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Maternal vitamin D sufficiency and reduced placental gene expression in angiogenic biomarkers related to comorbidities of pregnancy

Elizabeth V. Schulz [a] ✉, Lori Cruze [b], Wei Wei [c], John Gehris [d], Carol L. Wagner [a]

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Highlights

- Definition of maternal 25(OH)D sufficiency during pregnancy is reviewed.

- Placental changes in vascular disorders of pregnancy are discussed.

- Sufficient 25(OH)D concentrations in relation to placental expression is presented.

- Vitamin D_3 supplementation impact on placental gene transcription is highlighted.

- Improving outcomes of suspected vascular disorders of pregnancy is offered.

Abstract

Introduction

Maternal circulating 25-hydroxyvitamin D [25(OH)D] has been shown to optimize production of 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] during pregnancy at approximately 100 nmoles/L, which has pronounced effects on fetal health outcomes. Additionally, associations are noted between low maternal 25(OH)D concentrations and vascular pregnancy complications, such as preeclampsia. To further elucidate the effects of vitamin D activity in pregnancy, we investigated the role of maternal 25(OH)D, the nutritional indicator of vitamin D status, in relation to placental maintenance and, specifically, expression of placental gene targets related to angiogenesis and vitamin D metabolism.

Methods

A focused analysis of placental mRNA expression related to angiogenesis, pregnancy maintenance, and vitamin D metabolism was conducted in placentas from 43 subjects enrolled in a randomized controlled trial supplementing 400 IU or 4400 IU of vitamin D_3 per day during pregnancy. Placental mRNA was isolated from biopsies within one hour of delivery, followed by quantitative PCR. We classified pregnant women with circulating concentrations of <100 nmoles/L as deficient and those with ≥100 nmoles/L as sufficient. The value of each gene's change in the PCR cycle threshold (ΔCT), which is a relative measure of target concentration, was compared with maternal 25(OH)D concentrations <100 nmoles/L and ≥100 nmoles/L based on a two-sample Wilcoxon test.

Results

Soluble FMS-like tyrosine kinase 1 (sFlt-1) and vascular endothelial growth factor (VEGF) gene expression was significantly downregulated in the maternal subgroup with circulating 25(OH)D ≥100 ng/mL compared to the subgroup <100 ng/mL.

Discussion

Here, we report a significant association between maternal vitamin D status and the expression of sFlt-1 and VEGF at the mRNA level. Achieving maternal circulating 25(OH)D ≥100 nmoles/L suggests the impact of maternal vitamin D_3 supplementation on gene transcription in the placenta, thereby potentially decreasing antiangiogenic factors that may contribute to vascular pregnancy complications.

Keywords

Vascular endothelial growth factor; Preeclampsia; sFlt-1; Vitamin D; Placenta

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1. Introduction

Pregnancy has been shown to be a critical life stage in which dietary supplementation with vitamin D appears to have a pronounced effect on fetal health outcomes, including a reduction in the risks of premature labor/birth, and additional maternal comorbidities such as gestational diabetes, hypertensive disorders, and infection [1–6]. Little is known, however, about the role of maternal vitamin D sufficiency *on the fetus* and its role in pregnancy protection/maintenance throughout gestation. In a recent NICHD-sponsored, 6-year randomized, double-blind, placebo-controlled trial investigating serum concentrations of active, hormonal vitamin D during pregnancy, circulating maternal 25(OH)D concentrations were found to be optimized at 100 nmol/L (40 ng/mL), which is twice the level normally observed in non-pregnant women [7]. In the present study, we aimed to examine the effect of maternal vitamin D status on hormones with vital roles in placental development and maintenance.

The association of lower vitamin D concentrations with non-cardiovascular disease demonstrates a diverse range of pathologies in observational studies, including infectious diseases, obesity, bone health, cancer and multiple sclerosis [8–10]. Likewise, there is extensive evidence from laboratory studies to suggest that vitamin D influences the vascular system, supported by observational studies in humans revealing the association of vitamin D insufficiency with increased arterial stiffness and endothelial dysfunction in the conductance and resistance of blood vessels [11]. As endothelial dysfunction is a hallmark of pregnancy complications that potentially lead to premature labor and delivery, such as preeclampsia, we hypothesized that maternal circulating vitamin D concentrations may affect the expression of an array of genes linked with angiogenesis and the potential for placental insufficiency secondary to its abnormal vasculature.

