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Maternal 25-hydroxyvitamin D is inversely correlated with fetal serotonin.

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Abstract

Objective: Maternal vitamin D deficiency during pregnancy has been linked to impaired neurocognitive development in childhood. The mechanism by which vitamin D affects childhood neurocognition is unclear but may be via interactions with serotonin, a neurotransmitter involved in fetal brain development. In this study we aimed to explore associations between maternal and fetal vitamin D concentrations, and fetal serotonin concentrations at term.

Study design and measurements: Serum 25-hydroxyitamin D (25(OH)D, nmol/L) and serotonin (5-HT, nmol/L) concentrations were measured in maternal and umbilical cord blood from mother-infant pairs (n=64). Association between maternal 25(OH)D, cord 25(OH)D and cord serotonin was explored using linear regression, before and after adjusting for maternal serotonin levels. We also assessed the effects of siRNA knockdown of the vitamin D receptor (VDR) and administration of 10nM 1,25-dihydroxyvitamin D_3 on serotonin secretion in human umbilical vein endothelial cells (HUVECs) *in vitro*.

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Results: We observed an inverse relationship between both maternal and cord 25(OH)D concentrations with cord serotonin concentrations. The treatment of HUVECs with 1,25-dihydroxyvitamin D_3 *in vitro* decreased the release of serotonin (193·9±14·8 nmol/L vs. 458·9±317·5 nmol/L, control, $p<0·05$). Conversely,inactivation of VDR increased serotonin release in cultured HUVECs.

Conclusions: These observations provide the first evidence of an inverse relationship between maternal 25(OH)D and fetal serotonin concentrations. We propose that maternal vitamin D deficiency increases fetal serotonin concentrations and thereby contributes to longer-term neurocognitive impairment in infants and children.

Introduction:

Vitamin D deficiency during pregnancy, at least as defined by serum vitamin D levels (<50nmol/L) is endemic, present in ~20-85% of women, depending on country of residence, latitude, season, and other factors[1-3]. This may be important because maternal vitamin D deficiency has been linked with poorer health outcomes for both mother and baby. For example, vitamin D deficiency during pregnancy has been associated with increased risks of gestational diabetes (GDM), preeclampsia (PE), low birth weight, abnormal fetal skeletal development, pre-term birth, caesarean section and post-partum depression[4-9]. However, most recently, the associations between maternal vitamin D deficiency and adverse pregnancy outcomes have looked less convincing[10, 11]. What has remained consistent is the observation that maternal vitamin D status is associated with later childhood outcomes including diabetes, atopy and, in particular, learning outcomes[12-16].

Of particular concern is the growing evidence that vitamin D deficiency in early life, including *in utero*, may be linked to adverse neurodevelopmental and cognitive impairment[16-19]. For example, in a recent study of 363 infant mother pairs, from the China-Anhui Birth Cohort Study, the toddlers who were in the lowest quintile of cord blood 25(OH)D level had significantly lower mental and psychomotor development scores as assessed using the Bayley Scales of Infant Development than other toddlers[19]. Similarly, among 910 mother-offspring pairs from the Western Australian Pregnancy Cohort [Raine] Study, maternal vitamin D deficiency in mid-pregnancy was also associated with increased rates of neurocognitive difficulties and eating disorders at the age of 10[18]. In a large study of 960 women-infant pairs in rural Vietnam Hanieh *et al.* (2014) reported infants born to women who were vitamin D deficient in late pregnancy had significantly lower developmental language scores compared with those born to women who were sufficient for vitamin D[17].

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Despite these observational reports a biologically plausible mechanism whereby vitamin D might influence fetal neurocognitive development has not been obvious. That said, it is known that vitamin D has a number of neurological actions. It can activate spontaneous neuronal firing, modulate action potential duration, and increased intrinsic excitability and sensitivity to neurotransmitters as well as to neurotransmitter receptors such as GABA receptor and N-methyl-D-aspartate (NMDA) receptor[20]. Vitamin D can also activate a variety of signal transduction systems including calcium ion influx, the release of calcium ions from intracellular stores, the modulation of adenylate cyclase, phospholipase C (PLC), protein kinase C, protein kinase D, the mitogen-activated protein (MAP) kinases, and the rapidly accelerated fibrosarcoma (Raf) kinase pathways[21, 22]. It also regulates the actions of a variety of neurotransmitters including acetylcholine[23], dopamine[24], and serotonin[25].

