**VIA MEDICA**

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# Vitamin D deficiency in pregnancy may affect fetal thymus development

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## ABSTRACT

**Objectives:** The aim of our study was to evaluate the association of vitamin D deficiency (VDD) during pregnancy with thymus size in full-term fetuses.

**Material and methods:** In this prospective study, we evaluated mid-pregnancy serum 25-hydroxyvitamin $D_3$ (25(OH)$D_3$) concentrations. The fetal thymus size was measured by ultrasound in the third trimester. Neonatal 25(OH)$D_3$ levels were evaluated by umbilical cord blood sampling. Correlation of maternal and neonatal vitamin D levels and association between thymus size and both, maternal and neonatal vitamin D concentrations were investigated.

**Results:** Serum 25(OH) $D_3$ concentrations were within the normal range in 48 (29.8%) mothers and 10 (13.1%) newborns. A strong correlation between mid-pregnancy maternal and neonatal 25(OH)$D_3$ concentration (r = 0.8, p < 0.001) was found. A significant linear correlation was observed between both, maternal and neonatal 25(OH)$D_3$ concentrations and thymus perimeter length (r = 0.45, p = 0.04 and r = 0.43, p < 0.01, respectively). Both, maternal and fetal VDDs were associated with decreased thymus perimeter (p = 0.04, p = 0.03).

**Conclusions:** Vitamin D deficiency during pregnancy may be associated with smaller fetal thymus. Our data suggest that VDD in pregnancy may lead to systemic inflammatory response in the fetus.

**Key words:** pregnancy, vitamin D deficiency, fetal thymus size

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## INTRODUCTION

Recent studies have shown that, beyond its role in bone metabolism, calcium and phosphorus homeostasis, vitamin D exerts profound effects in the immune system [1, 2]. Studies in both, animal models and in humans have demonstrated that vitamin D deficiency (VDD) contributes to the exacerbation of both, infectious and autoimmune diseases through dysfunctional regulatory T cells [3, 4]. The function of regulatory T cells in inflammatory diseases of the neonatal and early childhood period (e.g. allergy to cow's milk, atopic eczema, asthma, and wheezing) is closely related to VDD during pregnancy [5, 6]. Umbilical cord T-cell receptors, which are direct indicators of T-Lymphocyte synthesis and, consequently, thymic output, are negatively correlated with vitamin D levels [7]. As a result,

a hypothesis has been advanced that the season in which a baby was born may influence autoimmune and atopic disorder risk in later life. Regardless, the underlying mechanism of the association between vitamin D and the development of the fetal immune system remains unclear.

The thymus is the primary site of T cell differentiation in early life. This maturation process is specific to the fetal period, but antigenic and environmental changes during that time may affect the programming of the immune system [8]. Moreover, the thymus has been shown to actively respond to systemic fetal inflammation during the intrauterine period [9, 10]. This non-specific anti-inflammatory response may be triggered by endogenous corticosteroids, systemic inflammatory mediators, and deficiency in

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the intake of proteins, minerals or vitamins [11–13]. Fetal thymus size may be a good marker of fetal systemic inflammatory response [14, 15]. Recent studies have revealed fetal thymus size to be positively correlated with the umbilical cord C-reactive protein (CRP) levels in cases with preterm premature membrane rupture (PPROM) and a strong predictor of neonatal sepsis. Additionally, it is suggested that thymus size is smaller in fetuses of preeclamptic mothers as compared to healthy controls, independently of coexisting SGA status and antenatal steroid use [16–18].

In our study, we evaluated the correlation between both, mid-pregnancy maternal and neonatal 25-hydroxy-vitamin $D_3$ (25(OH)$D_3$) concentration and fetal thymus size. We hypothesized that VDD during pregnancy might alter the immune system by affecting thymic development. Furthermore, restricted fetal thymus size is an accepted indicator of the systemic inflammatory response and VDD during pregnancy may cause fetal systemic inflammatory response.