Consistent with this hypothesis, multiple studies have shown associations between low maternal 25(OH)D concentrations and the risk of preeclampsia [12–14]. Mirzakhani H, et al. [15] demonstrated in their study of over 800 participants that higher maternal circulating vitamin D concentrations both at the start of the study (first trimester) and in late pregnancy were associated with a lower risk of preeclampsia [15]. There is still little known, however, about the effects of maternal circulating vitamin D concentrations on the fetus/fetal tissue; although new and developing studies are attempting to combat the controversy of vitamin D's relation to pregnancy outcomes. Al-Garawi A, et al. [16] recently described the gene expression profiles of healthy pregnancy women in the *Vitamin D Antenatal Asthma Reduction Trial* (VDAART). The conclusions of this study suggest maternal vitamin D levels influence transcriptional profiles and these alterations of the maternal transcriptome may contribute to fetal immune imprinting [16]. It is proposed that these transcriptional changes may be related to pregnancy comorbidities, including vascular complications. Women with preeclampsia (a known significant vascular complication of pregnancy) are recognized to be at great risk for adverse pregnancy outcomes with a 20-fold increased risk for maternal mortality and several-fold higher risk for neonatal morbidity and mortality, depending on the gestational age at delivery and the presence of growth restriction in the fetus [17]. Therefore, the placenta was chosen, due to its innately high vascularization and as the interface between maternal and fetal tissue, to determine how maternal vitamin D status affects both maternal and fetal tissue on a molecular level.

Our study primarily investigated the role of maternal vitamin D status on placental expression of target genes related to vascular complications of pregnancy; however, a total of three groups of target genes were chosen based on previously reported functions within the placenta. The first group chosen has critical function in the angiogenesis pathway related to pregnancy, and included vascular endothelial growth factor (VEGF), placental growth factor (PGF), and soluble fms-like tyrosine kinase 1 (sFlt-1). Additionally, regulatory genes known for their role in placental (and thus pregnancy) maintenance, which included progesterone receptor B (PRB), estrogen receptor 1 (ESR1), human chorionic gonadotropin β (hCGβ), and human placental

lactogen (hPL) were investigated. Finally, genes related to vitamin D metabolism, including vitamin D receptor (VDR), glucocorticoid receptor (GRα, aka NR3C1), 24-hydroxylase (CYP24A1), and CYP27B1 were evaluated. Of note, CYP27B1 is responsible for the production of $1,25(OH)_2D$ from $25(OH)D$, while CYP24A1 catalyzes the conversion of $1,25(OH)_2D$ into 24-hydroxlated products, constituting the degradation of the vitamin D molecule [18]. We hypothesized that increased mRNA expression of proangiogenic genes and decreased mRNA expression of antiangiogenic factors would be observed in vitamin D sufficient women compared to vitamin D deficient women.

2. Methods

2.1. Study design

This study was part of a randomized, placebo-controlled clinical trial (NCT 01932788) in which women provided informed consent and were followed from time of enrollment through delivery. The Institutional Review Board at the Medical University of South Carolina approved this study protocol (Pro 00020570). Enrolled mothers were 18–45 years of age who presented at 8-14 weeks' gestation with a singleton pregnancy. Exclusion criteria included: pre-existing calcium or uncontrolled thyroid/parathyroid disease, requiring chronic diuretic/cardiac medications, sickle cell disease, sarcoidosis, and inflammatory bowel disease. Mothers were randomized to receive placebo or 4000 IU/day vitamin D_3 plus the standard prenatal vitamin (containing 400 IU vitamin D_3). Mothers were followed monthly through delivery, which coincided with a total of six to seven visits prior to delivery, at which time placental biopsies were obtained. Of note, the treatment group was not known at the time of sample analysis due to study blinding. Table 1 highlights demographic data for the subjects whose placentas were utilized for the following study. Again, these forty-three women and their placentas were studied as a subset of the larger ongoing RCT, in which long term follow-up for the children of these pregnancies continues.

Table 1. Comparison of maternal and infant characteristics of women classified as 25(OH)D sufficient (≥100 nmoles/L) or deficient (<100 nmoles/L) prior to delivery.

