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Vitamin D3 and B12 supplementation in pregnancy

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ABSTRACT

Aim: To assess the efficacy of vitamin D3 or B12 supplementation during pregnancy.
Methods: Pregnant women at 6–14 weeks in the intervention arm received oral high dose intermittent vitamin D3 and/or low dose B12 supplementation if they had vitamin D or vitamin B12 deficiency. The control arm received prescribed dietary instruction only. An additional observational arm for those mothers at booking with normal vitamin D and vitamin B12 level was also recruited. All groups received standard care during pregnancy.
Results: The primary endpoint of either vitamin D or B12 at term was not met. At baseline 25% participants in both the interventional and control arms had severe D deficiency (<30 nmol/l), reducing to under 3.4% in both groups. No maternal differences in vitamin D or B12 levels were found at delivery between the intervention, control, or observational groups. No significant difference in any of the pregnancy or birth outcomes was observed between three groups.
Conclusions: In this study, oral supplementation of high dose intermittent vitamin D or low dose vitamin B12 regime failed to correct the relevant nutritional deficiencies in Bangladeshi pregnant women as per protocol. Both dietary supplementation and high dose vitamin D corrected severe vitamin deficiency.

1. Introduction

Deficiency of 25-hydroxyvitamin D (vitamin D) and vitamin B12 are very common worldwide. There is reasonable evidence from observational studies that low levels of vitamin D [1] is associated with the predisposition to chronic diseases. It has been hypothesized that supplementation with vitamin D may prevent type 2 diabetes (T2D) and cardiovascular disease

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(CVD). However, evidence from randomized clinical trials (RCTs) of vitamin D supplementation to prevent T2D or CVD have not provided convincing evidence of a protective effect [2,3].

Evidence shows that vitamin D deficiency is common during pregnancy, particularly in the Middle East and Asian, middle east and and North Africa [4]. Despite the reported high prevalence and risks of vitamin D deficiency during pregnancy, there only a few RCTs of vitamin D supplementation to optimize the vitamin D status during pregnancy, especially in such high-risk populations as Bangladesh [5,6].

The Pune Maternal Nutrition Study showed that maternal vitamin B12 deficiency is associated with hyperhomocysteinemia and low birth weight (LBW). Yajnik and colleagues have since shown an association between maternal B12 deficiency and increased insulin resistance and adiposity in the offspring, the first time that insulin resistance has been linked to a specific maternal nutritional deficiency [7].

In the first trimester of pregnancy vitamin B12 deficiency was found in 42% [8] and 15% [9] in Pakistan and Bangladesh, respectively. A meta-analysis of maternal vitamin B12 in pregnancy concluded that deficiency of B12 was associated with an increased risk of pre-term birth and LBW and thus the need for well-conducted RCTs of supplementation [10]. Although it has been proposed that low vitamin B-12 levels may be a risk factor for CVD and diabetes. A systematic review of cohort studies concluded that there was only limited evidence for such an effect [11].

LBW is an important public health problem and one of the strongest single risk factors for early neonatal mortality and morbidity [12], including DM and CVD in later life. Findings have shown that LBW is related to reduced insulin sensitivity. It has been recommended that reduced insulin sensitivity in LBW subjects' results from the adaptation to adverse in utero conditions during a critical period of development.

The prevalence of LBW is 15.5% globally, and 96.5% of LBW infants are born in developing countries [13,14]. Nonetheless, International guidelines are not consistent on the importance of correcting micronutrient deficiencies in pregnancy since there is a lack of quality RCTs in pregnancy to base such recommendations.

Given the increased prevalence of micronutrient deficiencies in developing and middle-income countries during pregnancy, there is an urgency to establish guidelines for recommendations during pregnancy for supplementing micronutrients. A new WHO guideline has commenced addressing all these issues [15], however, it still leaves significant knowledge gaps regarding ideal weight during pregnancy, notably for South Asian women. Thus, this study's objective was to conduct a complex intervention during pregnancy involving personalized micronutrient supplementation (vitamin D and B12) tailored to the participant's nutritional status. Maternal and delivery outcomes, including adverse outcomes, were evaluated in the intervention, control, and observational arms.

2. Materials and methods

2.1. Study design and participants

The GIFTs trial was a randomized open-label phase 2 pilot trial conducted from August 2014 to October 2015 Dhaka, Bangladesh. The trial was funded under the EU-FP7 (ISRCTN Number: 83599025) program. No support from any commercial entity was provided. Trial registration number is Trial reg NCT01924013 in 2013. All mothers were recruited in their first trimester (6–14 weeks of gestation followed until delivery as a follow-up of subjects. This clinical trial was a component of the EU funded GIFTS (Genomic and lifestyle predictors of feotal outcome relevant to diabetes and obesity and their relevance to prevention strategies in South Asian peoples) project (https://cordis.europa.eu/project/id/278917/reporting).

The protocol was approved by the Norwegian Medical Ethical Committee (East) (2013/845/REK South-East D) and Ethics Review Committee of the Diabetic Association of Bangladesh (BADAS-ERC/EC/13/00175). Research participation, confidentiality, and consent followed the Helsinki declaration, with local adaptation to allow both verbal and written instructions. Two major changes to the original protocol were made with ethical approval and the trial steering committee's knowledge. The original protocol specified

1. Randomization of participants defined by micronutrient deficiencies of either vitamin D or vitamin B12 into an intervention and a control group. The protocol was modified to allow the local investigator and trial co-chief investigator to recruit a second observational control group with normal levels of vitamin D and vitamin B12.
2. Vitamin D3 supplementation of 4000 IU/day for those participants with a total vitamin D level < 30 nmol/l and those with total vitamin D levels $\geq$ 30 nmol/L and < 75 nmol/L supplementation of 2000 IU/day. This was revised from a daily regime to a high dose intermittent bolus regime as described in the intervention arm below.

The trial was overseen by an Institutional Review Committee (IRC), GIFTS Trial Steering Committee (TSC) and GIFTS Data monitoring and ethics committee (DMEC). All our authors attest to the completeness and accuracy of the data and analysis and for the adherence of the trail.

All participants were recruited from five areas (Azimpur, Lalbag, Hazaribag, Kamrangirchar, and Keraniganj) in Dhaka city, Bangladesh, through the monthly household visit by Government Family Welfare Visitors (FWV). These areas included both urban and semi-urban areas. Pregnant women who were in the first trimester of their pregnancy (6–14 week gestation), aged 18–28 years, singleton pregnancy, conceiving without treatment of fertility, and willing to participate in the study were included in the study after taking the written consent. Women were excluded if they: had pre-pregnancy diabetes (previously diagnosed or by OGTT at 12–14 weeks gestation), had history of congenital malformations, had intolerance to any of the micronutrient supplements being administered in the trial, had prior history of hypercalcaemia or renal stones, serum calcium > 2.65 mmol/L, participation in

another trial/study likely to impact the results of this study, had CKD stage 4 or worse defined by eGFR (glomerular filtration rate) of 30 mL/min/1.73 m^2, had history of significant liver disease – AST > 3 × ULN (upper limit of normal) or bilirubin > 2.5xULN, had thyroid disease, active sarcoidosis, tuberculosis, or malignancy, used following medication: multivitamin supplements that includes B12, vitamin D2 or vitamin D3, thiazide diuretics, or oral corticosteroids in the past one month and had intention to only consider a home delivery.

