Prenatal vitamin D₃ supplementation suppresses LL-37 peptide expression in ex vivo activated neonatal macrophages but not their killing capacity

Rubhana Raqib¹*, Anna Ly², Evana Akhtar¹, Akhirunnessa Mily¹, Nandita Perumal², Abdullah Al-Mahmud¹, Rokeya Sultana Rekha¹,³, Abdullah Hel Baqui⁴ and Daniel E. Roth²

¹Nutritional Biochemistry Laboratory, Centre for Vaccine Sciences, International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Mobakalli, 1212 Dhaka, Bangladesh
²Department of Paediatrics, Hospital for Sick Children and University of Toronto, 555 University Avenue, Toronto, ON, Canada M5G 1X8
³Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm, Sweden
⁴Department of International Health, International Center for Maternal and Newborn Health, The Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205, USA

(Submitted 3 December 2013 – Final revision received 5 May 2014 – Accepted 22 May 2014 – First published online 4 August 2014)

Abstract
Vitamin D has regulatory effects on innate immunity. In the present study, we aimed to assess the effect of prenatal vitamin D₃ (vitD₃) supplementation on neonatal innate immunity in a randomised, placebo-controlled trial by evaluating cathelicidin (LL-37) expression and the killing capacity of macrophages. Healthy pregnant women (n 129) attending a clinic in Dhaka were randomised to receive either a weekly oral dose of 0·875 mg vitD₃ or placebo starting from 26 weeks of gestation up to delivery. Serum, plasma and monocyte-derived macrophages (MDM) were obtained from the cord blood. 25-Hydroxyvitamin D (25(OH)D) concentration was measured in serum. MDM were stimulated with or without Toll-like-receptor 4 ligand (TLR4L). Innate immune function was assessed by measuring LL-37 peptide levels in the culture supernatant of MDM by ELISA, LL-37 transcript levels by quantitative PCR, and ex vivo bactericidal capacity of MDM. vitD₃ supplementation did not increase LL-37 peptide levels in plasma or in the extracellular fluid of macrophages with or without TLR4L induction. However, stimulated intracellular LL-37 expression (ratio of stimulated:unstimulated MDM) was significantly reduced in the vitamin D group v. placebo (P=0·02). Multivariate-adjusted analyses showed that intracellular LL-37 peptide concentration from stimulated MDM was inversely associated with 25(OH)D concentration in serum (P=0·03). TLR4L stimulation increased the bactericidal capacity of MDM compared with the unstimulated ones (P=0·01); however, there was no difference in killing capacity between the two groups. A weekly dose of 0·875 mg vitD₃ to healthy pregnant women suppressed the intracellular LL-37 peptide stores of activated macrophages, but did not significantly affect the ex vivo bactericidal capacity of cord blood MDM.

Key words: Vitamin D₃; Antimicrobial peptides; Macrophages; Innate immunity; Neonates

Globally, vitamin D deficiency is common during pregnancy. However, epidemiological studies determining the associations between vitamin D status and adverse pregnancy outcomes such as pre-eclampsia, gestational diabetes, low birth weight, preterm labour and infectious diseases have shown conflicting results¹. Therefore, the benefits of routine vitamin D supplementation for improving pregnancy outcomes remain to be established².

Vitamin D plays an important role in defence against infectious diseases by modulating both innate and adaptive immune responses. All major immune cells, including T cells, B cells, neutrophils and antigen-presenting cells (monocytes, macrophages and dendritic cells), express the vitamin D receptor. Macrophages, the front-line responders to microbial infection, sense pathogen-associated molecular patterns by utilising pattern recognition receptors such as Toll-like receptors (TLR). TLR triggering induces Cytochrome p450 27B1 (CYP27B1), a vitamin D-activating enzyme that converts 25-hydroxyvitamin D (25(OH)D) into the active form (1,25-dihydroxyvitamin D; 1,25(OH)₂D), and vitamin D receptor expression³. The 1,25(OH)₂D–vitamin D receptor complex directly induces cathelicidin antimicrobial peptide (CAMP; gene encoding

Abbreviations: 1,25(OH)₂D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; AViDD, Antenatal Vitamin D in Dhaka; CBMC, cord blood mononuclear cell; CFU, colony-forming unit; ICDDB, International Centre for Diarrheal Disease Research, Bangladesh; ICF, intracellular fluid; LPS, lipopolysaccharide; MDM, monocyte-derived macrophages; TLR, Toll-like receptor; TLR4L, Toll-like receptor 4 ligand; vitD₃, vitamin D₃.

