g., counselling regarding feeding and immunizations)
- vi. Infant nasal swab specimen collection if criteria for acute respiratory illness are met [see Appendix for sub-study protocol, "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh"].
- vii. Maternal blood pressure (first postnatal visit only or if remains hypertensive)
- viii. One weekly visit (randomly assigned to 2, 4, 6, or 8 weeks after birth) will include infant anthropometry: length, weight and head circumference.

21) *All infants will have scheduled visits at 3 and 6 months postnatal. In addition, infants will have one additional set of anthropometric measurements (weight, length and head circumference) performed between birth and 8 weeks, but the specific timing of these additional visits will be randomly assigned at 2-week intervals. Random assignment of the supplementary anthropometry will be generated at the same time as the allocation sequence. Although dates of these visits will be scheduled in advance for each infant, the specific timing may be altered to accommodate participants' requests/schedules.

1. Postnatal/postpartum clinic visits (0 to 6 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	2	7 and 8 (3 months) 7,9 and 3C (6 months)

- i. All data collected during postnatal routine weekly visits (above)
- ii. Maternal food frequency questionnaire (at 6 months only)
- iii. Infant anthropometry: weight, length, and head circumference, mid-upper arm circumference, upper arm length, rump-to-knee length.
- iv. Maternal blood specimen collection (at 3 and 6 months)
- v. Maternal breast milk specimen collection (at 3 and 6 months)
- vi. Infant blood specimen collection (at 3 and 6 months), with test for anemia (at 6 months only)
- vii. Maternal and infant urine specimen collection (at 6 months only)
- viii. Infant nasal swab specimen collection if criteria for acute respiratory illness are detected [see Appendix for sub-study protocol, "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh"].

2. Postnatal/postpartum tri-monthly visits (9, 15, 18, and 21 months)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Home (or clinic)	4	10 and 4B (9 and 21 months)

- i. Questionnaire (brief) to document on-going participation, child health/vital status, and medical events.
- ii. Morbidity surveillance (infant)
- iii. Health promotion (e.g., counselling regarding feeding and immunizations)
- iv. Infant anthropometry: weight, length, and head circumference.
- v. Questionnaire (brief) to document household socioeconomic status and family characteristics, only during the first weekly visit (Form 4B)

3. Postnatal/postpartum primary endpoint visit (age 52 weeks)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	10 and 11

- i. All data collected during postnatal routine tri-monthly visits (above)
- ii. Maternal weight and height
- iii. Infant anthropometry: mid-upper arm circumference, upper-arm length, and rump-to-knee length in addition to the anthropometry listed above.
- iv. Infant blood specimen collection

4. Postnatal secondary endpoint visit (age 2 years)

<i>Site</i>	<i>Frequency</i>	<i>Form(s)</i>
Clinic	Once	10 and 12

- i. All data collected during postnatal routine tri-monthly visits (above)
- ii. Maternal weight and height
- iii. Infant anthropometry: mid-upper arm circumference, upper-arm length, and rump-to-knee length in addition to the anthropometry listed above.
- iv. Infant blood specimen collection

The timing of visits will be scheduled in advance; however, the actual dates of visits may be postponed (changed to later dates) to accommodate participants. Additional unscheduled encounters may occur if the participant contacts study personnel to report new symptoms or clinical concerns, to ask questions regarding study participation, or to advise of an upcoming absence from the study area.

b) Verbal and clinical data collection methods (including point-of-care tests)

1. **Questionnaires.** At every visit, questionnaires will guide study personnel in conducting structured, verbal, face-to-face interviews of the primary participant (mother). As a contingency, some data may be collected by phone (e.g., to confirm supplement consumption by participants who are temporarily outside of Dhaka). In some circumstances, other individuals may act as the respondents in the mother's absence (e.g., reporting a maternal or infant medical event).
2. **Blood pressure measurement.** Maternal systolic and diastolic blood pressure (BP) will be measured using an automated digital blood pressure monitor (Microlife 3BTO-AP or equivalent). Two readings will be taken at least one minute apart and recorded; if either diastolic or systolic measurements differ between the paired readings by >10 mmHg, a third measurement will be performed. For analysis, paired readings will be averaged; where a third reading was obtained, the single discordant reading will be excluded. BP will be measured at enrolment, 24 weeks, 30 weeks, and then weekly from 36 weeks until the first postpartum home visit; if hypertensive at postpartum

measurement, will continue to be monitored weekly unless BP normalizes. BP may be measured in the home or clinic.

- 3. Urine dipstick testing.** Urine dipsticks (Siemens Diagnostics Urinalysis Reagent Test Strips) will be used at enrolment and then after enrolment only if high blood pressure is detected (systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg) or if there are symptoms of dysuria (for leukocyte esterase).
- 4. Hemoglobin measurements.** At enrolment (as part of the eligibility screening process), maternal hemoglobin will be measured in a finger-prick blood sample using a handheld hemoglobinometer (Hb 201, Hemocue AB, Sweden). Infant haemoglobin will be measured at 6 months of age. If blood is not collected at the 6-month visit, hemoglobin testing will be offered at a subsequent visit (e.g., 12-month visit).
- 5. Adult (maternal and paternal) height and weight.** Weight and height will be measured in the clinic using standard methods adapted from the CDC NHANES manual⁶⁰, and employing a digital floor scale (Tanita HD318, Tanita Corporation, Japan or equivalent) and a stadiometer (Leicester Height Measuring device, Chasmors Ltd., England or equivalent). Measurements will be performed in duplicate. Third measurements will be taken if the following discrepancies are noted between the paired measures: > 0.5 kg for weight; > 2 cm for height.
- 6. Obstetric ultrasonography.** A second trimester ultrasonography will be performed at the time of enrolment by technicians at MCHTI using equipment available at the MCHTI (Just Vision 400, Toshiba, Japan; SONOACE X8, Medison, Korea). Technicians will be trained to collect specific data for study purposes, including number of fetuses, presentation, fetal heart rate, biparietal diameter, anterior-posterior thoracic diameter, crown-rump length, femoral length, abdominal circumference, placental position, amniotic fluid index, gestational age, fetal weight, presence/absence of severe oligohydramnios, and presence/absence of major anomalies.
- 7. Infant anthropometry.** Standardized procedures for infant anthropometry will be adapted from the Intergrowth-21 study manual (<http://www.intergrowth21.org.uk>). At each clinic visit, each infant will be measured independently by two study personnel, and the paired measurements will be compared; if they differ by more than the threshold values (7 mm for length; 5 mm for head circumference, upper arm length, knee-rump length, and mid-upper arm circumference; and, 50 g for weight), a second set of measurements will be performed and again compared. For all measurements except infant weight, if the second set differs by more than the threshold values, the procedure will be repeated a third time. Lengths and head circumferences will be recorded to the last completed unit (not to the nearest unit). The average (mean) of acceptable paired measures will be used in analysis.

- i. **Weight** will be measured in the clinic (or home) using a digital infant scale (Seca 334, Seca, Hamburg, Germany), to the nearest 5 g (up to 10 kg) and to the nearest 10 g (for > 10 kg).
 - ii. **Length** will be measured in the clinic or home using a wooden length board (Infant/Child ShorrBoard; Weigh And Measure, Olney, Maryland), to the last completed 0.1 cm (1 mm).
 - iii. **Head circumference, mid-upper arm circumference (MUAC), upper-arm length, and rump-to-knee length** will be measured at scheduled clinic visits using a soft measuring tape or caliper, to the last completed 0.1 cm (1 mm).
8. **Dietary recall:** Estimation of usual dietary intake of vitamin D, calcium, phosphorus, and phytates among trial participants (mothers) at enrolment and 6 months postpartum using a food frequency questionnaire.
9. **Neonatal examination:** As soon as possible after delivery, a physician will perform a standardized physical exam to document any congenital anomalies.
10. **Placental weight and dimensions:** After collection of cord blood, the umbilical cord and membranes will be removed from the placenta, the blood will be drained, and then the placenta will be weighed to the nearest 0.5 g (iBalance i2500, MyWeigh, Vancouver, Canada).
11. **Morbidity surveillance:** During routine weekly visits during Phase 1 (a & b), study personnel will review symptoms of possible infectious illness.
 - i. Maternal prenatal illness will be tracked using a symptom checklist at weekly prenatal visits. Temperature measurement and/or urine dip for leukocytes (indicator of urinary tract infection) will be performed in the home if clinical symptoms are suggestive of infection, based on standardized clinical algorithms.
 - ii. Infant episodes of diarrhea, acute respiratory infection (ARI) – subdivided as lower and upper tract subtypes, sepsis, or skin infections will be documented during the period from birth to 6 months of age. Conventional diarrhea and ARI case definitions will be based on maternal report of symptoms (e.g., cough, frequent stools, etc). When symptoms of ARI are reported (cough and/or difficulty breathing), the field worker will measure the infant’s temperature and observe for rapid breathing, chest indrawing, or clinical danger signs (using the World Health Organization Integrated Management of Child Illness algorithms). Infants who meet study criteria for an “acute respiratory infection” will further undergo **pulse oximetry** and **nasal swab specimen collection** [see Appendix for sub-study protocol, “Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh”].

Infants with suspected “pneumonia” (i.e., lower respiratory tract infection) or other serious infections will be referred to the hospital for assessment and treatment by a physician. The occurrence of other minor infections (e.g., skin infestations) and severe infections (e.g., meningitis) diagnosed during the follow-up period will be recorded.

12. Clinical and serious adverse event (SAE) reports. Details of all serious adverse events (i.e., hospitalizations, deaths) and sequelae are routinely recorded on SAE reporting templates as required by the local institutional regulations for adverse event monitoring. Where appropriate, details of hospitalizations are recorded on hospitalization Forms 19 and 19B. Records related to other types of clinical events may be generated by study physicians in the process of providing direct patient care and/or facilitating referral to other facilities or physicians; where appropriate, data are captured in structured clinical event case report forms (i.e., Forms 15, 16). Study physicians obtain data related to clinical events and SAEs from direct clinical assessments; notes, prescriptions or impressions of other treating physicians; reports of investigations; or, directly from participants/caregivers. Formal health records are generally unavailable in the trial setting. Data related to clinical encounters and SAEs will be retrospectively extracted from study physicians' notes, clinical event and hospitalization report forms, and SAE reports for incorporation into the trial database. Where necessary, de-identified data (including diagnostic images) will be post-hoc reviewed and/or adjudicated by study investigators to assign diagnostic codes or classifications.

c) Biological specimen collection and processing

Specimen Collection Schedule

Specimen	Phase 1a			Phase 1b		Phase 2a	Phase 2b
	Baseline	30 weeks	Delivery	3 months	6 months	12 months	24 months
Maternal urine	X		X		X		
Maternal venous blood	X	X	X	X	X		
Paternal venous blood	X						
Cord venous and arterial blood			X				
Placenta and cord tissue			X				
Infant venous blood				X	X	X	X
Infant urine					X		
Breast milk				X	X		
Infant nasal swab				X*	X*		

*Nasal swab is performed only if criteria for acute respiratory infection are met

Trained phlebotomists will collect maternal venous blood, cord venous and arterial blood (after delivery of the placenta), and infant specimens according to standard operating procedures. The following general principles will apply:

- Specimens will be routinely collected in the clinic (or hospital) setting; however, in cases in which a home delivery occurs and study personnel are present during the delivery or immediately afterwards, placenta and cord blood specimens may be collected in the home. Venous blood specimen collection and urine collection will not be routinely performed in the home.
- All specimen collection will precede the weekly administration of the study supplement (in phase 1).

- To limit the extent to which biomarkers are influenced by acute inflammatory responses, mothers and infants experiencing an episode of acute diarrhea or acute respiratory illness at the time of scheduled sampling will have specimen collection postponed by 7 days. However, this will not apply to baseline specimens, or delivery specimens (maternal urine, maternal blood, cord blood and placental specimens).
- Maternal blood specimens at 30-weeks are primarily to document serum calcium in individual women, as a safety parameter. Baseline and delivery specimens will enable analyses related to the change in serum biomarkers from baseline to end of pregnancy (e.g., change in vitamin D status). Specimens collected during phase 1 (up to 6 months of age) will be used for planned/budgeted biomarker analyses (see below), and any remaining sample tissue/volume will be banked in Dhaka. Infant blood specimens in phase 2 (i.e., those collected at 12 and 24 months of age) will be bio-banked for additional analyses based on funding availability, or as part of future sub-studies.
- Processing of blood samples for plasma and serum separation:

Specimen type	Vacutainer tube	Centrifugation	Storage/transport for batched analyses	Storage/transport for same/next-day analyses
Plasma	EDTA tube (lavender top), or trace element EDTA tube (royal blue top)	Immediately (within 30 minutes of blood draw).	To be placed in portable -80°C freezer immediately after centrifugation and aliquot preparation (0.25 mL aliquots for plasma, 0.5 or 1.5 mL aliquots for whole blood); then, transported to icddr,b and transferred to an upright -80°C freezer for long term storage.	Not applicable.
Serum	Serum tube (red top)	In 30 to 60 minutes after blood draw, kept at room temp before centrifugation.		Placed in 2-8°C refrigerator until transport in a 2-8°C cold box to icddr,b, where held at 2-8°C until analysis.
Whole blood	EDTA tube (lavender top), or trace element EDTA tube (royal blue top)	Removed from vacutainer immediately after blood draw and before centrifugation.		Not applicable.

- Whole blood aliquots (1.5 or 0.5 mL) will be drawn from EDTA tubes immediately after mixing (prior to centrifugation) for paternal blood, maternal delivery and cord blood samples.
- Serum and EDTA tubes will be centrifuged at low speed for 15 minutes, and the supernatant transferred by plastic micropipette in 0.25 mL aliquots into labelled microfuge tubes.

1. Maternal Urine: Participants will be instructed to collect a spot urine specimen into a sterile dry container at baseline, delivery, and 6 months post-partum. During delivery,

one specimen aliquot (1.5 mL) will be held at 2 to 8°C until it is transported in a 2 to 8°C cooler to the laboratory for same or next-day analysis at icddr,b. Other aliquots collected at baseline, delivery and 6 months will be placed in a portable -80°C freezer until transfer to icddr,b for long-term storage at -80°C.

2. **Maternal venous blood:** A phlebotomist will collect 3 mL venous blood in a serum tube and 6 mL in an EDTA tube, at baseline, 30 weeks gestation, delivery, 3 months postpartum and 6 months postpartum. The delivery sample will be optimally collected during labour (2nd stage or later); however, if this is not feasible, a postpartum specimen will be obtained within 24 hours of delivery. Specimens will be considered to have been collected 'at delivery' if drawn within +/- 24 hours of delivery. At delivery, a trace element tube (royal blue-top; with EDTA; 6 mL) will be used in place of the regular EDTA tube.
3. **Paternal venous blood:** A phlebotomist will collect 6 mL venous blood in an EDTA tube, at baseline. If the father is not able to contribute a blood sample at baseline, he can return at another time in the near future.
4. **Cord blood:** The umbilical cord will be clamped and cut, the specific technique and timing for which will be determined by the attending physician or birthing attendant (i.e., cord clamping time is outside of the study protocol). Within 10 minutes of delivery (up to a maximum of 30 minutes after delivery of the placenta), a site on the umbilical cord attached to the placenta will be cleansed using dry cotton gauze to wipe away any maternal blood. The umbilical vein will be cannulated and blood will be collected into the following collection tubes (in this order): 1) one 5 ml serum (red-top) tube, 2) one 10 mL EDTA (purple-top) tube, 3) one 6 ml trace element (blue-top) tube (with EDTA), and 4) one 10, 6, 4 or 2 mL EDTA (purple-top) tube (depending on the volume of blood collected). Attempts will be made to collect a total of about 20 to 40 mL of blood. If feasible, the umbilical artery will be cannulated and one 5 mL serum (red-top) tube will also be filled.
5. **Placental and cord tissue:** Following delivery of the placenta, large **placental specimens** from 2 quadrants of the placenta will be collected and then divided into three distinct specimen types.
 - 1) Full disc biopsies with ~0.5 cm thickness that will be stored in 10% formal saline at room temperature for later histopathological examination.
 - 2) Small tissue samples (~0.2 cm³) from the fetal surface of the placenta that will be stored in RNALater at 2 to 8°C for 48 hours and then -80°C for later epigenetic/gene expression studies.
 - 3) Small tissue samples (~0.2 cm³) from the maternal surface of the placenta that will be stored and used similarly to #2 above.Specimens from each quadrant will be collected and then pooled together to create samples that represent the entire placental disc (one pooled histopathology specimen and multiple pooled RNALater specimens).

Two distinct types of **cord tissue specimens** will be collected, both consisting of thin (~0.25 cm thick) cross-sections of the entire cord.

- 1) One cross-section stored in 10% formal saline with the full disc biopsies above at room temperature for later histopathological examination.
- 2) Cross-sections stored in RNALater at 2 to 8°C for 48 hours and then -80°C for later epigenetic/gene expression studies.

- 6. Infant venous blood collection:** During 4 clinical visits during the post-partum / infant follow-up phase, if the parent/caregiver gives permission a phlebotomist will collect a single venous blood specimen by venipuncture of a superficial arm, hand, leg, or foot vein. Samples will be collected into an EDTA tube and a serum tube. At 3 months, the total blood volume will be 3 mL (2 mL in EDTA tube, 1 mL in serum tube); at 6 months, the total blood volume will be 6 ml (4ml in EDTA tube, 2 ml in serum tube); at 1 and 2 years of age, the total blood volume will be 5 mL per draw (4 mL in EDTA and 1 mL in a serum tube). The following measures will be taken to enhance infant comfort during the procedure (based on parental preference):
- a. Swaddling
 - b. Breastfeeding, and/or
 - c. Oral dextrose/sucrose administration (2 mL of 12% dextrose or 24-25% sucrose solution applied to the tongue of infants within 2 minutes of the procedure).

For infant and maternal blood collection, a maximum of two attempts (where a single attempt is defined as having occurred if the skin is breached) will be made for each scheduled visit. If a sample cannot be obtained after two attempts, the study personnel will request that the participant return once more for another two attempts at the participant's convenience (typically within 1 week). If the two additional attempts are unsuccessful, no further attempts will be made until the next scheduled blood sample.

To directly benefit the infant, serum calcium at 3 months and hemoglobin, ferritin, calcium, creatinine, phosphorus, alkaline phosphatase and urine calcium:creatinine results at 6 months of age will be reported back to the study physician, and the results will be interpreted using standard clinical algorithms. Infants with iron-deficiency anemia will be treated, and referred for specialist care if necessary. Other biochemical abnormalities will be treated and/or referred as required on a case-by-case basis. Urinary phosphate will also be measured at the 6-month visit, but not used for clinical decisions as there are no established reference ranges for infancy. If a blood sample is not collected at the 6-month visit, blood and urine collection for the biochemistry panel (haemoglobin, ferritin, calcium, creatinine, phosphorus, alkaline phosphatase, urine calcium:creatinine) will be offered at a subsequent visit.

- 7. Infant urine specimen** will be collected at 6 months of age. An adhesive plastic urine collection bag will be placed on the infant's perineum for a 2-hour urine collection period. One specimen aliquot (1.5 ml) will be kept in a refrigerator at 2 to 8°C until it is transported in a 2 to 8°C cooler to icddr,b for next-day analysis. All

others will be placed in a portable -80°C freezer until transfer to icddr,b for long-term storage at -80°C.

8. **Breast milk samples** will be collected at 3 and 6 months postpartum according to the SOP developed for the MAL-ED study. Breast milk sample collection will be undertaken in a private setting, with appropriate sensitivity to the participant's comfort during the procedure. Specimen aliquots will be placed in a portable -80°C freezer until transfer to icddr,b for long-term storage at -80°C.
9. **Infant nasal swab specimens** will be collected at the time of acute respiratory illnesses from birth to 6 months of age [see Appendix for sub-study protocol, "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh"].

Approaches to potential variations in the specimen collection schedule:

- Missed or late specimens: Participants may occasionally be absent on scheduled days of specimen collection, or events such as national holidays or general strikes may require minor changes to sampling schedules. The following principles will guide specimen collection in case of late or missed specimen collection:
 - In general, 'late' specimens will be collected at the earliest opportunity following the missed visit.
 - A specimen will only be considered 'missed' if the next scheduled time of collection of the same specimen type is reached before the missed specimen can be collected.
- Specimens unsuitable for analysis: A small proportion of scheduled urine or blood specimens may be found to be unsuitable for analysis, mislabelled, etc. In these situations, study personnel will request collection of a single replacement specimen from the participant. Individual participants will not have replacement specimens requested on more than one occasion during the trial.
- Unscheduled sampling to monitor potential adverse events: According to the standardized safety monitoring algorithm, study personnel will occasionally collect unscheduled specimens from participants to either follow-up on initial results suggestive of hypercalcemia or on the basis of other clinical concerns. If a participant refuses follow-up sampling that has been advised for safety reasons, such that a specimen cannot be obtained within 7 days of the scheduled date or the repeat sample cannot be obtained within a time period that is sufficient to enable a decision regarding the safety of the next scheduled supplement dose, then supplementation may stop (for safety reasons) but other forms of observational follow-up may proceed as per the protocol (see 'Safety Monitoring' below).
- At-home blood collection: in cases when an initial biochemical abnormality was found and a repeat sample collection is not possible due to circumstances that prevent the participant to visit the clinic within the necessary time-period, at-home blood collection will be offered by the study team. If the participant permits the at-home collection, a trained phlebotomist will visit the home and collect the blood specimen, observing the same safety and hygienic protocols used at clinic visits.

b) Biomarker analyses

Biomarkers of the vitamin D-parathyroid axis (vitamin D metabolites, PTH fragments, PTHrP, serum calcium, and FGF-23) will be serially quantified through the pre- and postnatal periods.

- Vitamin D status:* Vitamin D status will be determined by the serum/plasma 25(OH)D concentration, which is a well-established biomarker⁶¹. Maternal, infant, and (if funding permits) paternal serum (or plasma) 25(OH)D will be assessed using state-of-the-art liquid chromatography tandem mass spectroscopy (LC-MS/MS) at the AFBM.
- Serum calcium:* Maternal serum calcium concentration is the primary biochemical safety outcome, and will be measured at scheduled intervals during the intervention phase (baseline, 30 weeks of gestation, delivery, 3 months postpartum, and 6 months postpartum). Serum calcium concentration in the cord vein and infants at 3 and 6 months will also be measured.
- Parathyroid hormone (PTH):* The PTH immunoassays currently used widely in clinical laboratories (so-called 2nd-generation assays) utilize antibodies specific to epitopes found in the middle of the whole PTH protein (84-amino acid length). These immunoassays cross-react with PTH fragments that do not include the extreme N-terminal amino acid sequence responsible for PPR binding (see **Figure 9**); these assays are conventionally referred to as *intact PTH* (iPTH) assays. Newer assays utilizing epitopes specific to the bioactive N-terminus (first 3 amino acids) enable specific identification of ‘whole PTH’ (wPTH) molecules that include the full 84-amino acid protein (PTH(1-84)). wPTH is increasingly recognized as a more biologically relevant marker; in a recent epidemiologic study of adults on renal dialysis, wPTH correlated more precisely with adult mortality than iPTH⁶². Since wPTH may represent a relatively greater fraction of iPTH in the context of parathyroid hyperactivity, a linear conversion factor cannot be implemented⁶³.

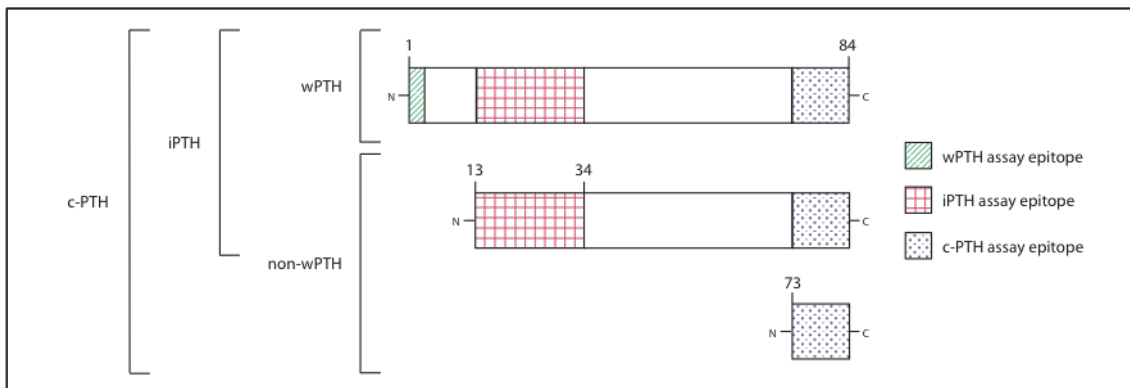


Figure 9: Schematic representation of the three types of PTH molecules to be assayed in the proposed study. Labeled brackets indicate the types of molecules that will be identified by each of 3 assays (c-PTH, iPTH, wPTH). The non-wPTH fraction will be calculated as c-PTH minus wPTH.