	Maternal 25(OH)D <100 nmoles/L	Maternal 25(OH)D ≥100 nmoles/L	Total	p-value*
	N (%)	N (%)	N (%)	
Ethnicity				0.04
African American	5 (11.6)	6 (13.9)	11 (25.6)	
Hispanic	6 (13.9)	11 (25.6)	17 (39.5)	
Caucasian	1 (2.3)	13 (20.2)	14 (32.5)	

American Indian	1 (2.3)	0 (0.0)	1 (2.3)	
Insurance				0.10
Private	2 (4.7)	13 (30.2)	15 (34.9)	
Medicaid	7 (16.3)	7 (16.3)	14 (32.6)	
Self-Pay	4 (9.3)	10 (23.3)	14 (32.6)	
Marital Status				0.76
Single	4 (9.3)	5 (11.6)	9 (20.9)	
Married	6 (13.9)	18 (41.9)	24 (55.8)	
Cohabitating	3 (7.0)	7 (16.3)	10 (23.3)	
	Mean ± SD	Mean ± SD	Mean ± SD	
Maternal age (years)	36.9 ± 4.2	28.9 ± 4.2	28.3 ± 4.3	0.31
Maternal BMI	30.3 ± 8.2	28.7 ± 8.1	29.2 ± 8.0	0.56
Maternal 25(OH)D baseline (ng/mL)	58.4 ± 22.7	71.9 ± 23.7	67.9 ± 23.9	0.10
Maternal 25(OH)D V6/7 (ng/mL)	60.9 ± 26.2	140.5 ± 31.2	116.6 ± 47.2	
Infant gestational age (weeks)	39.2 ± 1.2	38.5 ± 1.8	38.7 ± 1.7	0.24
Infant birth weight (grams)	3381 ± 562	3363 ± 517	3369 ± 524	0.64

*p-values are included to identify the association between maternal vitamin D status and other demographic variables of interest. Of note, African Americans and Hispanics are more likely to be vitamin D deficient (p = 0.043). The type of insurance (p = 0.104), maternal marriage status (p = 0.759), maternal age (p = 0.306), gestational age (p = 0.239), baseline 25(OH)D (p = 0.096), maternal BMI (p = 0.563), birthweight (p = 0.644) were not significantly different between the vitamin D sufficient and vitamin D deficient patients.

The maternal serum 25(OH)D concentrations obtained at the visit prior to delivery, either study visit 6 or 7 (abbreviated as V6/7), were separated into two categories: those <100 nmol/L (<40 ng/mL) and those ≥100 nmol/L (≥40 ng/mL). Based on optimized conversion of 25(OH)D to 1,25(OH)$_2$D at ≥100 nmol/L, mothers with total circulating 25(OH)D concentrations <100 nmol/L were defined as "deficient" and those with concentrations ≥100 nmol/L were defined as "sufficient" [1,7]. As is standard in clinical practice, the 25(OH)D concentration was utilized for serum measurements as it provides the best estimate of a patient's vitamin D status. We chose to study the differences between the two groups based on their 25(OH)D concentrations, rather than their randomized assignment to placebo vs treatment group secondary to the fact that our study participants remain blinded. As this study was a sub-aim of the RCT, the presented information will be released prior to unblinding of the overarching trial.

2.2. Placenta collection

From November 2013 through June 2014, placentae were collected, stored at 4 °C, and sampled within 1 h of delivery (n = 43). A convenience sample of forty three placentas were available from the larger overarching study cohort, due to the processing time limitation at delivery (within 1 h). Each placenta was sampled at four different sites using a standard biopsy punch (3 mm). Biopsy sites were approximately 5 cm from the umbilical cord insertion, 2 cm from each other, and avoided the placental edge, fibrous knots and large blood vessels. Each biopsy was a full thickness sample taken from the maternal through the fetal side of the placenta. Biopsies were washed briefly in 1X phosphate buffered saline (PBS), immediately placed in RNA*later* solution (Thermo Fisher Scientific, Waltham, MA) at 4 °C and stored at −20 °C until RNA isolation. The protocol was modified from those of Wyatt et al. and Pidoux et al. [19,20].

2.3. RNA isolation, reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from each biopsy with TRIzol reagent (Invitrogen Life Technologies, Waltham, MA) and purified with the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA was treated with ribonuclease-free deoxyribonuclease I (DNase I; Qiagen, Venlo, Netherlands) to remove any genomic DNA contamination. Concentrations and quality of RNA samples were evaluated by measuring optical density with a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) and by formaldehyde gel electrophoresis. One microgram (μg) of total RNA for the placental biopsy samples passing the RNA quality check were pooled for each subject and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Complementary DNA (cDNA) was stored at −20 °C until RT-qPCR analysis.