In particular, vitamin D can decrease the synthesis of serotonin in the brain[25-27]. This is likely to be important in fetal brain development because serotonin is a key modulator of neuronal cell proliferation, migration, and brain wiring during fetal and early postnatal life[28, 29]. Disruption of the serotonin signaling system results in abnormal cortical development and neuronal migration in animals[29] and serotonin is associated with a number of neuropsychiatric disorders, such as schizophrenia[30], affective disorders[31], anxiety[32], and autism in humans[25, 26, 33]. Serotonin is synthesized in two steps from tryptophan, an essential amino acid present in small amounts in dietary protein. Two distinct tryptophan hydroxylase (TPH) enzymes encoded by two different tissue specific genes, *TPH1* and *TPH2*, regulate this process. Vitamin D regulates the transcription of *TPH* both by gene activation and repression[26, 33]. If maternal vitamin D deficiency led to increased fetal serotonin levels then that would offer a plausible mechanism linking vitamin D deficiency with impaired neurocognitive development. To our knowledge there have been no human studies exploring the association between maternal and fetal vitamin D levels and fetal serotonin levels.

In planning this study, we hypothesized that (1) maternal vitamin D is inversely correlated with fetal serotonin concentrations, and (2) vitamin D decreases serotonin release in the fetal endothelium through a vitamin D receptor (VDR) dependent mechanism. To address these we set out to a) to determine the association between maternal serum 25(OH)D and fetal serotonin in mother-infant pairs collected from term uncomplicated pregnancies, and b) to investigate the mechanism by which maternal vitamin D may impact on fetal serotonin release using an *in vitro* model of cultured primary human umbilical vein endothelial cells

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(HUVECs).

Materials and Methods

Study Participants-Mother-Infant Pairs

We studied 64 mother-infant pairs from uncomplicated term pregnancies undergoing elective caesarean birth at Monash Medical Centre, Clayton and the Mercy Hospital for Women, Heidelberg, both in Victoria, Australia. Pregnancies from assisted reproductive technology, multiple gestations, or with a history of pregnancy complications (including preeclampsia, preterm birth, fetal growth restriction, high body mass-index (BMI>30), diabetes mellitus, hypertension, abnormal heart function, intrahepatic cholestasis of pregnancy, and moderate or severe anemia), smoking, alcohol consumption, chemical dependency (including use of selective serotonin reuptake inhibitors), stillbirth, or congenital abnormalities were excluded from the study. Maternal and infant information (maternal age, parity, maternal country of birth, baby gender, gestation and birth weight) were collected. Matching maternal and umbilical cord blood was obtained at the time of caesarean section for the subsequent measurement of serum 25(OH)D and serotonin. All women gave written and informed consent and with the approval of the Monash Health and Mercy Hospital Human Research Ethics Committees.

In vitro studies

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from uncomplicated term pregnancies (n=12) and cultured as previously described[34]. Briefly, cells were cultured and maintained in M199 tissue culture medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100□g/mL streptomycin and maintained in 5%CO_2. HUVECs were treated with 1,25$(OH)_2D_3$ (Calcitriol, Tocris Bioscience, Bristol, UK) prepared in 0·01% ethanol or vehicle control (0·01% ethanol as control) for 48h. At the end of the incubation period the conditioned media was collected for the measurement of serotonin. To silence *VDR* expression, cultured HUVECs were transfected with two independent Ambion® *VDR* siRNAs, referred to as si1 and si2 (Thermo Fisher Scientific, Waltham, MA, USA), using RNAiFect transfection reagent (Qiagen, Hilden, Germany) as described previously[35]. HUVECs were transfected with 100nM of siRNA for 48h. Non-targeting siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) served as a negative control (NC) for transfection experiments, whilst cells treated with transfection reagent only was used as a technical

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control (Mock control, MC). The effect of various concentrations of 1,25$(OH)_2D_3$ including 2.5nM, 5nM, 10nM and 20nM treatment of HUVEC on cell viability over 24h in culture was determined using a colorimetric CellTitre 96® MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide, Promega, Australia) assay as previously described (Murthi et al., 2016)[36], and the absorbance was read at 570nm on a Spectramax L Luminescence plate reader (Molecular Devices, Australia).