* Corresponding author: Dr R Raqib, fax +880 2881 2529, email rubhana@icddrb.org
LL-37) transcription and corresponding increases in the expression of the antimicrobial peptide, LL-37.\(^3\)

Antimicrobial peptides function as front-line host defence effector molecules, acting as both endogenous antibiotics and paracrine immunomodulators\(^3\). Human cathelicidin (the cleaved active component of which is LL-37) is synthesised by several immune cell types (neutrophils, macrophages, B cells, T cells, natural killer cells and mast cells) as well as by barrier/mucosal epithelial cells\(^6\). In response to some infections (diarrhoeal diseases and gonorrhoea), LL-37 has been found to be down-regulated\(^9\)–\(^12\), while in some other diseases, it is up-regulated (psoriasis)\(^13\). The localisation of LL-37 to skin\(^14\), vernix caseosa\(^15\) and breast milk\(^16\) suggests a potential role for the antimicrobial peptide in response to infection in young infants. Antimicrobial peptides, including LL-37, exhibited bactericidal activity against organisms responsible for bacterial infections in newborns\(^17\). They are up-regulated in tracheal secretions during neonatal lower respiratory tract infections\(^18\), suggesting that clinical or nutritional interventions to enhance the production of antimicrobial peptides may reduce the risk of neonatal infections.

Little is known regarding the effect of vitamin D supplementation during pregnancy on the synthesis of cathelicidin/LL-37 or other aspects of neonatal immune defences.

Therefore, in the present study, we aimed to evaluate the effect of prenatal vitamin D\(_3\) (vitD\(_3\)) supplementation on neonatal innate immune function. In a previous double-blind, placebo-controlled trial of vitD\(_3\) supplementation (0.875 mg/dose) during the third trimester of pregnancy on the synthesis of cathelicidin/LL-37 or other aspects of neonatal innate immune defences.

In the present trial cohort, we studied the effect of this substantial rise in vitamin D status on the expression of cord blood LL-37 peptide and mRNA transcript levels in stimulated macrophages and the killing capacity of neonatal macrophages \textit{ex vivo}.

### Methods

#### Study design and participants

The Antenatal Vitamin D in Dhaka (AViDD) study was a randomised, double-blind, placebo-controlled trial of vitamin D supplementation of pregnant women in the third trimester conducted in Dhaka, Bangladesh. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by the Ethics Committees of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and Johns Hopkins Bloomberg School of Public Health, Hospital for Sick Children (protocol no. PR-09058). Written informed consent was obtained from all eligible participants. Details of the trial design and primary biochemical outcomes have been published previously\(^19\). Briefly, 160 pregnant women were enrolled at the Shimantik Urban Primary Health Care Project Maternity Center, a non-governmental facility that provides basic antenatal and obstetric services in a low-income community. Pregnant women were included if they were aged between 18 and \(<35\) years, at a gestational age of 26–29 weeks, currently residing in Dhaka with plans to stay there throughout pregnancy and for at least 1 month after delivery, and had plans to deliver at the maternity centre. Study participants were allocated to receive a weekly dose of either 0.875 mg vitD\(_3\) (VitD group: cholecalciferol, Vigantol oil; Merck KGaA) or placebo oil (placebo group: Miglyol oil; Merck) until delivery. Of the 160 women enrolled in the AViDD trial, there were 129 mother–infant pairs (81%), sixty-four from the placebo group and sixty-five from the VitD group, with adequate biochemical outcomes have been published previously\(^19\).