2.2. *Randomization and masking*

Pregnant women with a nutritional deficiency were stratified into intervention and control arms by three nutrition categories (underweight, normal weight, and overweight), so that the number of women with different nutritional categories is distributed evenly. A computer-generated, simple randomization scheme was created independently by the trial statistician. Pregnant women without either vitamin D or vitamin B12 deficiency formed an observation arm of the study. Women with nutritional deficiencies, defined as vitamin D serum levels below 75 nmol/L [16] and/or vitamin B12 below 200 pg/ml [17], were randomized to a complex intervention arm with nutritional supplementation, intense lifestyle and general dietary advice given by dietitians (Intervention arm). Women in the randomized control arm received intensive lifestyle and dietary intervention tailored to address specific micronutrient deficiencies using available and affordable foods. All trial participants received iron and folate supplementation as per usual care. Because of the nature of the interventions, the intervention team could not be masked to allocation. However, the trial statistician was masked to the study groups during data analysis.

2.3. *Procedure*

Pregnant women were identified by a history of a missed menstrual cycle. A urine pregnancy test was done for confirmation of pregnancy. Women were then invited to visit MCHTI (Maternal and Child Health Training Institute), a tertiary Government hospital for antenatal care and registration. The duration of gestation was calculated from the date of the last menstrual period and ultra-sonographic examination at the time of recruitment. In case of disparity, ultrasonographic estimation was recorded. If a woman was diagnosed with gestational diabetes (GDM) their care was transferred to Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM 2, affiliated to the Diabetes Association of Bangladesh) in Dhaka. As per randomizations, below mentioned procedures were followed:

2.3.1. *Intervention arm*

As per obstetricians' standard care in Bangladesh, folate supplementation (400 mcg/day) throughout pregnancy and iron capsules from 2nd trimester (visit 2) were given as per obstetricians' standard care. In addition to supplementation, participants in the intervention arm were also advised on a prescribed dietary and lifestyle-based intervention developed in the same community. The dosage for vitamin D and vitamin B12 was similar to that used in other clinical trials for vitamin B-12 [18,19] and for vitamin D [5], although for the latter the dosage interval was longer.

The intervention group received supplementation of in the following doses throughout the perinatal period: vitamin D3 (cholecalciferol) supplementation was given as an Osteo-D oral solution with each 1 mL of solution contains Cholecalciferol BP (as vitamin D3) 5 mcg (200 IU). Supplementation was given to the following participants as:

- severe deficiency (defined by total vitamin D levels < 30 nmol/l) received an oral dose of 200,000 IU of vitamin D every 60 days (daily equivalent dose of 3,333 IU/day).

- insufficiency (defined by total vitamin D levels between 30 and 75 nmol/l) received an oral dose of 200,000 IU of vitamin D every 90 days (daily equivalent dose of 2,222 IU/day).

The oral vitamin D3 solution was routinely administered and controlled by trial personal and discontinued in the presence of hypercalcemia (serum calcium > 2.65 mmol/L) level, feotal or infant death, or new necessary medication that could alter vitamin D metabolism.

Vitamin B12 supplementation was given to those participants with a B12 level of < 200 pg/ml who received a 1x B126TM tablet every 14 days. Each film-coated tablet contains Thiamine Nitrate BP 100 mg, Pyridoxine Hydrochloride BP 200 mg, and Cyanocobalamin BP 200 mcg. Thus, the dose of B12 was equivalent to 14mcg daily. At the time of the trial, low doses of B12 are only available in Bangladesh combined with a multivitamin preparation.

2.3.2. *Control arm*

No oral supplements of vitamin D3 or B12 were provided. A trained dietician advised mothers on specific foods and amounts tailored to address specific micronutrient deficiencies in individuals and, in particular, vitamins D and B12. A practical demonstration with picture and pots were presented for the recognition of food items and the amount of food required to consume during the antenatal period—the dietician-maintained follow-up records in each visit. In addition to specific dietary advice, each participant in the control arm was also advised on a lifestyle-based intervention developed in the same community.

As per obstetricians' standard care in Bangladesh, folate supplementation (400 mcg/day) throughout pregnancy and iron capsules from 2nd trimester were given as per obstetricians' standard care.

2.3.3. *Observational arm*

Participants in this arm received 'standard' antenatal care as per guidelines used in Bangladesh. Folate supplementation (400 mcg/day) throughout pregnancy and iron capsules from 2nd trimester (visit 2) were given as per obstetricians' standard care.

2.3.4. Outcomes

Subjects were in regular contact with the project workers. Three sets of data were collected for mothers (a) visit 1 at inclusion (between 6 and 14 weeks of gestation), (b) visit 2 in the 2nd trimester between 24 and 28 weeks of gestation, and (c) visit 3 at delivery. A structural interview tool was used for the subjects. Four trained interviewers (including a physician and three dieticians) conducted interviews through an interview guide. Both at the training stage and in formulating the guides, the adequate emphasis was given on in-depth probing. Responses were recorded and noted down by interviewers. Detailed information regarding data collection was described previously [9]. In brief, subject information was collected by interview for demographic, socio-economic, history of chronic diseases, past pregnancy related history and family history of illness and pregnancy were recorded. Anthropometric measures, feotal growth and infant assessment were noted following the same procedure as described earlier. Further, biochemical, and clinical data including cord sample collection and analysis were performed similarly [9].

The primary endpoint as per original protocol was a composite based on maternal micronutrients at term by increasing total vitamin D repletion (>75 nmol/l) to 40% (compared to an estimated 10% repletion in the control arm) and decreasing vitamin B12 deficiency (<200 pg/ml) to 5% (compared to an estimated 15% in the control arm). The trial steering committee advised changing from a composite to co-primary endpoints separately for vitamin D and B12 repletion, as defined above. Secondary endpoints included feotal and maternal outcomes as listed in results (Tables 3 and 4).

Any participant who developed hypercalcaemia (corrected serum calcium > 2.65 mmol/L) during the study was withdrawn from further vitamin D supplementation but continued to be followed up for the duration of the trial. Data regarding adverse events were sent to the DMEC and reviewed by the TSC. In the event of compromised participant safety, they advised withdrawn from the study if a significant difference in the incidence of fatal/life-threatening events to mother or baby is demonstrated between groups with a p value < 0.029 (according to the Pocock stopping rule).