Commercially available assays now exist that utilize antibodies that bind truncated c-terminal fragments of PTH (c-PTH), thereby capturing virtually all PTH peptides, ranging from wPTH (i.e., PTH(1-84)) to peptides as short as the c-terminal 11 amino acids (i.e., PTH(73-84)). To capture the entire spectrum of vitamin D-parathyroid-growth

associations, we will quantify multiple PTH fractions in the proposed study. wPTH, c-PTH, and iPTH will be measured by ELISAs (Immutopics) which employ the same biotinylated capture antibody to bind PTH at amino acids 39-84. We considered other available methods for the multi-target assessment of PTH; but the Immutopics assay was selected based on assay characteristics, epitope specificity, and specimen volume efficiencies. Evidence suggests that non-wPTH (i.e., PTH that lacks the bioactive N-terminal region) has biological effects that are generally opposite to those of wPTH⁶⁴. High circulating concentrations of c-PTH relative to wPTH have been associated with growth faltering in some studies of children with end-stage renal disease⁶⁵. We will thus investigate whether relative suppression of maternal and/or infant c-PTH secretion mediates the effect of vitamin D supplementation on fetal growth.

- *FGF-23* is a relatively recently discovered bone-derived protein, the expression of which may be triggered by 1,25(OH)₂D and which may act as a phosphaturic factor in the context of hyperparathyroidism⁶⁶. In many Gambian children with calcium-deficiency rickets and elevated PTH, FGF-23 concentrations were above the expected range⁶⁷. We hypothesize that FGF-23 may be a biomarker of parathyroid hyperactivity. FGF-23 will be quantified in plasma using a 2nd-generation immunoassay that detects epitopes within the carboxyl-terminal (C) portion of the molecule (Immutopics).
- *Parathyroid hormone related peptide (PTHrP)*. Partially homologous to PTH, PTHrP is an established regulator of endochondral bone formation that is essential for long bone extension⁶⁸. In pregnancy, PTHrP is a critical regulator of maternal-fetal calcium flux²⁸. It is also speculated that PTHrP mediates postnatal infant growth since it is readily transferred from the mother to infant via breastmilk⁶⁹. In rats, antagonism of PTHrP during pregnancy causes fetal growth restriction⁷⁰ and, in a recent epidemiologic study, it was shown that fetal PTHrP concentrations were significantly lower in infants with IUGR compared to normal weight infants⁷¹. The systemic interplay between vitamin D, PTH and PTHrP in humans has not been well described, but animal studies suggest that elevations in PTH are associated with suppression of endochondral PTHrP expression⁷². We aim to test the hypothesis that maternal PTHrP production and its transfer via breast milk are mechanisms by which vitamin D supplementation enhances infant bone growth.
- *Urinary phosphate excretion*. PTH over-expression may increase urinary phosphate loss, as suggested by observations of children with hypophosphatemic rickets who manifest significant growth restriction and low serum phosphate concentrations⁷³. Infant urinary phosphate excretion will be measured and expressed as the phosphate-creatinine ratio and the renal maximum tubular reabsorption of phosphate per litre of glomerular filtration rate (TmP/GFR). It is also possible that severe parathyroid axis dysregulation may lead to end-organ PTH/PTHrP receptor (PPR) downregulation, which has been suspected in Indian toddlers with very low dietary calcium intake⁷⁴. Urinary cyclic AMP will be measured as a biomarker of PPR responsiveness.

Other biomarkers will be used to test mechanistic hypotheses during discrete developmental periods (see Figure 4 above):

- *Insulin-like growth factor (IGF) system.* In children with healing rickets, skeletal remineralization and the decline in PTH is accompanied by increases in circulating IGF-1 and IGF binding protein-3²¹. This observation is concordant with studies in animals in which secondary hyperparathyroidism is accompanied by diminished IGF-1 and IGFBP-3 secretion⁷⁵. IGFBP-3 appears to facilitate IGF-1 action, whereas binding of IGF-1 by IGFBP-1 may inhibit IGF-1 function. In epidemiologic studies, maternal IGFBP-1 concentrations were inversely associated birth weight (in Norway)⁷⁶, and elevated IGFBP-1 was found among small-for-gestational age newborns in Pakistan⁷⁷ as well as among stunted children in South Africa⁷⁸. We aim to explore whether suppression of fetal IGF-1 and IGFBP-3, as well as increased IGFBP-1 production, mediate the effect of maternal prenatal vitamin D supplementation on infant size. Thus, IGF system biomarkers will be studied in the perinatal period and early infancy via the cord blood sample and the first postnatal infant specimen.
- *Phosphates and phytates* may inhibit calcium absorption⁷⁹, and thus may interact with vitamin D and calcium intake. *Vitamin A* status may regulate PTH secretion⁸⁰. Also, retinols interact with vitamin D in the nucleus, via heterodimerization of the retinoic acid and vitamin D receptors. *Iron* status has been postulated to have a modifying effect on the vitamin D-parathyroid axis. Recently, in a study of Gambian children, hemoglobin (interpreted by the authors as a surrogate marker of iron status) was shown to be inversely associated with FGF-23⁸¹, suggesting that iron may modify the effects of dietary calcium deficits or vitamin D deficiency on bone mineral metabolism. *Cadmium and fluoride* may be found in high concentrations in the groundwater in some regions of South Asia. Cadmium has been postulated to adversely affect calcium absorption in Bangladeshi women⁸², and fluoride excess has been linked to rickets in some parts of India⁸³. *Folic acid* is of unique interest because of its potential to act on maternal-fetal vitamin D status via epigenetic regulation of enzymes involved in vitamin D metabolism⁸⁴. In fact, folic acid supplementation may improve vitamin D status independently of vitamin D based on observations in pregnant Nepali women⁸⁵, and emerging evidence suggests that folic acid may influence the bioavailability of vitamin D during pregnancy by affecting the methylation pattern of 24-hydroxylase (Cyp24A1), the enzyme that catabolizes 25(OH)D⁸⁴.

Maternal vitamin A status, folate, and cadmium exposure will be studied during the prenatal period; Serum retinol will be applied as a biomarker of maternal vitamin A status, and maternal folic acid intake will be considered to be reflected by the maternal serum folate concentration⁸⁶. Fluoride exposure will be studied among infants in the postnatal period.

- *Inflammatory cytokines.* Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) may directly restrict linear growth through effects on osteoclast activity⁸⁷. Inflammation may also drive parathyroid hyperactivity⁸⁸; the pro-inflammatory chemokine interleukin-8 (IL-8) may specifically promote PTH secretion⁸⁹. On the other hand, vitamin D may dampen placental inflammation⁹⁰, thereby facilitating fetal development by optimizing transplacental nutrient transfer. To address the potentially key role of vitamin D in the regulation of immunity during the perinatal period, we will evaluate associations among vitamin D dose, parathyroid activity, and maternal peripartum or cord blood biomarkers of inflammation (TNFalpha, IL-6, IL-8, C-reactive protein).

- To more broadly explain mechanisms of infant growth and related health outcomes in the trial cohort, a range of other blood and urine analytes related to growth, energy regulation, satiety, metabolism, angiogenesis, inflammation, and immune function will be measured in stored maternal, fetal (cord), placental, infant and paternal biospecimen aliquots, as funding permits. Targeted and untargeted metabolomic and proteomic approaches will be considered. Concentrations of environmental toxins (e.g., persistent organic pollutants) may also be measured. Such analyses will be limited to participants for whom written consent was obtained for use of stored specimens.

A schedule of analytes is shown in Appendix C. Selected analytes (described above) will be measured on a routine basis and reported back to study physicians. However, as a cost-saving measure, additional analytes will be measured in a subset of specimens in the context of a nested biochemical sub-study.

Blood/urine/breastmilk biomarker analyses will be performed at three laboratories: the *Clinical Biochemistry Laboratory* (CBL) at ICDDR,B in Dhaka, the *Nutritional Biochemistry Laboratory* (NBL) at ICDDR,B in Dhaka, and the *Analytical Facility for Bioactive Molecules* (AFBM) at the Hospital for Sick Children (SickKids) in Toronto.

Maternal serum calcium, maternal urine calcium:creatinine ratio at delivery, infant serum calcium at 3 months, and infant hemoglobin, ferritin, serum calcium, creatinine, phosphorus and alkaline phosphatase and urine calcium:creatinine results at 6 months of age will be reported back to the field in real-time and managed by study physicians according to specific protocols. All others assays will be performed as batched analyses, and not reported back to the field, as they will not have clearly-defined clinical implications.

c) Epigenetic analyses

In a subset of mother-infant pairs, DNA (and mRNA, if funding permits) will be extracted from blood samples (and placental specimens, if funding permits) using standard techniques as previously described⁹¹. Where possible, paternal whole blood specimens will be collected to enable the identification of allele-specific patterns. Genomic DNA will be subjected to bisulfite modification, which will convert unmethylated cytosine (C) to thymine (T) but leave methylated C intact. Bisulfite modified DNA will be hybridized on to the Illumina Infinium HumanMethylation450 Beadchip (or similar array). If funding permits, we may also conduct pyrosequencing to assess DNA methylation (at single nucleotide (CpG) resolution) at several consecutive CpG sites within a ~150 bp region at the promoters of two genes involved in vitamin D metabolism (CYP27B1 and CYP24A1) in maternal and/or cord blood (and paternal blood, if feasible). Gene expression (mRNA) from placental specimens (fetal tissue) will be assessed by RT-PCR, if funding permits. Correlations between DNA methylation patterns and mRNA transcript levels will enable tissue-specific inferences regarding gene expression. All available maternal, paternal, cord blood and placental specimens will be stored, and if additional funding permits, maternal, paternal and fetal/infant genome-wide methylation patterns on a larger cohort will be analysed. Such analyses will be limited to participants for whom written consent was obtained for use of stored specimens.

d) Nasal swab specimen analysis by PCR [see Appendix for sub-study protocol, “Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh”].

e) Data quality control

A range of quality assurance (QA) and quality control (QC) mechanisms will be established to ensure the highest feasible data quality.

Primary QA methods will include:

1. Development of detailed standard operating procedures (SOPs) for all field and laboratory components of the protocol.
2. Intensive training of study personnel, with competency assessments after initial training, and at least every 3 months thereafter.
3. Field-testing of data collection tools (e.g., questionnaires, anthropometry) prior to enrolment of trial participants.
4. Structured oversight of all activities by supervisory personnel, including review of all completed data forms.
5. Weekly clinical/field personnel meetings to review common errors, challenges, etc.
6. Weekly reports circulated to the PI and trial coordination team by the field team. The report will be manually generated by field staff and will contain trial metrics regarding accrual, withdrawal, protocol deviations/violations, and adverse events, QC metrics, and reports of equipment function and calibration. In addition, regular (bi-weekly) reports will be generated from the database itself (data queries); however, these may lag by ~2 weeks due to the time required for data entry.

7. Specific approaches to improve precision of the primary outcome data, including duplicate anthropometric measurements (see above).
8. Duplicate data entry and use of built-in range and consistency checks in the database.

Primary methods of QC will include:

1. Random spot observations of study procedures by supervising personnel, with the aim of providing real-time feedback to field personnel.
2. Repetition of selected items from structured interviews and other procedures (such as anthropometry) from a sub-sample of visits/encounters.
3. Random spot checks by supervisors to ensure home visits occurred as recorded.
4. Random spot checks of individual participant tablet counts to ensure concordance with recorded number of tablets administered.
5. Analysis of vitamin D3 content of tablets by high-performance liquid chromatography (HPLC) prior to trial initiation and at regular intervals throughout. In addition, masked sets of supplements will be sent to a 3rd party lab to verify that placebo and vitamin D tablets are labelled appropriately.

f) Safety monitoring

Participant safety during the intervention phase will be monitored by study personnel based on:

1. Weekly follow-up visits that will include the use of a checklist of symptoms that may indicate vitamin D toxicity or other medical concerns. Monitoring for maternal and infant clinical events during clinic-based and household visits will follow a standard algorithm. In general, severe symptoms (e.g., severe headache) or persistence of mild-moderate complaints (e.g., persistent low-grade lower back pain) will prompt referral to the study physician.
2. Maternal serum calcium measured at scheduled intervals during the intervention phase (baseline, 30 weeks of gestation, delivery, 3 months postpartum, and 6 months postpartum).

Serum calcium is the best available biomarker of vitamin D toxicity⁹²; therefore, hypercalcemia will be the primary biochemical safety parameter in this trial. Nonetheless, based on our own experience with doses equivalent to up to 5000 IU/day (see above) and previously published trials conducted in the USA using doses up to 4000 IU/day during pregnancy⁴¹ and up to 6400 IU/day during lactation⁴⁸, we do not anticipate any episodes of hypercalcemia or other supplement-related adverse events.

- '*Possible hypercalcemia*' will be defined as a single maternal serum calcium concentration >2.60 mmol/L. Serum calcium will be reported back to the study physician within 72 hours of specimen sampling (typically, reporting will occur within 48 hours). Any participant with possible hypercalcemia found through scheduled blood sampling will provide a second sample within 24 hours of the time that the study physician receives a report of an abnormal value. Abnormal serum calcium results will always be reported and confirmed before the time of the following week's dose (to enable a decision to be made

about withholding supplementation). Severe derangements in serum calcium will be managed as urgently as possible by a non-study physician at a referral facility.

- ‘*Confirmed hypercalcemia*’ will be defined as serum calcium concentration >2.60 mmol/L on two separate blood specimens, and will be considered the primary supplement-related adverse event. The second, confirmatory specimen is required to prompt the cessation of study supplementation due to the possibility of a laboratory error, but clinical management need not await the second assay if the participant is symptomatic.
- If a second (confirmatory) blood specimen cannot be obtained and the initial value of serum calcium is >2.80 mmol/l, the measurement will be repeated on the same specimen to ensure the high value is not due to measurement error and the case will be managed similarly to cases of “confirmed hypercalcemia”,.

If the repeat serum calcium is normal, then supplementation will continue and a further repeat serum calcium will be measured one week after the first abnormal result, to confirm that it remains within the normal range. In the unlikely event that a participant has confirmed hypercalcemia, study personnel will follow the clinical course until normalization of serum calcium or one month post-partum (whichever occurs later), although medical care (including antenatal, perinatal and neonatal care) and additional non-study biochemical monitoring will be managed by non-study physicians. If medical referral for hypercalcemia is either not accepted or not adhered to by the participant, or non-study physicians do not request serial follow-up serum calcium measurements, then biochemical monitoring deemed appropriate by the study physician (in addition to routine study biochemistry) will be offered to the participant to document resolution of hypercalcemia or provide further justification for referral to a specialist. Participants with mild and asymptomatic hypercalcemia may not require referral or treatment beyond the cessation of supplementation, and will continue to participate in study follow-up. The study physician will be responsible for ensuring that any participant with confirmed or suspected moderate-severe hypercalcemia is assessed at a hospital by a clinician with expertise in the treatment of hypercalcemia, either immediately if indicated clinically or as soon as possible after the second serum calcium result is reported as abnormal (if the participant is otherwise asymptomatic). This assessment, and hospital admission if necessary, will be undertaken at a private clinic/hospital in Dhaka, since the availability of specialist care is inconsistent at public facilities. Costs of care will be covered by the study.

Although infant serum calcium is not a primary safety indicator, a similar approach as described above will be used to manage above-range infant serum calcium values. However, the normal range of infant serum calcium is higher than in adults, and normal values may be as high as 3.05 mmol/L⁹³⁻⁹⁸. Since there are few reference data for infants specifically at 3 and 6 months, a more conservative threshold of >2.80 mmol/L will be used to define above-range infant values (at the 3- and 6-month visits) that will prompt further assessment. Above-range infant serum calcium values need not prompt cessation of study supplementation in the absence of maternal hypercalcemia, but will be evaluated on a case-by-case basis.

3. Maternal urine calcium:creatinine ratio at delivery.

In previous trials, we used regular monitoring of maternal urinary calcium:creatinine as a screening measure for vitamin D toxicity; however, this test is non-specific, and frequently led to the need for repeat testing, which was burdensome for participants and field staff. In no cases did an isolated calcium-creatinine ratio indicate vitamin D toxicity. Therefore, regular urine calcium:creatinine ratio will not be employed as a primary clinical safety monitoring tool in this study. However, we will measure urinary calcium:creatinine ratio among mothers at delivery, as a screening test for hypercalciuria, rather than overt vitamin D toxicity. Abnormal values (Ca:Crea>1 mmol/mmol) will prompt repeat testing, and either of the following criteria will be considered as a presumptive diagnosis of 'hypercalciuria'

- two consecutive urine samples with Ca:Crea>1 mmol/mmol, or
- one urine sample with Ca:Crea>1 mmol/mmol in the presence of persistent symptoms suggestive of possible uro/nephrolithiasis.

Participants with persistent symptoms of renal colic or hypercalciuria will be referred for renal ultrasound to assess for the presence of urolithiasis or nephrolithiasis (renal stones). Participants with uro- or nephrolithiasis will be referred to the DSMB for a decision regarding unblinding and possible discontinuation of the study supplement, to be decided on a case-by-case basis. Biochemical evidence of hypercalciuria alone will not trigger urgent DSMB review, but will be reviewed at regular intervals. However, participants with hypercalciuria and an absence of stones will undergo repeat urine calcium:creatinine assessment one month later; if hypercalciuria is persistent, a repeat ultrasound will be undertaken.

g) Methods to promote participant retention and complete follow-up

Participant retention will be primarily promoted through frequent interaction between field-level study personnel and the participants (weekly during phase 1). Adherence to the schedule of clinic visits will be facilitated by compensation of participants for costs of transportation and time away from the home and/or work (equivalent of approximately \$5 USD per visit). The typical per-visit transportation cost to and from the clinic (e.g., by rickshaw) is expected to be 100 to 200 Taka (~\$1.25 to \$2.50). For comparison, the average household income in this low-income area of Dhaka is estimated to be about ~10,000 Taka per month (~\$125/month). A similar level of remuneration is currently being used without complications in ongoing studies in Dhaka. It is generally perceived to be high enough to adequately compensate participants for out-of-pocket expenses and time spent for visits (in transit and at the clinic), but low enough to avoid unduly influencing women to participate. Also, the payment in cash at the time of each visit prevents any undue influence to continue in the study that could occur if payment was delayed. Separate reimbursement may be made to directly compensate for transportation costs required when pregnant participants are in labour, to maximize the probability that pregnant participants will deliver at the Clinic. In many cases, this will not involve payment to participants, but rather direct payment to hired drivers on behalf of participants. Participants may decline some or all of the payments without affecting study participation.

Efforts will be made to obtain complete follow-up data from all enrolled participants. If a participant experiences a serious adverse event (e.g., hospitalization), follow-up and scheduled data collection will continue to the extent that is possible. Even in cases in which supplementation is stopped, data collection may continue, to the extent that the participant continues to consent to a modified form of participation.

h) Definition and handling of protocol deviations and violations

The terms *protocol deviation* and *protocol violation* refer to incorrect actions or omissions by study personnel or omissions of study procedures due to external factors, rather than participant actions, omissions or decisions. The terms are operationally defined as follows, for the purposes of monitoring and reporting of the trial:

- **Protocol deviations** will be considered to be all types of non-compliance with the written protocol by study personnel that do not materially increase the level of risk for individual participants, and do not sufficiently compromise data integrity to the extent to that a participant would need to have supplementation discontinued or be withdrawn from the study.
- **Protocol violations** will be considered to be acts of omission or errors by study personnel that materially increase the risk to one or more participants and/or require discontinuation of study supplementation or, if necessary, withdrawal of the affected participant from the trial. The withdrawal from the study or discontinuation of supplementation may be justified by protocol non-compliance that seriously compromises the data integrity even if this did not present a serious risk to the individual (e.g., participant was enrolled who did not meet eligibility criteria).

For ease of operationalization, the terms ‘deviation’ and ‘violation’ will be considered mutually exclusive, rather than considering violations to be a subset of deviations. The distinction between deviations and violations is not inherently related to whether the act/omission was intentional or accidental. In some cases, a failure to follow the protocol correctly may occur because of events beyond the control of the investigators, staff and participants (e.g., missed visit due to severe weather storm). Such instances will be referred to as protocol deviations or violations, as deemed otherwise appropriate based on the definitions above.

All protocol deviations and violations will be tracked closely by study personnel and supervisors, and will be recorded using specific data forms. Protocol deviations will, by definition, not require withdrawal of the affected participants, but will be carefully documented. Deviations will be reported in real-time to the ethics boards to the extent that is required by their regulations (e.g., if associated with a serious adverse event). In some cases, review of protocol deviations will result in application for protocol and/or study document amendments. Conversely, protocol violations typically will lead to discontinuation of supplementation (if occurring during Phase 1), and in some cases, may lead to withdrawal of the affected participant from the trial. Protocol violations will be reported to the DSMB and ethics boards to the extent required by the boards’ regulations and requirements; an ethics board may only require immediate reporting of protocol violations that are associated with serious adverse events.

As noted above, protocol deviations and violations are errors/omissions by study personnel or those due to external factors. In contrast, participant actions or decisions that result in deviation from the protocol will be referred to as **protocol non-adherence**. This distinction is important because participants are free to voluntarily refuse procedures at any time. In general, protocol non-adherence will not result in withdrawal from the trial, but may in some cases result in discontinuation of study supplementation as a safety precaution; such instances are detailed above (e.g., refusal to discontinue the use of non-study vitamin D or calcium supplements).

19) Data management

Research personnel will record data on standardized data collection forms (paper-based). A site supervisor will review all forms for completeness and protocol deviations/violations before sending them to the data management center at icddr,b on a weekly basis. All completed forms will be scanned at the study office before the hard copies are sent to the Data Management Unit at icddr,b. Electronic (scanned) versions of all forms will also be regularly delivered to icddr,b for long-term storage.

The flow of data is shown schematically in the diagram below:

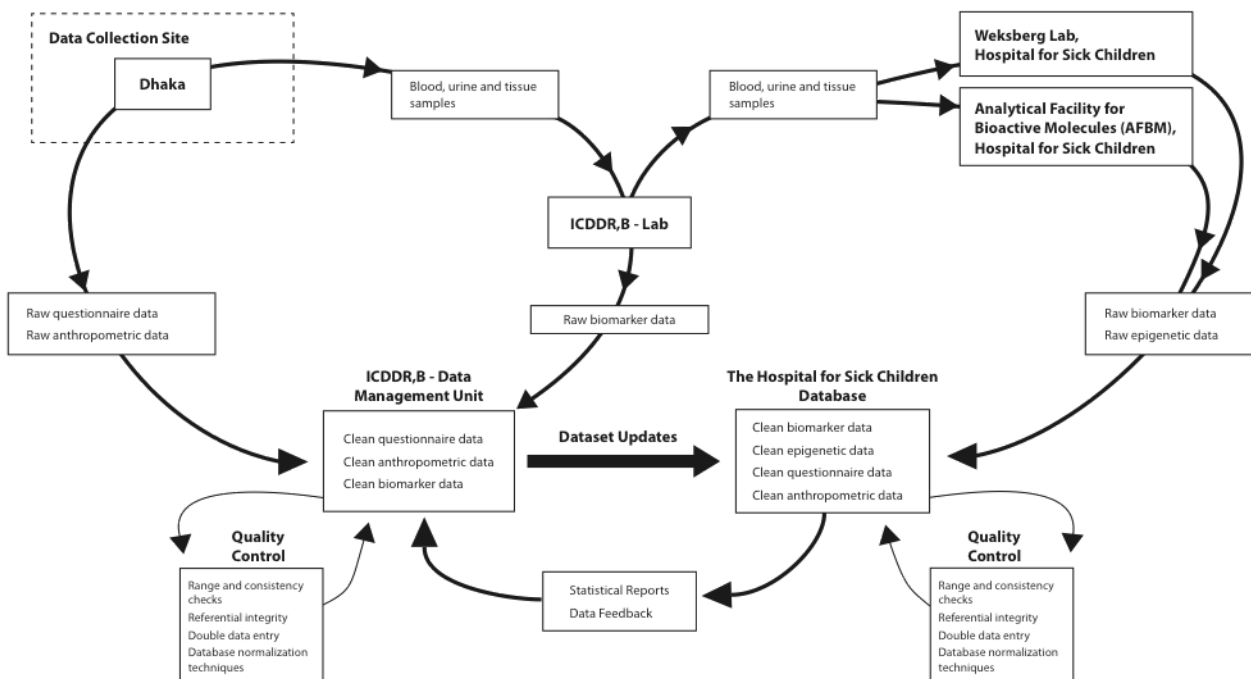


Figure 10: The flow of study data

The database will be designed using SQL Server (back-end) and entered using Visual Basic (front-end). A set of range and consistency checks will be built into the data capture system to

provide immediate feedback to data entry personnel regarding errors or inconsistent data. Double data entry will be used to further reduce the rate of data entry errors. Data queries to establish the integrity of the database will be generated on a monthly basis and circulated for review to the investigators.

20) Data analysis

a) Statistical methods (Aims #1, #2 and #3)

**A detailed data analysis plan is in development, and will be made available prior to the initiation of data analysis.*

The primary outcome measure – length-for-age z-score (LAZ)– will be derived from each length measure (cm) using the WHO child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Length at each visit will be based on the mean of paired measurements. LAZ at “one year” will be based on data from 52 to 60 weeks (364 to 420 days) postnatal (where day 0 is birth). Visit dates will be scheduled based on the number of weeks past the child’s birth date; therefore, the mode of ‘one year’ measurements should be 52 weeks (364 days) postnatal. If an infant has >1 length measurement during the period of 364 to 420 days, the measurement collected closest in time to day 364 will be used in the primary cross-sectional analyses; only one measurement per child will be used in the primary analysis.