#### Assessment of vitamin D status

Details of serum 25(OH)D measurements have been described elsewhere\(^19\). To assess vitamin D status, serum 25(OH)D was measured by HPLC–tandem MS; only 25(OH)D\(_2\) was detected in serum, not 25(OH)D\(_3\). As no standard classification of 25(OH)D concentrations in cord blood exists, for the purpose of the present study, we followed the classification of...
vitamin D sufficiency or deficiency based on previous publications\(^{20-23}\). All participants were grouped into four categories of 25(OH)D concentrations: (1) \(\geq 76\) nmol/l (high); (2) 50–75 nmol/l (moderate); (3) 30–49 nmol/l (low); (4) < 30 nmol/l (very low).

**Measurement of LL-37 peptide concentration**

LL-37 peptide concentration was measured by ELISA (Hyduc Biotechnology), according to the manufacturer’s recommendations. The lower limit of detection of the kit was 0·14 ng/ml, and the intra- and inter-assay CV were 4·96 and 7·89%, respectively. The concentration of LL-37 peptide measured in the supernatant was then normalised to per million macrophages.

**Quantitative real-time RT-PCR amplification of LL-37 mRNA**

RNA was extracted from macrophages using the RNeasy Mini Kit (Qiagen GmbH), according to the manufacturer’s instructions, and corresponding complementary DNA was synthesised using the SuperScript III First-Strand Synthesis System (Invitrogen). The \(\beta\)-CAMP gene encoding LL-37 transcripts relative to the housekeeping 18S rRNA was measured in triplicate from the complementary DNA samples by quantitative real-time RT-PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) and the 18S rRNA-housekeeping gene kit (Applied Biosystems). The sequences of forward and reverse primers for LL-37 transcripts were 5’-TCACCAGGATTGTGACTTCA-3’ and 5’-TGA GGGTCACGTCCCATAC-3’, respectively (Primer Express; Applied Biosystems). Results were analysed using the relative standard method\(^{91}\).

**Assay of monocyte-derived macrophage-mediated killing capacity**

The capacity of MDM to kill ingested bacteria was determined by quantifying the number of viable \(E.\) \(\text{coli}\) K-12 colonies (avirulent) in agar plates after a period of phagocytosis and removal of extracellular bacteria. MDM were infected with \(E.\) \(\text{coli}\) K-12 at a multiplicity of infection of 100 (100 bacteria per one macrophage) and incubated for 2h. Extracellular bacteria were removed by washing, and infected macrophages were cultured in media with 10% autologous plasma overnight. The infected macrophages were lysed with 0·3% saponin to release intracellular bacteria. Cell lysates were cultured on MacConkey agar plates (Becton Dickinson) overnight and viability of the bacteria was determined by counting the colony-forming units (CFU). A ‘relative CFU count’ was calculated for each participant as the ratio of CFU in LPS-stimulated to unstimulated cells, to account for inter-subject variations in the baseline (unstimulated) killing activity of macrophages.

**Statistical analyses**

Primary outcomes were fold difference in intracellular LL-37 peptide concentration (indicator of the effect of prenatal \(\text{vitD}_3\) supplementation on LPS-induced LL-37 synthesis in cord blood cells) and the relative CFU count (an indicator of the effect of \(\text{vitD}_3\) supplementation on LPS-induced neonatal macrophage killing activity). Secondary outcomes were unstimulated and LPS-stimulated LL-37 concentrations in the intracellular and extracellular fluid, LL-37 mRNA transcript copy numbers in MDM, and LL-37 concentration in plasma.