2.4. Statistical analysis

The Primary Endpoint was based on last maternal visit

1. Vitamin B12 deficiency (<200 pg/ml) 15% in the control group reducing to 5% in the intervention group (individually 160 in each group: alpha 0.05 and power 0.8)
2. Total vitamin D replete (>75 nmol/l) 10% increasing to 40% (individually 38 in each group alpha; 0.05 and power 0.8).

We also powered the sample size on feotal cord blood insulin, which was a secondary endpoint. Mean cord insulin in the pilot study was 8.8 μIU/ml (95% CI 7.9, 9.9) [9]. The standard deviation for cord insulin was 5.7 μIU/ml, and we estimate a mean difference of 1.5μIU/ml based on differences between South Asian and Western Caucasian cord insulin of 2 μIU/ml. A sample size of 372 is required in each group (372 in the intervention and 372 in the control groups) to give 90% power to detect a difference with 0.01.

To ensure baseline characteristics relevant to outcome measures are compared between groups. Groups was stratified into three subgroups based on BMI: <18.5 kg/m^2, 18.5–22.9 kg/m^2 and ≥ 23 kg/m^2 [9]. Previous experience from cross-sectional data in the same population [9] showed that 30% of women would be undernourished, 20% overnourished, and the remaining 50% will be normally nourished, hence the need to stratify for BMI.

Continuous data were presented as means and 95% CI (confidence interval), while categorical data as frequencies (n) and percentages (%). The ANOVA (Analysis of variance) was used to compare differences of means between different maternal and neonatal status, and pairwise comparisons between the groups were performed and corrected for multiple testing using Tukey's HSD. Chi-square test was used to test for the association between categorical variables. Both simple and multiple linear regression analyses were done for the prediction of maternal and neonatal birth outcomes. All analyses were done using IBM SPSS 24 and Stata SE 14.

3. Results

Each study participant had three visits during the study period. Visit 1 in the first trimester of their pregnancy between 6 and 14 weeks, visit 2 in second trimester pregnancy between 24 and 28 weeks, and visit three at delivery. A total of 915 women recruited after providing written consent (Fig. 1). After exclusion criteria 748 pregnant women remained and were randomized into intervention (n = 384) and control arms (n = 364). A further 113 women remained as the observational arm who had no nutritional deficiencies of either 25hydroxyvitamin D or vitamin B12. Of intervention arm (n = 384) cord samples were collected from 274 (71%) deliveries, from control arm (n = 364) cord samples were collected from 267 (73%) deliveries and the observational arm cord samples were collected from 78 (69%) deliveries. A total of 861 women were followed until deliveries, and cord samples were collected from 619 (72%) deliveries.

3.1. Baseline characteristics

Maternal characteristics at inclusion by three arms of the study are listed in table 1. As expected, the 25hydroxyvitamin D and vitamin B12 levels were significantly higher in the observational arm at inclusion than in the other two study arms. There were no further significant differences in maternal characteristics between the three study arms at inclusion.

3.2. Primary endpoint

The primary endpoint for differences between the randomized arms of the study for either 25hydroxyvitamin D or vitamin B12 replenishment as pre-specified were not met for maternal levels of either micronutrient at the final visit at delivery.

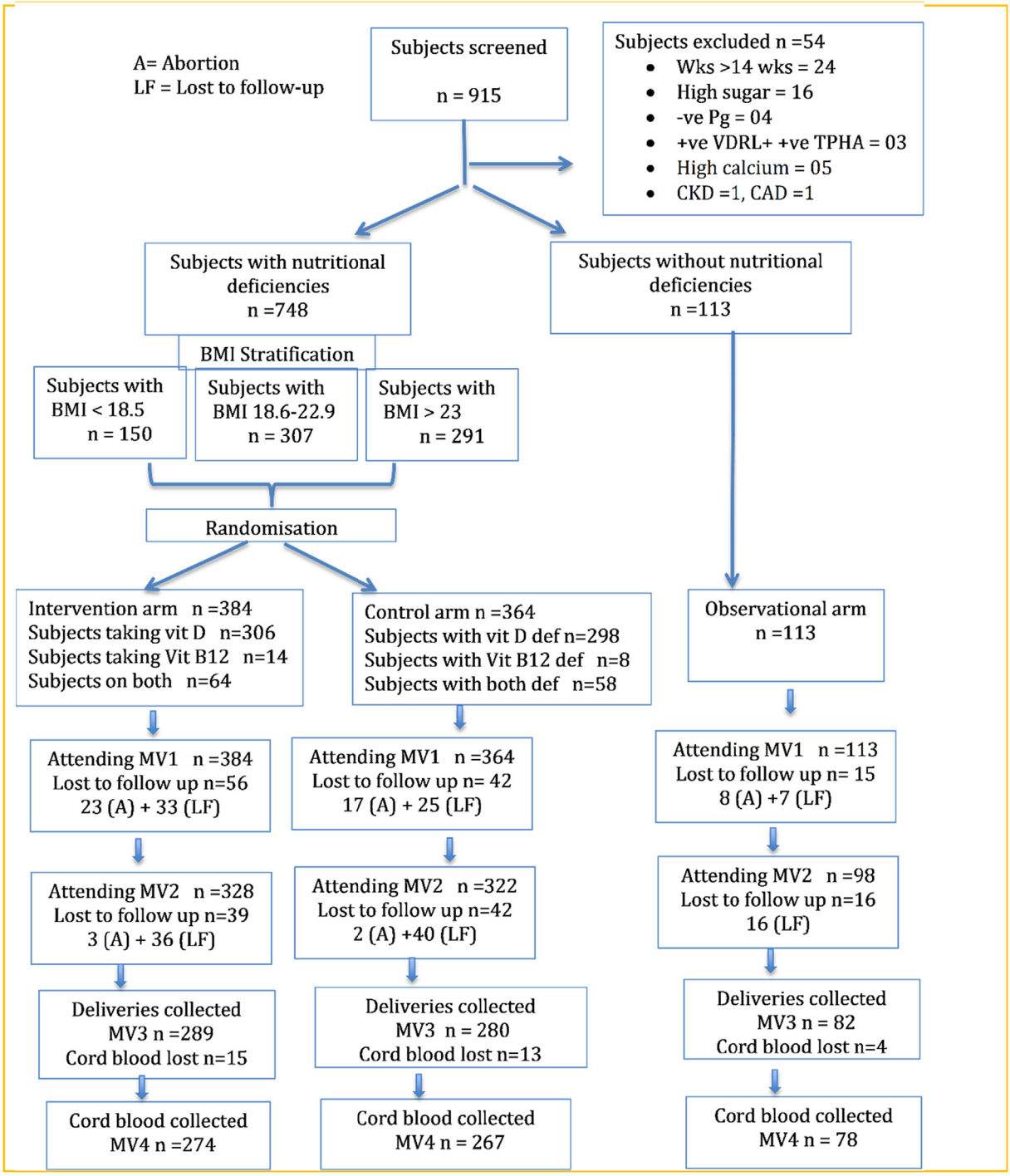

Fig. 1 – Screening, randomization, and follow-up. Pg = pregnancy. VDRL = venereal disease research laboratory. TPHA = treponema pallidum hemagglutination. CKD = chronic kidney disease. CAD = coronary artery disease. BMI = body mass index. MV1 = maternal visit 1 (at inclusion; 6–14 weeks). MV2 = maternal visit 2 (24–28 weeks). MV3 = maternal visit 3 (at delivery). MV4 = collection of cord blood at delivery.