Measurement of 25(OH)D *and Serotonin*

Serum concentrations of 25(OH)D (nmol/L) were measured using the DiaSorin 25(OH)D immunoassay on a Liaison analyser (Stillwater, MN). The intra-assay coefficient of variation (CV) was 9%. Serotonin, also referred to as 5-hydroxy tryptophan (5HT, nmol/L), was measured in maternal, cord sera and in the cultured media of the HUVEC cells using an enzyme-linked immunosorbent 5HT assay (ENZO Life Sciences, New York, USA). The intra-assay CV was 5·2%.

Real-time PCR

Total RNA from cultured HUVECs was extracted using the RNeasy Minikit as previously described [37]. cDNA was prepared from 2□g total RNA reverse-transcribed using Superscript III ribonuclease H-reverse transcriptase (Invitrogen, Australia) in a two-step reaction also as previously described[37, 38]. *VDR* and *TPH1* mRNA expression was determined using validated assays that consisted of a TaqMan® FAM™ labelled MGB probes (Thermo Fisher Scientific, USA) on an ABI Prism 7500 (Thermo Fisher Scientific, USA). Gene expression quantitation was performed as the second step in a two-step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) protocol according to the manufacturer's instructions. Gene expression quantitation for the housekeeping gene *18S* rRNA (VIC-labelled probe, Thermo Fisher Scientific, USA) was performed in the same reaction as described previously[37-40] and the gene expression differences was calculated according to the $2^{-\Delta\Delta CT}$ method [41].

Immunoblotting

Total cellular protein was extracted as previously described[37]. Immunoblotting was performed with 25 μg of total protein using a 10% SDS/PAGE and electroblotting onto a nitrocellulose membrane (Pal Gelman, Australia). The membrane was blocked with 5% (w/v) skim milk for one hour at room temperature, and followed by an overnight incubation in 0.05μg/μL rabbit anti-human polyclonal VDR (Santa Cruz Biotechnology, USA) at 4°C.

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Antibody binding was visualised using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (0.02µg/µL, Invitrogen/LifeTechnologies, Australia), followed by autoradiography using the ECL-Western Chemiluminescence Detection Kit (GE Healthcare, Australia). Immunoreactive VDR protein by scanning densitometry using loading control GAPDH (ImageQuant, GE Healthcare, Australia) as described previously[37].

Statistical analysis

All statistical analyses were undertaken using StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP. A p-value<0·05 (two-tailed) was considered statistically significant.

Mother-Infant Pairs

Continuous variables were assessed for normality. Descriptive statistics of the mother-infant pairs were tabulated. Spearman correlations between 25(OH)D, serotonin and maternal and baby characteristics were performed to identify potential confounders. The association between maternal 25(OH)D, cord 25(OH)D and cord serotonin was undertaken using linear regression before and after adjusting for maternal serotonin levels. Qnorm plots of the residuals were used to assess that linear regression model assumptions were met.

In vitro Studies

Serotonin concentrations were assessed for normality. Cell-viability and VDR mRNA expression following treatment of HUVEC with various concentrations of $1,25(OH)_2D_3$ compared with vehicle control (0.01% ethanol) was determined using one-way ANOVA. The difference in serotonin concentration before and after treatment with 10nM of $1,25(OH)_2D_3$, in the *VDR/NC/MC* inactivated was determined using a two-way ANOVA. Statistically significant interaction between the cell types and treatment groups were determined using a simple main effects analysis. Pairwise comparison with SIDAK adjustment for multiple comparisons was performed. All *in vitro* experiments were performed on 6 to 12 different occasions as independent experiments, with at least triplicate conditions for each treatment.

