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Original Paper

Maternal Vitamin D Deficiency and Fetal Programming – Lessons Learned from Humans and Mice

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Key Words
Vitamin D • Birth weight • Preterm delivery • Fetal programming • Glucose tolerance • Cardiovascular diseases

Abstract
Background/Aims: Cardiovascular disease partially originates from poor environmental and nutritional conditions in early life. Lack of micronutrients like 25 hydroxy vitamin D_3 (25OHD) during pregnancy may be an important treatable causal factor. The present study explored the effect of maternal 25OHD deficiency on the offspring. ***Methods:*** We performed a prospective observational study analyzing the association of maternal 25OHD deficiency during pregnancy with birth outcomes considering confounding. To show that vitamin D deficiency may be causally involved in the observed associations, mice were set on either 25OHD sufficient or insufficient diets before and during pregnancy. Growth, glucose tolerance and mortality was analyzed in the F1 generation. ***Results:*** The clinical study showed that severe 25OHD deficiency was associated with low birth weight and low gestational age. ANCOVA models indicated that established confounding factors such as offspring sex, smoking during pregnancy and maternal BMI did not influence the impact of 25OHD on birth weight. However, there was a significant interaction between 25OHD and gestational age. Maternal 25OHD deficiency was also independently associated with low APGAR scores 5 minutes postpartum. The offspring of 25OHD deficient mice grew slower after birth, had an impaired glucose tolerance shortly after birth and an increased mortality during follow-up. ***Conclusions:*** Our study demonstrates an association between maternal 25OHD and offspring birth weight. The effect of 25OHD on

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birth weight seems to be mediated by vitamin D controlling gestational age. Results from an animal experiment suggest that gestational 25OHD insufficiency is causally linked to adverse pregnancy outcomes. Since birth weight and prematurity are associated with an adverse cardiovascular outcome in later life, this study emphasizes the need for novel monitoring and treatment guidelines of vitamin D deficiency during pregnancy.

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Introduction

Fetal programming, a theory of developmental origins of disease, refers to adaptive processes of the fetus to adverse environmental cues during gestation, which can have long-term effects on physiology, metabolism and therefore health status of an individual. Until now numerous epidemiological studies were able to show that effects of fetal programming can translate into a low birth weight, which is an independent risk factor for hypertension, cardiovascular and metabolic diseases in later life [1]. More and more evidence is accumulating that preterm birth is a similarly important risk factor for an elevated adulthood morbidity and mortality [2]. Thus, more information is needed on environmental cues triggering fetal programming.

Vitamin D, a secosteroid, is involved in the regulation of calcium and phosphate homeostasis and mediates a multitude of effects on various cell systems and signalling pathways beyond mineral metabolism. Currently it is estimated that vitamin D modulates about 10% of the whole human genome [3], underlining a putative major role in developmental processes [4-6]. Vitamin D plays an important role during human pregnancy. In early phases of pregnancy vitamin D triggers the decidualisation of the endometrium, a process relevant for the implantation of the blastocyst [7]. At the end of the first trimester various hormones are required for the maintenance of pregnancy. Invitro studies suggest that vitamin D supports the expression and secretion of human choriongonadotropin, human placental lactogen, estradiol and progesterone by placental cells [8].

Vitamin D hypovitaminosis, especially among pregnant women, is a major public health problem with a very high prevalence ranging from 18 to 84%, depending on the country of residence and local clothing customs [9, 10]. 25 hydroxy vitamin D_3 (25OHD) is considered the storage form of vitamin D and plasma concentrations of 25OHD serve as a suitable indicator of the vitamin D status [8]. It is still unclear, whether the ideal level of 25OHD should differ from documented optimal levels for non-pregnant adults or if vitamin D should be routinely supplemented during pregnancy. Being pregnant and deficient for 25OHD could reduce maternal 25OHD levels even further, although results from observational studies are not completely clear, either showing no change or a decline of maternal 25OHD levels during pregnancy [11, 12].

25OHD deficiency may be an independent risk factor for pregnancy and birth complications [13]. Maternal complications range from decreased weight gain [14], primary cesarean sections [15], bacterial vaginosis [16], osteomalacia [17] to gestational diabetes [14], hypertension and pre-eclampsia [13]. In the newborn, maternal 25OHD deficiency has been linked to hypocalcemia, poor weight gain, impaired development and rickets [18]. Current literature suggests that there is association between 25OHD hypovitaminosis and adverse pregnancy outcomes such as impaired fetal growth and preterm birth, however with conflicting results [19-24].

Materials and Methods

Clinical study

All research involving human participants has been approved by the authors' Institutional Review Board (IRB) of the university hospital Charité, Berlin, Germany, and all clinical investigations were

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conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the participants. From 2007 to 2008 we invited a total of 547 mothers with singleton pregnancies delivering at Charité obstetrics department to participate in this prospective observational study. The study was approved by the local ethics committee. After obtaining written consent, structured interviews were performed with the mothers. Patients who fulfilled the criteria of a regular pregnancy were included regardless of the ethical background. However, the majority (n=480) were of Caucasian ethnicity. The remaining patients were of African, Asian, or Arabic heritage. Data from the so called "Mutterpass", documenting the results of follow ups during pregnancy were also collected. The following data were added to our database: age, ethnicity, education, income, body height, weight before and during different time points of pregnancy, gravidity, parity, diabetes mellitus before or during pregnancy, incidence of hypertension before and during pregnancy, smoking status before and during pregnancy, systolic and diastolic blood pressure measurements recorded during pregnancy and the mode of delivery. Biometric data of the newborn were collected during the routine postnatal examination. Gestational age at delivery was based on last menstrual period, anamnestically assessed during the first pregnancy examination. For the final statistical analyses extreme cases with neonatal gestational ages <28weeks and birth weights <900g were excluded. Following data of the newborn were added to the database: birth weight, birth length, head circumference, season of birth, child sex, Apgar score 5 minutes postnatally and Apgar score 10 minutes postnatally. Midwives collected maternal blood from a cubital vein in the delivery room or on the ward.