## MATERIAL AND METHODS
### Study design

The study design was reviewed and approved by the Local Ethics Committee. All study subjects provided written consent according to the procedures recommended by the 1975 Declaration of Helsinki Declaration (revised in Tokyo 2004). All study data were collected from September 2013 to March 2014. During the study period, there were 142 vaginal and 204 cesarean section deliveries in our clinic. After obtaining informed consent, healthy volunteers between 24 and 28 weeks of pregnancy attending routine antenatal examination were recruited for the study. Exclusion criteria were: intrauterine growth retardation, fetal anomaly, multi-fetal pregnancy, maternal hypertension, chronic liver or renal disease, gastrointestinal disease, rheumatic disease, current tobacco use, or any ongoing chronic medical therapies. The daily dose of 400 IU vitamin D supplements was recommended to all pregnant women.

We hypothesized that if vitamin D deficiency during pregnancy leads to a low-level chronic inflammatory response at fetal thymus unlike pre-eclampsia or chorioamnionitis, the evaluation of a relationship between vitamin D level in mid-pregnancy and fetal thymus size in late pregnancy may be a more appropriate approach. Therefore, maternal blood samples for the measurement of 25(OH)$D_3$ level were collected between 20–28 weeks of gestation and fetal thymus measurement was performed at 37–40 weeks of gestation. The study population included 204 pregnant women. Forty-two pregnant women were excluded in accordance to the exclusion criteria in the first step. A total of 162 pregnant women who met the inclusion criteria were enrolled in the study. Pregnant women were followed up until delivery. Ul-

trasound measurement of fetal thymus size was performed during 37–40 weeks of gestation. The ultrasound image quality was judged to be poor in 44 patients who, together with 20 patients who were diagnosed with pre-eclampsia, gestational diabetes, PPROM or preterm labor, as well as 20 pregnant women who were missing at monitoring, were excluded from the study in the second step. Finally, cord blood samples were taken from 76 neonates at delivery. Figure 1 represents a flow chart of the study design.

### Measurement of fetal thymus

A high resolution ultrasound device (Siemens; Acuson Anteres, Mountain View, CA 94043, USA) was used to perform all ultrasound measurements by two experienced radiologists with extensive qualifications in obstetric ultrasonography.

The fetal thymus was imaged in transverse at the point of the upper mediastinum adjacent to the three large cardiac vessels. The homogeneous structure within the anterior mediastinum was identified as the thymus (Figure 2A). Maximum transverse thymic diameter was defined as the diameter at the point perpendicular to the line connecting the spine and sternum and the complete perimeter was measured in the same plane (Figure 2B). Poor image quality was considered an exclusion criterion. An image was identified as poor quality under the following conditions: inability to identify the sternum and the spine, presence of multiple ribs on either side of the thorax, indicating obliquity, inability to identify the transverse thymic border [18].

A pair of observers, blinded to patient data as well as the assessment of the other observer, examined 40 out of 150 patients enrolled in the study to assess inter-observer reliability. Ultrasonic thymic perimeter and thymus/thorax diameter intra-observer reproducibility were 1.5–2.0% and 1.8–3.2%, respectively.

### Sample collection

Serum 25(OH)$D_3$ is a marker of both, dietary vitamin D intake and cutaneous synthesis of vitamin D. The normal range of serum 25(OH)$D_3$ in pregnant women has not been determined. Based on previous studies in non-pregnant women, VDD was defined as 25(OH)$D_3 \leq 20$ ng/mL (50 nmol/L). Mild vitamin D deficiency was defined as serum 25(OH) $D_3$ concentration of 10–20 ng/mL (25–50 nmol/L), whereas severe vitamin D deficiency was defined as serum 25(OH)$D_3$ concentration of $\leq 10$ ng/mL ($\leq 25$ nmol/L).