Results

Table 1 shows the characteristics of 64 mother-infant pairs. The majority of women were parous and Australian born. Elective cesarean was performed due to previous history of cesarean births. The median maternal and cord 25(OH)D concentrations at birth were

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63nmol/L (IQR 50·4-78·9) and 74nmol/L (47·2-108), respectively. The median maternal and cord serotonin concentrations at birth were 96ng/mL (46-202) and 41ng/mL (9-70), respectively. The scatterplots and correlations between maternal 25(OH)D and serotonin (5-HT) and cord 25(OH)D and 5-HT concentrations are shown in Figure 1. Maternal 25(OH)D concentrations were positively correlated with cord 25(OH)D concentrations (Panel A, rho=0·55, p<0·001). Maternal 5-HT concentrations were positively correlated with cord 5-HT concentrations (Panel B, rho=0.52, p<0·001). Maternal (rho=-0·24, p=0·06) and cord 25(OH)D concentrations (rho=-0·37, p=0·03) were both negatively correlated with cord serotonin concentrations (Panels C and D). There were no significant associations between maternal age, parity, ethnicity, smoking status, gender of baby, birth weight and gestation and cord serotonin concentrations.

The associations between maternal and cord 25(OH)D and cord 5-HT concentrations before and after adjusting for maternal serotonin levels are presented in Table 2. After adjustment for maternal 5-HT level, both maternal 25(OH)D and cord 25(OH)D concentrations remained negatively associated with cord serotonin concentrations. For every 1nmol/L increase in maternal 25(OH)D concentration there was an associated 0·35 nmol/L (95% CI -0·64,-0·08; p=0·01) decrease in cord 5-HT concentrations. For every 1nmol/L increase in cord 25(OH)D concentration there was an associated 0·37 nmol/L (95% CI -0·61,-0·13; p=0·003) decrease in cord 5-HT concentrations.

In order to assess whether $1,25(OH)_2D_3$ could directly alter the production of 5-HT in cultured HUVEC, we examined effects of $1,25(OH)_2D_3$ and *VDR* silencing on 5-HT secretion. Firstly, the effect of various concentrations ranging from 2.5nM to 20nM of $1,25(OH)_2D_3$ on HUVEC cell viability was determined. As shown in Figure 2A, a significantly increased HUVEC cell viability was observed following treatment of cultured HUVEC with 10nM $1,25(OH)_2D_3$ compared with untreated control ($F(4, 20) = 32.52$) $p<0.001$, n=6). Furthermore, in the same experiments, a significant increase in *VDR* mRNA was observed in HUVEC when treated at 10nM $1,25(OH)_2D_3$ compared with untreated control ($F(4, 20) = 39.26$) $p<0.001$, n=6, Figure 2B). Therefore, all subsequent experiments were performed with 10nM $1,25(OH)_2D_3$. 5-HT concentrations in the conditioned media of HUVEC cells were measured following treatment with 10 nM of $1,25(OH)_2D_3$ or vehicle control (control) alone over 48h in culture. 5-HT concentrations were significantly decreased in $1,25(OH)_2D_3$-treated HUVECs compared with control (203.30 ± 16.42 nmol/L, t=10.81,

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df=5, 1,25(OH)$_2$D$_3$-treated cells vs. 458·9 0 ± 42.47 nmol/L, t=12/38, df=5, control, p<0·001, n=6, Figure 2C).

Having shown that 1,25(OH)$_2$D$_3$ suppresses 5-HT, we investigated whether a *VDR* mediated molecular mechanism modulates 5-HT in HUVEC cells. As depicted in Figure 3, following inactivation with *VDR*si1 and *VDR*si2 demonstrated significant decreases of *VDR* at both the mRNA and protein levels over 48h in HUVEC cells. *VDR* mRNA was significantly reduced in *VDR* siRNA treated-cells compared with controls (Figure 3A). The mean difference in VDR mRNA compared with NC was 0.62 ± 0.033; p<0.001 for the *VDR*si1 treated cells; 0.73 ± 0.051, p< 0.001 for the *VDR*si2 transfected cells. The decreases in *VDR* mRNA were then further confirmed at the protein level using immunoblotting (Figure 3B). Significant decrease of VDR protein was observed in *VDR* siRNA treated cells compared to control siRNA-transfected cells. The mean difference in VDR protein compared with NC was 3098.0 ± 390.7, p<0.005 for *VDR*si1 treated cells; and 3412.0 ± 390.7, *VDR*si2 treated cells, p<0.005, Figure 3C). As both si1 and si2 showed consistent knockdown at the mRNA and protein levels, all subsequent analyses were performed with si2.