For the analysis of the primary trial efficacy outcome (LAZ at one year of age), mean LAZ will be compared across groups using analysis of variance (ANOVA), without adjustment for covariates. To assess the effect of *prenatal* vitamin D on mean LAZ at one year of age, we plan to perform five primary between-group analyses – each vitamin D dose versus placebo (3 pairwise comparisons), as well as comparisons between all adjacent vitamin D doses (2 pairwise comparisons). Because the primary hypotheses relate to differences between two groups, each analysis will be akin to an independent samples t-test. The primary effect measure for each comparison will be expressed as a mean difference between groups with 95% confidence intervals (95% CI). However, an overall alpha for statistical significance for all 5 comparisons will be 0.05 (two-sided), and the Holm test will be used to account for multiple testing. Therefore, even if the 95% CI for a particular mean difference does not include zero, it is possible that it may not be considered statistically significant when all 5 comparisons are presented together.

The ‘postpartum effect’ will be assessed by the comparison of mean LAZ at about one year of age between 28,000 IU/week postpartum versus placebo among women who received 28,000 IU/week antenatally, using a similar statistical approach. The nominal alpha for statistical significance will be 0.05 (two-sided test) for this analysis. Because there is only one primary pairwise comparison related to the post-partum effect, no adjustment for the multiplicity of outcomes is planned.

In the primary analysis, all infants with LAZ at one year will be analysed ‘as randomized’ (i.e., an intent-to-treat approach), irrespective of supplementation adherence, and without imputation for missing data (i.e., infants for whom LAZ is unavailable at one year of age). The primary analyses will not be adjusted for baseline covariates; however, sensitivity analyses will involve adjustment for any covariates that substantially differ at baseline (enrolment), if any. Several sensitivity

analyses will be performed: a per-protocol analysis (see 20c below); analysis in which LAZ will be assigned to preterm infants using the age corrected for gestational age at birth (see 20b below); multivariable adjustment for covariates that substantially differ across groups at baseline; and, a missingness analysis to understand the pattern of missing data (in particular to detect differential loss of data across groups), with multiple imputation methods to correct for these losses.

All longitudinal analysis of LAZ will employ generalized estimating equations (GEE) with robust variance estimation to account for within-subject correlation of repeated measures over time. Two secondary approaches will be used to examine the effects of vitamin D on linear growth. First, regression spline models, with knots at the major scheduled follow-up visit time points, will be used to analyse changes in LAZ over specific time intervals. Interaction terms for time and group allocation will be used to test between-group differences in the changes in LAZ during discrete time intervals. Second, participants will be classified as 'stunted' if LAZ < -2. Cross-sectional comparisons between groups with respect to the risk of stunting will be based on estimation of the unadjusted risk ratio, and use of a chi-square test to test the significance of a between-group difference in prevalence. Longitudinal analysis of the risk ratio for stunting will employ GEE, with a log link and binomial distribution.

With respect to the additional anthropometric outcomes, weight-for-age, weight-for-length, head circumference-for-age, and growth velocity for age z-scores will be similarly derived based on WHO growth standards. For some analyses, birth weight will be adjusted for sex and gestational age using the INTERGROWTH-21st standards⁹⁹ and a published country-specific fetal weight reference¹⁰⁰. Individually-customized fetal growth references have not yet been established to provide additional benefits beyond standardization for country/ethnicity¹⁰⁰.

Summary measures (e.g., means, frequencies, proportions, incidence rates) and effect estimates (i.e., regression coefficients) will be reported as point estimates and 95% confidence intervals. In general, risk ratios (RR) with corresponding confidence intervals will be used to compare dichotomous variables, and difference in means will be used for analysis of continuous variables. P-values will be reported to three decimal places with p-values less than 0.001 reported as <0.001. Statistical analyses will be performed using the Stata software package, with main analyses of primary outcomes performed in a blinded fashion.

b) Methods for subgroup or adjusted analyses

As noted above, extreme z-scores will be flagged based on the WHO Anthro software (< -6 SD or >6 SD for LAZ), and manually reviewed to ensure they are not the result of data recording or entry errors. Real values that are extreme outlying negative z-scores are expected to be predominantly contributed by infants who had early preterm births (<34 weeks gestation) and very low birth weight (VLBW, <1500 g). Therefore, the preferred approach to sensitivity analyses that address these outliers will be to stratify infants according to surrogate markers of the underlying biological processes leading to these outliers (i.e., early preterm versus term/ late preterm, or categories based on birth weight), rather than stratifying on the outcome itself (i.e., outlier LAZ or not). Although the primary analysis will *a priori* include all infants with one-year LAZ, on an intent-to-treat basis, the empiric distribution of LAZ and the influence of extreme

outliers will be explored. Sensitivity analyses will be conducted that, a) use age that is corrected based on gestational age at birth; b) exclude early preterm infants, c) include all infants but employ non-parametric methods (e.g., Mann-Whitney U test) or regression methods that are robust against outliers.

In addition, sub-group analyses will be considered based on the following covariates:

- gestational age at birth.
- Sex, to understand potentially variable impact of the intervention on the growth of boys versus girls
- Maternal baseline vitamin D status
- Maternal height
- Supplement adherence (see below)

c) Definition of analysis population relating to protocol non-adherence

The primary analysis will be an intent-to-treat (as randomized) analysis. A per-protocol analysis of the effect of prenatal supplementation will be performed that is restricted to participants who meet the following criteria for acceptable adherence during the prenatal period:

- Consumed at least 90% of all scheduled prenatal doses
- Had no episodes of reported consumption of non-study vitamin D or calcium

d) Analysis of the effect of vitamin D supplementation on infectious disease morbidity (Aim #4)

We will estimate incidence rates of infant postnatal respiratory and diarrheal illness episodes from birth to 6 months of age. Incidence rate ratios will be reported to quantify between-group differences in illness rates, assuming an appropriate count distribution (i.e., Poisson or negative binomial), and accounting for repeated events within the same child using generalized estimating equations (GEE). For further details of the analysis of the acute respiratory infection outcomes, see Appendix for the sub-study protocol, "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh".

e) Biomarker and epigenetic data analyses (Aims #5 and #6)

Among those mother-infants pairs with complete anthropometric datasets and appropriate sets of biological specimens for analyses of a range of biomarkers (outlined above), a range of observational mechanistic and epidemiological sub-studies will be undertaken. For example, structural equation modelling (path analysis) of biomarker data and newborn/infant anthropometry will be performed to obtain estimates of the epidemiological associations among mediators/modifiers of the vitamin D-parathyroid axis and fetal/infant growth.

For the epigenetic analyses, we will select approximately 100 mother-infant pairs who have sufficiently complete datasets and specimens for adequate for epigenetic analyses. Statistical analyses will focus on between-group differences in DNA methylation patterns.

Detailed approaches to the study design and statistical analyses to address aims #4, #5 and #6 are in development.

21) Monitoring

a) Data monitoring

A data and safety monitoring board (DSMB) will be constituted and authorized by the icddr,b Ethical Review Committee (ERC). An external international member of the trial steering committee (TSC) will also review reports submitted to the DSMB, and report to the TSC.

b) Interim analyses and stopping rules

There are no formal interim analyses of efficacy or stopping rules planned for this trial.

22) Harms

The primary clinical harm that can be caused by excessive vitamin D ingestion is hypercalcemia. Based on existing data in the study setting, the occurrence of hypercalcemia as a study-related adverse event is not anticipated in this trial. However, clinical and biochemical parameters will be monitored to detect this event. Maternal serum calcium will be monitored at regular intervals (see above), and the occurrence of the clinical features of hypercalcemia will be further monitored by asking participants about the following symptoms on a weekly basis:

- Decreased appetite
- Weight loss
- Vomiting
- Fever or chills
- Constipation
- Abdominal pain
- Excessive thirst
- More frequent urination
- Muscle weakness
- Back, arm, or leg pain
- Confusion
- Depression

Other potential discomforts or inconveniences in the trial include the following: 1) discomfort and the very low risk of bruising or infection from venous blood sampling; 2) the potential emotional effects of some questions we ask regarding health status and previous pregnancies, and 3) the time required for participation. All other procedures in this study, including the clinical assessment procedures, perinatal specimen collection, and neonatal examination present no more than a minimal risk.

With respect to the discomforts/risks related to blood drawing, we will take standard preventive measures, including universal infection control precautions, and we will employ trained

phlebotomists or nurses to complete these tasks. The amount of blood to be drawn is low and not harmful.

With respect to the potentially sensitive nature of some of the questions (e.g., asking about prior pregnancy loss), we will ensure participants are able to answer these questions in a private setting (without partner/husband present) and have an opportunity to talk about concerns regarding these issues if they arise after the questioning. Research assistants will be specifically trained to check whether participants were bothered or upset by any of the questions and study staff will be able to arrange for appropriate mental health referrals if necessary.

Reporting of adverse events will employ the following definitions:

Adverse event (AE): Any untoward medical occurrence experienced by a participant. An adverse event does not necessarily have to have a causal relationship with the study intervention. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom or disease temporally associated with the application of an intervention, whether or not considered related to the intervention.

Serious adverse event (SAE): An adverse event qualifies as *serious* if it is accompanied by any of the following complications or outcomes:

- Death
- Life-threatening complication, such that death was averted by medical or surgical interventions (the term "life-threatening" in this context refers to an event in which the patient was at risk of death *at the time of the event*; it does not refer to an event, which hypothetically might have caused death, had it been more severe).
- Inpatient hospitalization or prolongation of an existing hospitalization (other than for uncomplicated delivery).
- Persistent or significant disability/incapacity following the resolution of the acute event. Disability means a substantial disruption of one's ability to carry out normal life functions.
- *In infants:* a diagnosis of cancer, neurological disorder (stroke, seizure disorder, encephalopathy, or structural brain abnormality), or major congenital anomaly whether or not these required inpatient hospitalization.

A study physician will be responsible for coordinating the management and documentation of all suspected or confirmed adverse events reported by field personnel, comprising the following actions:

1. Organizing the medical assessment and management of participants with AEs, in coordination with MCHTI medical personnel who may or may not already be involved in patient care (e.g., they would be necessarily involved if a complication occurs during delivery), including the transfer to a tertiary-care facility if necessary (i.e., all SAEs will require care at a medical facility). All medical care (including diagnostics and treatment), including consultation or admission to a tertiary level hospital if indicated, will be free of charge to the participant or family. For those patients treated at medical facilities,

management will be guided by physicians not directly affiliated with the study and will be guided solely by the welfare of the patient.

2. Collection of urine and blood specimens as soon as possible for all participants with suspected or confirmed SAEs; these specimens will preferably be collected prior to medical interventions that would affect the interpretation of any biochemical tests (e.g., obtain a plasma calcium prior to initiation of diuretic therapy) if obtaining the specimen will not interfere in the indicated management of the patient. To the extent that is possible, environmental or genetic reasons for idiosyncratic reactions to vitamin D will be investigated through ancillary testing, but only upon receiving informed consent from the participant where appropriate.
3. Completion of necessary documentation for ethics boards.
4. Immediate (i.e., as soon as possible) reporting of all SAEs to the Dhaka-based principal investigator (or designate). Non-serious AEs will be discussed at least monthly with one designated co-investigator during the course of the study, to monitor patterns of multiple similar AEs not considered serious in individual.
5. Follow-up of the patient status in hospital, and forwarding relevant information to the study coordinator, until complete resolution of the AE.

The potential for a causal association between the study interventions and the SAE will be assessed according to the commonly-employed scheme below:

Determination of association between AE and supplementation

0	NO RELATIONSHIP	Inconsistent timely relationship (e.g., detection prior to participant starting supplementation) or evidence that a symptom, sign or laboratory finding is definitely related to an etiology other than the study supplements.
1	POSSIBLE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and a potential alternative etiology has not been investigated.
2	PROBABLE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and a potential alternative etiology is not apparent on the basis of limited investigations.
3	DEFINITE RELATIONSHIP	Clinical or biochemical data are consistent with an effect of the supplement and any possible alternative etiologies have been ruled out, or re-challenge with the suspected causative factor led to recurrence of the signs or symptoms.

Prompt reporting to the DSMB and ethics boards will be undertaken for *unanticipated* adverse events, the scope of which will include the following:

- An adverse event that is severe *and* considered to have a probable or very probable relationship to vitamin D supplementation (i.e., is considered a SAE at least probably caused by vitamin D) or another study procedure. SAEs that are explained by obstetric/perinatal complications seen in the study setting (e.g., obstructed labor) and not considered to be related to vitamin D supplementation or study procedures will not be considered 'unanticipated'.
- An atypical severe adverse event that is not readily explained by obstetric/perinatal or complications or other medical events unrelated to supplementation, even if the event

would not otherwise be considered a manifestation of vitamin D toxicity or another study procedure.

- A pattern of any adverse events (not necessarily defined as SAEs) that collectively change the risk-benefit calculation for participation.

23) Auditing

An external monitoring plan is planned but details are pending confirmation of funding for these activities.

Ethics and dissemination

24) Research ethics approval

Research ethics committee/institutional review board approval will be obtained from the Hospital for Sick Children Research Ethics Board (REB) and the icddr,b ethical review committee (ERC) prior to initiation of participant contact.

25) Protocol amendments

Any amendments to the protocol will be reviewed and approved by the SickKids REB and icddr,b ERC prior to changes to study procedures. As deemed necessary by the trial steering committee and/or ethical review boards, changes to the protocol will be communicated to the trial participants.

26) Consent or assent

The consent process and all accompanying documents will be in Bangla, the national language of Bangladesh that is universally spoken and understood in Bangladesh. Literate women or their family members will be encouraged to read the form aloud, under the supervision of study personnel. Alternatively, because of variable levels of literacy, consent documents will be read out loud to prospective participants by study personnel if necessary. The project coordinator and study physicians will be required to have successfully completed an on-line ethics training course. All study personnel will be trained to ensure their understanding of the importance of promoting free and voluntary consent.

The participants will be screened for exclusion criteria before providing written consent to participate in the study. A study physician will be primarily responsible for the informed consent process, and in all cases will confirm consent and respond to any questions from participants prior to completion of the process. The initial components of the consent process overlap with detailed eligibility assessment, and thus will be overseen by the physician. However, other trained study workers (i.e., paramedics or research assistants) may assist in providing detailed explanations of study procedures, risks, and benefits to the prospective participants. The informed consent process will be conducted at screening and/or baseline visit in a designated

room at the clinic. If a woman is interested in participating in the study, she will be given a consent form and asked to review it with her husband and/or family members. Prospective participants may take several days to consider participation, within the bounds of the gestational age inclusion criterion.

There will be four types of consent requested in this study

1. Primary study participation consent – this will encompass all maternal and infant activities.
2. Paternal consent – this will be limited to the involvement of the child's biological father, if available, for height/weight measurements and one blood specimen to be collected at or near the time of enrollment. Fathers may choose to participate in one or both of anthropometry and/or specimen collection.
3. Biorepository storage consent – Both mothers (after providing primary study participation consent) and fathers (after providing paternal consent) will be asked if they consent to long-term storage of their biological specimens. This is not a necessary component of either paternal or primary study participation consent.
4. Consent for infant nasal swab specimen collection and storage.

27) Confidentiality

All data will be collected in a manner that respects participants' privacy and confidentiality. We do not anticipate that any potentially sensitive information concerning health will be generated or disclosed in the context of this study.

28) Declaration of interests

The investigators do not have conflicts of interest to declare.

29) Access to data

The principal investigator and co-investigators will have access to the final trial dataset; there are no contractual agreements that limit such access for investigators.

30) Ancillary and post-trial care

Phase 1 (intervention phase): The study physician will assess all suspected adverse events or medical concerns. An obstetrician and pediatrician at MCHTI will be available for consultation. When medical issues cannot be resolved by MCHTI medical staff, or a situation requires urgent or critical care, referral will be made to a designated tertiary care hospital in Dhaka (Ad-Din Hospital). MCHTI physicians will be primarily responsible for arranging the referral, but study personnel will assist to ensure timely care. When possible, a study worker will accompany participants to the hospital, or meet the participant at the hospital (if the participant leaves directly from home rather than the clinic). In all cases, the study physician will follow and document the course of events, and obtain relevant information from the participant's medical records in order to document any adverse events fully. Costs of essential urgent/emergent medical care at the referral hospital will be covered by the study budget.

Phase 2 (observational phase): Medical care and referral during this period will only be facilitated by study physicians if a clinical issue is discovered at the time of a scheduled visit (e.g., severe malnutrition detected by anthropometry). Costs of initial referral for care will be covered by the study budget. However, medical care costs for problems that arise between scheduled visits will not be routinely covered, and will be considered on a case-by-case basis.

31) Dissemination plans

a) Dissemination policy

Results of the trial will be disseminated widely, without restriction, through scientific conferences and journal publications.

b) Authorship eligibility guidelines and any intended use of professional writers

Authorship eligibility will be based on the guidelines of the International Committee of Medical Journal Editors (http://www.icmje.org/ethical_1author.html).

c) Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

The full protocol will be made publicly available following review and approval by the trial steering committee, DSMB, and ethical review boards in Canada and Bangladesh.

There are no specific plans at present regarding the mechanisms by which the public will be granted access to datasets and statistical code; however, in principle, public access is supported by the investigators. Requests for datasets and statistical code will be reviewed on a case-by-case basis.

REFERENCES

1. Rajaratnam JK, Marcus JR, Flaxman AD, Wang H, Levin-Rector A, Dwyer L, et al. Neonatal, postneonatal, childhood, and under-5 mortality for 187 countries, 1970-2010: a systematic analysis of progress towards Millennium Development Goal 4. *Lancet* 2010; **375**: 1988-2008.
2. George K, Prasad J, Singh D, Minz S, Albert DS, Muliylil J, et al. Perinatal outcomes in a South Asian setting with high rates of low birth weight. *BMC Pregnancy Childbirth* 2009; **9**: 5.
3. de Onis M, Blossner M, Borghi E, Morris R, Frongillo EA. Methodology for estimating regional and global trends of child malnutrition. *Int J Epidemiol* 2004; **33**: 1260-70.
4. Victora CG, Adair L, Fall C, Hallal PC, Martorell R, Richter L, et al. Maternal and child undernutrition: consequences for adult health and human capital. *Lancet* 2008; **371**: 340-57.
5. Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, et al. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* 2008; **371**: 243-60.
6. Victora CG, de Onis M, Hallal PC, Blossner M, Shrimpton R. Worldwide Timing of Growth Faltering: Revisiting Implications for Interventions. *Pediatrics* 2010; **125**: e473-80.
7. Evans KN, Bulmer JN, Kilby MD, Hewison M. Vitamin D and placental-decidual function. *J Soc Gynecol Investig* 2004; **11**: 263-71.
8. Hollis BW, Pittard WB, 3rd. Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences. *J Clin Endocrinol Metab* 1984; **59**: 652-7.
9. Taylor SN, Wagner CL, Hollis BW. Vitamin D supplementation during lactation to support infant and mother. *J Am Coll Nutr* 2008; **27**: 690-701.
10. Roth DE, Shah MR, Black RE, Baqui AH. Vitamin D Status of Infants in Northeastern Rural Bangladesh: Preliminary Observations and a Review of Potential Determinants. *Journal of Health, Population and Nutrition* 2010; **28**: 458-69.
11. Roth DE, Mahmud AA, Raqib R, Akhtar E, Perumal N, Pezzack B, et al. Randomized placebo-controlled trial of high-dose prenatal third-trimester vitamin D3 supplementation in Bangladesh: the AViDD trial. *Nutrition Journal* 2013; **12**: 47.
12. Goltzman D. Emerging roles for calcium-regulating hormones beyond osteolysis. *Trends Endocrinol Metab* 2010; **21**: 512-8.
13. Rummens K, van Bree R, Van Herck E, Zaman Z, Bouillon R, Van Assche FA, et al. Vitamin D deficiency in guinea pigs: exacerbation of bone phenotype during pregnancy and disturbed fetal mineralization, with recovery by 1,25(OH)2D3 infusion or dietary calcium-phosphate supplementation. *Calcif Tissue Int* 2002; **71**: 364-75.
14. Finch SL, Rauch F, Weiler HA. Postnatal vitamin D supplementation following maternal dietary vitamin D deficiency does not affect bone mass in weanling guinea pigs. *J Nutr* 2010; **140**: 1574-81.
15. Kovacs CS. Vitamin D in pregnancy and lactation: maternal, fetal, and neonatal outcomes from human and animal studies. *Am J Clin Nutr* 2008; **88**: 520S-8S.
16. Rummens K, van Cromphaut SJ, Carmeliet G, van Herck E, van Bree R, Stockmans I, et al. Pregnancy in mice lacking the vitamin D receptor: normal maternal skeletal response, but fetal hypomineralization rescued by maternal calcium supplementation. *Pediatr Res* 2003; **54**: 466-73.
17. Kovacs CS, Woodland ML, Fudge NJ, Friel JK. The vitamin D receptor is not required for fetal mineral homeostasis or for the regulation of placental calcium transfer in mice. *American journal of physiology Endocrinology and metabolism* 2005; **289**: E133-44.
18. Soliman AT, Al Khalaf F, Alhemaiddi N, Al Ali M, Al Zyoud M, Yakoot K. Linear growth in relation to the circulating concentrations of insulin-like growth factor I, parathyroid hormone, and 25-hydroxy vitamin D in children with nutritional rickets before and after treatment: endocrine adaptation to vitamin D deficiency. *Metabolism* 2008; **57**: 95-102.

19. Weisberg P, Scanlon KS, Li R, Cogswell ME. Nutritional rickets among children in the United States: review of cases reported between 1986 and 2003. *Am J Clin Nutr* 2004; **80**: 1697S-705S.
20. Rajah J, Jubeh JA, Haq A, Shalash A, Parsons H. Nutritional rickets and z scores for height in the United Arab Emirates: to D or not to D? *Pediatr Int* 2008; **50**: 424-8.
21. Bereket A, Cesur Y, Ozkan B, Adal E, Turan S, Onan SH, et al. Circulating insulin-like growth factor binding protein-4 (IGFBP-4) is not regulated by parathyroid hormone and vitamin D in vivo: evidence from children with rickets. *J Clin Res Pediatr Endocrinol* 2010; **2**: 17-20.
22. Kumar GT, Sachdev HS, Chellani H, Rehman AM, Singh V, Arora H, et al. Effect of weekly vitamin D supplements on mortality, morbidity, and growth of low birthweight term infants in India up to age 6 months: randomised controlled trial. *BMJ* 2011; **342**: d2975.
23. Brooke OG, Brown IR, Bone CD, Carter ND, Cleeve HJ, Maxwell JD, et al. Vitamin D supplements in pregnant Asian women: effects on calcium status and fetal growth. *Br Med J* 1980; **280**: 751-4.
24. Brooke OG, Butters F, Wood C. Intrauterine vitamin D nutrition and postnatal growth in Asian infants. *Br Med J (Clin Res Ed)* 1981; **283**: 1024.
25. Kalra P, Das V, Agarwal A, Kumar M, Ramesh V, Bhatia E, et al. Effect of vitamin D supplementation during pregnancy on neonatal mineral homeostasis and anthropometry of the newborn and infant. *The British journal of nutrition* 2012; **108**: 1052-8.
26. 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* 2011: n/a-n/a.
27. Kovacs CS. Bone development in the fetus and neonate: role of the calciotropic hormones. *Curr Osteoporos Rep* 2011; **9**: 274-83.
28. Bond H, Dilworth MR, Baker B, Cowley E, Requena Jimenez A, Boyd RD, et al. Increased maternofetal calcium flux in parathyroid hormone-related protein-null mice. *J Physiol* 2008; **586**: 2015-25.
29. Waller S. Parathyroid hormone and growth in chronic kidney disease. *Pediatr Nephrol* 2011; **26**: 195-204.
30. Yamshchikov AV, Desai NS, Blumberg HM, Ziegler TR, Tangpricha V. Vitamin D for treatment and prevention of infectious diseases: a systematic review of randomized controlled trials. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* 2009; **15**: 438-49.
31. Roth DE, Shah R, Black RE, Baqui AH. Vitamin D status and acute lower respiratory infection in early childhood in Sylhet, Bangladesh. *Acta Paediatr* 2010; **99**: 389-93.
32. Ish-Shalom S, Segal E, Salganik T, Raz B, Bromberg IL, Vieth R. Comparison of daily, weekly, and monthly vitamin D3 in ethanol dosing protocols for two months in elderly hip fracture patients. *J Clin Endocrinol Metab* 2008; **93**: 3430-5.
33. De-Regil LM, Palacios C, Ansary A, Kulier R, Pena-Rosas JP. Vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev* 2012; **2**: CD008873.
34. Ross AC, Taylor CL, Yaktine AL, Del Valle HB. Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, Institute of Medicine. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: The National Academies Press; 2010.
35. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 2011; **96**: 53-8.
36. Shrimpton R, Huffman SL, Zehner ER, Darnton-Hill I, Dalmiya N. Multiple micronutrient supplementation during pregnancy in developing-country settings: policy and program implications of the results of a meta-analysis. *Food Nutr Bull* 2009; **30**: S556-73.

37. Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Washington: National Academy Press; 1997. Available from: http://www.nap.edu/openbook.php?record_id=5776.
38. Roth DE. Vitamin D supplementation during pregnancy: safety considerations in the design and interpretation of clinical trials. *J Perinatol* 2011; **31**: 449-59.
39. Sahu M, Das V, Aggarwal A, Rawat V, Saxena P, Bhatia V. Vitamin D replacement in pregnant women in rural north India: a pilot study. *Eur J Clin Nutr* 2009; **63**: 1157-9.
40. Yu CK, Sykes L, Sethi M, Teoh TG, Robinson S. Vitamin D deficiency and supplementation during pregnancy. *Clin Endocrinol (Oxf)* 2009; **70**: 685-90.
41. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: Double blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res* 2011; **26**: 2341-57.
42. Godel JC, Irvine J, Onyett H, Saylor K, Schroter H, Young M, et al. Vitamin D supplementation: Recommendations for Canadian mothers and infants. *Paediatrics & Child Health* 2007; **12**: 583-9.
43. ACOG Committee Opinion No. 495: Vitamin D: Screening and supplementation during pregnancy. *Obstet Gynecol* 2011; **118**: 197-8.
44. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *The Journal of clinical endocrinology and metabolism* 2011; **96**: 1911-30.
45. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am J Clin Nutr* 2003; **77**: 204-10.
46. Aloia JF, Patel M, Dimaano R, Li-Ng M, Talwar SA, Mikhail M, et al. Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration. *Am J Clin Nutr* 2008; **87**: 1952-8.
47. Roth DE, Mahmud A, Raqib R, Akhtar E, Black R, Baqui A. Pharmacokinetics of high-dose weekly oral vitamin D3 supplementation during the third trimester of pregnancy in Dhaka, Bangladesh. *Nutrients* 2013; **5**: 788-810.
48. Wagner CL, Hulsey TC, Fanning D, Ebeling M, Hollis BW. High-dose vitamin D3 supplementation in a cohort of breastfeeding mothers and their infants: a 6-month follow-up pilot study. *Breastfeed Med* 2006; **1**: 59-70.
49. Roth DE, Mahmud A, Raqib R, Black RE, Baqui AH. Pharmacokinetics of a single oral dose of vitamin D3 (70,000 IU) in pregnant and non-pregnant women. *Nutr J* 2012; **11**: 114.
50. Ilahi M, Armas LA, Heaney RP. Pharmacokinetics of a single, large dose of cholecalciferol. *Am J Clin Nutr* 2008; **87**: 688-91.
51. Vieth R. How to optimize vitamin D supplementation to prevent cancer, based on cellular adaptation and hydroxylase enzymology. *Anticancer Res* 2009; **29**: 3675-84.
52. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA* 2010; **303**: 1815-22.
53. Wagner D, Sidhom G, Whiting SJ, Rousseau D, Vieth R. The bioavailability of vitamin D from fortified cheeses and supplements is equivalent in adults. *J Nutr* 2008; **138**: 1365-71.
54. Islam MZ, Lamberg-Allardt C, Karkkainen M, Ali SM. Dietary calcium intake in premenopausal Bangladeshi women: do socio-economic or physiological factors play a role? *Eur J Clin Nutr* 2003; **57**: 674-80.
55. Arsenault JE, Yakes EA, Islam MM, Hossain MB, Ahmed T, Hotz C, et al. Very Low Adequacy of Micronutrient Intakes by Young Children and Women in Rural Bangladesh Is Primarily Explained by Low Food Intake and Limited Diversity. *The Journal of Nutrition* 2013; **143**: 197-203.

56. Hels O, Hassan N, Tetens I, Haraksingh Thilsted S. Food consumption, energy and nutrient intake and nutritional status in rural Bangladesh: changes from 1981-1982 to 1995-96. *Eur J Clin Nutr* 2003; **57**: 586-94.
57. World Health Organization. WHO child growth standards : length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age : methods and development. Geneva: World Health Organization; 2006. vii, 312 p. p.
58. World Health Organization., Development. WHONfHa. WHO child growth standards : head circumference-for-age, arm circumference-for-age, triceps skinfold-for-age and subscapular skinfold-for-age : methods and development. Geneva: World Health Organization; 2007. xvii, 217 p. p.
59. de Onis M, Onyango AW, Van den Broeck J, Chumlea WC, Martorell R. Measurement and standardization protocols for anthropometry used in the construction of a new international growth reference. *Food Nutr Bull* 2004; **25**: S27-36.
60. National Health and Nutrition Examination Survey: Center for Disease Control and Prevention; 2009. Available from:
http://www.cdc.gov/nchs/data/nhanes/nhanes_09_10/BodyMeasures_09.pdf.
61. Seamans KM, Cashman KD. Existing and potentially novel functional markers of vitamin D status: a systematic review. *Am J Clin Nutr* 2009; **89**: 1997S-2008.
62. Melamed ML, Eustace JA, Plantinga LC, Jaar BG, Fink NE, Parekh RS, et al. Third-generation parathyroid hormone assays and all-cause mortality in incident dialysis patients: the CHOICE study. *Nephrol Dial Transplant* 2008; **23**: 1650-8.
63. Kazama JJ, Omori K, Higuchi N, Takahashi N, Ito Y, Maruyama H, et al. Intact PTH assay overestimates true 1-84 PTH levels after maxacalcitol therapy in dialysis patients with secondary hyperparathyroidism. *Nephrol Dial Transplant* 2004; **19**: 892-7.
64. Murray TM, Rao LG, Divieti P, Bringhurst FR. Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl- terminal ligands. *Endocr Rev* 2005; **26**: 78-113.
65. Waller SC, Ridout D, Cantor T, Rees L. Parathyroid hormone and growth in children with chronic renal failure. 2005; **67**: 2338-45.
66. Martin A, David V, Quarles LD. Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev* 2012; **92**: 131-55.
67. Braithwaite V, Jarjou LMA, Goldberg GR, Jones H, Pettifor JM, Prentice A. Follow-up study of Gambian children with rickets-like bone deformities and elevated plasma FGF23: Possible aetiological factors. *Bone* 2012; **50**: 218-25.
68. Kronenberg HM. PTHrP and skeletal development. *Ann N Y Acad Sci* 2006; **1068**: 1-13.
69. Budayr AA, Halloran BP, King JC, Diep D, Nissenson RA, Strewler GJ. High levels of a parathyroid hormone-like protein in milk. *Proc Natl Acad Sci U S A* 1989; **86**: 7183-5.
70. Thota CS, Reed LC, Yallampalli C. Effects of Parathyroid Hormone Like Hormone (PTHrP) Antagonist, PTHrP7,34, on Fetoplacental Development and Growth During Midgestation in Rats. *Biology of Reproduction* 2005; **73**: 1191-8.
71. Briana DD, Boutsikou M, Baka S, Hassiakos D, Gourgiotis D, Marmarinos A, et al. N-terminal parathyroid hormone-related protein levels in human intrauterine growth restricted pregnancies. *Acta Obstetrica et Gynecologica Scandinavica* 2007; **86**: 945-9.
72. Liu J, Lv F, Sun W, Tao C, Ding G, Karaplis A, et al. The Abnormal Phenotypes of Cartilage and Bone in Calcium-Sensing Receptor Deficient Mice Are Dependent on the Actions of Calcium, Phosphorus, and PTH. *PLoS Genet* 2011; **7**: e1002294.
73. Zivcinkjak M, Schnabel D, Billing H, Staude H, Filler G, Querfeld U, et al. Age-related stature and linear body segments in children with X-linked hypophosphatemic rickets. *Pediatr Nephrol* 2011; **26**: 223-31.
74. Khadilkar A, Mughal MZ, Hanumante N, Sayyad M, Sanwalka N, Naik S, et al. Oral calcium supplementation reverses the biochemical pattern of parathyroid hormone resistance in underprivileged Indian toddlers. *Arch Dis Child* 2009; **94**: 932-7.
75. Sanchez CP, He YZ, Leiferman E, Wilsman NJ. Bone elongation in rats with renal failure and mild or advanced secondary hyperparathyroidism. *Kidney Int* 2004; **65**: 1740-8.

76. Asvold BO, Eskild A, Jenum PA, Vatten LJ. Maternal Concentrations of Insulin-like Growth Factor I and Insulin-like Growth Factor Binding Protein 1 During Pregnancy and Birth Weight of Offspring. *American Journal of Epidemiology* 2011; **174**: 129-35.
77. Akram SK, Carlsson-Skwirut C, Bhutta ZA, Söder O. Placental IGF-I, IGFBP-1, zinc, and iron, and maternal and infant anthropometry at birth. *Acta Paediatrica* 2011; **100**: 1504-9.
78. Mamabolo RL, Alberts M, Levitt NS, Delemarre-van de Waal HA, Steyn NP. Association between insulin-like growth factor-1, insulin-like growth factor-binding protein-1 and leptin levels with nutritional status in 1-3-year-old children, residing in the central region of Limpopo Province, South Africa. *Br J Nutr* 2007; **98**: 762-9.
79. Calvo MS, Kumar R, Heath H, 3rd. Elevated secretion and action of serum parathyroid hormone in young adults consuming high phosphorus, low calcium diets assembled from common foods. *J Clin Endocrinol Metab* 1988; **66**: 823-9.
80. Paik JM, Curhan GC, Forman JP, Taylor EN. Determinants of plasma parathyroid hormone levels in young women. *Calcif Tissue Int* 2010; **87**: 211-7.
81. Braithwaite V, Jarjou LMA, Goldberg GR, Prentice A. Iron status and fibroblast growth factor-23 in Gambian children. *Bone*.
82. Kippler M, Goessler W, Nermell B, Ekstrom EC, Lonnerdal B, El Arifeen S, et al. Factors influencing intestinal cadmium uptake in pregnant Bangladeshi women--a prospective cohort study. *Environ Res* 2009; **109**: 914-21.
83. Khandare AL, Harikumar R, Sivakumar B. Severe bone deformities in young children from vitamin D deficiency and fluorosis in Bihar-India. *Calcif Tissue Int* 2005; **76**: 412-8.
84. Novakovic B, Sibson M, Ng HK, Manuelpillai U, Rakyar V, Down T, et al. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem* 2009; **284**: 14838-48.
85. Christian P, Jiang T, Khatry SK, LeClerq SC, Shrestha SR, West KP, Jr. Antenatal supplementation with micronutrients and biochemical indicators of status and subclinical infection in rural Nepal. *Am J Clin Nutr* 2006; **83**: 788-94.
86. Green R. Indicators for assessing folate and vitamin B-12 status and for monitoring the efficacy of intervention strategies. *The American Journal of Clinical Nutrition* 2011; **94**: 666S-72S.
87. Stephensen CB. Burden of infection on growth failure. *The Journal of Nutrition* 1999; **129**: 534S-8S.
88. Sainaghi PP, Bellan M, Antonini G, Bellomo G, Pirisi M. Unsuppressed parathyroid hormone in patients with autoimmune/inflammatory rheumatic diseases: implications for vitamin D supplementation. *Rheumatology (Oxford)* 2011.
89. Angeletti RH, D'Amico T, Ashok S, Russell J. The Chemokine Interleukin-8 Regulates Parathyroid Secretion. *Journal of Bone and Mineral Research* 1998; **13**: 1232-7.
90. Liu NQ, Kaplan AT, Lagishetty V, Ouyang YB, Ouyang Y, Simmons CF, et al. Vitamin D and the regulation of placental inflammation. *J Immunol* 2011; **186**: 5968-74.
91. Choufani S, Shapiro JS, Susiarjo M, Butcher DT, Grafodatskaya D, Lou Y, et al. A novel approach identifies new differentially methylated regions (DMRs) associated with imprinted genes. *Genome Res* 2011; **21**: 465-76.
92. Hathcock JN, Shao A, Vieth R, Heaney R. Risk assessment for vitamin D. *Am J Clin Nutr* 2007; **85**: 6-18.
93. Ghoshal AK, Soldin SJ. Evaluation of the Dade Behring Dimension RxL: integrated chemistry system-pediatric reference ranges. *Clin Chim Acta* 2003; **331**: 135-46.
94. Chan MK, Seiden-Long I, Aytakin M, Quinn F, Ravalico T, Ambruster D, et al. Canadian Laboratory Initiative on Pediatric Reference Interval Database (CALIPER): pediatric reference intervals for an integrated clinical chemistry and immunoassay analyzer, Abbott ARCHITECT ci8200. *Clin Biochem* 2009; **42**: 885-91.
95. Kulasingam V, Jung BP, Blasutig IM, Baradaran S, Chan MK, Aytakin M, et al. Pediatric reference intervals for 28 chemistries and immunoassays on the Roche cobas 6000 analyzer--a CALIPER pilot study. *Clin Biochem* 2010; **43**: 1045-50.

96. Blasutig IM, Jung B, Kulasingam V, Baradaran S, Chen Y, Chan MK, et al. Analytical evaluation of the VITROS 5600 Integrated System in a pediatric setting and determination of pediatric reference intervals. *Clin Biochem* 2010; **43**: 1039-44.
97. Ridefelt P, Aldrimer M, Rodoo PO, Niklasson F, Jansson L, Gustafsson J, et al. Population-based pediatric reference intervals for general clinical chemistry analytes on the Abbott Architect ci8200 instrument. *Clin Chem Lab Med* 2012; **50**: 845-51.
98. Roizen JD, Shah V, Levine MA, Carlow DC. Determination of Reference Intervals for Serum Total Calcium in the Vitamin D-Replete Pediatric Population. *The Journal of Clinical Endocrinology & Metabolism* 2013; **98**: E1946-E50.
99. Villar J, Cheikh Ismail L, Victora CG, Ohuma EO, Bertino E, Altman DG, et al. International standards for newborn weight, length, and head circumference by gestational age and sex: the Newborn Cross-Sectional Study of the INTERGROWTH-21st Project. *Lancet* 2014; **384**: 857-68.
100. Mikolajczyk RT, Zhang J, Betran AP, Souza JP, Mori R, Gulmezoglu AM, et al. A global reference for fetal-weight and birthweight percentiles. *Lancet* 2011; **377**: 1855-61.

Appendices

32) Informed consent materials

See attached.

33) Biological specimens

		Phase 1a				Phase 1b			
		Maternal baseline	Maternal 30 weeks	Maternal delivery	Cord blood	Maternal 3 months	Infant 3 months	Maternal 6 months	Infant 6 months
Blood/ Serum/ Plasma	Calcium	X	X	X	X	X	X	X	X
	Phosphate								X
	Alkaline phosphatase								X
	Creatinine								X
	25(OH)D	X		X	X		X	X	X
	1,25(OH)2D	X		X	X			X	X
	Whole/bioactive PTH(1-84)	X		X	X			X	X
	C-terminal PTH(73-84)	X		X	X			X	X
	PTH(1-34) (intact PTH)	X		X	X			X	X
	PTHrP	X		X	X	X	X	X	X
	FGF-23	X		X	X			X	X
	IGF-1				X				X
	IGFBP-1 & 3				X				X
	Ferritin								X
	Retinol			X					
	Folate			X					
	Cadmium			X					
	C-reactive protein			X	X				
	IL-6, IL-8, TNF-alpha			X	X				
	<i>Biobank</i>	X	X	X	X	X	X	X	X
Urine	Creatinine			X					X
	Calcium			X					X
	Phosphate								X
	Fluoride								X
	Cyclic AMP								X
	<i>Biobank</i>	X		X					X
Breast milk	PTHrP					X		X	
	<i>Biobank</i>					X		X	
Nasal swab	<i>Respiratory viruses</i>						X*		X*

In addition, a single paternal blood specimen will be collected for epigenetic analyses.

*Nasal swab specimens will be performed at the time of onset of acute respiratory illness.

34) Sub-study protocols

“Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh” *[attached]*

Trial protocol amendments instituted after the initiation of participant recruitment^{a,b}

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
Version Number 3.0	04-Jul-14	#01	Incorporated sub-study protocol, entitled "Effect of prenatal vitamin D supplementation on acute respiratory infections in infants in Bangladesh".	To leverage the randomized trial design to draw inferences about the causal effect of maternal vitamin D supplementation on respiratory infectious disease morbidity and etiology in a large cohort of infants who will be prospectively followed in the home from birth to 6 months of age.
		#02	Study duration updated to reflect new timeline.	The project started in 2014 instead of 2013 due to a delay in study launch.
		#03	Institutions and contact information updated for investigators/study staff.	Updated to provide current information.
		#04	Small formatting and wording changes throughout protocol.	Updated to enhance clarity.
		#05	Umbilical cord specimens added to all specimen collection descriptions.	Plans for epigenetic analysis have changed after discussions with our collaborators. Umbilical cord tissue specimens are now a necessary component.
		#06	Nasal swab specimens added to all specimen collection descriptions.	Nasal swabs are a necessary component of the new respiratory infection sub-study.
		#07	Additional rule added to the "Missed and late doses" portion of the "Intervention description" section explaining that a maximum of two attempts at ingestion should be made for each scheduled dose.	To minimize the chance of over-supplementation and conserve tablets when participants may be ill and unable to ingest supplements.
		#08	Addition to the list of specific events that may lead to discontinuation of supplementation: "Onset of a medical condition or initiation of a medication following enrolment that the TSC (trial steering committee) concludes may reasonably predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia"	After discussions with the TSC this was added for safety reasons.
		#09	Clarification that the 2-year visits will be scheduled for when the child is 24 months of age (or 104 weeks old).	Updated to enhance clarity.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#010	Details added and minor content changes to the growth and safety outcomes summary table.	To enhance clarity and to include anthropometric data collection timepoints that had not been included in this section in the previous version of the protocol.
		#011	Clarifications regarding the list of events that would lead to stoppage of all study activities for that participant. This includes: 1) Participant (maternal) death will now lead to an automatic end of study activities only if maternal death occurs prior to delivery; 2) Fetal or infant death at any time will now lead to an automatic end of study activities; 3) The criteria for a participant being lost to follow-up was re-written to allow for data collection up to three months after the final scheduled visit at 2 years of age.	Changes were made to this list in order to minimize the amount of missing data and loss to follow-up.
		#012	Clarification that two positive tests for proteinuria during screening (initial test and a repeat test if positive) will be required to exclude a potential participant from the study	To minimize the chance of false positives during screening.
		#013	In the "Data collection" summary section, changes have been made to the specific forms used during each visit. The types of data collected will not change, but some of the forms and data collection procedures have been reformatted (e.g., Form 4B: Household Characteristics is being completed at 9 and 21 months in addition to during the first prenatal weekly home visit).	Data collection procedures have been re-arranged to make interactions with participants more efficient and less time consuming.
		#014	Additional information (e.g. scale type) has been added to the description of data collection for "placental weight and dimensions".	The plans for weighing placentas have been finalized after field-testing and discussions with our collaborators.
		#015	Details have been added to the descriptions of blood, urine, breast milk, umbilical cord, and placental tissue specimen processing, storage and transport.	Plans for specimen processing, storage and transport have been finalized after field-testing and discussions with our collaborators.
		#016	A brief description of how and when data forms will be scanned has been added to the "Data management" section.	Updated to enhance clarity.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
Version number 3.1	21-Apr-15	#017	Infant nasal swab consent was added to the list of the types of consent requested during the study. The existing "Biorepository consent" description was edited for clarity.	Updated to illustrate the addition of the new respiratory infection sub-study.
		#018	References have been added throughout the main study protocol to link it to the new respiratory infection sub-study.	Updated to illustrate the addition of the new respiratory infection sub-study.
		#01	Study personnel contact information was updated.	Updated to provide current information.
		#02	There were minor changes made in the infant anthropometry protocol: for infant length measurements, a wooden length board (Infant/Child ShorrBoard; Weigh And Measure, Olney, Maryland), will be used, replacing the Seca 417 (Seca Hamburg Germany) infantometer.	The wooden board was found more reliable and easier to use by the personnel.
		#03	Minor changes were made to the placenta specimen collection protocol to reflect practice. Samples are always collected from 2 quadrants per placenta instead of 2-4, and histopathology specimens are routinely fixed in formal saline instead of formalin at the collection site. In addition, the scale used to measure placenta weight was changed to iBalance (myWeigh, Vancouver Canada) from MXX-612 (Denver Instruments, Bohemia, NY) as the latter was not suitable to measure above 600 g.	Updated to reflect actual procedure.
		#04	The phrase "until she discontinues the non-study supplement" was added to the following statement regarding non-study supplement use (page 22): "A one-time warning will be given when non-study vitamin D or calcium supplementation is first reported; if the participant has not discontinued the non-study supplement at the time of the next weekly visit, the participant will be discontinued from further study supplementation until she discontinues the non-study supplement."	Clarification to indicate that study supplementation may resume if the participant discontinues non-study supplement use.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#05	Additional details have been added to the 'Statistical Analysis' section of the protocol.	To provide greater clarity on the study's analytical plan. These changes do not represent changes in data collection or introduce new outcomes, but merely aim to describe the analytical methods more accurately.
Version number 3.2	12-Nov-15	#01	Study personnel contact information has been updated.	Updated to reflect a change in study personnel.
		#02	Clarified that infant venous blood specimens are only collected if the parent/caregiver gives permission.	This clarifies that infant specimens are collected in only a sub-set of infants based on parental acceptance of the procedure.
		#03	We have expanded the panel of biochemical tests performed in real-time and reported back to physicians at the 3- and 6-month infant visits. The revised text is, "To directly benefit the infant, serum calcium at 3 months and hemoglobin, ferritin, calcium, creatinine, phosphorus, alkaline phosphatase and urine calcium:creatinine results at 6 months of age will be reported back to the study physician, and the results will be interpreted using standard clinical algorithms. Infants with iron-deficiency anemia will be treated, and referred for specialist care if necessary. Other biochemical abnormalities will be treated and/or referred as required on a case-by-case basis. Urinary phosphate will also be measured at the 6-month visit, but not used for clinical decisions as there are no established reference ranges for infancy."	The protocol originally indicated that tests other than ferritin and hemoglobin would be performed on a group of infants selected after the study phase 2b concluded, and did not specify that serum calcium results would be reported back to physicians. Because a smaller group of infants than expected have provided blood samples, we decided it was more efficient to perform these tests on all collected infant specimens, enabling physicians to receive results on an ongoing basis. Real-time reporting to physicians of the larger panel of tests may yield potential benefits to the infants. This change does not increase risks to the infants.
		#04	Clarified that 25-hydroxyvitamin D concentration may be measured in either serum or plasma, and that the analyte may be measured in maternal, infant and paternal specimens. The revised text is: " <i>Vitamin D status</i> : Vitamin D status will be determined by the serum/plasma 25(OH)D concentration, which is a well-established biomarker. Maternal, infant, and (if funding permits) paternal serum (or plasma) 25(OH)D will be assessed using state-of-the-art liquid chromatography tandem mass spectroscopy (LC-MS/MS) at the AFBM."	This does not represent a change in the original intent of the study, but adds clarifications about the use of plasma as well as paternal and infant samples. There is no change in the number of volume of specimens to be collected; therefore, there is no change to the risk to participants.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#05	Minor change to reflect decision that failure to obtain a repeat maternal blood sample does not always lead to cessation of supplementation.	In Sept., 2015 we conducted a thorough review of follow-up of above-range maternal serum calcium results. We identified 12 women who had elevated serum Ca (range 2.61-2.68 mmol/L) at delivery (n= 2), 3-months postpartum (n=9) and 6 months postpartum (n=1), but repeat sampling was not obtained due to participant refusal. In these cases, the participants continued supplementation (except in the one case when the value occurred at the 6 month visit). All 11 who continued with the supplementation had follow-up serum calcium measurements 3 months later. In all cases, the serum calcium had returned to the normal range (highest value 2.48 mmol/L). None of the events were associated with clinical symptoms or adverse events. Therefore, we concluded that in cases in which elevations in serum calcium are mild (<2.80 mmol/L) and asymptomatic, and repeat samples are declined by participants despite encouragement by study staff to obtain such samples, study supplementation may continue. This decision was reviewed and approved by the Trial Steering Committee on Oct 13, 2015.
		#06	The text on variation in blood collection schedules has been modified to include the option of blood collection at the home of the participant, limited to the cases when an abnormality was found in the initial blood sample and the participant is unable to visit the clinic to provide the confirmatory specimen. In these instances, proper hygienic and safety precautions will be taken, identical to those observed during clinic visits.	This addition reflects feedback from the field-site team; participants especially in the early postpartum period often face obstacles that prevent them from visiting the clinic for a repeat measurement. The field team suggested providing the option of at-home blood collection to promote proper diagnosis and clinical care for all participants.
		#07	Clarified that infant serum calcium will be measured at the 3-month visit in addition to the 6-month visit. The text now reads: "Serum calcium concentration in the cord vein and infants at 3 and 6 months will also be measured."	This is not a change from the original plan, but adds a clarification to the narrative section of the protocol. There is no change in the frequency/volume of blood collection, and thus no change in the risk to infants. As noted above, the result is reported back to physicians so may have benefit for the infants.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#08	Corrected typo in the name of the cyp24A1 enzyme.	Updated to correct a typo.
		#09	Edits to the text clarifies that stored specimens may be used for testing analytes beyond those specifically listed in preceding paragraphs. The additional text is: "To more broadly explain mechanisms of infant growth and related health outcomes in the trial cohort, a range of other blood and urine analytes related to growth, energy regulation, satiety, metabolism, angiogenesis, inflammation, and immune function will be measured in stored maternal, fetal (cord), placental, infant and paternal biospecimen aliquots, as funding permits. Targeted and untargeted metabolomic and proteomic approaches will be considered. Concentrations of environmental toxins (e.g., persistent organic pollutants) may also be measured. Such analyses will be limited to participants for whom written consent was obtained for use of stored specimens.	This is not a change from original plans, as the existing written consent process specifically addresses the use of stored specimens. There is no change in the frequency/volume of specimen collection, and thus no change in the risk to participants.
		#010	Clarified that some analytes are measured routinely and others are measured in stored specimens in selected samples. Also removed the term "case-control" study as stored specimens selected for analysis may not strictly correspond to a case-control design. The revised sections of text on this page are: "Selected analytes (described above) will be measured on a routine basis and reported back to study physicians. However, as a cost-saving measure, additional analytes will be measured in a subset of specimens in the context of a nested biochemical sub-study." "Maternal serum calcium, maternal urine calcium:creatinine ratio at delivery, infant serum calcium at 3 months, and infant hemoglobin, ferritin, serum calcium, creatinine, phosphorus and alkaline phosphatase and urine calcium:creatinine results at 6 months of age will be reported back to the field in real-time and managed by study physicians according to specific protocols."	Changes in relation to real-time infant specimen analysis are described above. The subset of infants from whom blood/urine samples are being collected will be used for the biochemical sub-study, and will thus correspond to a sub-cohort rather than a case-control design.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#011	The methods for epigenetic studies had been further defined. It was clarified that genome-wide methylation studies may be performed using maternal, paternal and fetal/infant specimens, and acknowledged that some of the work may only be possible if funding permits. Also changed the word “banked” to “stored”	This edit does not represent a major change from the original intent of the protocol; it adds more details incorporating new techniques that became available since the development of the original protocol. It also adds clarification that specimens from all three participants may be used, as reflected in the consent process, but that analyses will be limited by available funding. The use of the word “stored” implies that specimens for such analyses may not be part of a formal biobank.
		#012	The word “maternal” has been inserted, such that the sentence is now: “Possible hypercalcemia’ will be defined as a single maternal serum calcium concentration >2.60 mmol/L.”	This edit does not reflect any change in monitoring practices, but is just a clarification since the threshold of 2.60 as an upper limit of the acceptable range only applies to maternal rather than infant samples.
		#013	A sentence had been inserted to clarify the approach to those cases when a second blood sample could not be obtained and the initial serum calcium concentration is >2.80 mmol/L (moderate or severe hypercalcemia)-these cases will be managed similarly to cases of “confirmed hypercalcemia”.	The rationale for this change is described above. Participants, especially in the early postpartum period, might not be able to visit the clinic to provide a second blood sample; however, cases with moderate or severe hypercalcemia (concentration >2.80 mmol/L) or symptoms must have follow-up.
		#014	Additional paragraph to explain the approach to monitoring of infant serum calcium. The new text is: “Although infant serum calcium is not a primary safety indicator, a similar approach as described above will be used to manage above-range infant serum calcium values. However, the normal range of infant serum calcium is higher than in adults, and normal values may be as high as 3.05 mmol/L. Since there are few reference data for infants specifically at 3 and 6 months, a more conservative threshold of >2.80 mmol/L will be used to define above-range infant values (at the 3- and 6-month visits) that will prompt further assessment. Above-range infant serum calcium values need not prompt cessation of study supplementation in the absence of maternal hypercalcemia, but will be evaluated on a case-by-case basis.”	This does not represent a change from the original protocol, but is a clarification of the plan for interpreting and managing infant serum calcium values during the trial.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#015	Change in plan for biomarker sub-study designs and analyses. The revised text is: "Among those mother-infants pairs with complete anthropometric datasets and appropriate sets of biological specimens for analyses of a range of biomarkers (outlined above), a range of observational mechanistic and epidemiological sub-studies will be undertaken. For example, structural equation modeling (path analysis) of biomarker data and newborn/infant anthropometry will be performed to obtain estimates of the epidemiological associations among mediators/modifiers of the vitamin D-parathyroid axis and fetal/infant growth."	We no longer will limit biochemical analyses to a post-hoc selection of case and control participants; rather, we will perform such analyses using a sub-cohort of participants selected primarily on the basis of completeness of anthropometric data and specimen collection. This will enable a wider range of sub-studies not limited to the specific question that would drive case and control selection.
		#016	Modifications reflect changes in the analytical approach for the epigenetic studies. The revised text notes: "We will select approximately 100 mother-infant pairs who have sufficiently complete datasets and specimens for adequate epigenetic analyses. Statistical analyses will focus on between-group differences in DNA methylation patterns. Detailed approaches to the study design and statistical analyses to address aims #4, #5 and #6 are in development."	The specific analytical plans for these sub-studies are currently in development. However, the changes to the protocol do not affect specimen collection or handling, and are consistent with the original intent and the current consent form.
Version Number 3.3	28-Jun-16	#01	The paragraph on hemoglobin point-of-care testing was edited; a sentence was added to clarify that, if hemoglobin testing was missed at the 6-month visit, it will be offered to the participants at a later visit (preferably the 12-month visit). The text now reads (new text in italic): "At enrolment (as part of the eligibility screening process), maternal hemoglobin will be measured in a finger-prick blood sample using a handheld hemoglobinometer (Hb 201, Hemocue AB, Sweden). Infant haemoglobin will be measured at 6 months of age. <i>If blood is not collected at the 6-month visit, hemoglobin testing will be offered at a subsequent visit (e.g., 12-month visit).</i> "	The change allows us to offer delayed hemoglobin testing for infants who missed the 6-month test to maximize the number of study infants benefiting from hemoglobin testing. While this means a change in the schedule of infant blood collection it does not affect the total amount of blood collected from the infants during the study.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
		#02	The paragraph on blood biochemistry testing was edited; a sentence was added to clarify that, if blood biochemistry testing was missed at the 6-month visit, it will be offered to the participants at a later time. The text now reads (new text in italic): "To directly benefit the infant, serum calcium at 3 months and hemoglobin, ferritin, calcium, creatinine, phosphorus, alkaline phosphatase and urine calcium:creatinine results at 6 months of age will be reported back to the study physician, and the results will be interpreted using standard clinical algorithms... <i>If a blood sample is not collected at the 6-month visit, blood and urine collection for the biochemistry panel (hemoglobin, ferritin, calcium, creatinine, phosphorus, alkaline phosphatase, urine calcium:creatinine) will be offered at a subsequent visit.</i> "	The 6-month biochemistry panel has led to the detection of a number of early rickets cases in infants. These cases were asymptomatic and may not have otherwise been identified until more severe symptoms developed. The proposed change will permit delayed biochemistry testing in infants who missed testing at 6 months of age. While this means a change in the schedule of infant blood collection, it does not affect the amount of blood collected from the infants during the study. A script to inform and counsel participants about the opportunity to have the blood work is included with this amendment.
Version Number 3.4	05-Mar-17	#01	An additional paragraph was included to provide details about the records of clinical encounter and serious adverse event reporting in section 18b (Verbal and clinical data collection methods (including point-of-care tests)). The new paragraph (page 40) reads: "Clinical and serious adverse event (SAE) reports: Details of all serious adverse events (i.e., hospitalizations, deaths) and sequelae are routinely recorded on SAE reporting templates as required by the local institutional regulations for adverse event monitoring. Where appropriate, details of hospitalizations are recorded on hospitalization Forms 19 and 19B. Records related to other types of clinical events may be generated by study physicians in the process of providing direct patient care and/or facilitating referral to other facilities or physicians; where appropriate, data are captured in structured clinical event case report forms (i.e., Forms 15, 16). Study physicians obtain data related to clinical events and SAEs from direct clinical assessments; notes, prescriptions or impressions of other treating physicians; reports of investigations; or, directly from participants/caregivers. Formal health records are generally	Clinical encounters are recorded on various documents for clinical monitoring/therapeutic purposes. Moreover, serious adverse events are recorded on Serious Adverse Event (SAE) Reports as dictated by our partner organization (icddr,b) regulatory bodies. We propose to include these data sources into the MDIG trial dataset. The information represented in these documents is compiled by study physicians as part of our active clinical surveillance procedures in the trial. Original sources of information included prescriptions, physician's notes, reports of investigations, and verbal reports from physicians and families to adequately document all clinical events. The resulting document is a unique health record as hospital charts are not in use in Bangladesh; therefore, it would not be possible to gather this information retrospectively. The information is pertinent for several aspects of the study, for example, it will enable us to adjudicate and correctly report the number of rickets cases that occurred in the study, which is highly relevant in the context of vitamin D supplementation.