25OHD is an established indicator of the vitamin D status [8], and current guidelines advise against the measurement of the active form of vitamin D, 1,25-Dihydroxyvitamin D3 [25], due to its short blood half life and technical difficulties to measure it.

For the analysis of 25OHD heparinized collecting tubes were used and centrifuged at 2750g for 10 minutes at room temperature. Maternal plasma was then stored at -80°C for further analysis.

25OHD was measured in maternal plasma by Immundiagnostik AG (Bensheim) deploying a monoclonal antibody based quantitative ELISA assay. The measurable range was 1 nmol/L- 240nmol/L. External controls were used for internal quality assurance. As 37 mothers displayed 25OHD concentrations below the limit of detection, these values were adjusted to the limit of detection (1nmol/L) times 0.5, as recommended by the literature [26].

Animal experiment

All animal work has been conducted in accordance with national and international guidelines for the use of living animals and was approved by the Institutional Animal Care and Use Committee of the University of Tübingen, Germany and the state authorities of Baden-Württemberg. Male and female C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). The animals were kept at a temperature of 21±1°C, were subjected to a 12-hour light/dark cycle and received chow and water ad libitum. At 3 weeks, female mice were subjected either to a diet containing 10,000 I.U./kg vitamin D3 (Altromin, Lage, Germany) or to a diet containing <50 I.U./kg vitamin D3 (Altromin, Lage, Germany). Mice were mated 4-5 weeks later with males kept on a standard diet (Ssniff, Soest, Germany). The F1 generation consisted of 63 animals from 25OHD sufficient mothers and 57 animals from 25OHD insufficient mothers. The respective diet was continued for the female mice until the offspring was weaned. After weaning, all mice received standard diet (Ssniff, Soest, Germany). Weight was monitored weekly starting on the day of birth. At an age of 55-60 days, an intraperitoneal glucose tolerance test was performed. To this end, mice were fasted overnight. After i.p. injection of 20% glucose-containing saline solution (2 mg/g b.w. glucose), glucose levels were determined in tail vein blood with a glucometer (Roche, Mannheim, Germany) at the indicated time points. At an age of 55 days blood pressure was measured in offspring of 25OHD sufficient and insufficient mothers by tail cuff method (IITC Life Science, Woodlands Hills, CA, USA). The maternal 25OHD status was also assessed in 3 animals of each diet group, using a competitive protein binding assay (K2110; Immundiagnostik AG, Bensheim; Germany) for the measurement of 25OHD. Blood for 25OHD analysis was taken at the age of 105 ± 2 days when the mice had been subjected to the respective diet for 83 ± 2 days.

Statistical evaluation

Data were analyzed using SPSS version 20.0 (SPSS, Inc, Chicago, IL, USA). Unpaired t-test was used when comparing two groups. Bivariate correlation analysis was assessed to detect possible confounders relevant to be included into statistical models. Additional established confounding factors of birth

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weight [27-29], such as offspring sex, smoking during pregnancy and maternal height and weight were included into the models. ANCOVA analysis was then used to confirm relevant confounding variables that independently influence birth weight or gestational age and to adjust for these in different statistical models. Graphs regarding differences in birth weight were calculated and compiled with Graphpad Prism 5 (GraphPad Software Inc., La Jolla, California, USA). Data and graphs from the animal experiment were also calculated and compiled with Graphpad Prism 5. Data regarding weight gain and glucose tolerance in the animal experiment were calculated using two-way ANOVA with a Bonferroni posttest. Survival was assessed by employing a Kaplan-Meier curve comparison. Probability values <0.05 were considered significant. The authors had full access to the data and take full responsibility for its integrity.

Table 1.

Descriptive data of the mother and the newborn (n=547)	
Maternal age, y	30.9 ± 6.1
Maternal height, cm	166.8 ± 6.8
Body mass index before pregnancy, kg/m^2	22.7 ± 4.7
Mean weight 3rd trimester of pregnancy, kg	76.4 ± 14.0
Hypertension before/during pregancy, %	3.7/9.5
Diabetes mellitus before/during pregnancy, %	1.5/9.4
Mean systolic BP 3rd trimester of pregnancy, mm Hg	114.6 ± 10.8
Mean diastolic BP 3rd trimester of pregnancy, mm Hg	69.6 ± 7.3
Smoking before/during pregnancy, %	41.2/15.0
Ethnicity (Caucasian/other)	87.8/12.2
Income (low, medium, high)	37.5/37.9/24.5
25OHD concentration [nmol/L]	18.0 ± 19.0
Primigravida/primipara, %	41.7/56.9
Gestational age at delivery, days	271.1 ± 16.4
Birthweight, g	3244.3 ± 647.5
Birthlenght, cm	50.1 ± 3.7
Child head circumference, cm	34.6 ± 1.9
Child sex, male/female, %	52.1/47.9
Apgar score at 5 min	9.1 ± 1.4
Apgar score at 10 min	9.3 ± 1.4

Data are given as mean±SD or %

Results

Clinical study

Descriptive Data. Detailed descriptive statistics of the mothers and their newborns are displayed in Table 1.