The distributions of LL-37 and bacterial CFU counts are expressed as ranges, means and standard deviations and 95% CI, or medians and interquartile ranges for non-normally distributed variables. The fold difference in the intracellular concentration of LL-37 peptide was calculated for each participant as the ratio of LL-37 concentration in LPS-stimulated to unstimulated cells. Unstimulated and LPS-stimulated intracellular LL-37 peptide concentrations and fold difference in intracellular LL-37 peptide concentration did not follow normal distributions even after transformation; therefore, they were analysed by non-parametric statistics (Mann–Whitney U test). Bootstrap linear regression was used to verify inferences from non-parametric tests. Factors that have been previously reported in the literature to be associated with cord blood LL-37 concentration including cord serum 25(OH)D concentration, gestational age (categorised by preterm \(\leq 37\) weeks of gestation or term > 37 weeks of gestation), infant sex and delivery mode were assessed for associations with LL-37 concentration using the bootstrap procedure for unadjusted bivariate and adjusted multivariate regression analyses. Cord plasma LL-37 was log-transformed as data were not normally distributed, and between-group comparisons were analysed by Student’s \(t\) test. For the aforementioned LL-37 outcomes, sensitivity analyses that excluded LL-37 observations below the minimum detectable limit of the ELISA assay (<0·14 ng/ml) were also conducted. Participants were categorised into four groups based on 25(OH)D concentrations, as described previously; comparisons between groups with respect to the LL-37 peptide and transcript levels were evaluated by one-way ANOVA.

To calculate the relative CFU count, one CFU was added to all values due to multiple observations with bacterial colony counts of zero. Relative CFU counts did not follow a normal distribution and were therefore log-transformed before the analysis. The number of bacterial colonies that remained after treatment with or without LPS was considered as count data; however, because the Poisson distribution was a poor fit for the over-dispersed count data, comparisons were based on negative binomial regression models, using generalised estimating equations to account for the correlation between colony counts of LPS-stimulated and unstimulated cells within the subjects. The primary parameter of interest in the negative binomial generalised estimating equation model was the interaction between vitamin D supplementation and LPS stimulation on CBMC-derived macrophage killing activity. Statistical analyses were performed using Stata/IC 12.1 for Mac (StataCorp), and \(P \leq 0·05\) was considered significant.

**Results**

Of the 160 women enrolled in the AViDD trial, there were 129 mother–infant pairs (81%), sixty-four from the placebo group...
Antenatal vitamin D₃ and neonatal innate immunity

and sixty-five from the VitD group, with adequate volume of cord blood for immune function assays. The mean age (22·4 (SD 3·5) v. 22·4 (SD 3·5) years) and gestational age (27·73 (SD 1·1) v. 28·0 (SD 1·1) weeks) of the pregnant women in the VitD group were similar to those in the placebo group.

Vitamin D and LL-37 levels in cord blood

25(OH)D concentration ranged from 20 to 192 nmol/l in the study cohort, which was similar to the entire cohort as reported previously. Maternal 25(OH)D concentration in the VitD group (mean 101·52 (SD 30·67) nmol/l) was significantly increased at delivery compared with the placebo group (mean 40·30 (SD 17·83) nmol/l) (P<0·001) (Table S1, available online). The concentration of LL-37 in cord plasma ranged from 16·6 to 101·7 ng/ml. There was no significant difference in plasma LL-37 concentrations between the two groups based on serum 25(OH)D concentrations, LL-37 peptide expression in Toll-like receptor 4 ligand-stimulated macrophages (Table 1). Cord serum 25(OH)D was not significantly associated with LL-37 concentration in cord plasma (Table 2).

Vitamin D₃ supplementation suppressed intracellular LL-37 peptide expression in Toll-like receptor 4 ligand-stimulated macrophages

Concentrations of LL-37 ranged from 0·05 to 4·93 ng/ml in the ICF of unstimulated macrophages. No significant differences were observed between the VitD and placebo groups (Table 1). The bootstrap linear regression analysis did not show an effect of vitD₃ supplementation on unstimulated intracellular LL-37 concentration (mean between-group difference 0·12 ng/ml, 95 % CI −0·16, 0·39; P=0·41).