Version #	Version Date (D-M-Y)	Serial #	Protocol Modification	Rationale for Change
			unavailable in the trial setting. Data related to clinical encounters and SAEs will be retrospectively extracted from study physicians' notes, clinical event and hospitalization report forms, and SAE reports for incorporation into the trial database. Where necessary, de-identified data (including diagnostic images) will be post-hoc reviewed and/or adjudicated by study investigators to assign diagnostic codes or classifications."	We propose to extract information from the clinical encounter records and SAE reports in a de-identified manner. For this purpose, three data collection forms are proposed to introduce as new MDIG Trial documents. Consent to use this information is not possible to obtain as close to half of the MDIG trial participants are now discharged because they completed the trial or due to migration/loss to follow-up or because the participant or their infant is deceased.

^aSome minor protocol modifications made after the date of publication of the methods paper (July 2015)¹ may not be reflected in that manuscript.

^bThe protocol indicates incorrectly in some places that postpartum supplementation was to continue until 24-weeks post-partum; however, in fact postpartum supplementation was continued to 26-weeks postpartum. This duration was correctly reflected in the published Methods paper¹. It was an oversight that this error was not corrected in the protocol.

Reference

1. Roth DE, Gernand AD, Morris SK, Pezzack B, Islam MM, Dimitris MC, et al. Maternal vitamin D supplementation during pregnancy and lactation to promote infant growth in Dhaka, Bangladesh (MDIG trial): study protocol for a randomized controlled trial. *Trials* 2015; **16**: 300.

Statistical Analysis Plan

Randomized placebo-controlled trial of maternal vitamin D
supplementation during pregnancy and lactation to improve infant
linear growth in Dhaka, Bangladesh

“**M**aternal Vitamin **D** for **I**nfant **G**rowth (**MDIG**) trial”

Table of Contents

1. Introduction	4
2. Study Objectives	4
3. Design	5
3.1 Overview	5
3.2 Study Population	5
3.3 Expected Sample Size	5
3.4 Inclusion/Exclusion Criteria	5
3.4.1 Inclusion Criteria	5
3.4.2 Exclusion Criteria	5
4. Study Outcome Variables	6
4.1 Primary Outcome	6
4.2 Secondary Outcomes	6
4.3 Pregnancy and Safety Outcomes	6
5. Sequence of Planned Analyses	7
5.1 Interim Analyses	7
5.2 Final Analyses and Reporting	7
6. Statistical Methods	7
6.1 Analysis Principles	7
6.2 Incomplete Follow-Up, Missing Data, Outliers and Inconsistencies	8
6.2.1 Incomplete Follow-Up Data	8
6.2.2 Missing Outcome Data	8
6.2.3 Outliers and Inconsistencies	8
6.3 Derived and Computed Variables	8
6.3.1 Anthropometric Measurements	8
6.3.2 Maternal Characteristics at Enrolment	9
6.3.3 Gestational Age at Enrolment and Birth	10
6.3.4 Delivery Characteristics and Pregnancy Outcomes	10
6.3.5 Supplementation Duration and Adherence	10
6.3.6 Infant Feeding Practices	11
6.3.7 Safety Outcomes	11
7. Statistical Analyses	11
7.1 Trial Profile	11
7.2 Comparisons of Maternal Characteristics at Enrolment	12
7.3 Comparisons of Delivery Characteristics and Pregnancy Outcomes	12
7.3 Comparisons of Supplement Duration and Adherence	12
7.4 Comparisons of Maternal and Infant Biochemical Measures	12
7.5 Analysis of the Primary Outcome	13

7.6	Analysis of Secondary Anthropometric Outcomes	13
7.7	Comparisons of Infant Feeding Practices	14
7.8	Safety Outcomes	14
8	References.....	14

1. INTRODUCTION

Despite progress towards achieving reductions in child mortality in low-income countries of South Asia and sub-Saharan Africa, slower declines in fetal, infant and child rates of undernutrition in South Asia have been observed^{1,2}, suggesting that increased efforts are required to influence the high prevalence of stunting, defined as having a sex- and age-adjusted height or length less than 2 standard deviations below the median, as established by the World Health Organization growth standards.

In Bangladesh, about half of children younger than 5 years of age were afflicted by stunting in 2005³, with minimal improvements in 2007 (43%) and 2011 (41%) according to the Bangladesh Demographic Health Surveys. Early infant growth stunting and faltering⁴ despite breastfeeding suggests that the intrauterine nutritional, hormonal and metabolic environment during fetal development is also a strong determinant of early infant growth, which in turn, has long-term implications on infectious disease susceptibility, mortality, cognitive outcomes and social outcomes^{4,5}. However, to better design interventions to address the problem of stunting, increased understanding of the causal pathways to childhood stunting in low-income settings is required.

Vitamin D, parathyroid hormone (PTH), and parathyroid hormone-related peptide (PTHrP) function to maintain mineral homeostasis and modulate bone growth. In addition to its immunomodulatory actions in the placenta during pregnancy, maternal vitamin D is the sole source of vitamin D for the fetus during pregnancy and is often the sole source during early infancy through breastfeeding⁶, and therefore has potential implications on prenatal and postnatal growth. This trial of maternal pre- and postnatal vitamin D supplementation will test the hypothesis that vitamin D deficiency and consequent parathyroid gland hyperactivity increase the risk of fetal and early infant growth faltering in low-income settings, and in particular in Bangladesh, where biochemical vitamin D deficiency among women and young infants are highly prevalent⁷.

2. STUDY OBJECTIVES

1. To determine whether maternal prenatal oral vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week, administered as weekly doses) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh.
2. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.

3. DESIGN

3.1 Overview

This is a randomized, double-blind, placebo-controlled trial, using a parallel design with a 1:1 allocation ratio across 5 vitamin D3 dose-ranging groups: (1) prenatal placebo, postpartum placebo; (2) prenatal 4,200 IU/week, postpartum placebo; (3) prenatal 16,800 IU/week, postpartum placebo; (4) prenatal 28,000 IU/week, postpartum placebo; and (5) prenatal 28,000 IU/week, postpartum 28,000 IU/week.

3.2 Study Population

This is a single-site trial conducted in Dhaka, Bangladesh. The population of interest is pregnant women in the second trimester of pregnancy (17 to 24 completed weeks of gestation), recruited at the Maternal and Child Health Training Institute (MCHTI), commonly known as Azimpur Maternity Center, in Dhaka, Bangladesh, which covers a catchment area that includes Kamrangir char, Azimpur, Lalbag, and Hazaribag.

3.3 Expected Sample Size

A total of 1,300 participants are enrolled and were randomized into 5 groups of 260 women each. The goal is for a sample size of 220 analyzable participants per group, assuming up to 15% attrition from each group.

3.4 Inclusion/Exclusion Criteria

3.4.1 Inclusion Criteria

Patients were eligible for inclusion into the trial if all of the following criteria were met:

- Age 18 years and above
- 17 to 24 completed weeks of gestation (i.e., 17 weeks +0 days to 24 weeks + 0 days, inclusive) based on recalled last menstrual period (LMP) and/or ultrasound
- Intends to reside in the trial catchment area (including Hazaribag, Azimpur, Lalbag, and Kamrangirchar) for at least 18 months
- Provides written informed consent

3.4.2 Exclusion Criteria

Patients were ineligible for inclusion into the trial if any of the following conditions were met:

- History of any medical condition or medications that may predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia, including active tuberculosis or current therapy for tuberculosis, sarcoidosis, history of renal/ureteral stones, parathyroid disease, renal or liver failure, or current use of anti-convulsants
- High-risk pregnancy based on one or more of the following findings by point-of-care testing:
 - Severe anemia: hemoglobin <70 g/L assessed by Hemocue
 - Moderate-severe proteinuria: ≥ 300 mg/dl (3+ or 4+) based on urine dipstick
 - Hypertension: systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg
- High-risk pregnancy based on one or more of the following findings by maternal history and/or ultrasound:

- Multiple gestation
- Major congenital anomaly
- Severe oligohydramnios
- Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin containing calcium and/or vitamin D
- Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency
- Previous enrolment in the trial during a previous pregnancy

4. STUDY OUTCOME VARIABLES

4.1 Primary Outcome

The primary efficacy endpoint will be length-for-age z-score (LAZ) at one year of age. LAZ will be derived from each length measure (cm), in addition to the infant's exact age (in days) and sex, using the World Health Organization child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Length at each visit will be based on the mean of paired measurements. LAZ at "one year" will be based on data from 52 to 60 weeks (364 to 420 days) postnatal (where day 0 is birth). If an infant has more than one length measurement during the period of 364 to 420 days, the measurement collected closest in time to day 364 will be used; only one measurement per child will be used in the primary analysis.

4.2 Secondary Outcomes

- Weight-for-age z-score (WAZ) at one year of age
- Prevalence of stunting (LAZ below -2 SD) and wasting (WAZ below -2 SD) at one year of age
- Weight-for-length (WFL) at one year of age
- Head circumference for age z-score (HCAZ) at one year of age
- Raw length, weight, head circumference, upper-arm length and mid-upper arm circumference measures at one year of age
- LAZ, WAZ and HCAZ at birth (within 48 hours of birth)
- Maternal serum 25(OH)D concentration at 6 months postpartum; cord blood serum 25(OH)D concentration; and infant serum 25(OH)D concentration at 3 months and 6 months of age

4.3 Pregnancy and Safety Outcomes

- Placenta weight at delivery
- Gestational age at birth (in days), and percentage of births that were preterm (<37 weeks) and/or early preterm (<34 weeks)
- Confirmed maternal or infant hypercalcemia (serum calcium concentration > 2.60 mmol/L on two separate blood specimens, or serum calcium concentration > 2.80 mmol/L on a single blood specimen if a subsequent blood specimen could not be collected)
- Confirmed maternal hypercalciuria (two consecutive urine samples with calcium:creatinine ratio > 1 mmol/mmol, or one urine sample with Ca:Crea>1

mmol/mmol in the presence of persistent symptoms suggestive of possible uro/nephrolithiasis)

- Maternal urolithiasis or nephrolithiasis
- Maternal referral by study physicians for a suspected or diagnosed obstetric complication during prenatal period and up to 1 month postpartum
- Maternal Death
- Maternal serious adverse event
 - Hospitalization for any reason other than uncomplicated delivery
 - Intrauterine demise or delivery of a fetus that does not breathe or show any other evidence of life (stillbirth)
- Neonatal death (within first 28 days of life)
- Post-neonatal death (at any time during first year after 28 days)
- Infant serious adverse event
 - Infant hospitalization for any reason
 - Episode of skin infection, sepsis, diarrhoea or acute respiratory infection
 - Diagnosis of cancer, neurological disorder (stroke, seizure disorder, encephalopathy, or structural brain abnormality), or major congenital anomaly whether or not these required inpatient hospitalization

5. SEQUENCE OF PLANNED ANALYSES

5.1 Interim Analyses

An external Data and Safety Monitoring Board (DSMB) has monitored unblinded data for serious adverse events, baseline variables, and measures of study conduct and implementation on a regular basis. No interim efficacy analyses were planned or conducted.

5.2 Final Analyses and Reporting

All final planned analyses identified in the protocol and in this Statistical Analysis Plan will be performed only after the last infant has completed assessments scheduled for the 12-month study period.

6. STATISTICAL METHODS

6.1 Analysis Principles

All tests of the effect of treatment on outcomes (except analyses based on subsets) will be conducted as an intent-to-treat (as randomized) analysis, irrespective of supplementation adherence, and without imputation for missing data (i.e., infants for whom LAZ is unavailable at one year of age). Primary analyses will exclude data from mother-infant pairs when any one of the following events occur:

- Participant (maternal) death prior to delivery
- Fetal or infant death at any time
- Consent for all types of follow-up is withdrawn
- Participant is lost to follow-up (study staff determine conclusively that the participant cannot be contacted for the purposes of data collection for the duration of the period of

scheduled follow-up; or three months have passed since the scheduled but missed 12-month postnatal visit)

All tests of significance will be two-sided and primary analyses will not adjust for baseline covariates; however, sensitivity analyses will involve adjustment for any covariates that substantially differ at baseline (enrolment), if any.

6.2 Incomplete Follow-Up, Missing Data, Outliers and Inconsistencies

6.2.1 Incomplete Follow-Up Data

Participants with serial avoidance or refusal of supplementation, or with inconsistent follow-up visits are not withdrawn completely from the study if the participant agrees to the follow-up procedures (e.g. sample collection, anthropometry, etc.) and are included in the primary analysis if a 52 to 60 week measurement is contributed.

6.2.2 Missing Outcome Data

For the primary outcome, infants who do not have a measurement taken between 52 and 60 weeks of age will be excluded from the analysis.

6.2.3 Outliers and Inconsistencies

Outliers in measures of growth outcomes will be flagged as biologically implausible z-scores based on the World Health Organization Anthro software (<-6 SD or >6 SD for LAZ, >5 SD or <-6 SD for WAZ, >5 SD or <-5 SD for HCAZ, and <-5 SD or >5 SD for WFL). Temporal inconsistencies in growth measures, in which length and head circumference are assumed to be constantly increasing and weight cannot decline greater than or equal to 10%, will be checked for each infant. If outliers or inconsistencies cannot be resolved by manual data review, then the measurements for that time point will be dropped.

6.3 Derived and Computed Variables

6.3.1 Anthropometric Measurements

Paired measurements are collected for each length, weight, length, upper-arm length, mid-upper arm circumference and head circumference measure. Subsequent paired measurements, up to a total of three sets of paired measurements, are collected if the measurements differed by more than the threshold values: 7 mm for length; 50 g for weight; 5 mm for head circumference, upper arm length and mid-upper arm circumference. The average of the acceptable paired measures will be calculated and used in analyses.

Manual data review will be initiated if only a single measurement was collected for anthropometry, or if all available paired measurements for a given measure differed by more than the threshold value. If unresolved, the single measurement or average of the paired measurements, respectively, will be included in the primary analysis; however, sensitivity analyses will be conducted to exclude measurements which failed quality control procedures.

LAZ, WAZ, WFL and HCAZ at one year of age will be derived from the corresponding growth parameter calculated as a mean of paired measurements, infant's exact age (in days) and sex using the World Health Organization child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Dichotomous variables to describe prevalence of

stunting and wasting at one year of age will be generated using a cut-off of -2 SD for LAZ and WAZ, respectively.

For anthropometric measures at birth, LAZ, WAZ and HCAZ will be computed using the Intergrowth 21st Neonatal Standards to account for gestational age at birth, and measures must have been taken within 48 hours of birth.

6.3.2 Maternal Characteristics at Enrolment

Maternal level of education was assessed in Form 3A (Baseline Survey) using two questions:

2.	What sort of education have you completed? আপনি কী প্রকার শিক্ষা সমাপ্ত করেছেন?	School স্কুল.....1 Madrasha মাদ্রাসা.....2 → go to Q4 প্র.৪ এ যাও School and madrasha স্কুল ও মাদ্রাসা.....3 Neither কোনটিই নয়.....4 → go to Q4 প্র.৪ এ যাও Unknown জানিনা.....9 → go to Q4 প্র.৪ এ যাও	
3.	If you attended school, what is the highest class you completed at that level? আপনি স্কুলে গিয়ে থাকলে স্কুলের কোন শ্রেণী পর্যন্ত পড়ালেখা শেষ করেছেন?	No schooling স্কুলে যায়নি.....00 Class One প্রথম শ্রেণী.....01 Class two দ্বিতীয় শ্রেণী.....02 Class Three তৃতীয় শ্রেণী03 Class Four চতুর্থ শ্রেণী04 Class Five পঞ্চম শ্রেণী05 Class Six ষষ্ঠ শ্রেণী06 Class Seven সপ্তম শ্রেণী07 Class Eight অষ্টম শ্রেণী08 Class Nine নবম শ্রেণী09	S.S.C এস.এস.সি. (মাধ্যমিক) 10 H.S.C এইস.এস.সি. (উচ্চ মাধ্যমিক)11 University Graduate বিশ্ব বিদ্যালয় স্নাতক12 Master degree মাস্টার ডিগ্রী.....13 MBBS/Engineer/PhD এম.বি.বি.এস./প্রকৌশলী/পি.এইচ.ডি.....14 Unknown অজানা.....99

Level of education will be categorized as (1) “no schooling” if neither school nor madrasha were attended; (2) “some or completed primary education” if only madrasha was completed, or the highest class completed was class nine or lower; (3) “some or completed secondary education” if highest class completed was S.S.C or H.S.C; or (4) “some or completed tertiary education” if highest class completed was university graduate, Master degree or MBBS/engineer/PhD.

Primary daytime occupation of the mother at first weekly prenatal visit after enrolment was assessed in Form 4B (Household Characteristics):

1.	What is your primary daytime occupation? দিনের বেলায় আপনার প্রধান পেশা কি?	Homemaker গৃহকর্ম.....1 Garment factory তৈরি পোশাক6 শিল্প কারখানা.....2 Private business / গৃহকর্মী.....7 ব্যক্তিগত ব্যবসা3 Teacher শিক্ষক.....4 Student ছাত্র-ছাত্রী.....5 Professional (পেশাজীবী)6 Servant / maid-servant কর্মচারী / গৃহকর্মী.....7 Other: অন্যান্য:8 Don't know জানা নাই9
----	--	--

Primary daytime occupation will be categorized as (1) “homemaker”; or (2) “other”, which will include garment factory, private business, teacher, student, professional, and servant/maid-servant.