Table 2 shows detailed descriptive statistics of the study population grouped according to the severity of 25OHD deficiency. Cut-off values were chosen as follows: below the level of detection (<1 nmol/L; n=37), severe deficiency (≥1 to <25 nmol/L; n=361), moderate deficiency to sufficiency (≥25nmol/L; n=149). Significant group differences were calculated using ANOVA or Pearson's chi-squared test.

In the studied cohort we observed strong differences in maternal 25OHD plasma concentrations, when grouping according to the level of education (Figure 1A; low education: 10.9±1.3; medium education: 18.1±1.1; high education: 20.9±1.5; p=0.0003; ±SEM). Additionally sorting maternal height according to the severity of 25OHD deficiency revealed highly significant differences in maternal height (Figure 1B; <1nmol/L: 163.9±1.2cm; ≥1to<25 nmol/L: 166.3±0.4cm; ≥25 nmol/L: 168.9±0.5cm; p<0.001; ±SEM). We also observed significant differences of 25OHD plasma concentrations, when data were analyzed according to ethnicity (Caucasian; other; 19.3±0.9nmol/L vs. 9.7±1.1nmol/L; p<0.001; ±SEM) or season of birth (summer; other; 25.5±1.6nmol/L vs. 14.0±0.8nmol/L; p<0.001; ±SEM). As the above mentioned stratification did not show any group differences between severe deficiency (≥1 to <25 nmol/L) and moderate deficiency to sufficiency (≥25 nmol/L) in terms of birth weight and gestational age, further analyses were performed using only two groups, either below or above the level of 25OHD detection.

Birth weight and gestational age. A highly significant difference in birth weight (2791.4±776.1g vs. 3277.2±625.4g; p<0.001; ±SEM; Figure 2A) and gestational age

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Table 2. Descriptive Data of the Mother/Child Pairs grouped according to Severity of 25OHD Deficiency (n=547).

Maternal characteristics	Term maternal plasma 25(OH)D (n=547)			
	<1 nmol/L (n =37)	≥1 to <25 nmol/L (n = 361)	≥25 nmol/L (n = 149)	Significant group differences
Maternal age, y	29.8 ± 7.0	30.9 ± 6.3	31.1 ± 5.4	n.s
Ethnicity (Caucasian, other), %	83.8/16.2	84.8/15.2	96.0/4.0	p=0.002
Education (low,medium, high), %	27.0/45.9/27.0	16.9/47.1/36.0	6.0/48.3/45.6	p=0.002
Income (low, medium, high),%	54.1/32.4/13.5	39.3/37.3/23.4	29.3/40.8/29.9	p=0.039
Maternal height, cm	163.9 ± 7.2	166.3 ± 6.9	168.9 ± 5.7	p<0.0001
Maternal weight before pregnancy, kg	58.1 ± 9.2	63.9 ± 15.1	62.7 ± 11.3	p=0.053
Body mass index before pregnancy, kg/m2	21.7 ± 3.3	23.1 ± 5.1	22.0 ± 3.8	p=0.023
Mean weight 3rd trimester, kg	69.4 ± 9.6	77.1 ± 14.8	76.4 ± 12.3	p=0.007
Hypertension before/during pregnancy, %	0/2.7	3.6/10.8	4.7/8.1	n.s.
Diabetes mellitus before/during pregnancy, %	2.7/10.8	1.7/10.9	0.7/5.4	n.s.
Mean systolic blood pressure 3rd trimester, mmHg	112.9 ± 13.0	114.5 ± 11.4	115.3 ± 8.7	n.s.
Mean diastolic blood pressure 3rd trimester, mmHg	66.9 ± 7.2	69.5 ± 7.5	70.3 ± 6.8	p=0.043
Smoking before/during pregnancy, %	37.8/18.9	41.6/16.1	41.2/12.8	n.s.
Plasma 25OHD, [nmol/L]	below level of detection	9.8 ± 6.4	42.5 ± 19.5	p<0.0001
Primigravida/primipara, %	35.1/45.9	41.6/55.4	43.6/63.1	n.s./p=0.02
Season of birth (summer; other; %)	16.2/83.8	29.1/70.9	55.7/44.3	p<0.0001
Mode of delivery (spontaneous, vag. OP; c-section;)	43.2/8.1/48.6	60.7/9.1/30.2	59.7/5.4/34.9	n.s
Gestational age at delivery, d	260.1 ± 21.8	272.0 ± 15.4	271,6 ± 16.3	p<0.0001
Birthweight, g	2791.4 ± 776.1	3286.2 ± 640.7	3255.3 ± 588.3	p<0.0001
Birthlenght, cm	47.7 ± 4.8	50.4 ± 3.5	49.9 ± 3.7	p<0.0001
Child head circumference, cm	33.5 ± 2.7	34.5 ± 1.8	34.5 ± 1.9	n.s.
Child sex, male/female, %	51.4/48.6	51.5/48.5	53.7/46.3	n.s.
Apgar score at 5 min	8.3 ± 1.9	9.2 ± 1.2	9.0 ± 1.5	p=0.001
Apgar score at 10 min	8.9 ± 1.8	9.4 ± 1.3	9.3 ± 1.4	n.s.

(260.1±3.6 days vs. 271.9±0.7 days; p<0.001; ±SEM; Figure 2B) was observed between mothers with 25OHD levels below and above the threshold of detection, 1 nmol/L.