3.3. *25Hydroxyvitamin D*

25hydroxyvitamin D levels at booking (MV1) were low in both the intervention (42.8 nmol/l) and control arms (43.7 nmol/l) of the study (Table 1 and Fig. 2A). At 24–28 weeks (visit 2) these levels significantly rose in both the intervention arm (62.3 nmol/l; $p < 0.001$) and in the control arm (65.4 nmol/l; $p < 0.001$). There was no statistical difference between the intervention and control arms. At delivery (visit 3), the levels of 25hydroxyvitamin D levels fell to (intervention arm 54.4 nmol/l and control arm 54.2 nmol/l. Nevertheless, levels of 25hydroxyvitamin D in the 3rd visit was significantly higher than the baseline (Visit 1), both for the intervention and control arm $p < 0.001$. At visit three, vitamin D levels were

Table 1 – Baseline characteristics of the participants by three arms of randomization.

	Intervention	Control arm	Observational arm	P value
Number	384	364	113	
Age (years)	22.41 (22.01–22.81)	22.63 (22.18–2 3.09)	22.49 (21.75–23.22)	0.776
Gestation weeks	9.85 (9.54–10.17)	9.79 (9.49–10.9)	9.28 (8.8–9.75)	0.632
Years of education	7.25 (6.84–7.65)	7.06 (6.65–7.48)	7.42 (6.71–8.12)	0.670
Monthly income (BDT)	14,247 (13260–15235)	14,761 (13742–15780)	14,487 (12664–16310)	0.776
Leisure time physical activity (hrs/wk)	4.3 (4.0–4.5)	4.3 (4.0–4.5)	4.3 (3.6–4.6)	0.813
Height (cm)	1.51 (1.50–1.52)	1.50 (1.50–1.51)	1.51 (1.50–1.52)	0.173
Weight (kg)	80.0 (78.9–81.1)	80.1 (79.9–82.0)	80.1 (78.5–82.4)	0.986
BMI (kg/m2)	22.0 (21.6– 22.4)	22.30 (21.0–22.7)	22.0 (21.3–22.7)	0.609
Waist (cm)	80.1 (78.9–81.1)	80.9 (79.9–82.1)	80.5 (78.5–82.5)	0.484
Hip in cm	91.2 (90.4–92.1)	91.6 (90.8–92.4)	91.5 (90.0–92.9)	0.831
Waist Hip Ratio	0.88 (0.87–0.88)	0.88 (0.87–0.89)	0.88 (0.87–0.89)	0.480
Systolic BP	100.4 (99.2–101.5)	100.2 (99.0–101.3)	98.9 (97.0–100.9)	0.491
Diastolic BP	67.9 (67.0–68.7)	67.4 (66.6–68.2)	68.3 (66.8–69.8)	0.531
Fasting Plasma Glucose (mmol/L)	4.93 (4.87–5.00)	4.88 (4.82–4.94)	4.93 (4.82–5.05)	0.505
2 h Plasma Glucose (mmol/L)	6.72 (6.60–6.88)	6.76 (6.61–6.91)	6.90 (6.66–7.17)	0.476
Vitamin D (nmol/l)	42.8 (40.6–45.1)	43.7 (41.6–46.2)	115.9 (105.9–125.9)	<0.001
Vitamin B12 (pg/ml)	333.4 (318.2–348.6)	320.5 (306.0–335.1)	360.9 (337.5–384.3)	0.033
Folate (ng/ml)	8.2 (7.7–8.8)	8.0 (7.6–8.5)	8.2 (7.4–9.1)	0.769
Homocystin (μmol/l)	7.02 (6.75–7.28)	7.07 (6.77–7.37)	6.85 (6.43–7.27)	0.751
RBC folate (ng/ml)	192.6 (184.2–201.0)	194.5 (185.7–203.3)	211.9 (195.3–228.4)	0.098
Calcium (mg/dl)	8.43 (8.33–8.52)	8.43 (8.33–8.54)	8.50 (8.34–8.74)	0.549
Cholesterol (mmol/l)	4.09 (4.00–4.17)	3.99 (3.97–4.13)	4.12 (3.97–4.27)	0.621
Triglyceride (mmol/l)	1.33 (1.28–1.39)	1.32 (1.27–1.37)	1.37 (1.27–1.47)	0.600
HDL-C (mmol/l)	1.17 (1.15–1.19)	1.16 (1.14–1.18)	1.14 (1.10–1.17)	0.292
LDL-C (mmol/l)	2.31 (2.23–2.39)	2.28 (2.21–2.35)	2.34 (2.19–2.48)	0.753

Data are presented as mean (95% CI). Biochemical assays were all measured in serum. P value for multiple group comparisons. BMI = body mass index, BP = blood pressure, CI = confidence interval, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol.

similar in all arms (intervention, control, and observation arm). In the observational (Arm 3) the 25hydroxyvitamin D levels were high at baseline on booking 115.9 nmol/l but fell significantly over the next visits, which was 80.7 nmol/l (2nd visit) and 54.9 nmol/l (3rd visit) $p < 0.001$ (Fig. 2A). Nonetheless, while at baseline (visit 1) 24.7% and 24.5% of participants the interventional and control arms respectively had severe 25-hydroxyvitamin D deficiency < 30 nmol/l; (Fig. 2B) at the 2nd visit these values had gone down to 3.0% and 3.4% and this was maintained at 3rd visit (delivery) with a value of 2.4% and 3.9%. No individuals in the observational arm at any visit had severe 25hydroxyvitamin D deficiency.

3.4. *Vitamin B12*

The study found 20.3% vitamin B12 deficiency at baseline (booking visit MV1) with no significant differences between intervention and control arms (Table 1). At 24–28 weeks (MV2), deficiency in vitamin B-12 significantly decreased in both intervention ($p < 0.001$) and control arms ($p < 0.001$). But no differences between the intervention and control arms was observed. At delivery, (MV3) vitamin B12 reverted to levels similar to the baseline ($p = 0.600$). In the observational arm with no deficiency at baseline, levels of deficiency progressively increased such that at delivery (MV3), there was no difference between the intervention, control, and observational arms (Fig. 2C).