In TLR4L-stimulated macrophages, LL-37 concentration in the ICF ranged from 0·07 to 7·72 ng/ml. The distribution of TLR4L-induced intracellular LL-37 concentrations was slightly lower in the VitD group than in the placebo group, although the difference did not reach statistical significance as determined by the non-parametric Mann–Whitney U test (P=0·09; Table 1). Similarly, the results of the bootstrap linear regression analysis revealed that the mean decrease in TLR4L-induced intracellular LL-37 concentrations in the VitD v. placebo group did not reach statistical significance (mean 0·31 ng/ml, bootstrap 95 % CI −0·64, 0·023; P=0·068).

Overall, individual fold differences in intracellular LL-37 concentration ranged from 0·12 to 3·64. Fold differences in the VitD group were lower, on average, than those in the placebo group, and the difference between the distributions was significant as determined by the non-parametric Mann–Whitney U test (Table 1). However, when group means were compared using bootstrap regression, the effect of vitD₃ supplementation on the fold difference in intracellular LL-37 concentration did not reach statistical significance (P=0·15). Moreover, there was no significant interaction between vitD₃ supplementation and TLR4L stimulation in terms of their effects on mean intracellular LL-37 concentration (data not shown).

Cord serum 25(OH)D was not significantly associated with LL-37 concentration in the unstimulated ICF, or fold difference in intracellular LL-37 concentration (Table 2). However, 25(OH)D in cord serum was inversely related to TLR4L-induced intracellular LL-37 in both unadjusted bivariate and adjusted multivariate analyses (Table 2). For every 10 nmol/l increase in cord serum 25(OH)D concentration, TLR4L-induced intracellular LL-37 concentration decreased by 0·05 ng/ml (P=0·03). No significant associations were observed between LL-37 concentration and preterm birth, delivery mode or infant sex (Table 2). Excluding low LL-37 values in the sensitivity analysis did not change the inferences from any of the above-mentioned analyses (data not shown). Upon stratifying the participants into four groups based on serum 25(OH)D concentrations, LL-37 peptide expression was significantly lower in the VitD group than in the placebo group.

### Table 1. Antimicrobial peptide LL-37 concentration in umbilical cord plasma and cord blood mononuclear cell culture, overall and by treatment group (Medians and interquartile ranges (IQR); geometric means and 95 % confidence intervals)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Overall* (n 129)</th>
<th>Vitamin D (n 63)</th>
<th>Placebo (n 66)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median IQR</td>
<td>Median IQR</td>
<td>Median IQR</td>
<td></td>
</tr>
<tr>
<td>Cord blood plasma‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geometric mean</td>
<td>41·2 (39·8, 43·7)</td>
<td>41·1 (37·7, 44·8)</td>
<td>41·4 (38·1, 44·8)</td>
<td>0·91</td>
</tr>
<tr>
<td>95 % CI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>0·5 (0·4–0·9)</td>
<td>0·5 (0·4–0·9)</td>
<td>0·6 (0·4–0·9)</td>
<td>0·43</td>
</tr>
<tr>
<td>Stimulated cells</td>
<td>0·5 (0·4–0·9)</td>
<td>0·5 (0·4–0·9)</td>
<td>0·6 (0·4–1·1)</td>
<td>0·09</td>
</tr>
<tr>
<td>Fold difference§</td>
<td>1·02 (0·8–1·3)</td>
<td>0·96 (0·8–1·2)</td>
<td>1·1 (0·9–1·4)</td>
<td>0·02</td>
</tr>
<tr>
<td>Extracellular fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>1·92 (1·4–3·4)</td>
<td>1·9 (1·46–3·5)</td>
<td>1·84 (1·4–3·1)</td>
<td>0·52</td>
</tr>
<tr>
<td>Stimulated cells</td>
<td>1·96 (1·4–3·5)</td>
<td>2·2 (1·35–3·6)</td>
<td>1·90 (1·40–3·6)</td>
<td>0·73</td>
</tr>
<tr>
<td>mRNA transcripts‖</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>124·86 (63·7–232·9)</td>
<td>141·3 (85·5–217·8)</td>
<td>99·8 (55·6–249·9)</td>
<td>0·54</td>
</tr>
<tr>
<td>Stimulated cells</td>
<td>171 (104·5–321·6)</td>
<td>176·4 (108·6–369·9)</td>
<td>171·279 (102·9–318·1)</td>
<td>0·91</td>
</tr>
</tbody>
</table>