6.3.3 *Gestational Age at Enrolment and Birth*

Gestational age (GA) at enrolment is captured on Form 1A (Screening) is determined based on recalled last menstrual period (LMP) and/or ultrasound (captured on Form 1B, Ultrasound Screening). If there is a difference of >10 days between gestational age dated using the recalled LMP and second trimester ultrasound, the estimated GA will be adjusted as per the second trimester ultrasound; otherwise, if the difference is ≤10 days, GA estimation will be based on recalled LMP.

If there is more than one ultrasound, GA estimation should be based on the earliest of the ultrasounds for which a written report is available. If the earliest ultrasound was performed in the 1st trimester, and there is a difference of >5 days between gestational age dated using the recalled LMP and 1st trimester ultrasound, the estimated GA will be adjusted as per the 1st trimester ultrasound; otherwise, if the difference is ≤5 days, GA estimation will be based on recalled LMP.

A best estimate for date of LMP will be generated by subtracting the estimated gestational age in days from the date in which gestational age was calculated. Gestational age at birth will be calculated by the difference in days between the best estimate for date of LMP and date of birth. Gestational age at birth will also be categorized as (1) preterm if gestational age is less than 37 weeks (259 days); or (2) term if gestational age is equal to or greater than 37 weeks (259 days).

6.3.4 *Delivery Characteristics and Pregnancy Outcomes*

Season of birth will be categorized as (1) “spring” for births which occurred during the months of March to May; (2) “summer” for births which occurred during the months of June to August; (3) “fall” for births which occurred during the months of September to November; and (4) “winter” for births which occurred during the months of December to February.

6.3.5 *Supplementation Duration and Adherence*

Time on study will be calculated from the sum of the number of weeks spent enrolled in the study during the prenatal and postnatal period. Infants who completed a 1-year anthropometric measurement are assumed to have spent 52 weeks on study during the postnatal period. The amount of time on study during the prenatal period will be calculated from the difference between gestational ages at birth and enrolment.

Participants who did not have a delivery registered in the study due to discharge, withdrawal or loss to follow-up are assumed to not have spent time on the study during the postnatal period. The date of discharge or withdrawal, or if unavailable, the date of the last completed weekly prenatal interview will be used to determine the amount of time spent on the study.

For participants who were not discharged from the study but did not complete a 1-year follow-up visit, time spent on the study during the postnatal period will be determined from the date of the last completed weekly postnatal interview or trimonthly postnatal visit. Number of weeks for each participant will be rounded down to the nearest integer.

Total vitamin D administered will be calculated for from the product of the assigned dosage in the treatment group and the number of supplement doses administered.

Supplement adherence will be calculated from the number of doses received divided by the number of doses scheduled, multiplied by 100 to generate a percentage.

6.3.6 *Infant Feeding Practices*

Measures of exclusive breastfeeding will follow the WHO definition, in which the infant “receives only breast milk” and “no other liquids are given – not even water – with the exception of oral rehydration solution, or drops/syrups of vitamins, minerals or medicines”⁸.

Duration of exclusive breastfeeding will be captured as a continuous variable, in weeks, as well as a categorical variable for the age at discontinuation of exclusive breastfeeding: (1) ≤ 1 month of age; (2) >1 and ≤ 2 months of age; (3) >2 and ≤ 4 months of age; and (4) >4 months of age. Age at which formula and age at which animal source foods was introduced will be categorized in a similar nature.

6.3.7 *Safety Outcomes*

Infant clinical events are captured in Form 16 (Infant Clinical Event Form) and hospitalizations are captured in Form 19 (Hospitalization Outcome Form). Infant serious adverse events are subcategorized as (1) “hyperbilirubinemia/jaundice only” if the primary diagnosis on Form 16, and primary admitting diagnosis and primary discharge diagnosis on Form 19 all state “hyperbilirubinemia/jaundice” (code = 58) and secondary diagnoses are unavailable; (2) “neonatal sepsis/serious bacterial infection” if the primary discharge diagnosis on Form 19 states “sepsis/serious bacterial infection (incl. meningitis)” (code=63) and date of hospital admission is within 28 days of birth; and (3) “other”.

Infant deaths will be subcategorized as those occurring within the neonatal period (within 28 days of birth) and the post-neonatal period (after 28 days of birth and before 1 year of age).

7. STATISTICAL ANALYSES

7.1 Trial Profile

The progression of patients through the stages of provisional screening, detailed screening, enrolment, allocation, follow-up and analysis of primary outcome will be summarized in a CONSORT flow diagram. The number and percentage of patients who did not meet the trial’s inclusion criteria, declined to participate, were lost to follow-up or excluded from analyses will be provided by treatment group.

The number of protocol violations and deviations will be compared across treatment groups using Pearson’s chi-squared test.

7.2 Comparisons of Maternal Characteristics at Enrolment

Maternal characteristics at enrolment will be summarized by treatment group. For categorical variables, frequencies and percentages will be provided, and Pearson's chi-squared test will be conducted to assess the balance of distributions across treatment groups. Percentages will be calculated among participants whose information is not missing, potentially generating denominators less than 260. In these cases, the denominator will be specified in the body or as a footnote of the table. The following characteristics will be represented as categorical variables: marital status (married / not married); level of education (no schooling / some or completed primary education / some or completed secondary education / some or completed tertiary education); and primary occupation (homemaker / other).

7.3 Comparisons of Delivery Characteristics and Pregnancy Outcomes

Comparisons of delivery characteristics and pregnancy outcomes will be conducted by the same method as for maternal characteristics at enrolment. Categorical variables will include total deliveries registered (live births / intrauterine demise or stillbirths); gestational age at birth (preterm / term); mode of delivery (vaginal birth / caesarean section); infant sex (female / male); and season at birth (spring / summer / fall / winter).

Continuous parametric variables will include gestational age at birth (week); placenta weight (g); birth weight (g); length at birth (cm); head circumference at birth (cm); WAZ at birth; LAZ at birth; and HCAZ at birth. Continuous non-parametric variables will include gestational age at birth (week).

7.4 Comparisons of Supplement Duration and Adherence

Means, standard deviations, medians and ranges (minimums and maximums) will be presented by treatment group for time on study (week), total supplement doses administered, total vitamin D administered (IU) and adherence (%). ANOVA and Kruskal-Wallis test by rank will be conducted to assess equality of means and equality of medians, respectively, for each variable.

The proportion of participants who received all of their scheduled doses will be computed for each treatment group, and a test of the equality of proportions will be conducted using Pearson's chi-squared test.

7.5 Comparisons of Maternal and Infant Biochemical Measures

Means and standard deviations will be presented for all biochemical measures, and comparisons across treatment groups will be conducted using ANOVA. Comparisons will be conducted for maternal serum 25(OH)D concentrations at baseline, delivery, 3 months postpartum and 6 months postpartum; serum 25(OH)D concentration in cord blood; infant serum 25(OH)D concentrations (mmol/L) at 3 months and 6 months of age; maternal calcium concentrations (mmol/L) at baseline, delivery, 3 months postpartum and 6 months postpartum; infant calcium concentrations (mmol/L) at 3 months and 6 months of age; and maternal urinary calcium-creatinine ratios (mmol/mmol) at delivery.

7.6 Analysis of the Primary Outcome

The primary outcome, LAZ at one year of age, will be presented using means and standard deviations, and will be compared across the five treatment groups without adjustment for covariates using ANOVA. Sensitivity analyses will include an analysis adjusting for characteristics which differed across treatment groups at enrolment or delivery.

To assess the effect of prenatal vitamin D supplementation on LAZ at one year of age, five pairwise comparisons will be conducted using t-tests: 4,200 IU/week versus placebo; 16,800 IU/week versus placebo; 16,800 IU/week versus 4,200 IU/week; 28,000 IU/week versus placebo; and 28,000 IU/week versus 16,800 IU/week. Differences in means with 95% confidence intervals will be presented. Statistical significance for all 5 comparisons will be tested at an overall alpha of 0.05 (two-sided), and the Holm test will be used to account for multiple comparisons.

To assess the effect of postnatal vitamin D supplementation on LAZ at one year of age, one pairwise comparison will be conducted using a t-test: 28,000 IU/week postpartum versus placebo among women who received 28,000 IU/week antenatally. Differences in means with 95% confidence intervals will be presented. An alpha level of 0.05 will be used and no adjustment for the multiplicity of outcomes is planned since there is only one pairwise comparison related to the post-partum effect.

Sensitivity analyses will include analyses adjusting for characteristics which differed across treatment groups at enrolment or delivery. A generalized linear model with treatment allocation designated as a categorical variable will be used to contrast the parameter estimates for each treatment group.

7.7 Analysis of Secondary Anthropometric Outcomes

Calculations of means and standard deviations, and assessment of differences across treatment groups using ANOVA will be conducted for WAZ, WFL and HCAZ at one year of age; raw length, weight, head circumference, upper-arm length and mid-upper arm circumference at one year of age; and LAZ, WAZ and HCAZ within 48 hours of birth.

Prevalence of stunting and wasting, calculated as the proportion of infants with LAZ and WAZ score below -2 SD, respectively, will be compared across treatment groups using Pearson's chi-squared tests.

7.8 Comparisons of Infant Feeding Practices

Mean, standard deviation, median and range for length of exclusive breastfeeding (week) will be presented by treatment group, and ANOVA and Kruskal-Wallis test of ranks will be conducted to test equality of means and medians, respectively, across treatment groups.

Frequencies and percentages will be presented, and Pearson's chi-squared tests will be conducted to assess distribution across treatment groups for proportion of infants who initiated breastfeeding within 1 hour of birth; age at discontinuation of exclusive breastfeeding; proportion of infants who have ever been given formula; age at introduction of formula; and age at introduction of animal source foods.

7.9 Safety Outcomes

Frequencies, percentages and Pearson's chi-squared tests will be used to describe and compare the proportion of mothers or infants who experienced an adverse event and the distribution of serious adverse events across treatment groups. Among mothers, the number of participants who experienced hypercalcemia; hypercalciuria; urolithiasis/nephrolithiasis; a serious adverse event; and death will be assessed by treatment group. Among infants, the number of participants who experienced hypercalcemia; hypercalciuria; a serious adverse event, further stratified as hyperbilirubinemia/jaundice only, neonatal sepsis/serious bacterial infection or other; neonatal death; and post-neonatal death will be assessed by treatment groups.

The number of serious adverse events among mothers and infants, further stratified among infants as hyperbilirubinemia/jaundice only, neonatal sepsis/serious bacterial infection or other, will be described as a proportion of the sum of that event across all treatment groups.

8. REFERENCES

1. George K, Prasad J, Singh D, *et al.* Perinatal outcomes in a South Asian setting with high rates of low birth weight. *BMC Pregnancy Childbirth* 2009; **9**: 5.
2. de Onis M, Blössner M, Borghi E, Morris R, Frongillo EA. Methodology for estimating regional and global trends of child malnutrition. *Int J Epidemiol* 2004; **33**: 1260–70.
3. Black RE, Allen LH, Bhutta ZA, *et al.* Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* 2008; **371**: 243–60.
4. Victora CG, Adair L, Fall C, *et al.* Maternal and child undernutrition: consequences for adult health and human capital. *Lancet (London, England)* 2008; **371**: 340–57.
5. Dewey KG, Begum K. Long-term consequences of stunting in early life. *Matern Child Nutr* 2011; **7 Suppl 3**: 5–18.
6. Shin JS, Choi MY, Longtine MS, Nelson DM. Vitamin D effects on pregnancy and the placenta. *Placenta* 2010; **31**: 1027–34.
7. Roth DE, Shah MR, Black RE, Baqui AH. Vitamin D status of infants in northeastern rural Bangladesh: preliminary observations and a review of potential determinants. *J Health Popul Nutr* 2010; **28**: 458–69.
8. Kramer MS, Kakuma R. Optimal duration of exclusive breastfeeding. *Cochrane database Syst Rev* 2012; **8**: CD003517.

Statistical Analysis Plan

Randomized placebo-controlled trial of maternal vitamin D
supplementation during pregnancy and lactation to improve infant
linear growth in Dhaka, Bangladesh

“**M**aternal Vitamin **D** for **I**nfant **G**rowth (**MDIG**) trial”

Table of Contents

1. Introduction	4
2. Study Objectives	4
3. Design	5
3.1 Overview	5
3.2 Study Population	5
3.3 Expected Sample Size	5
3.4 Inclusion/Exclusion Criteria	5
3.4.1 Inclusion Criteria	5
3.4.2 Exclusion Criteria	5
4. Study Outcome Variables	6
4.1 Primary Outcome	6
4.2 Secondary Growth Outcomes	6
4.3 Pregnancy and Safety Outcomes	6
5. Sequence of Planned Analyses	8
5.1 Interim Analyses	8
5.2 Final Analyses and Reporting	8
6. Statistical Methods	8
6.1 Analysis Principles	8
6.2 Incomplete Follow-Up, Missing Data, Outliers and Inconsistencies	8
6.2.1 Incomplete Follow-Up Data	8
6.2.2 Missing Outcome Data	9
6.2.3 Outliers and Implausible Values	9
6.3 Derived and Computed Variables	9
6.3.1 Anthropometric Measurements	9
6.3.2 Maternal Characteristics at Enrolment	10
6.3.3 Gestational Age at Enrolment and Birth	11
6.3.4 Delivery Characteristics and Pregnancy Outcomes	11
6.3.5 Supplementation Duration and Adherence	12
6.3.6 Infant Feeding Practices	13
6.3.7 Safety Outcomes	14
7. Statistical Analyses	16
7.1 Trial Profile	16
7.2 Maternal Characteristics at Enrolment	17
7.3 Comparisons of Delivery Characteristics and Pregnancy Outcomes	18
7.4 Comparisons of Supplement Duration and Adherence	18
7.5 Comparisons of Maternal and Infant Biochemical Measures	18
7.6 Analysis of the Primary Outcome	19

7.7	Analysis of Secondary Anthropometric Outcomes	21
7.8	Comparisons of Infant Feeding Practices	22
7.9	Safety Outcomes	22
7.10	Quality Control of Anthropometry Measures	23
8.	References	23

1. INTRODUCTION

Despite progress towards achieving reductions in child mortality in low-income countries of South Asia and sub-Saharan Africa, slower declines in fetal, infant and child rates of undernutrition in South Asia have been observed^{1,2}, suggesting that increased efforts are required to influence the high prevalence of stunting, defined as having a sex- and age-adjusted height or length less than 2 standard deviations below the median, as established by the World Health Organization growth standards.

In Bangladesh, about half of children younger than 5 years of age were afflicted by stunting in 2005³, with minimal improvements in 2007 (43%) and 2011 (41%) according to the Bangladesh Demographic Health Surveys. Early infant growth stunting and faltering⁴ despite breastfeeding suggests that the intrauterine nutritional, hormonal and metabolic environment during fetal development is also a strong determinant of early infant growth, which in turn, has long-term implications on infectious disease susceptibility, mortality, cognitive outcomes and social outcomes^{4,5}. However, to better design interventions to address the problem of stunting, increased understanding of the causal pathways to childhood stunting in low-income settings is required.

Vitamin D, parathyroid hormone (PTH), and parathyroid hormone-related peptide (PTHrP) function to maintain mineral homeostasis and modulate bone growth. In addition to its immunomodulatory actions in the placenta during pregnancy, maternal vitamin D is the sole source of vitamin D for the fetus during pregnancy and is often the sole source during early infancy through breastfeeding⁶, and therefore has potential implications on prenatal and postnatal growth. This trial of maternal pre- and postnatal vitamin D supplementation will test the hypothesis that vitamin D deficiency and consequent parathyroid gland hyperactivity increase the risk of fetal and early infant growth faltering in low-income settings, and in particular in Bangladesh, where biochemical vitamin D deficiency among women and young infants are highly prevalent⁷.

2. STUDY OBJECTIVES

1. To determine whether maternal prenatal oral vitamin D3 supplementation (4,200 IU/week, 16,800 IU/week, or 28,000 IU/week, administered as weekly doses) versus placebo increases or decreases infant length at 1 year of age in Dhaka, Bangladesh.
2. To determine if maternal postpartum vitamin D3 supplementation (28,000 IU/week) versus placebo increases or decreases length at 1 year of age among infants born to women who received vitamin D 28,000 IU/week during pregnancy.

3. DESIGN

3.1 Overview

This is a randomized, double-blind, placebo-controlled trial, using a parallel design with a 1:1 allocation ratio across 5 vitamin D3 dose-ranging groups: (1) prenatal placebo, postpartum placebo; (2) prenatal 4,200 IU/week, postpartum placebo; (3) prenatal 16,800 IU/week, postpartum placebo; (4) prenatal 28,000 IU/week, postpartum placebo; and (5) prenatal 28,000 IU/week, postpartum 28,000 IU/week.

3.2 Study Population

This is a single-site trial conducted in Dhaka, Bangladesh. The population of interest is pregnant women in the second trimester of pregnancy (17 to 24 completed weeks of gestation), recruited at the Maternal and Child Health Training Institute (MCHTI), commonly known as Azimpur Maternity Center, in Dhaka, Bangladesh, which covers a catchment area that includes Kamrangir char, Azimpur, Lalbag, and Hazaribag.

3.3 Expected Sample Size

A total of 1,300 participants are enrolled and were randomized into 5 groups of 260 women each. We aim for no more than 15% attrition per group, translating to at least 220 analyzable participants per group to be included in the primary analysis.

3.4 Inclusion/Exclusion Criteria

3.4.1 Inclusion Criteria

Patients were eligible for inclusion into the trial if all of the following criteria were met:

- Age 18 years and above
- 17 to 24 completed weeks of gestation (i.e., 17 weeks + 0 days to 24 weeks + 0 days, inclusive) based on recalled last menstrual period (LMP) and/or ultrasound
- Intends to reside in the trial catchment area (including Hazaribag, Azimpur, Lalbag, and Kamrangirchar) for at least 18 months
- Provides written informed consent

3.4.2 Exclusion Criteria

Patients were ineligible for inclusion into the trial if any of the following conditions were met:

- History of any medical condition or medications that may predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia, including active tuberculosis or current therapy for tuberculosis, sarcoidosis, history of renal/ureteral stones, parathyroid disease, renal or liver failure, or current use of anti-convulsants
- High-risk pregnancy based on one or more of the following findings by point-of-care testing:
 - Severe anemia: hemoglobin <70 g/L assessed by Hemocue
 - Moderate-severe proteinuria: ≥ 300 mg/dl (3+ or 4+) based on urine dipstick
 - Hypertension: systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg
- High-risk pregnancy based on one or more of the following findings by maternal history and/or ultrasound:

- Multiple gestation
- Major congenital anomaly
- Severe oligohydramnios
- Unwillingness to stop taking non-study vitamin D or calcium supplements or a multivitamin containing calcium and/or vitamin D
- Currently prescribed vitamin D supplements as part of a physician's treatment plan for vitamin D deficiency
- Previous enrolment in the trial during a previous pregnancy

4. STUDY OUTCOME VARIABLES

4.1 Primary Outcome

The primary efficacy endpoint will be length-for-age z-score (LAZ) at one year of age. LAZ will be derived from each length measurement (cm), in addition to the infant's exact age (in days) and sex, using the World Health Organization child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Length at each visit will be based on the mean of paired measurements. LAZ at "one year" will be based on data from 52 to 60 weeks (364 to 420 days) postnatal (where day 0 is birth). If an infant has more than one length measurement during the period of 364 to 420 days, the measurement collected closest in time to day 364 will be used; only one measurement per child will be used.

4.2 Secondary Growth Outcomes

- Prevalence of stunting (LAZ below -2 SD) and wasting (WFL below -2 SD) at one year of age
- Weight-for-age z-score (WAZ) at one year of age
- Weight-for-length (WFL) at one year of age
- Head circumference for age z-score (HCAZ) at one year of age
- Mid-upper arm circumference for age z-score (MUACAZ) at one year of age
- BMI-for-age z-score (BMIAZ) at one year of age
- Raw crown-to-heel length, weight, head circumference, upper-arm length, mid-upper arm circumference and rump-to-knee length measurements at one year of age
- LAZ, WAZ and HCAZ at birth (within 48 hours of birth)
- Linear growth velocity (length and LAZ) from birth to one year of age
- Proportion of term infants (≥ 37 weeks) born with low birth weight (< 2500 g)
- Proportion of infants born with low birth weight (< 2500 g)
- Proportion of infants born small for gestational age ($< 10^{\text{th}}$ percentile for WAZ)

4.3 Pregnancy and Safety Outcomes

- Maternal serum 25(OH)D concentration at delivery, 3 months postpartum and 6 months postpartum; cord blood serum 25(OH)D concentration; and infant serum 25(OH)D concentration at 3 months, 6 months, and one year of age
- Maternal serum intact parathyroid hormone (iPTH) concentration at delivery and 6 months postpartum

- Gestational age at birth (in days), and percentage of births that were early preterm (<32 weeks), preterm (≥ 32 weeks to <37 weeks), term (≥ 37 weeks to <42 weeks) or postterm (≥ 42 weeks)
- Intrauterine demise or stillbirth
- Caesarean section
- Confirmed hypercalcemia
 - Women: Serum calcium >2.6 mmol/L in two consecutive samples, or >2.8 mmol/L in one sample when a subsequent (confirmatory) sample could not be obtained
 - Infants: serum calcium >2.8 mmol/L in two consecutive blood samples
- Possible hypercalcemia
 - Women: Serum calcium >2.6 mmol/L in one sample and ≤ 2.6 mmol/L in a repeat sample; or serum calcium >2.6 mmol/L and ≤ 2.8 mmol/L in one sample when a subsequent sample could not be obtained
 - Infants: Serum calcium >2.8 mmol/L in one sample and ≤ 2.8 mmol/L in a repeat sample; or serum calcium >2.8 mmol/L in one sample when a subsequent sample could not be obtained
- Confirmed hypercalciuria
 - Women: Urinary calcium: creatinine ratio >1 mmol/mmol in two repeat samples
 - Infants: Urinary calcium: creatinine >2.42 mmol/mmol in two repeat samples
- Possible hypercalciuria
 - Women: Urinary calcium: creatinine ratio >1 mmol/mmol and ≤ 1 mmol/mmol in a repeat sample; or calcium: creatinine ratio >1 mmol/mmol in one sample when a subsequent sample could not be obtained
 - Infants: Urinary calcium: creatinine >2.42 mmol/mmol in one sample and ≤ 2.42 mmol/mmol in a second repeat sample; or calcium: creatinine >2.42 mmol/mmol in one sample when a subsequent sample could not be obtained
- Any maternal clinical event from enrolment to 6 months postpartum
- Maternal urolithiasis or nephrolithiasis
- Maternal hospitalization from enrolment to 6 months postpartum
- Maternal death
- Gestational hypertension
- Frequency of self-reported symptoms among mothers (decreased appetite; vomiting; fever or chills/rigors; constipation; diarrhea; abdominal pain; cough; difficult or fast breathing; excessive thirst; frequent urination; burning sensation or pain in genital tract; muscle weakness; back pain or cramps; leg pain or cramps; arm pain or cramps; mental confusion; depressed mood; severe headache; blurry vision during the day; difficulty seeing at night; fainting or loss of consciousness; convulsions; vaginal bleeding; malodorous or coloured vaginal discharge; clear vaginal fluid; swelling of hands or feet; bruising; bleeding from mouth, rectum or in urine; yellow coloration of skin or eyes; labour pains or contractions; fetal movement (normal); and any fall, injury or trauma)
- Any infant clinical event from birth to one year of age
- Congenital anomaly
- Infant rickets (radiologically-confirmed)
- Infant neurological disabilities
- Infant hospitalization for any reason during the neonatal and post-neonatal period

- Neonatal death (within first 28 days of life)
- Post-neonatal death (at any time during first year after 28 days)
- Frequency of caregiver-reported symptoms among infants (fever or “too hot” to the touch; “too cold” to the touch; too tired or sleepy; convulsion or unresponsive; unusual cry or sounds; poor feeding; not feeding at all in the past 6 hours; not gaining enough weight; not passed any urine in the past 6 hours; cough; runny nose; nasal congestion (“stuffy nose”); fast breathing; difficulty breathing; wheeze or whistling in the chest; diarrhea; blood in the stool; vomiting; skin rash; red, oozing and/or swollen eye(s); red or oozing umbilicus; and injury or trauma)

5. SEQUENCE OF PLANNED ANALYSES

5.1 Interim Analyses

An external Data and Safety Monitoring Board (DSMB) has monitored unblinded data for serious adverse events, baseline variables, and measures of study conduct and implementation on a regular basis. No interim efficacy analyses were planned or conducted.

5.2 Final Analyses and Reporting

All final planned analyses identified in the protocol and in this Statistical Analysis Plan will be performed only after the last infant has completed assessments scheduled for the 12-month study period.

6. STATISTICAL METHODS

6.1 Analysis Principles

All tests of the effect of treatment on outcomes (except analyses based on subsets) will be conducted as a complete-case, intent-to-treat (as randomized) analysis, irrespective of supplementation adherence, and without imputation for missing data (i.e., infants for whom LAZ is unavailable at one year of age). Primary analyses will exclude data from mother-infant pairs when any one of the following events occur:

- Participant (maternal) death prior to delivery
- Fetal or infant death at any time prior to one year of age
- Consent is withdrawn
- Participant is lost to follow-up (study staff determine conclusively that the participant cannot be contacted for the purposes of data collection for the duration of the period of scheduled follow-up)

All tests of significance will be two-sided and primary analyses will not adjust for baseline covariates; however, sensitivity analyses will involve adjustment for baseline covariates.