To demonstrate the effect of *VDR*-dependent mechanism on 5-HT concentrations, firstly, the 5-HT concentrations released into the conditioned media by cultured HUVEC cells following treatment with either *VDR*si2 or NC siRNA over 48h were measured. As illustrated in Figure 4A, the mean concentrations of 5-HT in the control NC cells were 470±101 nmol/L compared with 965±139 nmol/L in the *VDR*si2 inactivated cells (p=0·002). Secondly, in the same experimental setup, following *VDR*si2 transfection in the cultured HUVECs, the difference in 5-HT release was measured upon further treatment with 10nM of 1,25(OH)$_2$D$_3$ in MC, NC and *VDR*si2 treated cells. Statistical differences were determined using a two-way ANOVA. We identified a statistically significant interaction between the cell types and treatment with 10nM of 1,25(OH)$_2$D$_3$ (F=25.8; p<0.001, Figure 4A). The effect of *VDR*si2 transfection and treatment with 10nM of 1,25(OH)$_2$D$_3$ on 5-HT concentration was determined using a simple main effects analysis. Treatment of cells with 1,25(OH)$_2$D$_3$ influenced 5-HT in all cell types (F(1,66) = 70.8; p<0.001 for the MC cells; F(1,66) = 9.7; p=0.003) for the NC cells; and F(1,66) = 176.1; p<0.001 for the *VDR*si2 transfected cells). Pairwise comparison with SIDAK adjustment for multiple comparisons was performed. The mean difference in 5-HT was -296 ± 35.14; p<0.001 in the MC cells; -109.3 ± 35.14; p=0.003 for the NC cells; and -466.3 ± 35.15; p<0.001 for the *VDR*si2 transfected cells.

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Furthermore, in the same experiment, the mRNA expression of the key enzyme, tryptophan hydroxylase 1 (*TPH1*), which is important for 5-HT production in HUVECs, was measured (Figure 4B). We identified a significant interaction between cell group and treatment with 10nM of $1,25(OH)_2D_3$ ($F=137.8;p<0.001$). The effect of *VDR*si2 transfection and treatment with 10nM of $1,25(OH)_2D_3$ on *TPH1* mRNA was determined using a simple main effects analysis. Treatment with $1,25(OH)_2D_3$ influenced *TPH1* mRNA levels in the *VDR*si2 ($F(1,20)=14.1$; $p<0.001$) cells but not the control NC cells $F(1,20)=0.9;p=0.36$). Pairwise comparison with SIDAK adjustment for multiple comparisons was performed. The mean differences in *TPH1* mRNA for the *VDR*si2 cells was 2.2 ± 0.18; $p<0.001$.

Discussion

Here we show that both maternal and fetal vitamin D levels are inversely correlated with fetal serotonin levels. We also show that $1,25(OH)_2D_3$ treatment suppresses serotonin secretion from umbilical vein endothelial cells and that silencing of VDR in those cells increases serotonin secretion. That $1,25(OH)_2D_3$ treatment dose-dependently increased VDR mRNA in HUVEC cells suggests a VDR-dependent mechanism on serotonin concentration. We believe that these observations provide a biologically plausible mechanism to explain how maternal vitamin D deficiency might increase fetal serotonin levels and thereby impair fetal and early childhood neurodevelopment. This is of course eminently testable through a randomized clinical trial of maternal vitamin D supplementation.

Animal model studies have demonstrated that prenatal vitamin D-deficiency leads to alterations in brain morphology and genes related to neuronal survival and dopamine synthesis in the offspring[42, 43]. Many observational studies have linked maternal vitamin D deficiency with impaired neurodevelopmental outcomes in the offspring, in quite diverse social settings including both high and low income countries[17, 44]. However, a plausible biological mechanism to explain why vitamin D deficiency might impair human fetal brain development has been lacking. The lack of such a mechanism has weakened calls for both routine supplementation and confirmatory large-scale, and expensive, randomized clinical trials. That changed with the recent suggestion by Patrick and colleagues[25] that vitamin D regulation of serotonin may be such a mechanism.