RT-qPCR analysis was performed on biopsy samples. Complementary DNA was analyzed in triplicate by RT-qPCR amplification using an iCycler MyIQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each 15-μL DNA amplification reaction contained 10 mM Tris-HCl (pH 7.84), 50 mM KCl, 3 mM MgCl$_2$, 200 μM dNTPs, 0.5% Tween-20, 0.8%

glycerol, 2% DMSO, 200-fold dilution of SYBR Green (Invitrogen, Waltham, MA), 0.01 µM Fluorescein Calibration Dye (Bio-Rad, Hercules, CA), 0.2 µM of each primer, 0.01 U/µL AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 2-times diluted cDNA. RT-qPCR amplification conditions included an enzyme activation step of 95 °C (5 min) followed by 30-45 cycles of 95 °C (15 s) and a primer specific combined annealing/extension temperature (30 s). Based on primer optimization runs, 30 cycles were utilized for expression data for the target gene hPL but otherwise 45 cycles of 95 °C (15 s) were utilized for all other target genes. The specificity of amplification was confirmed by the melt-curve analysis.

Triplicate data for each gene were averaged and mRNA expression levels were determined by the comparative C_T method (also known as the $2^{-\Delta\Delta CT}$ method, or for individual data points $2^{-\Delta CT}$). Primer sequences, annealing/extension temperatures and GenBank accession numbers are reported in Table 2.

Table 2. Target Gene Data.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Annealing/extension temp (°C)
VEGF	CTTGCTGCTCTACCTCCACCAT	ATGATTCTGCCCTCCTCCTTCT	62
PGF	CCTACGTGGAGCTGACGTTCT	TCCTTTCCGGCTTCATCTTCT	60
sFlt1	GGGAAGAAATCCTCCAGAAGAAAGA	GAGATCCGAGAGAAAACAGCCTTT	64
PRB	TCGGACACCTTGCCTGAAGT	CAGGGCCGAGGGAAGAGTAG	60
ESR1	AGATCTTCGACATGCTGCTGGCTA	AGACTTCAGGGTGCTGGACAGAAA	67.6
hCGβ	CTACTGCCCCACCATGACCC	TGGACTCGAAGCGCACATC	63.2
hPL	GCATGACTCCCAGACCTCCT	TGCGGAGCAGCTCTAGATTGG	63.2
CYP24A1	CATCATGGCCATCAAAACAAT	GCAGCTCGACTGGAGTGAC	60
CYP27B1	CACCCGACACGGAGACCTT	TCAACAGCGTGGACACAAACA	60
GRα (NR3C1)	CTTGGATTCTATGCATGAAGTGGTT	TTGGAAGCAATAGTTAAGGAGATTTTC	61.6
VDR	TGGCTTTCACTTCAATGCTATGA	CGTCGGTTGTCCTTGGTGAT	62

2.4. Statistical analysis

NormFinder software (Aarhus, Denmark) was utilized to identify the normalization gene out of the internal control data [21]. GAPDH was identified as having the greatest stability value. Allowing for ease of presentation as well as the ability to present the data as 'fold change' in expression (see Eq. (1)), the aforementioned ΔCT quantification method was used. The ΔCT was identified as the difference between the average cycle threshold of the target gene and the internal control (GAPDH). To be consistent, analyses were based on analyzing Real time PCR data based on ΔCT supported by Yuan et al. [22]. This equation and its derivation have been previously reported in Applied Biosystems User Bulletin No. 2 (P/N 4303859) [23].

We applied the $2^{-\Delta CT}$ for analysis to correlate the individual data points, rather than compare between two treatment groups, as the $\Delta\Delta CT$ equation is typically utilized. By this method, we were able to compare gene expression in two different samples (vitamin D deficient vs sufficient mothers) and relate this to an internal control gene (GAPDH).

$$\text{Foldchange} \begin{aligned} &= 2^{-\Delta CT} \\ &= 2^{-(CTt\ arg\ etgene - CT\ int\ ernalcontrol)} \end{aligned}$$

By using the above equation/method for analysis, the data may be interpreted as the expression of the target gene relative to the internal control gene. The data were then separated into the two categories of maternal vitamin D status (i.e. deficient vs sufficient) for comparative purposes. Demographics between the vitamin D sufficient and vitamin D deficient mothers were compared based on two-sample Wilcoxon test and Fisher's Exact test (see Table 1). The expression of the eleven genes (ΔCT values) were compared between the vitamin D sufficient and vitamin D deficient mothers based on two-sample Wilcoxon test. ΔCT values for the vitamin D sufficient and vitamin D deficient group were summarized using their median and inter quantile range (IQR). The associations between 25(OH)D concentration and gene expression were examined using Spearman's rank correlation test. Multiple regression models were performed by regressing ΔCT values on 25(OH)D concentration, maternal age, BMI and race/ethnicity for each of the eleven genes. Data were analyzed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Effect of maternal 25(OH)D concentrations on angiogenic factors (VEGF, PGF and sFlt-1)

Thirteen women were defined as vitamin D deficient and thirty as vitamin D sufficient at V6/7 (see Table 1). The results of the two-sample Wilcoxon test revealed the ΔCT values for VEGF and sFlt-1 are significantly different between the vitamin D <100 nmol/L and the vitamin D ≥100 nmol/L group at 0.05 level.