Maternal blood samples were collected within 20–28 weeks of gestation and neonatal blood was collected from the umbilical cord after delivery and clamping. Serum was separated from whole blood and stored at –30ºC until analyzed. Isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure
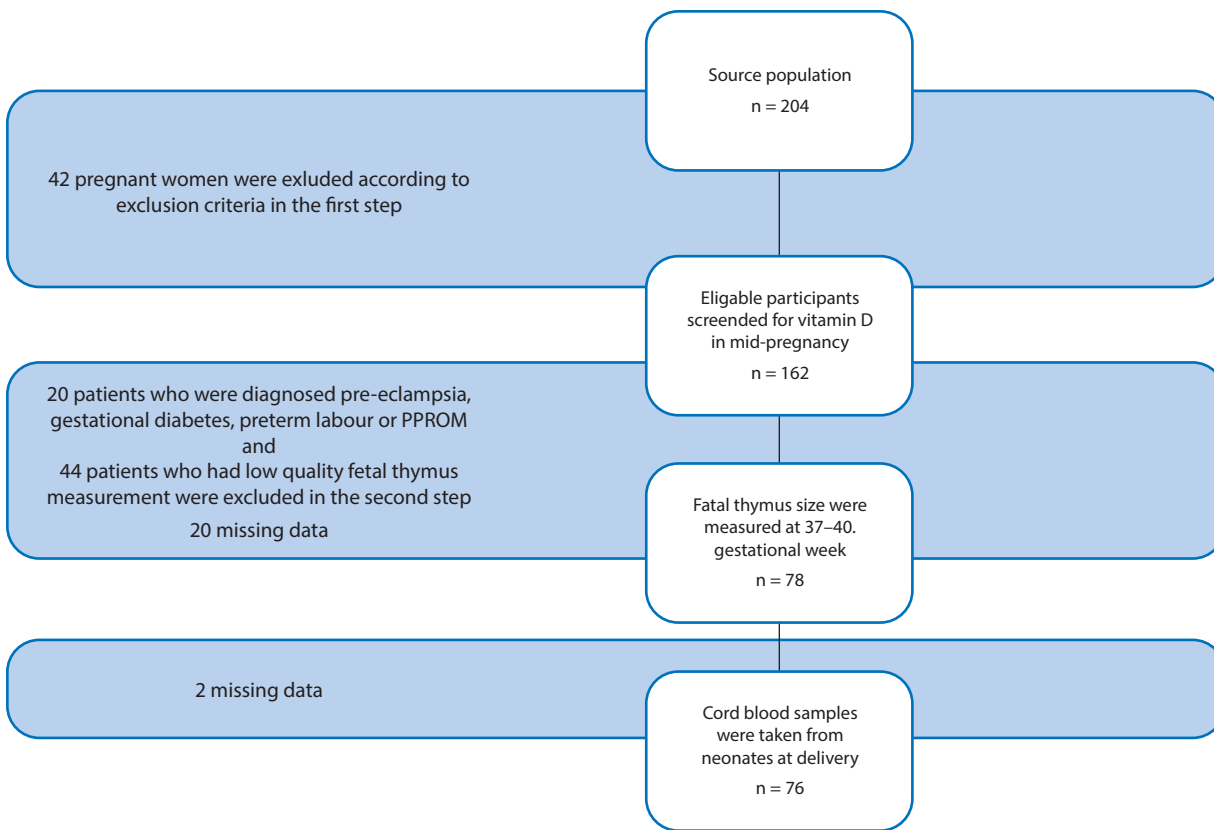
**Source population**
n = 204

42 pregnant women were exluded according to exclusion criteria in the first step

**Eligible participants screened for vitamin D in mid-pregnancy**
n = 162

20 patients who were diagnosed pre-eclampsia, gestational diabetes, preterm labour or PPROM
and
44 patients who had low quality fetal thymus measurement were excluded in the second step
20 missing data

**Fatal thymus size were measured at 37–40. gestational week**
n = 78

2 missing data

**Cord blood samples were taken from neonates at delivery**
n = 76

**Figure 1.** Flow chart of the study design. PPROM — preterm premature membrane rupture
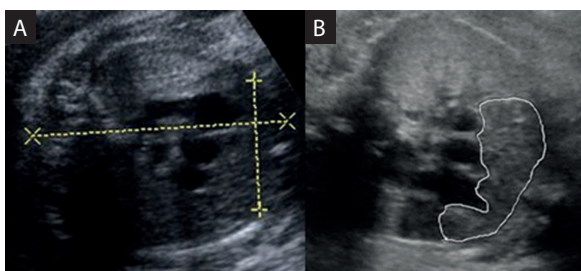