3.5. *Secondary outcomes*

Table 2 showed the maternal micronutrient levels at different maternal visit intervention arm, control arm (with deficiency), and observational arm (without deficiency). The proportion of women with ferritin deficiency was low in all study arms (10–14%) at baseline (visit 1), which reduced further to 1–4% by delivery (visit 3). Folate deficiency was uncommon through pregnancy, starting from 2.7 to 5.2% at booking to 0–1.8% at delivery. Similarly, few women had high homocysteine levels throughout pregnancy 0–3%. However, levels increased from booking to subsequent visits (low levels 19–21% at booking rising to 44–45% at delivery).

Table 3 showed mothers with the lowest BMI category (BMI < 18.5 kg/m^2) had the highest weight gain in all BMI categories than their initial weight at inclusion. However, overweight (≥23 kg/m^2) mothers remained heavier at delivery even though they had the lowest change in body weight during the pregnancy period. None of the pregnant mothers irrespective of their BMI at inclusion attained the ideal weight gain set by the WHO.

There were only a few significant differences in maternal or feotal outcomes irrespective of the arm of the study (Table 4). In the observation arm, a lower feotal glucose was observed ($p = 0.04$), and the feotal red cell folate was lowest in the intervention arm ($p = 0.03$). There were differences in

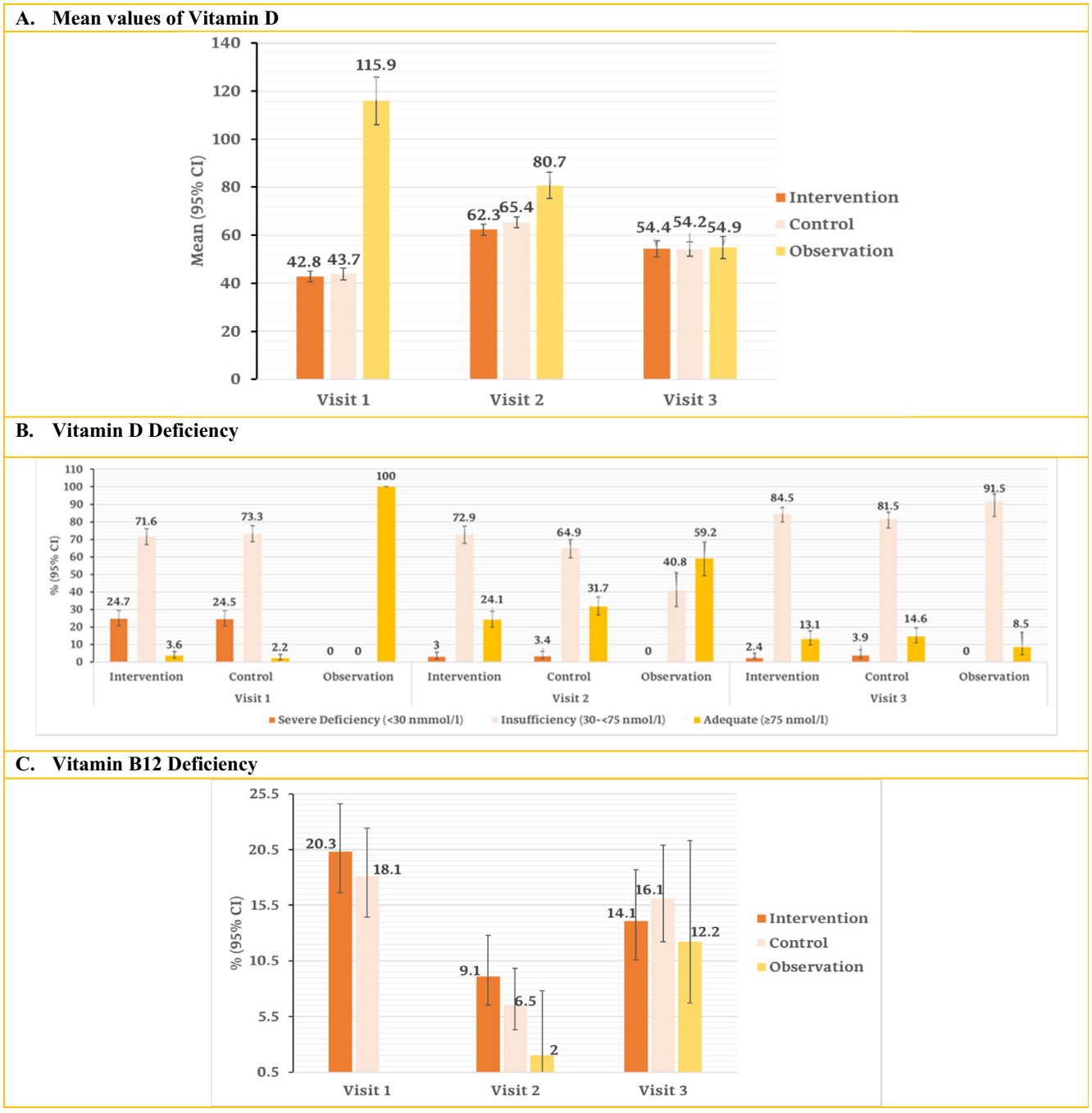

Fig. 2 – Levels of vitamin D and vitamin B12 at visit 1 (at inclusion – 6–14 weeks), visit 2 (24–28 weeks) and at visit 3 (at delivery) by three arms of randomization. Fig. 1A shows the mean vitamin D with 95% confidence interval at Visit 1 (at inclusion – 6–14 weeks), Visit 2 (24–28 weeks) and at Visit 3 (at delivery) by three arms of randomization. Fig. 1B shows the prevalence of vitamin D deficiency including severe (<30 nmol/l), insufficiency (30–<75 nmol/l) and adequate (≥75 nmmol/l) with 95% confidence interval at Visit 1, Visit 2 and at Visit 3 (at delivery) by three arms of randomization. Fig. 1C shows the prevalence of vitamin B12 deficiency (<200 pg/ml) with 95% confidence interval at Visit 1, Visit 2 and at Visit 3 (at delivery) by three arms of randomization.

feotal 25 hydroxyvitamin D levels between arm the highest in the control arm and lowest in the observational arm.

The outcome of the intervention on mean birth weight in different categories of maternal BMI is seen in Fig. 3. The left panel shows the overall mean birth weight in the three study arms, regardless of maternal BMI. No significant difference in mean birth weight was observed at any given maternal BMI for the intervention. However, mothers with higher BMI appear to give birth to heavier infants.

Adverse events of vitamin D and B12 supplementation are detailed in Table 5. No significant differences in the frequencies of adverse events were found between the study arms.

Table 2 – Maternal micronutrient levels at visit 1 (at inclusion – 6–14 weeks), visit 2 (24–28 weeks) and at visit 3 (at delivery) by three arms of randomization.