For VEGF, the median values are 2.7 (IQR: 2.2, 3.0) and 3.6 (IQR: 2.9, 4.6) in the vitamin D <100 nmol/L group and the vitamin D >100 nmol/L group, respectively. For sFlt-1, the median ΔCT values are −3.7 (IQR: −4.1, −3.6) and −2.7 (IQR: −3.6, −1.9) in the vitamin D <100 nmol/L group and the vitamin D>100 nmol/L group, respectively.

The expression of sFlt-1, PRB, hPL, and GRα were also associated with mother's baseline vitamin D status. At baseline, thirty-six women were vitamin D deficient and seven women were vitamin D sufficient. A two-sample Wilcoxon test revealed the values for sFlt-1, PRB, hPL and GRα are significantly different between the vitamin D <100 nmol/L and the vitamin D ≥100 nmol/L group at 0.05 level. For sFlt-1, the median ΔCT values are −3.6 (IQR: −4.0, −2.5) and −1.6 (IQR: −2.5,-1.2) in the vitamin D <100 nmol/L group and the vitamin D>100 nmol/L group at V1, respectively. Spearman's rank correlation tests revealed that higher baseline 25(OH)D concentration is associated with higher ΔCT values of VEGF ($r = 0.30$, p = 0.050), PGF ($r = 0.30$, p = 0.047), sFlt-1 ($r = 0.31$,p = 0.041), PRB ($r = 0.36$, p = 0.017) and GRα ($r = 0.31$, p = 0.045). Table 3 summarizes each gene's values in the vitamin D deficient and sufficient group based on their median and interquartile range [(IQR) representing the 25th and 75th percentiles].

Table 3. The effect of vitamin D deficiency[*] on placenta gene expression (ΔCT).

|	Vitamin D deficient at V1 Median(IQR)	Vitamin D sufficient at V1 Median(IQR)	p-value	Vitamin D deficient at V6/7 Median(IQR)	Vitamin D sufficient at V6/7 Median(IQR)	p-value
VEGF	3.1(2.2,4.0)	4.3(3.6,5.0)	0.068	2.7(2.2,3.0)	3.6(2.9,4.6)	**0.014**
PGF	−3.5(−4.0,-2.4)	−2.3(−2.6,-0.5)	0.056	−3.4(−4.0,-2.9)	−2.8(−3.9,-1.7)	0.191
sFlt-1	−3.6(−4.0,-2.5)	−1.6(−2.5,-1.2)	**0.010**	−3.7(−4.1,-3.6)	−2.7(−3.6,-1.9)	**0.008**
PRB	7.8(6.7,9.0)	10.0(9.2,10.7)	**0.021**	7.3(6.5,8.5)	8.7(7.1,9.5)	0.113
ESR1	7.9(7.4,8.5)	9.4(8.3,10.0)	0.069	7.6(6.8,8.3)	8.3(7.5,9.3)	0.061
hCGb	−6.3(−7.6,-5.5)	−4.5(−5.9,-3.8)	0.065	−6.4(−7.2,-6.0)	−5.9(−7.6,-4.6)	0.355
hPL	−14.4(−14.9,-13.3)	−13.4(−13.8,-12.7)	**0.031**	−13.7(−14.6,-13.4)	−14.2(−14.9,-13.2)	0.509
CYP24A1	5.6(4.5,6.3)	7.0(5.3,7.5)	0.237	5.6(5.0,6.4)	5.8(4.7,7.0)	0.765
CYP27B1	7.7(6.8,8.5)	8.3(8.0,10.0)	0.097	7.6(6.7,8.4)	8.1(7.0,9.0)	0.451
GRα	0.6(−0.5,1.2)	2.0(1.8,2.2)	**0.022**	0.2(−1.3,1.1)	1.0(−0.1,2.0)	0.128
VDR	7.4(6.9,8.4)	8.6(7.3,8.9)	0.236	7.2(7.1,8.2)	7.7(6.9,8.5)	0.853

[*]25(OH)D <100 nmol/L.