**Figure 2.** Sonogram of the thymus at the level of the 3-vessel view within the fetal thorax. **A.** Thymic-thoracic ratio. Tymus diameter was measured as its greatest width perpendicular to a line connecting the spine and sternum. **B.** Thymic perimeter

25(OH) D$_3$ concentration. The NIST SRM 972 (Chromsystems, DE) calibrators were used.

BMI [weight (kg)/height (m)$^2$] was calculated by measuring the height and the body weight at maternal vitamin D sampling.

### Statistical analysis

SPSS (15.0) for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The ANOVA test was used to test for differences within the groups. Factors associated with VDD were evaluated using multiple linear regression. The Pearson's correlation test was used to evaluate the relation-ship between thymus perimeter and diameter, as well as maternal and neonatal 25(OH)D$_3$ concentrations. Differences in thymus perimeter according to cord blood 25(OH)D$_3$ concentration were evaluated using ANOVA. The p-value of 0.05 was established as the threshold of statistical significance.

### RESULTS

Mean age, BMI, and week of pregnancy were 29.5 ± 4.3 years, 28.8 ± 2.6, and 38.7 ± 0.9 weeks, respectively. Average maternal serum 25(OH)D$_3$ concentration was 15.06 ± 6.9 ng/mL and mean umbilical cord serum 25(OH)D$_3$ concentration was 12.32 ± 4.8 ng/mL. A total of 48 mothers (29.8%) and 10 newborns (13.1%) had serum 25(OH)D$_3$ concentrations within the normal range. Fifty-seven mothers (35.1%) and 26 (34.2%) newborns had mild vitamin D deficiency, whereas 57 (35.1%) mothers and 40 (52.6%) newborns had severe vitamin D deficiency. The basic characteristics of the study group and the 25(OH) D$_3$ results are summarized in Table 1.

A strong statistical correlation between umbilical cord serum 25(OH)D3 concentration and maternal serum 25(OH) D3 concentration (r = 0.8, p < 0.001) was found (Figure 3).

Measurements of the fetal thymus perimeter were significantly correlated with the thymic-thoracic ratio (r = 0.4, p < 0.01).

**Table 1.** General characteristics and vitamin D status of the mothers and newborns in the study

| | Mean (± SD) | |
|---|---|---|
| Age (years) | 29.5 ± 4.3 | |
| Mean gestational week (maternal vitamin D sampling) | 25 ± 0.5 | |
| Mean gestational week (thymus measurement) | 38.7 ± 0.9 | |
| BMI | 28.8 ± 2.6 | |
| | Mothers<br>n = 162 | Newborns<br>n = 76 |
| Severe deficiency (≤ 10 ng/mL) (n,%) | 57, 35.1 | 40, 52.6 |
| Mild deficiency (10–20 ng/mL) (n,%) | 57, 35.1 | 26, 34.2 |
| Normal level (≥ 20 ng/mL) (n,%) | 48, 29.8 | 10, 13.1 |
| Average level ± SD (ng/mL ) | 15.06 ± 6.9 | 12.32 ± 4.8 |

SD — standard deviation, BMI — body mass index

**Table 2.** The relationship thymus perimeter with 25(OH)D3 concentrations in both maternal serum at mid-pregnancy and umbilical cord serum at delivery

| | Thymus perimeter | p | | Thymus perimeter | p |
|---|---|---|---|---|---|
| Severe deficiency, umbilical cord (≤ 10 ng/mL, n = 40) | 76.9[b] | | Severe deficiency, maternal serum (≤ 10 ng/mL, n = 29) | 78.3[a] | |
| Mild deficiency umbilical cord (10–20 ng/mL, n = 26) | 83.7[b] | **0.03** | Mild deficiency maternal serum (10–20 ng/mL, n = 26) | 85.1[b] | **0.04** |
| Normal level, umbilical cord (≥ 20 ng/mL, n = 10) | 94[a] | | Normal level, maternal serum (≥ 20 ng/mL, n = 19) | 92.9[c] | |