Variables	Visit 1 (at inclusion: 6–14 weeks)			Visit 2 (24–28 weeks)			Visit 3 (at delivery)		
	Intervention (n = 384)	Control (n = 364)	Obs (n = 113)	Intervention (n = 328)	Control (n = 322)	Obs (n = 98)	Intervention (n = 289)	Control (n = 280)	Obs (n = 82)
Ferritin (ng/ml)									
• Low (<20)	54 (14.1)	48 (13.2)	11 (9.7)	67 (17.4)	54 (14.8)	9 (8.0)	3 (0.8) *	13 (3.6) *	1 (0.9) *
• Normal (≥20)	328 (85.4)	316 (86.8)	102 (90.3)	221 (57.6)	226 (62.1)	73 (64.6)	270 (70.3) **	254 (69.8) **	75 (66.4) **
Folate (ng/ml)									
• Low (<3)	15 (3.9)	19 (5.2)	3 (2.7)	3 (0.8)	6 (1.6)	1 (0.9)	–	–	2 (1.8)
• Normal (3–<17)	340 (88.5)	325 (89.3)	103 (91.2)	234 (60.9)	226 (62.1)	67 (59.3)	173 (45.1) **	187 (51.4) **	51 (45.1) **
• High (≥17)	29 (7.6)	20 (5.5)	7 (6.2)	52 (13.5)	48 (13.2)	14 (12.4)	100 (26.0) ***	80 (22.0) ***	24 (21.2) ***
Homocysteine (μmol/l)									
• Low (<5)	74 (19.3)	69 (19.0)	24 (21.2)	127 (33.1)	125 (34.3)	37 (32.7)	168 (43.8) *	165 (45.3) *	51 (45.1) *
• Normal (5–<15)	301 (78.4)	284 (78.0)	88 (77.9)	161 (41.9)	153 (42.0)	47 (41.6)	107 (27.9) **	102 (28.0) **	26 (23.0) **
• High (≥15)	8 (2.1)	11 (3.0)	–	2 (0.5)	2 (0.5)	–	–	1 (0.3)	–
RBC Folate (ng/ml)									
• Low (<140)	95 (24.7)	90 (24.7)	17 (15.0)	7 (1.8)	2 (0.5)	2 (1.8)	50 (13.0) *	40 (11.0) *	11 (9.7)
• Normal (≥140)	287 (74.7)	273 (75.0)	96 (85.0)	320 (83.3)	320 (88.5)	95 (84.1)	224 (58.3) **	227 (62.4) **	66 (58.4) **

Data are presented as number (%). Intervention arm (with deficiency) and control arm (with deficiency either for vitamin D or B-12) Observational arm (without deficiency at inclusion).
† are isolated cases with vitamin B-12 deficiency.
*Differences in proportion of women with low levels of micronutrient at delivery compared to the proportion at inclusion ($P < 0.05$).
**Differences in proportion of women with normal levels of micronutrient at delivery compared to the proportion at inclusion ($P < 0.05$).
***Differences in proportion of women with high levels of micronutrient at delivery compared to the proportion at inclusion ($P < 0.05$).

Table 3 – Mean weight change of the pregnant mothers from inclusion to 2nd trimester and at delivery in different BMI categories.

BMI	Visit 1			Visit 2			Visit 3			Total change	Ideal wt gain
	Intervention	Control	Obs	Intervention	Control	Obs	Intervention	Control	Obs		
<18.5	39.7 ± 3.7	39.1 ± 4.0	39.7 ± 4.0	45.1 ± 4.4	46.0 ± 4.8	46.9 ± 5.2	47.7 ± 5.3	50.4 ± 5.6	51.5 ± 6.0	9.8 ± 4.0	13 to 18
18.5 . 22.9	47.0 ± 4.6	47.2 ± 4.8	47.0 ± 4.6	52.2 ± 5.6	52.5 ± 5.1	52.5 ± 5.8	55.3 ± 5.7	56.1 ± 5.7	55.4 ± 6.0	8.4 ± 3.8	11 to 16
≥23	60.4 ± 7.0	59.2 ± 7.6	58.9 ± 5.5	63.6 ± 7.4	63.2 ± 8.4	63.4 ± 6.2	67.4 ± 8.1	66.4 ± 8.7	66.3 ± 6.9	6.8 ± 3.9	5 to 9

Data are presented as mean (±2 SD). BMI = body mass index, SD = standard deviation, Obs = observation.

Hypercalcemia was seen in 3.1% in the intervention arm, 0.06% in the control arm, and 1.8% in the observational arm.

4. Discussion

We observed a very high prevalence of vitamin D deficiency in our population with a significant number of women with severe vitamin D deficiency (<30 mmol/l). Numerous studies have shown a high prevalence of vitamin D deficiency across tropical countries with an abundance of sunlight exposure and dietary sources of vitamin D (fatty fish and vegetables). In Bangladesh, people are exposed to sunlight throughout the year, with an average of about 10 h [20]. In urban Indian men, >1h of casual midday sunlight exposure daily was required to maintain serum 25(OH)D concentrations above 50 nmol/L, and > 2 h of occasional sunlight exposure was needed to maintain 25(OH)D concentrations above 75 nmol/L [21]. Therefore, the higher prevalence rate of vitamin D deficiency deserves closer scrutiny, including its diagnostic threshold in different populations. However, we cannot exclude that 'other' factors in some ethnic groups may influence vitamin D levels. Indeed, we have previously shown that betel nut chewing inhibits 25hydroxyvitamin D-24-hydroxylase reduces the active vitamin D3 [22]. Nevertheless, this was limited by it being a small study involving only 41 subjects from Bangladesh residing in East London, UK, and may therefore represent a type 1 error.

We failed to demonstrate, per protocol, that we could replenish 25hydroxyvitamin D levels in pregnancy to normal levels throughout pregnancy using a high dose of oral vitamin D3 given as an intermittent bolus in this Bangladeshi population. Interestingly, 25hydroxyvitamin D did rise similarly in both the intervention and control arms during pregnancy with the highest levels at 24–28 weeks, but significantly higher levels than booking were seen at delivery. Thus, specific nutritional advice of micronutrient deficiency in the control arm was as efficacious as the oral vitamin D3 supplementation regime that we employed in the intervention arm. And indeed, the highest levels of 25hydroxyvitamin D were found in the feotal samples at birth from this arm of the study compared to both the intervention and observational arms. These results were analyzed without those with isolated deficiency of Vitamin D (n = 14) of 370 in the intervention arm. The results did not change with or without the inclusion of these (n = 14).

Among the strengths of our study was that in both the intervention arm vitamin (vitamin D3 bolus replacement) and the control arm (specific nutritional advice) severe 25hydroxyvitamin D deficiency was almost completely corrected in the second and third trimesters. Further, the trial included rather a large number of pregnant women (n = 861) and most importantly, the trial completed 75% of the deliveries (n = 641). A weakness of our study that may have been that we chose to use an intermittent bolus approach to vitamin D supplementation rather than weekly or daily administration However, it was thought that this approach in countries such as Bangladesh would be more advantageous for higher compliance, improved recording and to increase concordance in further studies.