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Serotonin acts as a developmental signal in the immature brain, prior to the time it assumes its role as a neurotransmitter[45]. Using *in vitro* and *in-vivo* animal models serotonin has been shown to influence a variety of processes during brain development, including inhibition of outgrowth of its own neurons and neurogenesis in target neurons[46]. The serotonin system of the central nervous system modulates numerous physiologic functions including regulation of the stress response and behavioral traits, such as aggression, fear, and anxiety[23]. Accordingly, dysregulation of serotonergic neurotransmission is implicated as a contributing cause of several psychiatric disorders including attention-deficit hyperactivity disorder, autism, bipolar disorder, depression, and schizophrenia and in antisocial, obsessive-compulsive, and suicidal behaviors[26].

Although there is no direct evidence to suggest that serotonin in the blood reflects the levels in the human brain, animal model studies provide evidence for the direct regulation of brain serotonin via the serotonin transporter. For example, Nakatani et al., (2008) have reported that augmented brain serotonin crosses the blood-brain barrier through the serotonin transporter in the rat[47]. On the contrary Lee et al. (2007)[48], using a mouse model, reported that circulating serotonin and brain serotonin are separately regulated. Therefore, it is important to understand the mechanisms by which the brain serotonin level is regulated.

We believe that there are two possible biological mechanisms by which Vitamin D may have a direct or indirect effect on serotonin synthesis. Firstly, vitamin D may differentially regulate the transcription of *TPH1* or *TPH2* by binding to the vitamin D receptor response element, *VDRE*[19]. As demonstrated in this study *TPH1* mRNA was significantly increased in HUVEC cells following VDR inactivation compared with HUVECs treated in combination with $1,25(OH)_2D_3$ treated cells. Therefore, it is possible that elevated expression of *TPH1* as a consequence of low vitamin D levels may cause changes in *TPH1* activity to act as a tryptophan trap, thus causing an imbalance in tryptophan catabolism in the placenta resulting in an excess of serotonin and a relative deficiency of kynurenine[25]. Thus, an excess of serotonin leads to an autoimmune response attacking the fetal brain, causing an imbalance toward inflammation and autoimmunity[18, 19]. However, this deserves further investigation.

Secondly, disruption of serotonin signaling pathways including administration of certain direct or indirect serotonin agonists, combinations of serotonin enhancing drugs such as 5-HT precursors, 5HTP (tryphophan/tryptamine); use of inhibitors of monoamine oxidase (MAO-

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A/B-I); releasers of serotonin (para-chloroamphetamine); direct serotonin receptor agonists; selective serotonin uptake inhibitors (SSRI); genetic ablation of serotonin synthesising enzyme, TPH; serotonin receptors, have all shown to cause neuropathology in experimental animal models[46].

Limitations

Our study has a number of limitations. Regarding the samples used in this study, firstly, the cross-sectional design does not allow causation to be shown, however, the *in vitro* studies suggest a possible temporal association. Secondly, all our samples were collected from women at term Caesarean section prohibiting the ability to comment on associations earlier in gestation or the affect Caesarean delivery may have on circulating serotonin levels. Schulpis et al. (2008)[49] have reported that maternal serotonin was unaltered at the time of labour in vaginal delivery, however, in post-delivery maternal blood and in the cord blood, an increase in serotonin was associated with the activation of the neuroendocrine system and the participation of skeletal and uterine muscles. Therefore, effects of the mode of delivery on maternal and cord blood serotonin in our study are unknown. Thirdly, we have excluded complicated pregnancies and therefore potentially excluded those with the lowest vitamin D levels, thus our study may have introduced sampling bias, however, such bias would only underestimate rather than over-estimate the true association between maternal vitamin D and fetal serotonin.

Regarding the *in vitro* model system, HUVECs were used to model fetal endothelium and to study the effect of maternal vitamin D deficiency on fetal development. These cells are commonly used to study changes in function that are apparent at birth as they are the first point of exposure of fetal endothelial cells to the blood after placental exchange between maternal and fetal circulations. Future studies, using neuronal cells (astrocytes), are required to study the direct effect of vitamin D and serotonin on neurocognitive deficits. Finally, whether the infants and children from the pregnancies included in this study displayed normal neurocognitive development is unknown.

Conclusions

In this study, we report for the first time that low maternal vitamin D is associated with increased fetal serotonin. Our *in vitro* studies have demonstrated that treatment of HUVECs with $1,25(OH)_2D_3$ suppressed serotonin and silencing of *VDR* increased serotonin, possibly

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by *VDRE* activation of *TPH* gene transcription. While our study has shown a possible temporal association in term uncomplicated pregnancies, we have not investigated the neurocognitive outcomes in infants included in this study. Future longitudinal study addressing these limitations is now therefore warranted.