* LL-37 peptide concentrations are expressed as ng/ml and mRNA as copy numbers.
† P value was based on the non-parametric Mann–Whitney U test, unless otherwise indicated.
‡ Vitamin D group n 64 and placebo group n 65.
§ Fold difference was calculated as the ratio of LL-37 concentration in stimulated to unstimulated cells.
‖ Vitamin D group n 42 and placebo group n 37.
Effect of prenatal vitamin D₃ supplementation on LL-37 mRNA transcription

There was no significant effect of vitamin D supplementation on LL-37 transcript copy number in unstimulated or TLR4L-stimulated CBMC (Table 1), nor was there an interaction between vitamin D supplementation and TLR4L stimulation (data not shown). TLR4L stimulation increased LL-37 transcript levels in both groups. Stratifying the participants based on serum 25(OH)D concentrations did not show any significant differences among the four groups (data not shown).

Effect of prenatal vitamin D₃ supplementation on bacterial killing capacity of activated macrophages

The macrophage killing assay was performed on 112 cord blood cell cultures (fifty-six from the placebo group and fifty-six from the VitD group) since adequate cells were not available from the other seventeen participants. Overall, the range of viable bacterial counts after the MDM-mediated E. coli killing was 0 to 2.50 × 10⁴ CFU for unstimulated cells and 0 to 2.25 × 10⁵ CFU for TLR4L-stimulated cells, and the relative CFU counts ranged from 0 to 1.25 × 10³. The regression analysis did not show any significant effect of vitD₃ supplementation on the relative CFU count, or on the relative CFU counts in unstimulated and TLR4L-stimulated macrophages (Table 3). However, TLR4L stimulation augmented bacterial killing in both groups. Stratifying the participants based on serum 25(OH)D concentrations did not show any difference among the four groups (data not shown).

Discussion

Despite the potent effect of vitD₃ supplementation (0.875 mg/week) during the third trimester of pregnancy on maternal–infant vitamin D status(39), the present study showed that prenatal vitD₃ supplementation did not yield significant benefits in terms of indicators of innate immune function. In fact, we observed that vitD₃ supplementation may have slightly suppressed intracellular LL-37 peptide synthesis in activated cord blood macrophages, suggesting a potential immunosuppressive effect. Furthermore, we did not find any significant influence of vitD₃ supplementation on the bactericidal capacity of macrophages with or without TLR4L activation.

In vitro studies using primary monocytes/macrophages cultured in 25(OH)D-sufficient sera or culture media containing the adequate form of vitamin D (1,25(OH)₂D₃) have shown that vitamin D mediates the induction of cathelicidin (LL-37) and β-defensin 4 transcripts in macrophages(5,24,25). In vitro addition of 1,25(OH)₂D₃ or 25(OH)D₃ in cord blood cultures also showed increased TLR-independent or -dependent cathelicidin mRNA expression(35). In the present study, in vivo vitD₃ concentrations in the ICF of TLR4L-stimulated MDM were significantly reduced in the high (>76 nmol/l), moderate (50–75 nmol/l) and low (30–49 nmol/l) groups (Fig. 1(b)).

Table 2. Unadjusted and adjusted multivariate associations between LL-37 concentration (in cord plasma and intracellular fluid) and cord serum 25-hydroxyvitamin D (25(OH)D), gestational age, infant sex and delivery mode

(β-Coefficients and 95% confidence intervals)