Table 4 – Maternal and foetal outcomes by three arms of randomization.

Variables	Intervention (n = 284)	Control arm (n = 276)	Observation (n = 81)	P values
Maternal outcomes				
GDM (2nd trimester)[1]	60 (21.1)	68 (24.6)	13 (16.0)	0.23
Anaemia at delivery[1]	8 (2.8)	12 (4.4)	3 (3.7)	0.62
Pregnancy induced HTN[1]	17 (6.0)	18 (6.5)	5 (6.17)	0.18
Caesarean Section 1	169 (59.5)	166 (60.1)	53 (65.4)	0.64
Fasting Glucose at delivery (mmol/l)[2]	6.61 (6.60–6.62)	6.58 (6.57–6.59)	6.43 (6.42–6.44)	0.66
Foetal Outcomes				
Gestational week at delivery[2]	38.9 (38.7–39.2)	38.9 (38.7–39.1)	38.8 (38.4–39.2)	0.86
Preterm delivery (<37 Weeks) [1]	22 (7.8)	17 (6.2)	2 (2.5)	0.25
Neonatal death[1]	4.0 (1.4)	3.0 (1.1)	1.0 (1.2)	0.94
LBW[1]	52 (17.9)	41 (14.6)	16 (19.5)	0.44
SGA[1]	29 (10.2)	20 (7.3)	8 (9.9)	0.45
Birth weight (kg) [2]	2.79 (2.74 –2.84)	2.82 (2.77–2.87)	2.78 (2.68–2.87)	0.59
Length at birth (cm) [2]	49.6 (49.3–49.9)	49.5 (49.2–49.8)	49.4 (48.8–50.1)	0.90
Weight by height[2]	11.4 (11.2–11.6)	11.5 (11.3–11.7)	11.4 (11.0–11.7)	0.62
MUA[2]	10.1 (9.9–10.3)	10.1 (9.9–10.2)	10.2 (9.5–10.8)	0.90
Apgar score[2]	7.94 (7.88–8.01)	7.96 (7.91–8.01)	7.94 (7.85–8.03)	0.89
Ponderal index[2]	2.31 (2.26–2.36)	2.33 (2.29–2.38)	2.31 (2.22–2.40)	0.78
Micro-Nutrients				
Vitamin D (nmol/l) [2]	50.7 (47.5–54.0)	57.0 (53.1–60.9)	44.5 (39.9–48.9)	0.01
Ferritin (ng/ml) [2]	166.3 (146.1–186.6)	142.1 (125.7–158.6)	156.8 (128.7–184.9)	0.18
Vitamin B-12 (pg/ml) [2]	329.6 (314.3–344.9)	351.9 (334.8–369.1)	355.7 (321.9–389.5)	0.11
Calcium (mg/dl) [2]	8.15 (8.09–8.20)	8.17 (8.11–8.23)	8.24 (8.13–8.35)	0.36
RBC Folate (ng/ml) [2]	245.5 (230.7–260.3)	272.5 (256.3–288.7)	277.3 (245.2–309.5)	0.03
Leptin (ng/ml)[2]	6.2 (5.7–6.7)	8.7 (4.1–13.4)	5.3 (4.4–6.1)	0.40
Homocysteine (μmol/l) [2]	4.7 (4.5–4.9)	4.9 (4.7–5.1)	4.8 (4.3–5.3)	0.57
Clinical				
Cord Blood Glucose (mmol/l) [2]	4.6 (4.4–4.8)	4.6 (4.4–4.7)	4.2 (3.9–4.4)	0.04
Cord Insulin (μIU/ml) [2]	7.6 (7.2–8.1)	8.2 (7.6–8.9)	7.9 (7.0–8.8)	0.35
Cholesterol (mmol/l) [2]	1.40 (1.35–1.46)	1.47 (1.41–1.53)	1.56 (1.29–1.85)	0.10
Triglyceride (mmol/l) [2]	0.49 (0.47–0.52)	0.49 (047–0.52)	0.48 (0.43–0.52)	0.78
HDL-C (mmol/l) [2]	0.77 (0.73–0.81)	0.78 (0.74–0.85)	0.70 (0.63–0.76)	0.13
LDL-C (mmol/l) [2]	0.53 (0.49–0.57)	0.60 (0.55–0.65)	0.64 (0.52–0.75)	0.04
Folate (ng/ml) [2]	15.7 (15.1–16.3)	15.2 (14.6–15.8)	14.8 (13.6–16.1)	0.32

Data are presented as [1]number (%) or [2]mean (95% CI) where appropriate. Biochemical assays were all measured in serum. P value for multiple group comparisons. CI = confidence interval, GDM = gestational diabetes mellitus, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, MUAC = mid upper arm circumference, SGA = small for gestational age.