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Table 1. Variables in 64 mother-infant pairs.

	N=64
Maternal age	33·20 (4·5)[1]
Parous	48 (76%)[3]
Country of birth	
Australian	44 (69%)[3]
Asian	16 (25%)[3]
Other	4 (6%)[3]
Maternal vitamin D	63 (50·4-78·9)[2*]
Maternal serotonin	544·80 (261·05-1146·35)[2*]

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Cord vitamin D	74 (47·2-108) [2*]
Cord serotonin	230·97 (51.07-397.25) [2*]
Gestation at birth	38·60 (1.00) [3]
Infant gender-Female	31 (48%)
Infant birth weight	3349·00 (422·00) [1]

[1]Mean (SD)

[2]Median (IQR)

[3]Number (%)

* nmol/L

Table 2. Associations between maternal and cord 25(OH)D and cord 5-HT concentrations before and after adjusting for maternal serotonin (5-HT) concentrations.

	Univariate β co-efficient (95% CI)	**P Value**	**Multivariate β co-efficient (95% CI)**	**P Value**
Maternal 25(OH)D	-1·98 (-3·74, -1·92)	0·03	-1·98 (-3·63, -0·45)	0·01
Cord 25(OH)D	-0·91 (-3·74, 0·73)	0·004	-2·09 (-3·46, -0·73)	0·003

Multivariate linear regression with maternal serotonin in the model.

Figure Legends

Figure 1.

Vitamin D (25(OH)D, nmol/L) and serotonin (5-HT, nmol/L) concentrations were measured in maternal and cord serum from mother-infant matching pairs (n=64) using immunoassays. The scatterplots and correlations between maternal 25(OH)D and 5-HT and cord 25(OH)D and 5-HT are shown in Panels A-D. The association between maternal 25(OH)D, cord 25(OH)D and cord 5-HT was undertaken using linear regression before and after adjusting for maternal 5-HT levels. A p-value<0·05 (two-tailed) was considered statistically significant.

Figure 2.

The effect of various concentrations ranging from 2.5nM to 20nM of $1,25(OH)_2D_3$ or vehicle control alone on **A.** HUVEC cell viability was measured using the MTT assay and the absorbance read at 570nm. **B**. *VDR* mRNA relative to *18S* rRNA as measured by real-time PCR as described in the methods section. Significant differences in cell viability or *VDR*

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mRNA expression following treatment with various concentrations of 1,25$(OH)_2D_3$ or vehicle control was determined by one-way ANOVA. **C.** Serotonin (5-HT) concentration (nmol/L) in the conditioned media of HUVEC cells was measured over 48h in culture following treatment with 10nM of 1,25$(OH)_2D_3$ or vehicle control alone. The difference in 5-HT concentration between untreated and 1,25$(OH)_2D_3$-treated cells was determined using a paired t-test. A p-value<0·05 (two-tailed) was considered statistically significant.

Figure 3.

A. Cultured HUVEC were treated with *VDR* siRNAs, si1 and si2 for 48h. *VDR* mRNA relative to *18S rRNA* was determined using real-time PCR. Significant decrease was observed in both si1 and si2 treated HUVEC cells compared with *NC* (p<0·05, n=6). **B.** Cultured HUVECs were treated with *VDR* siRNAs, si1 and si2 for 48h. VDR immunoreactive protein (51 kDa) and loading control GAPDH (36 kDa) was determined using Western immunoblotting. **C.** Semi-quantitative analyses of VDR immunoreactive protein relative to GAPDH showed a significant decrease in both si1 and si2 treated HUVEC cells compared with NC (p<0·05, n=6).

Figure 4.