Based on recent evidence from a systematic review [28] that showed associations between maternal height, birth weight and preterm birth and the strong association of

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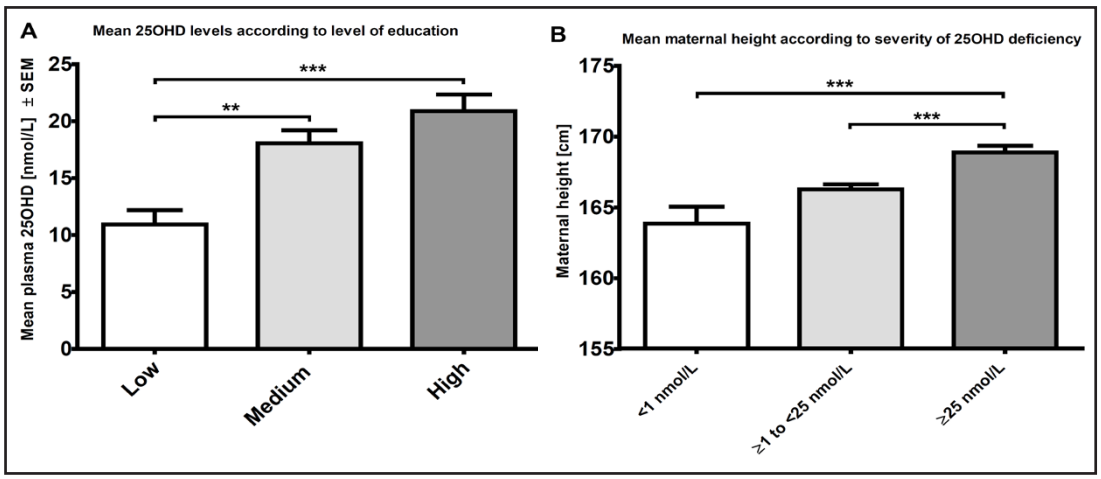

Fig. 1. Maternal 25OHD plasma concentrations according to education and height. **A:** Maternal 25OHD plasma concentration [nmol/L] according to the level of education (low education: 10.9±1.3; medium education: 18.1±1.1; high education: 20.9±1.5; Data are given as mean ± SEM;); **B:** Maternal height according to the severity of 25OHD deficiency (<1 nmol/L: 163.9±1.2cm; ≥1 to <25 nmol/L: 166.3±0.4cm; ≥25 nmol/L: 168.9±0.5cm; p<0.001 Data are given as mean ± SEM;). ***p<0.001; **p<0.01.

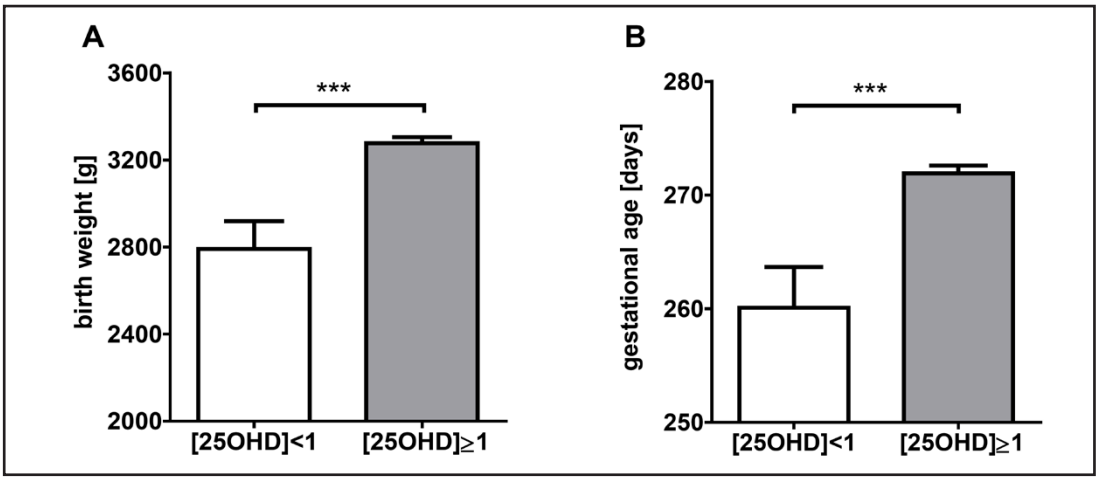

Fig. 2. Birth weight and gestational age according to chosen 25OHD cutoff. **A:** Birth weight in grams (2791.4±776.1 g vs. 3277.2±625.4 g; p<0.001; Data are given as mean ± SEM;); **B:** Gestational age in days (260.1±3.6 days vs. 271.9±0.7 days; p<0.001; Data are given as mean ± SEM;) of mothers with 25OHD levels below and above the threshold of detection, 1nmol/L.

maternal height and 25OHD plasma concentration in the observed cohort, maternal height and weight were used as separate confounders instead of BMI for the analysis of birth weight and gestational age.

ANCOVA analysis confirmed previous reports [27-29] that gestational age at delivery, the child's sex, maternal height, weight, and smoking during pregnancy are important variables independently associated with birth weight (Model A, Table 3). A highly significant impact of 25OHD on birth weight was shown in an ANCOVA model considering grouped 25OHD levels (below or above the level of detection), child sex, maternal height and weight before pregnancy, smoking during pregnancy, maternal ethnicity (Caucasian; other), education (low; medium; high;), season of birth (summer; other;) and mode of delivery (spontaneous; vaginal operation; c-section;) (Model B, Table 3).