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted models</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>Bootstrap 95% CI</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Cord plasma LL-37 (ng/ml)</td>
<td>−0.204</td>
<td>−0.773, 0.365</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Cord serum 25(OH)D*</td>
<td>−2.308</td>
<td>−5.533, 4.916</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Infant sex†</td>
<td>3.290</td>
<td>−1.569, 8.146</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Delivery mode</td>
<td>−3.925</td>
<td>−9.249, 1.399</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Unstimulated intracellular LL-37 (ng/ml)</td>
<td>−0.016</td>
<td>−0.058, 0.025</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Cord serum 25(OH)D*</td>
<td>−0.245</td>
<td>−0.796, 0.306</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Infant sex†</td>
<td>0.049</td>
<td>−0.229, 0.327</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Delivery mode</td>
<td>−0.134</td>
<td>−0.444, 0.177</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Stimulated intracellular LL-37 (ng/ml)</td>
<td>−0.049</td>
<td>−0.090, −0.008</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Cord serum 25(OH)D*</td>
<td>−0.240</td>
<td>−0.772, 0.293</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Infant sex†</td>
<td>0.122</td>
<td>−0.202, 0.446</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Delivery mode</td>
<td>−0.264</td>
<td>−0.371, 0.188</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Intraacellular fold difference in LL-37§</td>
<td>−0.015</td>
<td>−0.046, 0.015</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Cord serum 25(OH)D*</td>
<td>−0.050</td>
<td>−0.260, 0.160</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Infant sex†</td>
<td>−0.028</td>
<td>−0.224, 0.168</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Delivery mode</td>
<td>−0.006</td>
<td>−0.211, 0.198</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

* Cord serum 25(OH)D was scaled by 10 nmol/l.
† Gestational age was categorised as preterm (<37 weeks of gestation) or term (>37 weeks of gestation).
‡ Infant sex is female v. male.
§ Fold difference was calculated as the ratio of stimulated:unstimulated cells.
supplementation did not affect LL-37 mRNA levels in neonatal macrophages with or without activation. The main difference between the other reports and the present study is that here vitD₃ was given orally to pregnant women and autologous plasma was used instead of adding 1,25(OH)₂D₃ or 25(OH)D₃ exogenously in the macrophage culture. However, it is noteworthy that TLR4L activation was associated with increased LL-37 transcript levels in macrophages cultured in the presence of serum 25(OH)D of the moderate group (50–75 nmol/l) compared with those with poorer vitamin D status (30–49 or <30 nmol/l). This finding was substantiated by Walker et al. (23) who reported that TLR4L-stimulated cord blood monocytes showed higher LL-37 mRNA in the presence of 25(OH)D₃ compared with 25(OH)D₃ <30 nmol/l. They further showed that 25(OH)D concentration <50 nmol/l was linked with diminished TLR4L-mediated induction of CYP27B1 enzyme, the key enzyme responsible for the conversion of 25(OH)D to the active form in macrophages, and diminished TLR4L-mediated induction of cathelicidin. However, they also showed reduced cathelicidin mRNA levels in the presence of 25(OH)D >75 nmol/l compared with 25(OH)D <30 nmol/l. This further showed that 25(OH)D concentration >50 nmol/l was linked with diminished TLR4L-mediated induction of CYP27B1 enzyme, the key enzyme responsible for the conversion of 25(OH)D to the active form in macrophages, and diminished TLR4L-mediated induction of cathelicidin. However, they also showed reduced cathelicidin mRNA levels in the presence of 25(OH)D >75 nmol/l compared with 25(OH)D >50 nmol/l (23). Similarly, in the present study, LL-37 transcript levels declined in the high (≥76 nmol/l) group compared with the moderate (50–75 nmol/l) group. These findings suggest that 25(OH)D concentration >75 nmol/l suppresses the induction of LL-37 transcript levels.