Additionally, we analyzed the fold change in expression for each of these angiogenic factors. The mean(sd) for PGF of $2^{-\Delta CT}$ (vitamin D deficient group) is 12.5(7.4) and the mean(sd) $2^{-\Delta CT}$ (vitamin D sufficient group) is 11.5(11.5). When compared to the vitamin D deficient

group, the mothers who were vitamin D sufficient demonstrated a downregulation in PGF by 1.1 fold. There is no statistically significant difference in PGF expression between the two groups ($pval = 0.72$).

For VEGF, however, the mean(sd) of $2^{-\Delta CT}$ (vitamin D deficient group) is 0.20(0.12) and the mean(sd) of $2^{-\Delta CT}$ (vitamin D sufficient group) is 0.11(0.10). When compared to the vitamin D deficient group, the mothers who were vitamin D sufficient demonstrated a downregulation in VEGF by 1.7 fold. There is a significant difference in VEGF expression between the two groups ($pval = 0.04$) at a significance level of 0.05.

Likewise, the mean(sd) of $2^{-\Delta CT}$ (vitamin D deficient group) is 15.6(8.8) and the mean(sd) of $2^{-\Delta CT}$ (vitamin D sufficient group) is 9.2(8.1) for sFlt-1. When compared to the vitamin D deficient group, the mothers who were vitamin D sufficient demonstrated a downregulation in sFlt-1 by 1.7 fold. There is a significant difference in sFlt-1 expression between the two groups ($pval = 0.03$) at a significance level of 0.05.

Fig. 1 demonstrates the fold change conversion for each target gene as compared with the vitamin D sufficiency. In other words, the mean ΔCT values of each target gene in the vitamin D deficient group were obtained and the mean ΔCT values of each target gene in the vitamin D sufficient group were obtained; the two groups were then compared (based on fold change analysis) to see if a mother's status (i.e. vitamin D sufficiency) could be correlated with decreased or increased expression of that gene (i.e. up/downregulation).
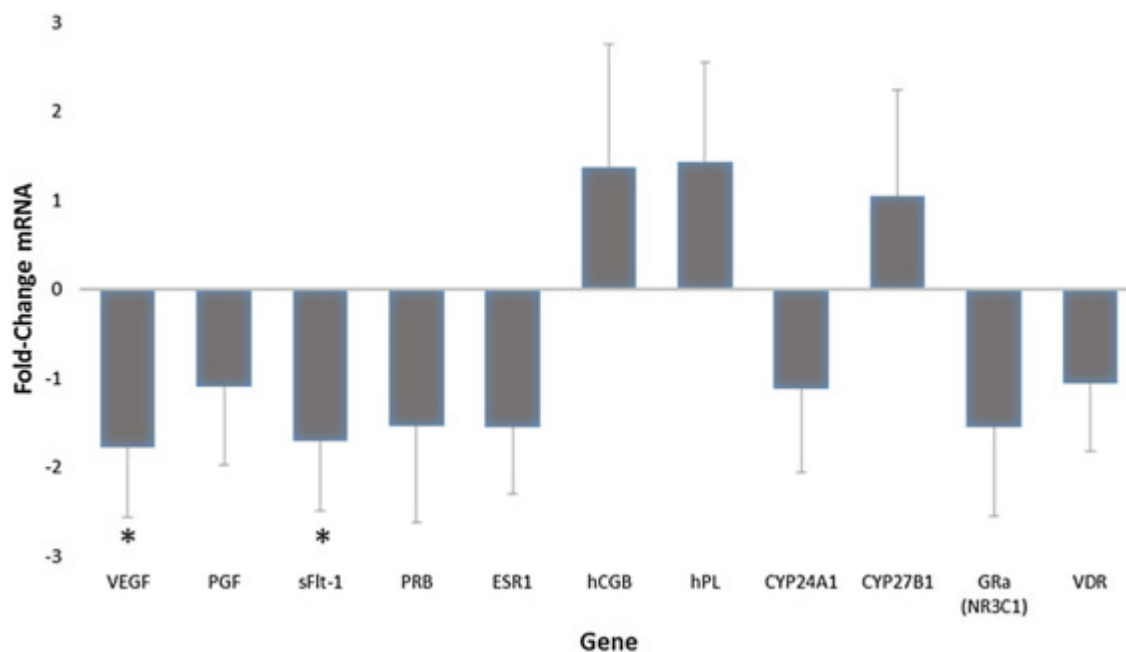
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Fig. 1. Placental target gene expression in vitamin D sufficient cohort as compared to deficient women. Calculated fold change difference between vitamin D deficient vs sufficient groups. Shown negative values

indicate downregulation and positive values indicate upregulation in women who were sufficient. Comparatively, vitamin D sufficiency resulted in downregulation of all but three of the genes tested. Results represent the fold change +/− standard deviation of each target gene. Genes with significant results are shown with an asterisk (*).