p < 0.05 statistical significance
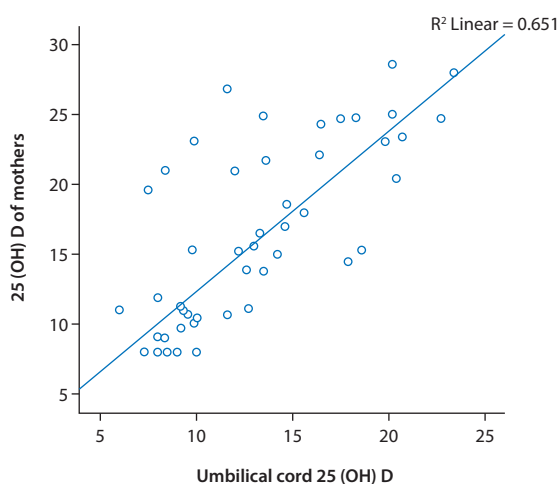


**Figure 3.** The relationship between serum 25(OH)D$_3$ concentrations of mothers and in umbilical cord serum at delivery (Pearson Correlation coefficient = 0.8, p < 0.01)
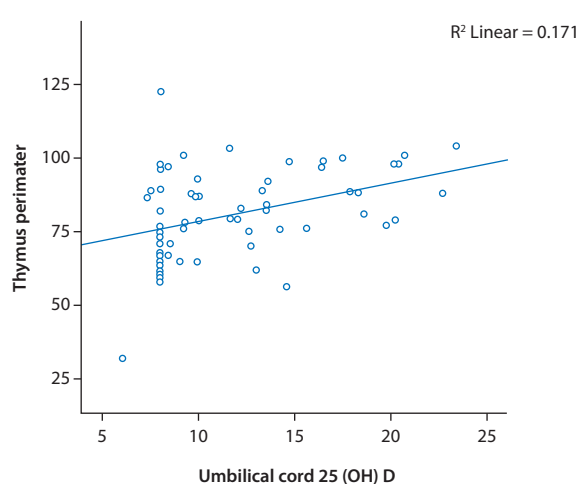


**Figure 4.** The relationship between thymus perimeter and 25(OH)D$_3$ concentrations in umbilical cord serum at delivery (Pearson Correlation coefficient = 0.4, p < 0.01)

A statistically significant linear correlation between both, maternal serum and umbilical cord 25(OH)D$_3$ concentration and thymus perimeter was observed (r = 0.45, p = 0.04 and r = 0.43, p < 0.01, respectively) (Figure 4).

Thymus perimeter differed significantly with regard to the level of both, maternal and fetal VDD (≤ 10, 10–20 and ≥ 20 ng/mL) (p = 0.04, p = 0.03) (Table 2).

## DISCUSSION

In this prospective study, we investigated the correlation between both, mid-pregnancy maternal and neonatal vitamin D levels and fetal thymus size. We found a statistically significant correlation between vitamin D level and thymus perimeter (r = 0.45, p = 0.04 and r = 0.43, p < 0.01, respectively). Moreover, both maternal and neonatal VDD levels were associated with decreased thymus perimeter (p = 0.04, p = 0.03). To the best of our knowledge, this has been the first study to evaluate the effect of VDD on thymus size in humans. The relationship between vitamin D and thymus has been investigated in two animal studies and their results are conflicting. Harvey et al., revealed that pregnant rats with VDD exhibit increased growth of central but not peripheral immune organs of the fetal thymus and leukocytes obtained from these fetuses demonstrated a pro-inflammatory phenotype [19]. These researches suggested that vitamin D may suppress growth hormone at the cellular level and an increase in fetal thymus weight might be associated with increased expression of insulin-like growth factor II (IGF-II). In a study conducted by Yurdakök et al., rats with VDD were not different from healthy controls in terms of thymus weight and histology [20].