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Table 3. A: ANCOVA analysis of the association between gestational age and birth weight (in g) as dependent variable; **B:** ANCOVA analysis of the association between maternal 25OHD and birth weight (in g) as dependent variable; **C:** ANCOVA analysis of the association between maternal 25OHD, gestational age and birth weight (in g) as dependent variable; **D:** ANCOVA analysis of the association between 25OHD and gestational age at birth (weeks) as dependent variable; **E:** ANCOVA analysis of the association between 25OHD and APGAR scores 5 minutes postpartum as dependent variable.

Model (adjusted R^2) and Independent Variables	Partial Eta squared	Observed power	*P*
A (0.590)			
Gestational age at delivery, d	0.557	1.000	<0.0001
Child sex (m/f)	0.027	0.968	<0.0001
Height before pregnancy, cm	0.017	0.866	0.002
Weight before pregnancy, kg	0.016	0.843	0.003
Smoking during pregnancy	0.024	0.950	<0.0001
Mode of delivery (spontaneous, vag. OP; c-section;)	0.002	0.155	0.349
B (0.100)			
Grouped 25OHD [<1 vs. ≥ 1 nmol/L]	0.020	0.914	0.0009
Child sex (m/f)	0.007	0.517	0.045
Height before pregnancy, cm	0.003	0.231	0.221
Weight before pregnancy, kg	0.015	0.814	0.004
Smoking during pregnancy	0.003	0.254	0.195
Ethnicity (caucasian; other;)	0.003	0.229	0.224
Season of birth (summer; other;)	0.002	0.171	0.315
Education (low; medium; high;)	0.005	0.361	0.109
Mode of delivery (spontaneous, vag. OP; c-section;)	0.045	0.999	<0.0001
C (0.591)			
Gestational age at delivery, d	0.547	1.000	<0.0001
Grouped 25OHD [<1 vs. ≥ 1 nmol/L]	0.002	0.160	0.337
Child sex (m/f)	0.029	0.977	<0.0001
Height before pregnancy, cm	0.015	0.817	0.004
Weight before pregnancy, kg	0.018	0.871	0.002
Smoking during pregnancy	0.017	0.852	0.003
Ethnicity (caucasian; other;)	0.004	0.318	0.137
Season of birth (summer; other;)	0.002	0.156	0.345
Education (low; medium; high;)	0.001	0.091	0.552
Mode of delivery (spontaneous, vag. OP; c-section;)	0.002	0.129	0.414
D (0.143)			
Grouped 25OHD [<1 vs. ≥ 1 nmol/L]	0.026	0.962	<0.0001
Weight before pregnancy, kg	0.015	0.817	0.004
Mean weight 3rd trimester, kg	0.039	0.996	<0.0001
Mean systolic blood pressure 3rd trimester, mmHg	0.038	0.996	<0.0001
Height before pregnancy, cm	0.005	0.377	0.100
Smoking during pregnancy	0.001	0.094	0.538
Ethnicity (caucasian; other;)	0.000	0.050	0.978
Season of birth (summer; other;)	0.000	0.076	0.634
Education (low; medium; high;)	0.003	0.240	0.211
Mode of delivery (spontaneous, vag. OP; c-section;)	0.059	1.000	<0.0001
E (0.131)			
Grouped 25OHD [<1 vs. ≥ 1 nmol/L]	0.009	0.609	0.026
Gestational age at delivery, d	0.045	0.999	<0.0001
Mode of delivery (spontaneous, vag. OP; c-section;)	0.046	0.999	<0.0001
Child sex (m/f)	0.004	0.336	0.124
Ethnicity (caucasian; other;)	0.006	0.438	0.071
Season of birth (summer; other;)	0.000	0.077	0.629
Education (low; medium; high;)	0.000	0.068	0.690

However, additionally adding gestational age at time of birth to the model diminished a direct influence of 25OHD levels on birth weight but gave rise to the question whether maternal 25OHD levels impact on birth weight by influencing gestational age at birth (Model C, Table 3).

Employing bivariate correlation analysis and literature research, confounders that impact on gestational age were identified [30-32]. To investigate if maternal 25OHD levels below the level of detection impact on gestational age at birth, another ANCOVA model

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was created. The model included 25OHD levels (below or above the level of detection), mean systolic blood pressure in the third trimester of pregnancy, maternal height and weight before pregnancy, mean weight in the third trimester of pregnancy, smoking during pregnancy, maternal ethnicity (Caucasian; other), education (low; medium; high;), season of birth (summer; other;) and mode of delivery (spontaneous; vaginal operation; c-section;). Our data show that maternal 25OHD levels below the level of detection impact on gestational age at birth strongly and statistically highly significantly (Model D, Table 3).

Furthermore another ANCOVA model using Apgar score 5 minutes postnatally as dependent variable was created. After adjusting for gestational age, mode of delivery, child sex, maternal ethnicity (Caucasian; other), education (low; medium; high;) and season of birth (summer; other;) a significant interaction between maternal 25OHD levels and Apgar score 5 minutes postnatally remained (Model E, Table 3).

Animal experiment

25OHD sufficient mothers gave birth to an average of 7.4±0.5 (SEM) pups, 25OHD deficient mothers to an average of 7.3±0.5 (SEM) pups. Litter size did not differ significantly. Offspring from 25OHD sufficient mothers weighed 1.29±0.02 g (SEM), offspring from 25OHD insufficient mothers 1.30±0.02 g (SEM). There were no significant differences in birth weight. However, beginning on day 15, the offspring of 25OHD deficient mothers weighed significantly less than the offspring from 25OHD sufficient mothers (Figure 3A).