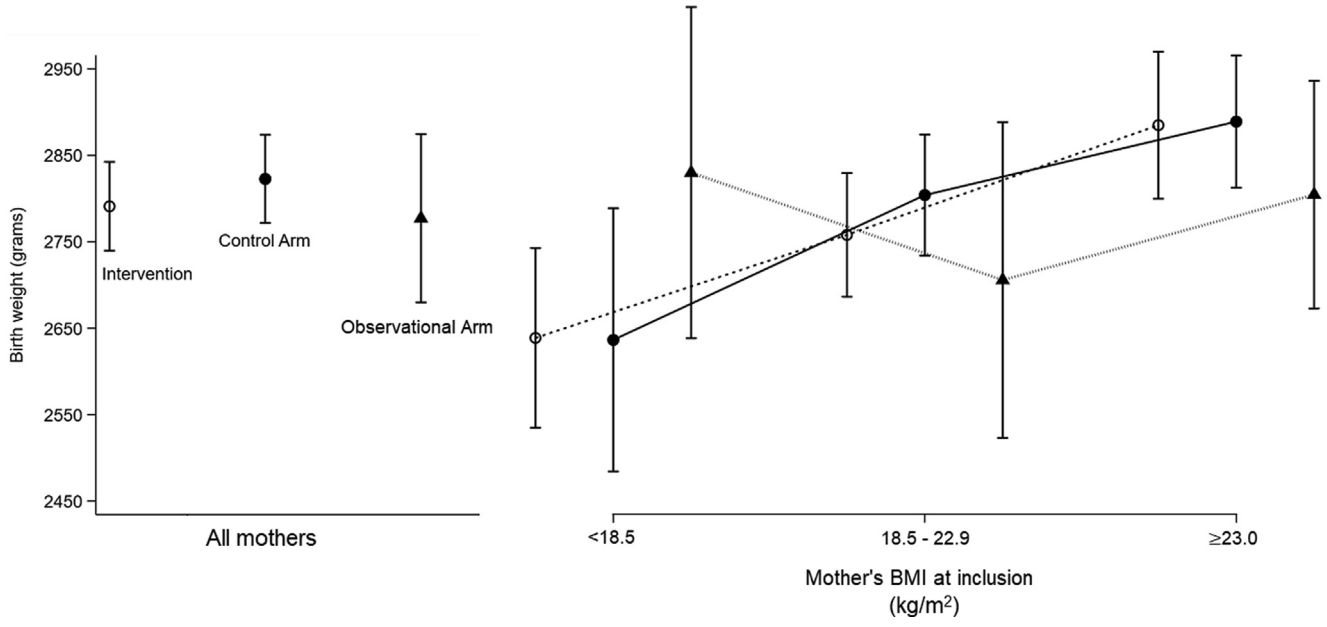

Fig. 3 – The effect of the intervention on birth weight in different categories of the maternal body mass index (BMI) at inclusion.

Table 5 – Serious Adverse Events (SAE) and Adverse Events (AE) during the trail related to vit D3 and B12 supplementations.

Classification	Description	Total events or reaction	Intervention and control arms (n = 748)				Observational arm (n = 113) normal antenatal care (n = 113)	Types of care	Status
			On Vit-D (n = 306) *	On Vit B complex (n = 14) *	On Vit-D + Vit B complex (n = 64) *	On lifestyle advice only (n = 364) **			
SAE	Neonatal death	8	4	0	0	3	1	Hospitalization	Cord sample collected
SAE	Miscarriage	53	21	0	5	19	8	Hospitalization	Withdrawn
SAE	P/V bleeding	11	4	0	0	5	2	Hospitalization	Improve
AE	Itching	14	6	1	1	6	0	OPD	Improve
AE	abdominal pain	14	7	1	0	5	1	OPD	Improve
AE	UTI	8	2	0	1	3	2	OPD	Improve
AE	Fever	7	1	0	1	3	2	OPD	Improve
AE	Headache	5	1	0	3	1	0	OPD	Improve
AE	Vomiting	12	1	0	3	1	7	OPD	Improve
AE	Diarrhoea	14	5	0	2	6	1	OPD	Improve
AE	Leaking water	2	1	0	0	1	0	OPD	Improve
AE	Joint pain	7	2	0	2	3	0	OPD	Improve
AE	tiredness	10	1	0	2	7	0	OPD	Improve
AR	Hypercalcemia	16	9	1	2	2	2	Normal care	Stop medication
AE	*Unrelated accidental maternal death	1	1	0	0	0	0	Death	Fell 3 floors in a lift that malfunctioned

Abbreviation: P/V bleeding, per vaginal bleeding; UTI, urinary tract infection; OPD, outpatient door.

* = randomized intervention arm.

** = randomized control arm.

No difference was seen in secondary outcomes between the intervention and control arms. Nonetheless, since there was no difference in 25hydroxyvitamin D levels throughout the study, perhaps this observation was not surprising. It might be considered a weakness of our study that the we gave tailored dietary advice to our control group rather than 'usual care'. However, given the high levels of vitamin D deficiency in pregnancy in our population and especially of those with levels below < 30 mmol/l (severe deficiency) we considered it unethical not to give specific dietary advice to this group; indeed this became a strength of our study since the control group attained similar levels to the intervention group. We did however, include an observational group representing those without vitamin D and B12 deficiency. In earlier meta-analyses of observational studies [23] and mostly small trials [6,24] some effect was seen on secondary endpoints that we measured. But in contrast in trials more adequately powered in areas characterized by vitamin D deficiency and feotal growth restriction [25,26] no effects were seen of vitamin D supplementation including in Bangladesh [5].

The fall of 25hydroxyvitamin D levels in the observational group from the booking (selected with normal levels of 25hydroxyvitamin D) throughout pregnancy was unexpected. However, it may be partially accounted for by haemodilution seen in pregnancy. A further strength of our study was that in both the intervention arm vitamin (vitamin D3 bolus replacement) and the control arm (specific nutritional advice) severe 25hydroxyvitamin D deficiency was almost completely corrected in the second and third trimesters.

Amongst weakness was that we chose to use an intermittent bolus approach to vitamin D supplementation rather than weekly or daily administration. In assessing 25hydroxyvitamin D deficiency it might be considered a weakness of our study that we did not use reference values adjusted for pregnancy; however, these normal ranges are not established in Bangladesh and are not in common use internationally. Normal values in pregnancy have been published that would indicate a progressive fall of over 67% between booking and delivery but that only partially explains our findings

We also failed, per protocol, to correct vitamin B12 deficiency in either the intervention or control groups. As with 25hydroxyvitamin D3, we did not account for varying ranges in pregnancy for B12 levels.

Evidence suggests that poor maternal nutritional status contributes to the occurrence of LBW [27]. Intervention with supplementing mothers with multiple micronutrients during pregnancy was conducted to evaluate the effect on birth outcomes [28,29]. Meta-analyses suggest that supplementation with micronutrients during pregnancy reduces the occurrence of LBW [30]. An observational study in Pune, India, showed a positive association between birth weight and the frequency of consumption of milk by the mothers in early gestation, and of green leafy vegetables and fruit in late pregnancy, with the latter associations being stronger in lighter and thinner women [31]. However, this finding was not replicated in a study in Mumbai Maternal Nutritional Project, India, where a micronutrient-rich supplement did not increase standard ultrasound measures of feotal size and growth at any stage of pregnancy [32,33].

The data has a greater implication worldwide for pregnancy care in the light of controversies related to universal vitamin D supplementation during pregnancy. Further, the data can be used for future studies.

In conclusion, although we failed to meet our primary endpoints per protocol, we have shown that specific nutritional advice can successfully correct severe 25hydroxyvitamin D deficiency in pregnancy that has important clinical implications for the population studied. Additional work is needed to interpret the vitamin D and B12 requirements of pregnant women, including its requirement in different pregnancy stages and the optimal timing of supplementation. This outline should consider different lifestyles, body types, and baseline vitamin D status and the diagnostic threshold for vitamin D3 requiring therapeutic action. Our results, using high dose intermittent bolus vitamin D3, supports the WHO position, not to recommend routine vitamin D supplementation based on current normal ranges during pregnancy and highlights the issue of severe vitamin D deficiency < 30 nmol/l).

Author Contributions

AK, GAH and AH planned the study, designed the protocol, and edited the manuscript. BB involved in designing the protocol, carried out the field work, performed the statistical analysis, data management and writing the manuscript. TS and LQN carried out the field work, collected the data and helped in writing the manuscript. IAM performed the statistical analysis and data management. ZH performed laboratory measurements. SJS, IJ, and NC helped in writing the manuscript. All authors have read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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