Growing evidence shows increased inflammatory processes in patients with low vitamin D status, but little is known about this relationship during pregnancy [21, 22]. A recent study revealed that early pregnancy vitamin D status is associated with some inflammatory biomarkers in mid-pregnancy [23]. In a recent animal study, researchers showed that maternal VDD leads to aberrant inflammatory responses in the fetus [24]. Preeclampsia is the most extreme point of systemic inflammatory response and offers a good opportunity to assess the inflammatory process in pregnancy. Two recent studies have demonstrated that second trimester thymus size in preeclamptic pregnant women is smaller than in healthy controls [18, 25]. The researchers claimed that preeclampsia triggers an acute-phase reaction in the fetus, leading to activation of the hypothalamo-pituitary-adrenal (HPA) axis and cortisol release. Endogenous cortisol subsequently binds to its receptor in cortical thymocytes and induces apoptosis and thymic involution. Interestingly, some resent studies found that maternal vitamin D deficiency was associated with severe preeclampsia [26, 27]. Similarly, decreased thymus size is observed more frequently in cases of chorioamnionitis and thymus size is more sensitive than CRP in predicting histological chorioamnionitis [28]. Unlike in animal studies, we found a thymic involution in pregnant women with vitamin D deficiency. The inflammatory process which occurs as a result of VDD may have led to a reduction in the thymus size.

Recent studies have revealed that maternal and fetal VDD is common throughout the world [29, 30]. In our study, we observed normal $25(OH)D_3$ serum concentrations in 48 mothers (29.8%) and 10 neonates (12.1%). Fetal vitamin D is supplied by the mother and umbilical cord vitamin D levels are correlated with maternal vitamin D levels [31]. Similarly, we observed a positive correlation between cord blood and mid-pregnancy maternal vitamin D levels (r = 0.8, p < 0.01). The high prevalence of vitamin D deficiency during pregnancy increases the importance of studies investigating the effect of vitamin D on fetal programming. Recent studies have implicated that vitamin D may have an important, multifaceted role in the development of fetal lungs, brain, and bone [5, 8, 32]. Similarly, the results of our study suggested that VDD during pregnancy can affect fetal programming of thymus.

Ultrasound image quality has dramatically increased, and assessment of thymus size has become feasible in most human pregnancies. In a study comparing ultrasonography with magnetic resonance (MR), ultrasonographic evaluation was found to have similar sensitivity to MR [33]. Ultrasonic measurements of fetal diameter are closely correlated with measurements of thymic volume obtained during autopsy [34]. The fetal thymus was imaged in transverse at the point of the upper mediastinum adjacent to the three large cardiac vessels. This image is relatively easy to obtain during the second trimester [18, 25]. Obtaining a suitable transverse image is difficult in the third trimester. That is why the image quality did not meet our expectations in 44 of 162 pregnant women included in our study. In a study conducted by De Leon-Luis et al., concordance of 95% was observed for thymus circumference and thymus/thorax diameter. That measurement was not influenced by fetal sex [35]. Our study also revealed a positive correlation between thymus circumference and thymus/thorax diameter (r = 0.44, p < 0.01). Therefore, we used the thymus diameter as the thymus size for subsequent statistical analysis.

Several important limitations apply to our study. First, the study group included a small sample of pregnant women and studies with a larger and more diverse study populations are necessary. Secondly, while we included mature neonates and we examined fetal thymic-thoracic ratio in our study, we did not investigate the association between fetal weight and fetal thymus measurements. Finally, the effect of different doses of vitamin D on thymic size was not evaluated. For future studies, we suggest that randomized controlled trials measure fetal thymic size during the second trimester in mothers receiving vitamin D replacement.

In conclusion, fetal VDD is a common occurrence worldwide. VDD during pregnancy can affect fetal programming of the thymus. Furthermore, these data suggest that vitamin D deficiency during pregnancy may lead to systemic inflammatory response in the fetus. Thus, more research attention and patient awareness of the role of vitamin D in immune development is necessary.

*Conflict of interests*

The authors declare that they have no conflict of interest.

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