Figure 3B shows Kaplan Meier curves of offspring from 25OHD sufficient mothers in comparison to offspring from 25OHD deficient mothers. Most of the animals died before weaning from unknown causes. The rest of the animals (n=1) died suddenly from unknown causes. A log-rank test between the two curves revealed a significantly higher mortality in offspring from 25OHD deficient mothers ($p=0.0014$).

At an age of 55-60 days, an intraperitoneal glucose tolerance test was performed. Offspring of 25OHD deficient mothers had significantly higher blood glucose levels 15 (272.7±6.97mg/dl vs. 307.4±8.31mg/dl, $p<0.01$), 30 (261.0±8.54mg/dl vs. 289.4±7.69mg/dl, $p<0.05$) and 45 minutes (209.0±8.93mg/dl vs. 235.1±7.09 mg/dl, $p<0.05$) after the intraperitoneal glucose load (Figure 3C; Data are given ± SEM;). Blood pressure was also assessed in offspring from 25OHD sufficient and insufficient mothers. Offspring of 25OHD sufficient mothers showed a mean systolic blood pressure of 94.4 ± 2.3 (SEM) mmHg and offspring from 25OHD deficient mothers a mean systolic blood pressure of 92.5 ± 2.1 (SEM) mmHg. There was no statistically significant difference in measured systolic blood pressure. To see whether or not the respective diets resulted in maternal 25OHD sufficiency and insufficiency, 25OHD was measured in 3 animals of each diet group. 25OHD deficient mice displayed serum 25OHD concentrations of 4.83±0.94 (SEM), whereas 25OHD sufficient animals had serum concentrations of 83.58±6.39 (SEM) nmol/L.

Discussion

The present study demonstrated in a large, apparently healthy pregnancy cohort that there is a relationship between the maternal 25OHD status and birth weight of the newborn, i.e., low maternal 25OHD levels are associated with low birth weight. A more detailed analysis revealed that low 25OHD levels are linked to low birth weight by controlling gestational age. Additionally it was shown that low maternal 25OHD levels also impact on the APGAR score of the newborn. To demonstrate that low maternal 25OHD status causes phenotypic effects in the offspring, an animal study was performed. Offspring of 25OHD sufficient mice was compared to offspring of 25OHD insufficient mice. Offspring of 25OHD insufficient mice showed a significantly stunted postnatal growth and a higher mortality than offspring of 25OHD sufficient mothers. A glucose tolerance test performed at the end of the experiment showed that offspring born to 25OHD insufficient mice display an impaired glucose tolerance. Since birth weight and prematurity are associated with an adverse renal and cardio-

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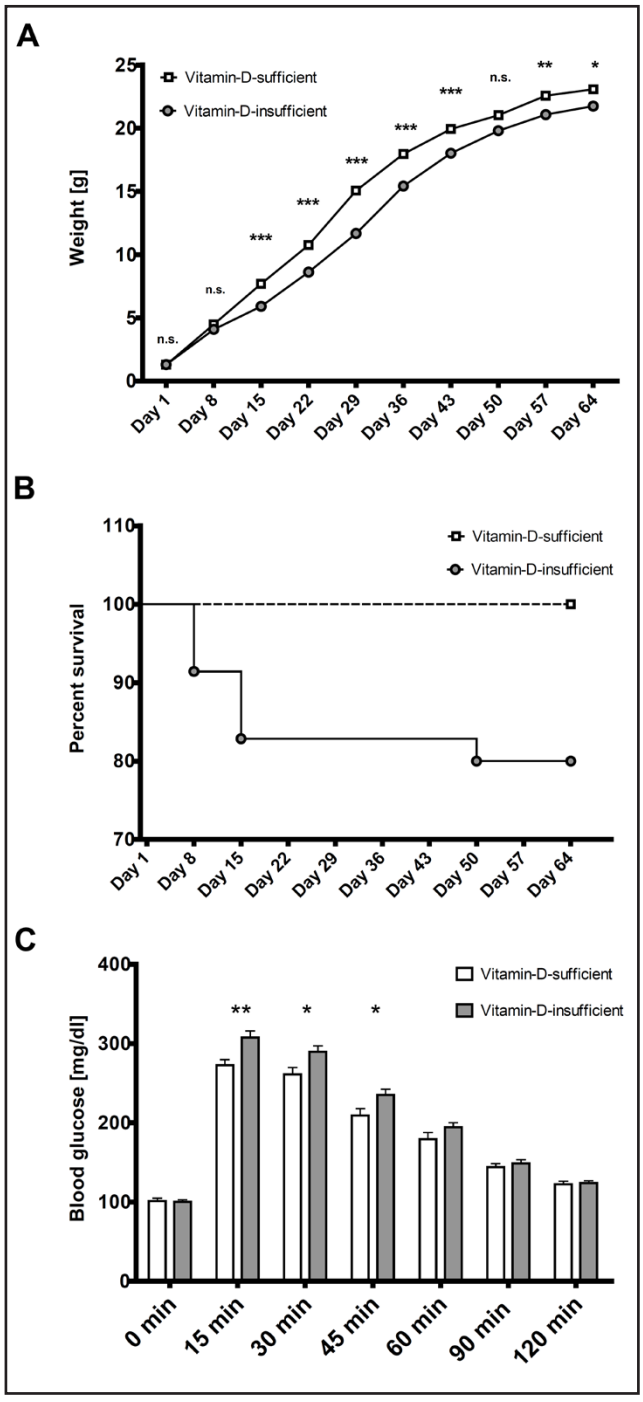