The effect of baseline 25(OH)D on placental gene expression was examined in multiple regression models adjusting for maternal age, BMI and race/ethnicity. As shown by Table 4, the concentration of baseline 25(OH)D is associated with VEGF expression after considering other variables in the model at a significance level of 0.05. Baseline 25(OH)D is not significantly associated with the expression of other genes we considered after adjusting for maternal age, maternal baseline BMI and race/ethnicity. The concentration of 25(OH)D at V6/7 is not associated with any of the eleven genes we considered at a significance level of 0.05, adjusting the effect of maternal age, maternal baseline BMI and race/ethnicity.

Table 4. The effect of baseline 25(OH)D concentration on VEGF expression adjusted for maternal age, maternal baseline BMI and maternal race/ethnicity.

Variables	Estimate	Std. Error	p-value
25(OH)D at baseline	0.05	0.02	0.048
Maternal Age	0.00	0.04	0.921
Maternal baseline BMI	0.01	0.03	0.584
African American		Reference	
Caucasian	0.67	0.53	0.218
Hispanic	0.94	0.47	0.055

While there is expected to be some baseline level of upregulation in many of these target genes during pregnancy and higher expression of some of these target genes (VEGF and sFlt-1) in vascular disorders of pregnancy, the maternal serum 25(OH)D status (i.e. sufficiency) appears to have a significant effect on downregulating the overall expression of these genes as compared to deficient women. There was a small, although not significant, decrease in PGF expression in the sufficient group.

3.2. Effect of maternal 25(OH)D concentrations on placental maintenance (PRB, ESR1, hCGβ, hPL) and vitamin D metabolism (CYP24A1, CYP27B1, GRα, VDR)

As shown in Table 3, the results of the analysis did reveal a significant association between baseline (V1) 25(OH)D concentrations and certain genes related to placental maintenance, to include PRB (pval = 0.02) and hPL (pval = 0.03), as well as GRα (pval = 0.02), whose role is

known in vitamin D metabolism. However, the analysis did not reveal a significant difference for the mRNA expression of ESR1 or hCGβ between the group with circulating 25(OH)D <100 nmol/L vs. ≥100 nmol/L group. Likewise, no significant difference was found in other specific genes involved in vitamin D metabolism, including CYP24A1, CYP27B1, or VDR.

4. Discussion

The major finding of our study was a statistically significant association between maternal vitamin D sufficiency (as defined by a total circulating 25(OH)D concentration >40 ng/mL) and decreased expression (downregulation) of VEGF and sFlt-1 as compared to the expression in deficient mothers, which is the first report of this association. Notably, maternal vitamin D sufficiency at baseline and at the last visit prior to delivery (V6/7) were both associated with decreased expression of sFlt-1 at 0.05 level. Maternal vitamin D sufficiency at baseline and at last visit were also associated with decreased expression of VEGF. The expression of PRB, hPL and GRα were also significantly associated with vitamin D deficiency at baseline. We also observed an association between baseline maternal 25(OH)D concentrations and the expression of VEGF, PGF, sFlt-1, PRB and GRα at a significance level of 0.05.

Due to our limited sample size, none of these associations were significant after the Bonferroni adjustment for multiple testing, however, our results can still provide insights regarding the role of vitamin D in placenta gene regulation. Also, the Bonferroni adjustment is known to be conservative especially when the tests are correlated. In our paper, we found significant correlations among the expression of the eleven genes we considered. For example, the expression of sFlt-1 was associated with GRα ($r = 0.77$, $p < 0.001$) and the expression of VEGF was associated with PRB ($r = 0.80$, $p < 0.001$). Therefore, the Bonferroni approach will lead to overly conservative results for our study.

We can reason from this data set that if our mothers were vitamin D sufficient (≥100 nmol/L), their expression of sFlt-1 was lower than the expression of sFlt-1 in those mothers who were vitamin D deficient. This reduction in expression, with maternal vitamin D sufficiency, suggests the ability to potentially decrease sFlt-1 expression during pregnancy. Reduction in sFlt-1 demonstrates the potential for vitamin D_3's role in decreasing antiangiogenic factors that may contribute to vascular pregnancy complications, such as preeclampsia.