6.2 Incomplete Follow-Up, Missing Data, Outliers and Inconsistencies

6.2.1 *Incomplete Follow-Up Data*

Participants with serial avoidance or refusal of supplementation, or with inconsistent follow-up visits are not withdrawn completely from the study if the participant agrees to the follow-up procedures (e.g. sample collection, anthropometry, etc.) and are included in the primary analysis if a 52- to 60-week measurement is contributed.

6.2.2 Missing Outcome Data

For the primary outcome, infants who do not have a measurement taken between 52 and 60 weeks of age will be excluded from the analysis.

6.2.3 Outliers and Implausible Values

Outliers will be defined as: (1) Biologically implausible z-scores based on the World Health Organization Anthro software (<-6 SD or >6 SD for LAZ; >5 SD or <-6 SD for WAZ; >5 SD or <-5 SD for HCAZ, WFL and MUACAZ); and (2) Aberrations from individual growth trajectories identified through a 'residual method', described briefly below. Implausible values refer to anthropometric measurements that are temporally inconsistent with previous measurements. Length, head circumference, upper-arm length and rump-knee length are assumed to be constantly increasing through serial measurements, while weight may fluctuate slightly between successive measurements. Any decreases in length, head circumference, upper-arm length and rump-knee length will be identified as implausible measurements, whereas any declines in weight $\geq 10\%$ between two successive measurements will be considered implausible.

To identify outliers based on the residual method, linear regression will be used to fit a straight line through an individual's age-standardized z-score (Z) as a function of age (t): $Z_{ij} = \beta_{0i} + \beta_i t_{ij} + \epsilon_{ij}$; where " i " is the i^{th} individual and " j " reflects the j^{th} time point. Based on the model predicted for each individual, jackknife residuals will be calculated, and measurements with an absolute jackknife residual ≥ 5 will be flagged as outliers for length, weight, and head circumference, and measurements with an absolute jackknife residual ≥ 7 will be flagged as outliers for mid-upper arm circumference. A similar procedure will be applied to raw anthropometric data when z-scores are not available (rump-knee length, upper-arm length), except the raw anthropometric measurement (Y) will be regressed on the square root of age ($t^{1/2}$): $Y_{ij} = \beta_{0i} + \beta_i t_{ij}^{1/2} + \epsilon_{ij}$; where " i " reflects the i^{th} individual and " j " reflects the j^{th} time point. Based on the model predicted for each individual, jackknife residuals will be calculated and measurements with an absolute jackknife residual ≥ 7 will be flagged as outliers. The purpose of identifying outlier is to prompt manual data review and reconciliation. Values will be corrected if the review of data forms and study logs provide information that enables such corrections. Measurements identified as outliers which cannot be reconciled will remain in all analyses unless there is sufficient evidence to indicate they are a result of measurement or data entry error.

When implausible values due to temporal inconsistencies between two consecutive measurements are flagged, jackknife residuals for each of the two time points will be compared and the measurement with the largest absolute jackknife residual will be considered the incorrect measurement. Implausible values will be dropped from analysis unless they can be reconciled and corrected through manual review.

6.3 Derived and Computed Variables

6.3.1 Anthropometric Measurements

Paired measurements are collected for each length, weight, head circumference, upper-arm length, mid-upper arm circumference and rump-to-knee length measure. Subsequent paired measurements, up to a total of three sets of paired measurements, are collected if the measurements differed by more than the threshold values of 7 mm for length, 50 g for weight, and 5 mm for head circumference, upper arm length, mid-upper arm circumference and rump-to-knee length. The average of the acceptable paired measures will be calculated and used in analyses. BMI will be calculated as $\text{weight}(\text{kg})/\text{height}(\text{m})^2$.

Manual data review will be initiated if only a single measurement was collected for anthropometry, or if all available paired measurements for a given measure differed by more than the threshold value. If unresolved, the single measurement or average of the paired measurements, respectively, will be included in the primary analysis.

LAZ, WAZ, WFL, BMIAZ, HCAZ and MUACAZ at one year of age will be derived from the corresponding growth parameter calculated as a mean of paired measurements, infant's exact chronological age (in days) and sex using the World Health Organization child growth standards and the STATA igrowup package (<http://www.who.int/childgrowth/software/en/>). Dichotomous variables to describe prevalence of stunting and wasting at one year of age will be generated using a cut-off of -2 SD for LAZ and WFL, respectively.

For length, weight and head circumference measurements taken within 48 hours of birth, LAZ, WAZ, and HCAZ will be computed using the Intergrowth-21st Neonatal Standards to account for gestational age at birth.

For anthropometric measurements among pre-term infants, the Intergrowth-21st International Postnatal Growth Standards for Preterm Infants will be used up to 64 weeks post-menstrual age.

Gestational age-corrected age will also be calculated and z-scores will be generated using the World Health Organization child growth standards. GA-correction will be applied to only preterm infants, and will be calculated using the following formula:

$$\text{GA-corrected age} = \text{Chronological age} - (280 - \text{Gestational age})$$

6.3.2 *Maternal Characteristics at Enrolment*

Maternal educational attainment was assessed in a baseline survey. Level of education will be categorized to be consistent with the Bangladesh DHS survey.

Primary daytime occupation of the mother at the first weekly prenatal visit after enrolment was assessed in a household characteristics questionnaire. Primary daytime occupation will be categorized as (1) "homemaker"; or (2) "other", which will include garment factory, private business, teacher, student, professional, servant/maid-servant, other and don't know.

To assess household socio-economic status, an asset index will be constructed using principle component analysis. Information from a baseline survey on household characteristics will be used to assess the ownership of the following 19 items: private toilet, electricity, radio, TV, mobile phone, non-mobile phone, fridge, almirah/wardrobe, table, chair, electric fan, DVD/VCD player, autobike, rickshaw/van, bicycle, motorcycle/motor scooter/tempo/CNG, livestock/herds/farm animals/poultry, homestead, and land. The first principle component will be used to assign each individual an asset score, with lower scores reflecting ownership of fewer items (i.e., less relative wealth) and higher scores being indicative of greater relative wealth. Using the distribution of asset scores in the study population, quintiles will be formed and participants will be categorized into 1 of 5 categories.

Gravidity, defined as the total number of pregnancies including the current pregnancy, will be reported. Parity, defined as the number of pregnancies which have occurred at gestational age 20 weeks or longer, will be calculated from the number of miscarriages or abortions subtracted from the total number of pregnancies. If the total number of pregnancies is 1 (including the current pregnancy), then parity will be 0.

Maternal height and weight at baseline will be calculated using the average value of the paired measurements. In instances where differences between paired measures is >2 cm for height and >0.5 kg for weight, a third measurement will be taken. Maternal height and weight will be calculated as the average of the two of three measurements with the smallest absolute difference between the two.

Month of enrolment will be categorized as (1) March-May; (2) June-August; (3) September-November; (4) December-February.

Maternal haemoglobin at enrolment will be measured in a finger-prick blood sample using a handheld hemoglobinometer (Hb 201, Hemocue AB, Sweden).

6.3.3 Gestational Age at Enrolment and Birth

Gestational age (GA) at enrolment, captured in a screening questionnaire, will be determined based on recalled last menstrual period (LMP) and/or ultrasound report. If there is a difference of >10 days between gestational age dated using the recalled LMP and second trimester ultrasound, the estimated GA will be adjusted as per the second trimester ultrasound; otherwise, if the difference is ≤10 days, GA estimation will be based on recalled LMP.

If there is more than one ultrasound, GA estimation will be based on the earliest of the ultrasounds for which a written report is available. If the earliest ultrasound was performed in the 1st trimester, and there is a difference of >5 days between gestational age dated using the recalled LMP and 1st trimester ultrasound, the estimated GA will be adjusted as per the 1st trimester ultrasound; otherwise, if the difference is ≤5 days, GA estimation will be based on recalled LMP.

A best estimate for date of LMP will be generated by subtracting the estimated gestational age in days from the date in which gestational age was calculated. Gestational age at birth will be calculated by the difference in days between the best estimate for date of LMP and date of birth. Gestational age at birth will also be categorized as (1) “early preterm” if gestational age is less than 32 weeks (224 days); (2) “preterm” if gestational age is equal to or greater than 32 weeks and less than 37 weeks (259 days); (3) “term” if gestational age is equal to or greater than 37 weeks (259 days) and less than 42 weeks (294 days); or (4) “postterm” if gestational age is equal to or greater than 42 weeks (294 days).

6.3.4 Delivery Characteristics and Pregnancy Outcomes

Delivery outcomes among all participants will be categorized as: (1) “live birth”; (2) “intrauterine demise or stillbirth”; (3) “maternal death prior to delivery”; (4) “lost to follow-up prior to delivery”. Mode of delivery will be categorized as vaginal birth or Caesarean section.

Location of delivery will be categorized as having occurred at: (1) “hospital or clinic” (MCHTI, or hospital or clinic other than MCHTI); (2) “home” ;(3) “other”.

Congenital anomalies will be classified post-hoc by pediatricians on the Trial Steering Committee based on available clinical data collected by study physicians.

Month of birth will be categorized as (1) March-May; (2) June-August; (3) September-November; (4) December-February.

Term low birth weight will be defined as having a birth weight, measured within 48 hours of birth, of less than 2,500 g and having been born at ≥ 37 weeks gestational age. Low birth weight will be defined as a birth weight, measured within 48 hours of birth, of less than 2,500 g.

6.3.5 Supplementation Duration and Adherence

The number of completed weekly monitoring visits will be calculated for the prenatal and postpartum period. A completed weekly monitoring visit will be defined by one in which the mother was present for the interview or the interview could be conducted over the phone.

The number of supplement doses administered will be calculated from both directly observed and unobserved doses. This will be determined through a manual review of supplementation logs by two independent reviewers, each of whom is a study physician. Any discrepancies between the two independent reviews will be flagged and a third physician will review the supplementation logs to resolve the discrepancy.

Total vitamin D administered will be calculated from the product of the assigned dosage in the treatment group and the number of supplement doses administered, stratified by the prenatal and postpartum period for each participant.

Supplement adherence will be calculated from the number of doses received divided by the number of doses scheduled, multiplied by 100 to generate a percentage, stratified by the prenatal and postpartum period for each participant.

For the majority of participants in which infant date of birth (DOB) is not missing (i.e., excluding participants who died, withdrew from the study, or were lost to follow up prior to giving birth), the number of scheduled doses in the prenatal period will be calculated as the number of weeks between enrolment and infant DOB. A subset of infants will be born on a day in which a regular prenatal visit is scheduled, such that the prenatal visit may not occur if the participant is already in labour. Participants who give birth on the day of a scheduled prenatal visit and do not complete a prenatal visit on the same day will be identified, and scheduled prenatal doses will be calculated as the number of weeks between enrolment and infant DOB, minus 1 week. Among women who die or are lost to follow up prior to giving birth, the number of scheduled doses in the prenatal period will be calculated as the number of weeks between enrolment and maternal death or date of last contact, respectively.

The number of scheduled doses in the postpartum period will be set to 26 for all participants who are included in the complete case analysis. Among participants who exit the study prior to delivery, deliver a stillborn baby, or are lost to follow up and not successfully contacted at least once after delivery, the number of postpartum scheduled doses will be set to 0. Among participants who are formally discharged between delivery and 6 months postpartum (due to infant death, loss to follow up), the number of postpartum scheduled doses will be calculated as the number of weeks between infant DOB and the date the participant is discharged from the study. For participants who are successfully contacted at least once during the postpartum period but are not officially discharged from the study before 6 months postpartum, the number of scheduled doses in the postpartum period will be determined as the number of weeks between infant DOB and the date in which the participant is last successfully contacted for a weekly postpartum interview.

Using the metric generated for supplement adherence, the proportion of participants who received 100% of scheduled doses, $\geq 90\%$ of scheduled doses and $\geq 80\%$ of scheduled doses will be calculated for the prenatal and postpartum period.

The proportion of tablets consumed under direct observation will also be estimated as the number of home visits in which a supplement was consumed divided by the total supplementation doses administered, stratified by the prenatal and postpartum period.

Three different indicator variables (yes / no) will be generated to identify participants who meet the various definitions of following the supplementation regime per-protocol: (1) per-protocol during the prenatal period, in which participants consume at least 90% of all scheduled doses and no episodes of reported consumption of non-study vitamin D or calcium during the prenatal period; (2) per-protocol during the postpartum period, in which participants consume at least 90% of all scheduled doses and no episodes of reported consumption of non-study vitamin D or calcium during the postpartum period; and (3) per-protocol throughout the whole study, in which participants consume at least 90% of all scheduled doses and no episodes of reported consumption of non-study vitamin D or calcium.

6.3.6 Infant Feeding Practices

Measures of exclusive breastfeeding will follow the WHO definition, in which the infant “receives only breast milk” and “no other liquids are given – not even water – with the exception of oral rehydration solution, or drops/syrups of vitamins, minerals or medicines”⁸.

Duration of exclusive breastfeeding will be captured as a continuous variable, in weeks. Deviations from exclusive breastfeeding during the first week of life will be permitted, given that the infant returns to being exclusively breastfed in the second week. To address the issue of missing data due to incomplete weekly visits:

- If an infant was exclusively breastfed prior to the incomplete visit(s) and no longer exclusively breastfed immediately after the incomplete visits, then the exact duration of exclusive breastfeeding will be calculated as the mid-point of the missing visit(s) (e.g. if an infant reported being exclusively breastfed during the previous 7 days before the scheduled visit for week 12, missed the scheduled visit for week 13, and then reported

being not exclusively breastfed during the previous 7 days before the scheduled visit for week 14, the duration of exclusive breastfeeding will be 12.5 weeks)

- If an infant was exclusively breastfed prior to and immediately after the incomplete visits, then the infant is assumed to have been exclusively breastfed during the period for the incomplete visit should have covered
- If an infant was exclusively breastfed up until a given and all subsequent visits (up until week 26) were incomplete, then the duration of exclusive breastfeeding will be calculated as the midpoint of that missing period (e.g. if an infant reported being exclusively breastfed during the previous 7 days before the scheduled visit for week 12, and all visits from week 13 to week 26 were incomplete, then the duration of exclusive breastfeeding will be 19 weeks)

Feeding patterns to 3 months and to 6 months of age will be categorized as (1) “exclusive breastfeeding”; (2) “predominant breastfeeding” if the infant’s main source of nourishment has been breast milk but may have received other liquids (water, sugar water, honey, or other non-milk, non-formula liquid); (3) “partial breastfeeding” if the infant is receiving infant formula, animal, powdered or condensed milk or semi-solid foods in addition to breastmilk; (4) “no breastfeeding” if the infant is not being breastfed; or (5) “unable to classify” if the scheduled visits for weeks 12 and 13 (when reporting the 3 month time period) or 25 and 26 (when reporting the 6 month time period) were not completed.

A categorical variable will be generated to capture whether infant formula was ever given (yes/no) during the first six months of age. A participant must have had at least 13 weekly visits completed in order to be classified as never having been given infant formula during the first six months of age. Minimum data requirements do not apply if the participant was ever given infant formula.

6.3.7 Safety Outcomes

Clinical encounters and hospitalizations for mothers and infants were reported in clinical event and hospitalization questionnaires.

Among women, clinical encounters (any clinical event, including hospitalizations), urolithiasis/nephrolithiasis, hospitalizations, gestational hypertension and death from enrolment to 6 months postpartum will be reported. For clinical encounters, urolithiasis/nephrolithiasis and hospitalizations, the number and proportion of women who ever had the outcome, the rate at which the outcome occurred (reported per 1,000 completed weekly visits), and the total number of occurrences of the outcome will be reported. In reporting the rate at which these outcomes occurred, the amount of person-time contributed by each participant will be calculated as the total number of weekly visits completed during the prenatal and postpartum period. If a participant experienced any outcome during a given week but the scheduled visit for that week was not completed, then the participant is still assumed to have contributed a week of person-time. In addition, the frequency and proportion of women who had gestational hypertension or died will also be reported.

Women will be classified as having gestational hypertension based on one or more of the following three criteria:

- Elevated blood pressure detected in routine prenatal monitoring: Maternal blood pressure is measured at baseline, 24 weeks gestation, 30 weeks gestation, and weekly from 36 weeks gestation to delivery. At each visit, measurements are repeated at least twice, providing a minimum of two systolic and two diastolic measurements per participant per visit. Women will be classified as having hypertension if, at a single visit, at least two systolic and/or two diastolic measurements are above 140/90 mmHg, respectively (e.g.1., Set 1: 142/70 mmHg, Set 2: 145/75 mmHg; e.g.2., Set 1:130/90 mmHg, Set 2: 125/95 mmHg). On the rare occasion that only one set of measurements is recorded at a visit, then a single measurement above 140/90 mmHg will be used to diagnose hypertension. As a data cleaning step, instances in which pulse pressure (difference between systolic and diastolic blood pressure) is <10 mmHg, the measurement will be dropped due to biological implausibility.
- Clinical encounter with reasonable evidence of hypertension and/or management of hypertension: Clinical encounters in which pre-eclampsia is diagnosed will be flagged and reviewed for evidence of hypertension/preeclampsia and/or management of hypertension (e.g., prescription for hypertension medication).
- Serious adverse event (SAE) with evidence of hypertension and/or management of hypertension: Women who experience any hypertension-related serious adverse event in the prenatal period will be classified as having gestational hypertension

Deaths, clinical encounters and hospitalizations among infants will be summarized as described above for women, but events will be stratified to those that occurred during the neonatal period (within 28 days of birth), from 29 days to <6 months of age and from 6 to 12 months of age. To calculate the rate at which clinical encounters or hospitalizations occurred, weekly postpartum visits up until 4 weeks of age will contribute to the calculation of person-time for the neonatal period and visits from 5 weeks to 6 months will contribute to the calculation of person-time for the period between 29 days and <6 months of age. A clinical encounter rate or hospitalization rate will not be calculated for the period beyond 6 months because weekly monitoring visits stop at this time. As previously described, if a participant has a clinical encounter or hospitalization during a given week, then it will automatically contribute towards the calculation of person-time, even if the scheduled weekly visit for that given week is not completed.

The number and proportion of infants with x-ray confirmed rickets, determined through physician-reviewed x-ray reports, and permanent neurological disabilities, determined post-hoc by paediatricians on the Trial Steering Committee based on available clinical data from study physicians, from birth to one year of age will also be reported. The denominator for x-ray confirmed rickets will be the number of infants with a complete panel of serum calcium, alkaline phosphatase, and inorganic phosphorous results before one year of age (abnormal biochemical results would prompt an x-ray). This includes infants who were routinely screened for rickets at a 6-month clinical visit, infants who had delayed rickets screening (after 6 months but before one year of age), and infants who had biochemical results for any other reason (e.g., clinical follow-up) before one year of age. The denominator used to calculate proportion of infants with neurological disabilities will be the number of live births.

Primary diagnoses received by women during any clinical encounter, and primary discharge diagnoses received by women during a hospitalization will be summarized by whether or not a woman ever received the diagnosis, and the number of times a woman received the diagnosis. Only clinical encounters and hospitalizations during the prenatal period and up to 6 months postpartum will be considered for women. Primary diagnoses during a clinical encounter and primary discharge diagnoses during a hospitalization among infants will be reported in a similar manner. For infants, diagnoses will be reported from birth to <6 months of age, and 6-12 months of age.

For reported symptoms among mothers, the timing of the symptoms will be assessed as having occurred during prenatal follow-up or during postpartum follow-up. Symptoms during the prenatal follow-up period will be further assessed for having occurred at baseline; during the second trimester of pregnancy (≥ 13 weeks and < 27 weeks gestation); or during the third trimester of pregnancy (≥ 27 weeks gestation). The number of times the symptom was reported and whether or not the symptom was ever reported by a given participant will be calculated. The week of gestation during which the symptom was reported will be calculated by dividing the number of days between date of last menstrual period and date of weekly prenatal interview by 7, with rounding down to the nearest integer. A visit is considered complete if a given participant reports the presence or absence of any symptom during the interview.

For reported symptoms among infants, the timing of the symptoms will be assessed as having occurred during the neonatal period (≤ 4 weeks of age); or during the post-neonatal period (> 4 weeks of age). The number of times the symptom was reported and whether or not the symptom was ever reported by the caregiver will be calculated. A visit is considered complete if the presence or absence of any symptom is reported by the caregiver during the interview.

7. STATISTICAL ANALYSES

7.1 Trial Profile

The progression of patients through the stages of provisional screening, detailed screening, enrolment, allocation, follow-up and analysis of primary outcome will be summarized in a CONSORT flow diagram.

During provisional screening, women may be deemed ineligible due to:

- not being pregnant;
- being less than 18 years of age;
- being < 17 weeks or > 24 weeks pregnant;
- not residing or intending to reside in the trial catchment area for at least 18 months; or
- refusing to participate.

Although women may be ineligible in provisional screening for more than one reason, the highest order exclusion criteria (following the order listed above) will be selected, such that the total number of ineligible women will equal the sum of the number of women who met each exclusion criteria.

During detailed screening, women may be deemed ineligible due to:

- having a history of a medical condition that may predispose to vitamin D sensitivity, altered vitamin D metabolism, and/or hypercalcemia;
- having multiple gestation, major congenital anomaly or severe oligohydromnios;
- being prescribed vitamin D supplementation or unwillingness to stop taking non-study vitamin D or calcium supplementation;
- having been enrolled in the trial previously;
- hypertension (SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg) at screening;
- severe anemia (Hb < 70 g/L) at screening; or
- moderate-severe proteinuria (urine protein ≥ 300 mg/dL) at screening;

in addition to the reasons listed for ineligibility during provisional screening. Women may leave at any point during the screening process, and these women are deemed ineligible due to refusing to participate. Similar to the approach used to report provisional screening ineligibility, the highest order reason for ineligibility (in the order outlined above) will be selected.

Two women who met the ineligibility criteria were incorrectly enrolled into the study and subsequently discharged as protocol violations. These two participants are excluded from all analyses (including those for characteristics at baseline), making the total sample size $N = 1298$.

7.2 Maternal Characteristics at Enrolment

Maternal characteristics at enrolment will be summarized by treatment group. Percentages will be calculated among participants whose information is not missing, potentially generating denominators less than the number of women enrolled into the given treatment arm. In these cases, the denominator will be specified in the body or as a footnote of the table. The following characteristics will be represented as categorical variables: marital status (married / not married); level of education (no schooling / primary incomplete / primary complete / secondary incomplete / secondary complete or higher); primary occupation (homemaker / other); asset index (1/2/3/4/5) and month of enrolment (March-May / June-August / September-November / December-February). Comparisons between groups will be made using Chi-squared or Fischer's tests.

Continuous parametric variables, including height (cm), weight (kg), haemoglobin (g/L) and baseline serum 25(OH)D concentration (nmol/L), will be presented using means and standard deviations. Medians and ranges will be used to describe continuous non-parametric variables, including age (years), gravidity, parity and gestational age at enrolment (weeks). ANOVA and Kruskal-Wallis tests will be used to make comparisons across treatment arms for parametric and non-parametric variables, respectively.

A similar table will be presented in the supplementary material and will include only participants included in the complete-case intention-to-treat analysis (i.e., excluding those with missing infant length measurements at one year of age). An additional supplementary table will describe and compare characteristics of participants included in the complete case analysis to participants not included in the complete-case analysis. T-tests, Wilcoxon rank-sum tests, Chi-squared tests, or Fisher's exact tests will be conducted to assess whether there are any differences between these two groups. This table will not be stratified by treatment arm.

7.3 Comparisons of Delivery Characteristics and Pregnancy Outcomes

Delivery characteristics and pregnancy outcomes will be compared between groups. For categorical variables, frequencies and percentages will be reported, and Chi-squared or Fischer's exact tests will be conducted to assess the distributions across treatment groups. Categorical variables will include: deliveries outcomes (live births / intrauterine demise or stillbirths / maternal death prior to delivery / loss to follow up during pregnancy); gestational age at birth (early preterm / preterm / term/ postterm); mode of delivery (vaginal birth / caesarean section); congenital anomaly (yes / no); location of delivery (hospital or clinic / home / other); infant sex (female / male); month of birth (March-May / June-August / September-November / December-February); term low birth weight (yes / no); low birth weight (yes / no); and small for gestational age (yes / no).

Continuous parametric variables, presented as means and standard deviations, will include serum 25(OH)D concentration at delivery (nmol/L); birth weight (g); length at birth (cm); head circumference at birth (cm); WAZ at birth; LAZ at birth; and HCAZ at birth. Comparisons between groups will be conducted using analysis of variance (ANOVA). Continuous non-parametric variables will be presented as medians and ranges and include gestational age at birth (weeks). Between-group comparisons will be conducted using the Kruskal-Wallis test by ranks.