Although most in vitro studies (5,23,25–29) have shown an increase in cathelicidin mRNA expression after exposure to vitD₃, none of these studies measured the cathelicidin/LL-37 peptide concentration in the intracellular or extracellular compartment of macrophages. Liu et al. (53) demonstrated the presence of cathelicidin peptide in macrophages by flow cytometry, immunofluorescence staining and the surface-enhanced laser desorption ionisation time-of-flight method, but LL-37 peptide concentration in macrophages was not measured. Only one study in Canada reported the in vivo effects of vitD₃ supplementation (30) that corroborated our findings, showing that 8 months of supplementation with vitD₃ significantly decreased serum LL-37 levels compared with pre-supplementation levels in healthy participants. They did not find any significant difference in TLR2/1L-induced LL-37 peptide concentration (assessed by Western blot and densitometry) in MDM between pre- and post-vitD₃ supplementation. The decrease in peptide concentration in the ICF of macrophages following TLR4L activation in the VitD group may have been due to a post-transcriptional regulatory block. It is important to acknowledge that this effect was quantitatively small, not consistent across multiple statistical approaches, and not mirrored by

### Table 3. Effect of prenatal vitamin D supplementation v. placebo on the number of viable Escherichia coli colony-forming units (CFU) after culture with cord blood macrophages, with and without Toll-like receptor 4 ligand stimulation

(Medians and interquartile ranges (IQR); ratios of CFU counts and 95 % confidence intervals)

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Overall (n 112)</th>
<th>Vitamin D (n 56)</th>
<th>Placebo (n 56)</th>
<th>Ratio of CFU counts*</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated cells</td>
<td>1775</td>
<td>378–4385</td>
<td>1685</td>
<td>445–1113</td>
<td>1858</td>
<td>282–4385</td>
</tr>
<tr>
<td>LPS-stimulated cells</td>
<td>505</td>
<td>80–2083</td>
<td>440</td>
<td>43–1825</td>
<td>1008</td>
<td>128–2508</td>
</tr>
<tr>
<td>Relative CFU count†</td>
<td>0·55</td>
<td>0·2–1·03</td>
<td>0·47</td>
<td>0·13–1·1</td>
<td>0·68</td>
<td>0·36–1·03</td>
</tr>
</tbody>
</table>

LPS, lipopolysaccharide.

* Based on the negative binomial regression model for comparison between the vitamin D and placebo groups.

† Relative CFU count for each participant was calculated as the ratio of CFU in LPS-stimulated to unstimulated cells.

‡ Based on the t test for comparison between the vitamin D and placebo groups, using log-transformed data.
The dose and duration of vitD₃ supplementation as well as genetic polymorphisms may modify treatment responses.

There are several studies reporting the effects of vitamin D supplementation on infectious diseases, particularly in lung diseases in children. However, there is little conclusive evidence from controlled trials about the effect of vitamin D supplementation on the risk of infectious diseases in early infancy.

In conclusion, our findings showed that prenatal vitD₃ supplementation with a weekly dose of 0.875 mg in the last trimester may have slightly reduced intracellular LL-37 peptide concentration, but did not adversely affect the antibacterial activity of macrophages in neonates. We did not find mechanistic evidence of a promising beneficial effect of prenatal vitD₃ supplementation on newborn innate immunity. However, vitamin D has complex immune-regulatory roles, indicating the need for further short- and long-term studies to evaluate the effects of prenatal vitamin D exposures on infant and child immune responses.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114514001512

Acknowledgements

The authors express their gratitude to the pregnant women who participated in the present study and to the staff of Shimantik (non-governmental organisation) for their efforts in the implementation of the AViDD trial. The present study was supported by the Thrasher Research Fund (Salt Lake City, UT), the Swedish Agency for Research Cooperation with Developing Countries (Sida/SAREC Agreement support) and the ICDDR,B. The ICDDR,B acknowledges with gratitude the commitment of all donors to its research efforts.

The contributions of the authors are as follows: R. R. and D. E. R. conceived and designed the study plan; R. R., D. E. R. and A. H. B. obtained funding; A. A.-M. supervised the field activity and collected the data; E. A., A. M. and R. S. R. performed the laboratory experiments; E. A., A. L., N. P. and R. S. R. performed the statistical analysis; R. R. and D. E. R. drafted the manuscript. All the authors revised and approved the final manuscript.

There is no conflict of interest.

References

Antenatal vitamin D₃ and neonatal innate immunity

915