The association between high levels of sFlt-1 and maternal development of preeclampsia has been well demonstrated in both laboratory and clinical studies [24–29]. As an antiangiogenic factor, sFlt-1 is known to cause vasoconstriction and endothelial damage in the placenta, thereby inducing a cascade of placental insufficiency, intrauterine fetal growth restriction and vascular comorbidities of pregnancy, such as preeclampsia. Likewise, PGF is often decreased in these disease processes, leading to the well cited relationship and diagnostic predictive value of the sFlt-1/PGF ratio. A higher ratio of sFlt-1 to PGF has been associated with an increased risk of preeclampsia [30–33].

Despite a small and insignificant decrease in the overall expression of PGF in women who were vitamin D sufficient compared to those who were deficient, the results of our study yielded evidence to support a relatively high expression in PGF in this cohort. More importantly, our study revealed the potential at sufficient concentrations of circulating 25(OH)D ≥100 nmol/L to reduce gene expression in sFlt-1, which may provide important clinical data to warrant maternal 25(OH)D monitoring/optimization throughout pregnancy—especially in women predisposed to vascular pregnancy complications. Our results revealed decreased expression of VEGF in women who were considered vitamin D sufficient, which has previously not been described. This may reflect a lower need for new vasculature toward the end of pregnancy/third trimester, as increased vascularization and expression of VEGF is most important in early gestation [34].

This was a candidate gene study, based on existing evidence of the roles of the selected genes in the biological pathways of vitamin D regulation, placental maintenance and vascular complications of pregnancy. Of course, there are limitations to the subjective nature of the selection of these genes secondary to our team's specific interests, as there are a host of additional genes that could have been chosen for analysis. Further investigation of the role of vitamin D supplementation on placental vascular development and regulation throughout pregnancy is warranted in a larger population, as the independent contribution of a single gene, as compared to the influence and contribution of many other genes, is difficult to assess in this small sample size. However, our genes of interest and the results we obtained provide the foundation for future studies to expound on the effects of maternal 25(OH)D concentrations in relation to the development of vascular compromise/complications of pregnancy.

In terms of clinical limitations of this study, patient compliance/variation of vitamin D concentrations are innate to the study protocol. Each mother in the study obtained a pill count at each individual appointment throughout the course of the study. If the study individuals were not at least 75% compliant with their supplement, they were exited from the study. As the study remains blinded, treatment allocation remains unknown; however, maternal circulating 25(OH)D concentrations were measured on a monthly basis for safety reasons and are the best indicator of vitamin D status [35].

Additionally, placental biopsy sampling yields a possible laboratory limitation. Pidoux et al. describes the intra- and inter-placental variability that may occur as a result of full thickness placental biopsies [20]. This was a key reason for the specifications of biopsy sampling sites among our population; however, the limits of processing biopsies that encompass both maternal and fetal tissue remain. As each placental sample was frozen, stored and homogenized using the same method, it is unlikely that this added to the potential discrepancy of mRNA expression variability, especially amongst those genes for which mRNA expression was not statistically significant between the two maternal groups. It is unclear whether no (or little) change in mRNA expression could indicate an increase on the maternal tissue but a decrease on the fetal side, or vice versa. While there are reports of histological separation of

placental tissue at the fetal and maternal interface, to our knowledge technology does not yet exist for gross sample separation.

While this study revealed an early molecular basis in favor of vitamin D sufficiency during pregnancy, there is a great deal of work to be done for clinical relevancy. For example, protein analysis is needed to corroborate the mRNA results. Additionally, we expect the results of the overarching clinical study to provide a wealth of knowledge as to the maternal and neonatal outcomes of the supplemented mothers. Two of the women, out of the total 43 we observed, experienced preeclampsia with resultant preterm deliveries at 35 weeks and 36 + 3 weeks, respectively. While the sample size of this study is large enough to detect placental gene expression changes, it was not large enough to detect associative gene expression changes *with clinical outcome*.

4.1. Conclusions

Our data may serve to demonstrate the impact of maternal vitamin D_3 supplementation on gene transcription in the placenta. The results are suggestive of the possibility to modify placental gene expression to improve placental health for those women at risk for suspected vascular disorders of pregnancy. This is the first step to further elucidate the role of maternal supplementation with vitamin D_3 in factors relating to placental health.

Author contributions

E. Schulz, L. Cruze, and C. Wagner were responsible for designing and carrying out the experiments. J. Gehris assisted in carrying out the early laboratory preparatory work and experiments. W. Wei analyzed and interpreted the data. E. Schulz and C. Wagner further interpreted the data and drafted the manuscript.

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