Fig. 3. Growth curves, survival and glucose tolerance of 25OHD sufficient and insufficient mice. **A:** Growth curves of offspring from 25OHD sufficient (white squares) and insufficient (grey circles) mothers. ***p<0.001; **p<0.01; *p<0.05; n.s.: not significant; **B:** Kaplan Meier curves of offspring from 25OHD sufficient mothers in comparison to offspring from 25OHD deficient mothers. There was a significantly higher mortality in offspring from 25OHD deficient mothers (log-rank test; p=0.0014); **C:** Results from the intraperitoneal glucose tolerance test comparing offspring from 25OHD sufficient to offspring from 25OHD insufficient mothers. Offspring of 25OHD deficient mothers had significantly higher blood glucose levels 15 (272.7 ± 6.97mg/dl vs. 307.4± 8.31mg/dl), 30 (261.0±8.54mg/dl vs. 289.4 ± 7.69mg/dl) and 45 minutes (209.0 ± 8.93mg/dl vs. 235.1±7.09 mg/dl) after the intraperitoneal glucose load. Data are given as mean ± SEM; **p<0.01; *p<0.05.

vascular outcome in later life, this study emphasizes the need for novel monitoring and treatment guidelines of 25OHD during pregnancy to improve cardiovascular outcome in later life.

The finding that very low maternal 25OHD plasma concentrations levels are associated with low birth weight, is in line with current literature [22, 23]. However, ambiguous data on the influence of 25OHD on birth weight are published as some studies did not detect an impact of low 25OHD on birth weight [21] .

Recent systematic reviews point out that the association between low maternal 25OHD concentrations and birth weight is genuine [33, 34]. Our study adds a different, not yet performed approach for the assessment of the impact of 25OHD on birth outcomes. Patients were stratified into groups depending on their 25OHD plasma concentration (<1 nmol/L; ≥1 to<25 nmol/L; ≥25 nmol/L) differently from other studies, because of the very high frequency of 25OHD deficiency in the analyzed cohort. Most other studies used different stratification, regarding higher gestational levels of 25OHD already as insufficiency [33]. In meta-analyses it was shown that the chosen cut-off value is important to detect effects of

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25OHD on anthropometric measurements [33]. The time point of blood sampling might be one explanation why such a high frequency of 25OHD deficiency was observed. It is well known that 25OHD deficiency is a major common public health problem [9, 10, 35]. It was also shown that 25OHD levels decline at the end of pregnancy [12]. Given these two factors, it is possible that the time point of blood sampling at term is in part responsible for the observed very low 25OHD levels. Although the frequency of patients with 25OHD levels considered low was exceptionally high, this present study was able to distinguish a group of extremely deficient mothers that were at high risk for impaired birth outcomes.

Results of this clinical study suggest that extremely low maternal 25OHD concentrations impact on birth weight by shortening the duration of gestation. This observation is in line with literature. Very recent studies were able to show similar results. In a multicenter U.S. study by *Bodnar et al.*, it was observed that gestational serum 25OHD concentrations above 30nmol/L are associated with a 20%–30% reduction in the risk of spontaneous preterm birth compared with 25OHD concentrations less than 30 nmol/L [36]. Different from the present study, blood samples were taken at 26 weeks gestation or earlier and the observation was only made in non-Caucasian mothers. The authors mentioned though, that it is possible that there were not enough cases of white women with 25OHD levels less than 30 nmol/L in their cohort to detect an effect [36]. A limitation of the just mentioned study is that blood samples were already collected around 50 years ago between 1959 and 1965 and might have shown signs of analyte deterioration [36, 37]. Another study assessing the 25OHD status of prematurely and term born newborns was able to demonstrate that newborns born before 32 weeks of gestation were at higher risk of having insufficient 25OHD levels [38]. The results of our study, together with previous reports [36, 38, 39] underline an involvement of 25OHD in controlling gestational age. Biologically, it would be plausible that low gestational maternal 25OHD levels are involved in triggering shortening of gestation. It is known that low gestational 25OHD concentrations are associated with bacterial vaginosis [16] and infection is one risk factor of preterm labour [40]. Vitamin D is also involved in gene regulation important for trophoblast invasion and angiogenesis [41], other important factors involved in preterm labour [42]. In the above mentioned study by *Bodnar et al*. it was shown in histologic examinations of the placenta, that low maternal 25OHD concentrations are associated with both inflammatory and vascular placental lesions [36]. Another study investigated the effect of 25OHD deficiency on birth weight in relation to placental weight, a known factor impacting on birth weight. It was shown that 25OHD deficiency is associated with low birth weight, but independently of placental weight [43].

We observed differences in maternal height, according to the severity of 25OHD deficiency in this study. The more deficient mothers were for 25OHD, the smaller they were. To the best of our knowledge, this observation was only made by two other groups before [44, 45]. Given the involvement of 25OHD in bone metabolism it was suggested that long term 25OHD deficiency might impact on longitudinal bone growth [44]. It was also hypothesized that bone mineral content and density during times of 25OHD deficiency are maintained in normal ranges at the expense of body height [45]. In context with data from a recent meta analysis, that showed an association between maternal short stature and preterm birth, these associations warrant further attention by future studies [28].