7.4 Comparisons of Supplement Duration and Adherence

Continuous, non-parametric variables, including number of completed weekly monitoring visits, total supplementation doses administered, total vitamin D administered, adherence, and proportion of tablets consumed under direct observation, will be reported using medians and interquartile ranges. Categorical variables will be reported as frequencies and percentages and include the proportion of participants who received 100%, $\geq 90\%$, and $\geq 80\%$ of their scheduled doses. All variables will be reported for the prenatal and postpartum period separately. To compare continuous nonparametric variables, the Kruskal-Wallis test by ranks will be used, while Chi-squared tests will be used to compare categorical variables. No statistical test will be used to compare total vitamin D administered between the five treatment groups.

7.5 Comparisons of Maternal and Infant Biochemical Measures

Serum 25(OH)D (nmol/L) concentrations for women at delivery, 3 and 6 months postpartum, in cord blood, and among infants at 3, 6 and 12 months of age will be reported as means and standard deviations. Participants will also be categorized using various cut-offs of vitamin D status and will be reported as frequencies and percentages. Serum calcium concentrations (mmol/L) for women (at baseline, 30 weeks gestation, delivery, 3 and 6 months postpartum), infants (at 3 and 6 months of age), and calcium in venous cord blood will be reported as means and standard deviations. Maternal urinary calcium: creatinine ratio (mmol/mmol) at delivery, infant urinary calcium: creatinine ratio (mmol/mmol) at 6 months of age, maternal iPTH (pmol/L) at enrolment, delivery and 6 months postpartum will be reported as medians and interquartile ranges. Comparisons between treatment groups will be conducted using ANOVA for parametric variables (25(OH)D, serum calcium), and Kruskal-Wallis tests for non-parametric variables

(urinary calcium: creatinine ratio, iPTH). Categorical variables (cut-offs of 25(OH)D status) will be compared across treatment arms using Chi-squared or Fischer's tests.

The proportion of women and infants with confirmed and possible hypercalcemia and hypercalciuria (see definition provided in 4.3 Safety Outcomes) will be reported. For women, hypercalcemia will be reported in both the prenatal and postpartum period (where the prenatal period includes measurements taken at delivery), while maternal hypercalciuria will only be reported in the prenatal period because urine calcium was not routinely monitored during the postpartum period. The denominator for all equations will be the number of women per treatment arm with at least one available serum calcium or urinary calcium: creatinine measurement. Comparisons across treatment arms will be conducted using Chi-squared or Fischer's tests.

7.6 Analysis of the Primary Outcome

The primary outcome, LAZ at one year of age, will be presented using means and standard deviations, and will be compared across the five treatment groups using ANOVA.

To assess the effect of prenatal vitamin D supplementation on LAZ at one year of age, five pairwise comparisons will be conducted using t-tests: 4,200/0 IU/week versus placebo; 16,800/0 IU/week versus placebo; 16,800/0 IU/week versus 4,200/0 IU/week; 28,000/0 IU/week versus placebo; and 28,000/0 IU/week versus 16,800/0 IU/week. Differences in means with 95% confidence intervals will be presented. Statistical significance for all 5 comparisons will be tested at an overall alpha of 0.05 (two-sided), and the Holm test will be used to account for multiple comparisons. To isolate the effect of *prenatal* vitamin D supplementation, the group receiving 28,000 IU/week in the postpartum period will be excluded from this analysis.

To assess the effect of postpartum vitamin D supplementation on LAZ at one year of age, one pairwise comparison will be conducted using a t-test: 28,000 IU/week postpartum versus placebo among women who received 28,000 IU/week antenatally. Differences in means with 95% confidence intervals will be presented. An alpha level of 0.05 will be used and no adjustment for the multiplicity of outcomes is planned since there is only one pairwise comparison related to the postpartum effect.

Sensitivity analyses will include (1) using z-scores assigned based on GA-corrected age rather than chronological age; (2) per-protocol analyses; (3) subgroup and interaction analyses; (4) adjustment for baseline characteristics; and (5) use of multiple imputation to address primary outcome data.

For per-protocol analyses, comparisons will be restricted to participants who receive at least (1) 90% of all scheduled doses for the overall test of difference across treatment groups (ANOVA) and no consumption of non-study vitamin D or calcium; (2) 90% of prenatal doses for the five pairwise comparisons assessing the prenatal effect and no consumption of non-study vitamin D or calcium in the prenatal period; or (3) 90% of postpartum doses for the single pairwise comparison assessing the effect of postpartum supplementation and no consumption of non-study vitamin D or calcium in the postpartum period .

The following variables will be used to define subgroups and be included as interaction terms in the models: gestational age at birth, dichotomized as preterm (<37 weeks gestational age) and term (≥ 37 weeks gestational age); infant sex (male / female); maternal baseline serum 25(OH)D concentration, dichotomized as those who are deficient using a cut-off of <30 nmol/L vs. ≥ 30 ; maternal height, dichotomized at the median (<151 cm or ≥ 151 cm); and maternal supplement adherence, dichotomized as those who were per-protocol and not per-protocol. Linear regression models, stratified by each of the subgroups, and contrasts based on treatment assignment will be conducted to assess the overall effect of treatment assignment on LAZ at one year of age within the subgroup. A linear regression model with an interaction term between treatment assignment and the subgroup will be generated, and contrasts will be performed on the interaction term to assess the overall significance of this interaction effect across treatment arms. For pairwise comparisons, significance of single interaction (since only one comparison is being conducted per model) will be presented.

To adjust for baseline characteristics, separate linear regression models will be conducted for each baseline characteristic, in which only one covariate is being controlled for at a time. The coefficient estimate (and 95% CI) and the significance of the coefficient will be presented for each covariate. Contrasts will be conducted to test the difference in LAZ at one year of age across treatment groups after adjusting for the covariate included in the model. Two p-values for each covariate will be presented: $p_{\text{covariate}}$ which reflects the association between LAZ at one year and the covariate of interest, and p_{group} which reflects the overall difference in LAZ at one year of age after adjusting for the single baseline characteristic.

The marginal effect of treatment group on LAZ will be assessed using generalized estimating equations to model all LAZ as a function of age. A restricted cubic spline model will be used, in which knots will be set at 91, 182 and 273 days of age (3, 6, and 9 months). Interaction terms between treatment group and all age terms will be included in the model to allow for differences in slopes between treatment arms. The marginal effect of treatment group on LAZ at birth, 3 months, 6 months, 9 months and 12 months of age, as well as on overall LAZ will be assessed. The predicted mean LAZ will be presented for the treatment group that received a placebo dose in the prenatal and postpartum period and the mean difference (compared to the placebo group) in LAZ will be presented for all other treatment groups.

To handle missing LAZ at one year of age, multiple imputation by chained equations (MICE) will be used. The imputation model will include the main exposure (treatment assignment), outcome (LAZ at one year), and auxiliary variables which are related to missingness and/or LAZ at one year of age. Variables hypothesized to be related to missingness include number of living children, marital status, paternal occupation, asset index, maternal education, and whether the participant's face is regularly covered (as a proxy for religion), while variables hypothesized to be related to LAZ at one year of age include maternal height, gestational age at birth, LAZ at 6 months, LAZ at 9 months, infant sex, and asset index. Although additional LAZ measurements at different time points will also likely be related to LAZ at one year, they will not be considered due to the high degree of collinearity.

To assess whether a variable is associated with missingness, a new variable called 'Missing' will be generated, whereby women with missing LAZ at one year will be coded as 1 and women with LAZ at one year will be coded as 0. To determine whether auxiliary variables will be included in the imputation model based on their relationship to missingness, Chi-squared or Fischer's exact tests will be used for categorical variables and t-tests or Wilcoxon-rank sum tests will be used for continuous variables, using a liberal p-value of ≤ 0.1 for inclusion. To determine whether a continuous auxiliary variable is related to LAZ at one year of age, the strength of correlation will be evaluated with R^2 values, using a cut-off of 0.3 for inclusion in the imputation model. The relationship between categorical auxiliary variables and LAZ at one year will be determined using Chi-squared or Fischer's exact tests, using a p-value of ≤ 0.1 for inclusion. Using the above criteria, asset index, maternal height, LAZ at 6 months, LAZ at 9 months, and infant sex were related to missingness or LAZ at one year of age and were included in the imputation model.

The dataset will be arranged in wide format, such that each participant will have one row of data. Using a MICE approach, all variables included in the imputation model (treatment assignment, LAZ at one year, asset index, maternal height, LAZ at 6 months, LAZ at 9 months, infant sex) will be imputed if data are missing. Continuous variables will be imputed using linear regression and binary variables will be imputed using logistic regression. Fifty imputed datasets will be generated. We will impute data for: (1) All participants with missing LAZ at one year, except two women who were identified as protocol violations, to generate complete datasets for 1298 mother-infant pairs; (2) Participants with missing LAZ at one year, *among live births only and infants who did not die before one year of age*, and excluding two participants identified as protocol violations.

The estimation model will use linear regression to determine the association between treatment assignment and LAZ at one year, with a contrast post-estimation statement added to test the overall difference in mean LAZ at one year of age between treatment groups. Although the primary analysis will use an ANOVA test, ANOVA is not an available estimation model in Stata and linear regression results in identical inferences when no covariates are included in the model and the contrast statement is included.

7.7 Analysis of Secondary Anthropometric Outcomes

Calculations of means and standard deviations, and assessment of differences across treatment groups using ANOVA will be conducted for WAZ, WFL, BMIAZ, HCAZ and MUACAZ at one year of age. Pairwise comparisons, as described for the primary outcome, will also be conducted for WAZ, WFL, BMIAZ, HCAZ and MUACAZ at one year of age. Raw crown-to-heel length, weight, head circumference, upper-arm length, mid-upper arm circumference, and rump-to-knee length at one year of age, stratified by infant sex, will be presented using means and standard deviations and compared across groups using ANOVA. Similarly, birth length, weight and head circumference (not stratified by sex), and LAZ, WAZ and HCAZ at birth (within 48 hours of delivery) will be presented using means and standard deviations and compared across treatment groups using ANOVA. Median (min and max) age (in days) at measurement of one year anthropometry will be presented.

Prevalence of stunting and wasting at one year of age, term low birth weight, low birth weight and small for gestational age (at birth) will be presented using frequencies and percentages and compared across treatment groups using Chi-squared tests.

7.8 Comparisons of Infant Feeding Practices

Median and range for length of exclusive breastfeeding (weeks) will be presented by treatment group, and Kruskal-Wallis test of ranks will be conducted to test equality of medians across treatment groups.

Frequencies and percentages will be presented, and Chi-squared tests will be conducted to assess distribution across treatment groups for proportion of infants who initiated breastfeeding within 1 hour of birth; breastfeeding status up to 3 months of age; breastfeeding status up to 6 months of age; and proportion of infants who have ever been given formula.

7.9 Safety Outcomes

Frequencies, percentages and Chi-squared tests or Fisher's exact test will be used to describe and compare the proportion of women or infants who ever had a clinical encounter, urolithiasis/nephrolithiasis (among women only), hospitalization, gestational hypertension (among women only), rickets and neurological disabilities (among infants only), or death. In addition, frequencies will be used to describe the total number of clinical encounters, hospitalizations and urolithiasis/nephrolithiasis (among women only), in each treatment group. Incidence rates and zero-inflated negative binomial models for count data will be used to describe and compare the rate of clinical events, hospitalizations, and urolithiasis/nephrolithiasis (among women only) across treatment arms.

To describe and compare the proportion of women or infants who ever received a primary diagnosis (for clinical encounters) or primary discharge diagnosis (for hospitalizations), frequencies and percentages and Chi-squared or Fisher's tests will be used. The frequency of receiving a primary diagnosis or primary discharge diagnosis will also be reported by treatment assignment.

For reported symptoms among mothers, the number and proportion of times the symptom was reported (where the total number of visits is the denominator) in each treatment arm, and the number and proportion of women ever having reported the symptom in each treatment arm will be calculated. Timing of reported symptoms will be stratified by those reported at baseline, during second trimester pregnancy, during third trimester pregnancy, and during postpartum follow-up. Since participants can only report symptoms once at baseline, metrics related to the number of times the symptom was reported will not be assessed. Reported labour pains or contractions, and fetal movement will only be assessed for the prenatal period. Symptoms will be presented based how much the distribution of the reported symptoms differed across treatment arms, from symptoms which differed the greatest to those which differed the least. Ranking will be established via the following method:

1. The greatest absolute difference in proportions (for both number of times a symptom was reported and number of women ever having reported the symptom) between any given active treatment arm and the placebo group will be calculated.

2. Within each reported time period (baseline versus second trimester versus third trimester versus postpartum) and the two types of proportions calculated (counts versus ever), each symptom will be ranked according to the greatest absolute difference calculated in step 1. Therefore, each symptom should receive 7 separate ranking numbers (or 6 for symptoms reported only during the prenatal period).
3. The average ranking will be calculated to provide an overall rank.

Similar analytical methods will be applied to the reported symptoms among infants.

7.10 Quality Control of Anthropometry Measures

To provide a summary of the quality control procedures undertaken during data quality and cleaning, measurements (crown-to-heel length, weight, head circumference, upper arm length, mid-upper arm circumference, rump-knee length) will be flagged if:

- The difference between the first set of paired measurements is greater than the established threshold difference (0.7 cm for length; 50 g for weight; 0.5 cm for head circumference, upper-arm length, mid-upper arm circumference and rump-knee length)
- Difference between the final set of paired measurements is greater than the established threshold difference
- Only one measurement is taken
- The measurement is biologically implausible according to the cut-offs in the WHO Child Growth Standards (<-6 SD or >6 SD for LAZ; >5 SD or <-6 SD for WAZ; >5 SD or <-5 SD for HCAZ, WFL and MUACAZ)
- The measurement is flagged as an outlier based on the residual method
- The measurement is inconsistent with a measurement taken at a previous or later time point

The number of instances that measurements are flagged for the above reasons will be reported, after stratifying by measurement time point (birth, 2-8 weeks, 3 months, 6 months, 9 months, 12 months).

The technical error of measurement will also be calculated for each type of measurement at each time point, based on the following formula:

$$TEM = \sqrt{\frac{\sum d^2}{2n}}$$

Where d is the difference between paired measurement, and n is the number of observations at a given measurement time point.

8. REFERENCES

1. George K, Prasad J, Singh D, *et al.* Perinatal outcomes in a South Asian setting with high rates of low birth weight. *BMC Pregnancy Childbirth* 2009; **9**: 5.
2. de Onis M, Blössner M, Borghi E, Morris R, Frongillo EA. Methodology for estimating regional and global trends of child malnutrition. *Int J Epidemiol* 2004; **33**: 1260–70.
3. Black RE, Allen LH, Bhutta ZA, *et al.* Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* 2008; **371**: 243–60.
4. Victora CG, Adair L, Fall C, *et al.* Maternal and child undernutrition: consequences for adult health and human capital. *Lancet (London, England)* 2008; **371**: 340–57.

5. Dewey KG, Begum K. Long-term consequences of stunting in early life. *Matern Child Nutr* 2011; **7 Suppl 3**: 5–18.
6. Shin JS, Choi MY, Longtine MS, Nelson DM. Vitamin D effects on pregnancy and the placenta. *Placenta* 2010; **31**: 1027–34.
7. Roth DE, Shah MR, Black RE, Baqui AH. Vitamin D status of infants in northeastern rural Bangladesh: preliminary observations and a review of potential determinants. *J Health Popul Nutr* 2010; **28**: 458–69.
8. Kramer MS, Kakuma R. Optimal duration of exclusive breastfeeding. *Cochrane database Syst Rev* 2012; **8**: CD003517.

Amendments to the statistical analysis plan from the original to final plan

Section	Amendment
4.2	<ul style="list-style-type: none"> • Changed the title of section 4.2 from “Secondary Outcomes” to “Secondary Growth Outcomes” • Additional growth outcomes were added: mid-upper arm circumference for age z-score (MUACAZ) at one year of age, BMI-for-age z-score (BMIAZ) at one year of age, raw rump-to-knee length measured at one year, linear growth velocity (length and LAZ) from birth to one year of age, term low birth weight, low birth weight, and proportion of infants born small for gestational age • Maternal, infant and cord blood 25(OH)D outcomes removed from this section to reflect updated title
4.3	<ul style="list-style-type: none"> • Added maternal, infant and cord blood 25(OH)D outcomes to this section. Additional time points (mothers at baseline and 3 months postpartum, infants at 12 months of age) in which maternal and infant 25(OH)D are measured and are to be reported are included. • Updated the categorization of gestational age at birth from reporting only preterm and early preterm infants to also report term infants (≥ 37 weeks to < 42 weeks gestation) and postterm infants (≥ 42 weeks gestation) • Provided a more detailed definition of maternal and infant confirmed and possible hypercalcemia and hypercalciuria • Additional outcomes were added: maternal iPTH at delivery and 6 months postpartum, gestational hypertension, infant rickets and neurological disabilities, C-section, maternal and infant symptoms • Removed placenta weight and “maternal referral by study physician for suspected or diagnosed obstetric complication during prenatal period and up to 1 month postpartum” • Noted that maternal clinical encounters and hospitalizations (serious adverse events) from enrolment to 6 months will be reported, and all infant clinical encounters and hospitalizations from birth to one year of age will be reported
6.1	<ul style="list-style-type: none"> • Procedures describing the adjustment of baseline characteristics as a sensitivity analysis were changed from only adjusting for characteristics that substantially differ between treatment arms to adjusting for all baseline covariates
6.2.3	<ul style="list-style-type: none"> • Provided a description of an additional method (termed the ‘residual method’) used to identify and handle anthropometry outliers and implausible measurements.
6.3.1	<ul style="list-style-type: none"> • Updated to acknowledge that paired measurements were also collected for rump-to-knee length • Included a formula to calculate BMI • Removed the sentence “however, sensitivity analyses will be conducted to exclude measurements which failed quality control procedures” because preliminary analyses (while still blinded to treatment assignment) found only a small proportion of anthropometric measurements in which only 1 measurement was taken or the difference in paired measurements was above the designated threshold • Changed the sentence ““LAZ, WAZ, WFL and HCAZ at one year of age will be derived from the corresponding...” to “LAZ, WAZ, WFL, BMIAZ, HCAZ and MUACAZ at one year of age...” to reflect the additional anthropometric outcomes

	<ul style="list-style-type: none"> • Described an additional method in which z-scores among pre-term infants will be calculated using gestational-age corrected age
6.3.2	<ul style="list-style-type: none"> • Removed the screenshot of the study questionnaire in which maternal education was reported. Noted that the categorization of maternal education will be consistent with the Bangladesh DHS survey • Removed the screenshot of the study questionnaire in which maternal daytime occupation was addressed. • A description of the methods that were planned to be used to create an asset index were added • Added descriptions of how gravidity, parity, maternal height and weight, month of enrolment, and hemoglobin are derived and reported
6.3.3	<ul style="list-style-type: none"> • The categorization of gestational age at birth was modified to include early preterm and postterm, in addition to preterm and term
6.3.4	<ul style="list-style-type: none"> • Changed “Season of birth” to “month of birth” • Additional delivery characteristics and pregnancy outcomes added: delivery outcomes (live birth, intrauterine demise/stillbirth, maternal death prior to delivery, lost to follow-up prior to delivery), mode of delivery (vaginal birth/ C-section), location of delivery (hospital or clinic/ home/other), congenital anomalies, term low birth weight, low birth weight, and small for gestational age
6.3.5	<ul style="list-style-type: none"> • Provided detail on how the time on study during the prenatal and postpartum period was to be determined • Added a statement to indicate that the number of supplements consumed will be determined through a manual review of supplementation logs by study physicians • Noted that supplementation adherence will also be reported using three cut-offs (participants who consume 100%, $\geq 90\%$, and $\geq 80\%$ of doses) • Defined the various ‘per-protocol’ variables
6.3.6	<ul style="list-style-type: none"> • Refined the definition of duration of exclusive breastfeeding and described how missing data due to incomplete weekly visits was to be handled • Added some information about how feeding patterns were to be described to 3 and 6 months of age (categorized as exclusive breastfeeding, predominant breastfeeding, partial breastfeeding, no breastfeeding, and unable to classify) • Instead of reporting age at which a formula was given, decided to report whether or not a formula was ever given • No longer reporting the age at which animal source foods were introduced
6.3.7	<ul style="list-style-type: none"> • Described how maternal clinical encounters, urolithiasis/nephrolithiasis, and hospitalizations from enrolment to 6 months postpartum are to be reported as the proportion of women who ever had an outcome, the rate at which the outcome occurred, and the total number of occurrences of the outcome. Described how person-time for rates will be calculated • Provided similar explanations for infant clinical encounters, deaths, and hospitalizations. Removed information pertaining to the categorization of infant serious adverse events (hyperbilirubinemia/jaundice, neonatal sepsis/serious bacterial infection, sepsis/serious bacterial infection within 28 days of birth) • Provided detail on how gestational hypertension was to be defined and reported • Described how x-ray confirmed rickets was to be determined • Added information on how clinical diagnoses and symptoms will be reported for mothers and infants

7.1	<ul style="list-style-type: none"> • Added provisional and detailed screening exclusion criteria to this section, and described how this will be reported in the CONSORT diagram • Removed the sentence “The number of protocol violations and deviations will be compared across treatment groups using Pearson’s chi-squared test” because there were very few protocol violations/deviations
7.2	<ul style="list-style-type: none"> • Added asset index and month of enrolment to the list of categorical variables • Added hemoglobin and maternal baseline 25(OH)D as continuous parametric, and removed age, gestational age at enrolment (because both variables were assumed to be non-parametric) and body mass index (decided to instead report maternal weight and height) from this list • Added age (years) to the list of non-parametric variables that will be described using medians and ranges • Described an additional analysis in which baseline characteristics will be compared between participants included in the complete case analysis to those not included in the complete case analysis
7.3	<ul style="list-style-type: none"> • Changed the outcome “total deliveries registered (live births / intrauterine demise or stillbirths) to “deliveries outcomes (live births / intrauterine demise or stillbirths / maternal death prior to delivery / loss to follow up during pregnancy)”. • Changed “gestational age at birth (preterm/term)” to gestational age at birth (early preterm/preterm/term/postterm)” • Changed “season of birth” to “month of birth” • Added additional dichotomous (yes/no) variables: congenital anomaly, term low birth weight, low birth weight, small for gestational age • Continuous parametric variables: removed placenta weight, added delivery 25(OH)D • Removed gestational age at birth from the list of parametric variables
7.4	<ul style="list-style-type: none"> • Modified this section to reflect an updated approach to describe and compare supplementation adherence between groups: “Continuous, non-parametric variables, including number of completed weekly monitoring visits, total supplementation doses administered, total vitamin D administered, adherence, and proportion of tablets consumed under direct observation, will be reported using medians and interquartile ranges. Categorical variables will be reported as frequencies and percentages and include the proportion of participants who received 100%, $\geq 90\%$, and $\geq 80\%$ of their scheduled doses. All variables will be reported for the prenatal and postpartum period separately. To compare continuous nonparametric variables, the Kruskal-Wallis test by ranks will be used, while Chi-squared tests will be used to compare categorical variables. No statistical test will be used to compare total vitamin D administered between the five treatment groups”.
7.5	<ul style="list-style-type: none"> • Noted that 25(OH)D will be categorized using different cut-offs of vitamin D status and comparisons across arms will be conducted using Chi-squared or Fischer’s tests • The approach to report and compare calcium-creatinine ratios across treatment arms changed. The updated version states that calcium-creatinine will be reported using medians and IQRs (to reflect the non-parametric distribution) and comparisons across groups will be conducted with Kruskal-Wallis tests. Reporting of infant urinary Ca:Cr at 6 months also included in the revised plan. • Maternal iPTH at delivery, enrolment, and 6 months postpartum was added

	<ul style="list-style-type: none"> • A description of how maternal and infant confirmed/possible hypercalcemia/hypercalciuria are to be reported and compared across groups was added
7.6	<ul style="list-style-type: none"> • Clarified the specific prenatal and postpartum pairwise comparisons • Added a detailed description of various sensitivity analyses
7.7	<ul style="list-style-type: none"> • Modified this section to include additional anthropometric outcomes at one year of age (BMIAZ, MUACAZ, raw rump-to-knee length) • Noted that raw anthropometric comparisons at one year will be stratified by infant sex • Added a statement noting that raw weight, length and head circumference at birth will be reported • Noted that the median (min and max) age (in days) of infants at the time of their one-year anthropometry will be reported
7.8	<ul style="list-style-type: none"> • Revised the analytic approach to report and compare exclusive breastfeeding (weeks) across treatment groups to reflect the distribution of this variable by reporting medians and ranges and using Kruskal-Wallis test • The list of categorical feeding pattern variables was revised to include: initiated breastfeeding within 1 hour of birth; breastfeeding status up to 3 months of age; breastfeeding status up to 6 months of age; and proportion of infants who have ever been given formula
7.9	<ul style="list-style-type: none"> • We expanded our reporting of maternal and infant clinical encounters and serious adverse events (hospitalizations). Instead of only reporting serious adverse events, we decided to report all clinical encounters (rate and proportion of women/infants who ever experienced a clinical encounter) using Chi-squared tests and zero-inflated log-binomial models • Removed the statistical approach for comparing hypercalcemia/hypercalciuria from this section; instead, it is described in section 7.4 • Instead of just reporting hyperbilirubinemia/jaundice only, neonatal sepsis/serious bacterial infection, decided to report all infant and maternal diagnoses • Added gestational hypertension, rickets, neurological disabilities to the list of variables that will be compared • A detailed description of how maternal and infant diagnoses and symptoms will be reported and compared was added
7.10	<ul style="list-style-type: none"> • Anthropometry quality control section added