To assess whether or not the impact of low maternal 25OHD levels is not just associative but causative, an animal experiment mimicking the clinical situation was performed. Mice were put on diets insufficient or sufficient for vitamin D_3 and mated with male mice on standard diets. Maternal 25OHD insufficiency was confirmed by measuring plasma levels. The F1 generation was explicitly characterized to detect possible effects of developmental programming, which can be linked to low birth weight [1] or prematurity [2, 46-48]. Results from the animal experiment indicated a direct involvement of maternal 25OHD in gestation, birth outcomes and fetal programming of adult disease in later life. No statistical significant differences were observed regarding birth weight. This is in line with our clinical data, since only women with extremely low 25OHD concentrations (<1 nmol/L) had offspring with substantially reduced birth weight. If the maternal 25OHD concentration was in the range

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of that seen in the animal study in the 25OHD deficient group, the effect on birth weight in the human study was minor. Other groups investigating the effects of gestational 25OHD deficiency in rodent models on the offspring did not see differences in birth weight either [5]. One study even demonstrated that offspring from 25OHD deficient mice is significantly larger at day E14 and E18 than offspring from sufficient mothers. This weight difference was not present at time of birth and reversed after birth, with offspring from 25OHD deficient mice being significantly lighter from postnatal day 14 on [49], an effect that was also observed in the present study.

Beyond birth weight, the offspring of 25OHD deficient mothers presented a strongly altered phenotype with a significantly reduced postnatal growth rate until the end of the study and an increased mortality. The observed higher mortality in offspring of 25OHD deficient mothers supports the concept of low gestational 25OHD levels triggering a shortening of gestation as prematurity is strongly associated with an elevated mortality [50, 51]. The observation that low gestational 25OHD also causes prematurity in rodent models is supported by a study from *Liu et al.* which focused on prenatal morphology of offspring from 25OHD deficient mice. They showed that maternal gestational 25OHD deficiency leads to a higher rate of premature births, that is even comparable to established knockout models of prematurity [49, 52].

A hallmark of fetal programming is the development of symptoms of metabolic syndrome in later life, i.e. development of hypertension and changes in glucose and lipid metabolism [1]. To detect symptoms of metabolic syndrome in this study, blood pressure and glucose tolerance were assessed in offspring from 25OHD sufficient and insufficient dams. No statistical significant differences were seen in systolic blood pressure, maybe because the time point of measurement, at postnatal day 55, was too early. In a study in rats investigating the consequences of low gestational vitamin D on kidney development it was shown that offspring of mothers receiving diets insufficient for Vitamin D_3 started to develop significantly higher systolic blood pressure than offspring from mothers on sufficient diets at an age of 3 months [5]. The glucose tolerance test, which was also performed at an age of 55-60 days showed that offspring from 25OHD deficient mothers had an impaired glucose tolerance. This is quite remarkable as these mice were to be seen as pre-adult animals at the time of the test. This result fits to newer concepts of fetal programming generated by large epidemiological studies. These indicated that regardless of the setting, the most prominent feature of fetal programming is a modification of insulin signaling and that low birth weight is already at time of birth associated with elevated surrogate parameters of insulin resistance [53, 54]. It has been shown that 25OHD deficiency is associated with impaired insulin secretion, reduced glucose homeostasis, increased risk of metabolic syndrome and type 2 diabetes [55, 56].

However, evidence remains inconclusive, as data from randomized controlled trials on 25OHD supplementation failed to show positive effects of 25OHD supplementation on the incidence of diabetes, glucose control and insulin resistance in general populations [57]. Our data in mice demonstrate for the first time that vitamin D programs glycemic control –a key element of the metabolic syndrome. However, the underlying mechanism (impaired insulin secretion or increased insulin resistance) needs to be addressed in clamp studies.

Our study investigated the effects of low gestational 25OHD levels on birth outcomes in a clinical and an experimental setting. Results from both approaches indicated an important role of low gestational 25OHD on birth weight, preterm delivery and postnatal life. Additionally, data from the animal experiment demonstrated that low gestational 25OHD levels also elicit effects on developmental programming and cause an impairment of glucose tolerance in later life.

The molecular pathways causing fetal programming are linked to epigenetic modifications. With respect to vitamin D, it is known that vitamin D interacts with the epigenome on several different levels. Important genes involved in vitamin D signaling and metabolism, such as the vitamin D receptor and the enzymes 25-hydroxylase, 1α-hydroxylase and 24-hydroxylase possess large CpG islands in their promoter regions and can be regulated

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by DNA methylation [58]. Beyond that, multiple genes encoding for chromatin modifiers and remodelers, such as histone demethylases of the Jumonji C-domain-containing-proteins and lysine-specific demethylase families are primary targets of the vitamin D receptor and its ligands [58]. There is also evidence that certain VDR ligands evoke DNA demethylating effects [58].

The impact of low 25OHD levels on birth outcomes such as preterm delivery and birth weight is of medical and socioeconomic relevance given the large body of evidence that both, low birth weight and prematurity can alter disease risk in adult life [1, 2, 46-48]. Vitamin D deficiency during pregnancy leads to an exposure of the growing fetus to inadequate levels of vitamin D, which is an essential factor for a normal development. It is now well-established that the antecedents of cardiovascular disease can originate very early in life. Endocrine factors like maternal vitamin D deficiency as seen in the current study but also alterations of the fetal and maternal renin–angiotensin system have the potential to program long-term vulnerability to hypertension, metabolic- and cardiovascular disease [59, 60].

Conclusion

This study demonstrates that maternal 25OHD in human pregnancy is associated with offspring birth weight. The effect of 25OHD on birth weight is most likely mediated by vitamin D controlling gestational age. Results from an animal experiment suggest that gestational 25OHD insufficiency is causally linked to increased perinatal mortality and impaired glucose tolerance.

It is well established that both low birth weight and also prematurity are associated with an adverse renal and cardiovascular outcome in later life. Since 25OHD deficiency is a treatable condition, the current study emphasizes the need for novel monitoring and treatment guidelines of 25OHD during pregnancy to improve cardiovascular outcome in later life.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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