A. Serotonin concentrations (5-HT, nmol/L) were measured in conditioned media from cultured HUVEC when treated with *VDR* siRNA (si2) and non-targeted siRNA (*NC*). Serotonin, 5-HT concentrations were assessed for normality. Following *VDR* inactivation, the difference in 5-HT concentration in the presence of 1,25$(OH)_2D_3$ in *VDR* inactivated cells or in NC treated cells was determined using a two-way ANOVA. Further statistical analyses for interaction between the cell types and treatment with 10nM of 1,25$(OH)_2D_3$ on 5-HT concentration was determined by using a simple main effects analysis. Pairwise comparison with SIDAK adjustment for multiple comparisons was performed. A p-value<0·05 was considered statistically significant for n=12 independent experiments. **B**. Tryptophan hydroxylase 1 (*TPH1*) mRNA was measured in cultured HUVEC when treated with *VDR* siRNA (si2) and non-targeted siRNA (*NC*). The difference in *TPH1* mRNA between *NC* and *VDR* inactivated cells was determined using a paired t-test. A p-value <0·05 (two-tailed) was considered statistically significant for n=6 independent experiments. Following *VDR* inactivation, the difference in *TPH1* mRNA in the presence of 1,25$(OH)_2D_3$ in *VDR* inactivated cells or in NC treated cells was determined using a two-way ANOVA. Further statistical analyses for interaction between the cell types and treatment with 10nM of

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581 1,25$(OH)_2D_3$ on *TPH1* mRNA was determined by using a simple main effects analysis.
582 Pairwise comparison with SIDAK adjustment for multiple comparisons was performed. A p-
583 value <0·05 was considered statistically significant for n=6 independent experiments.

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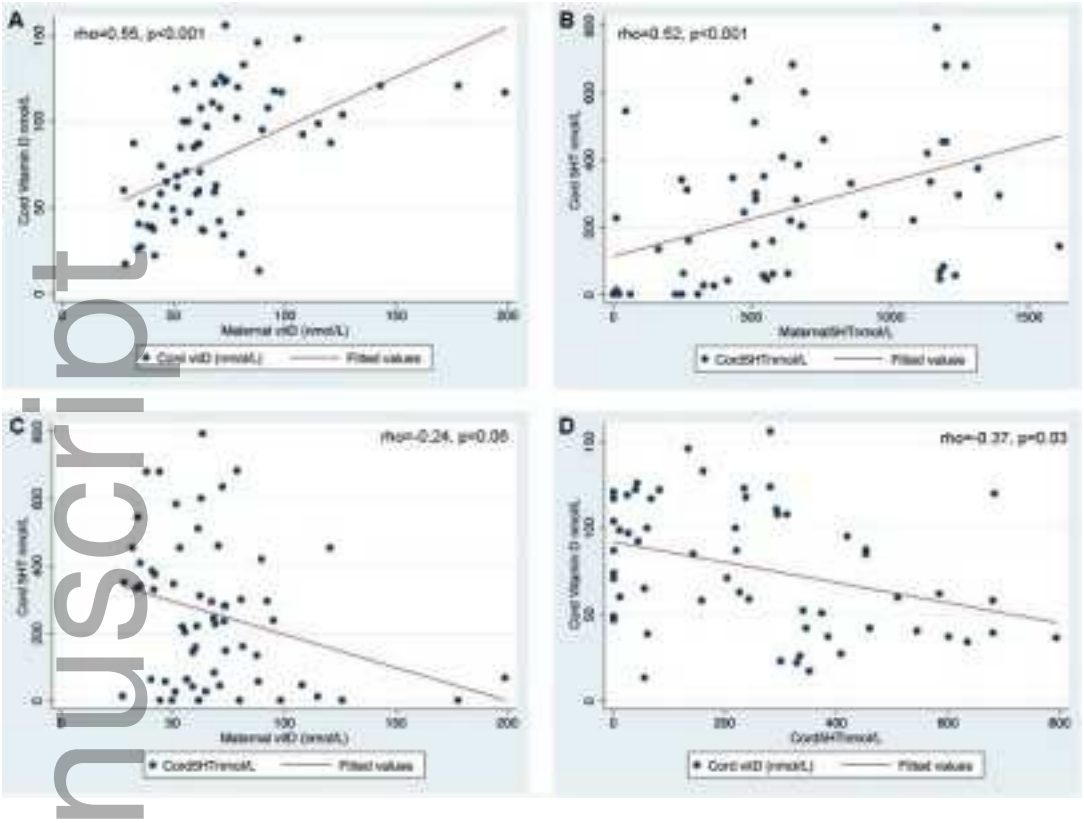

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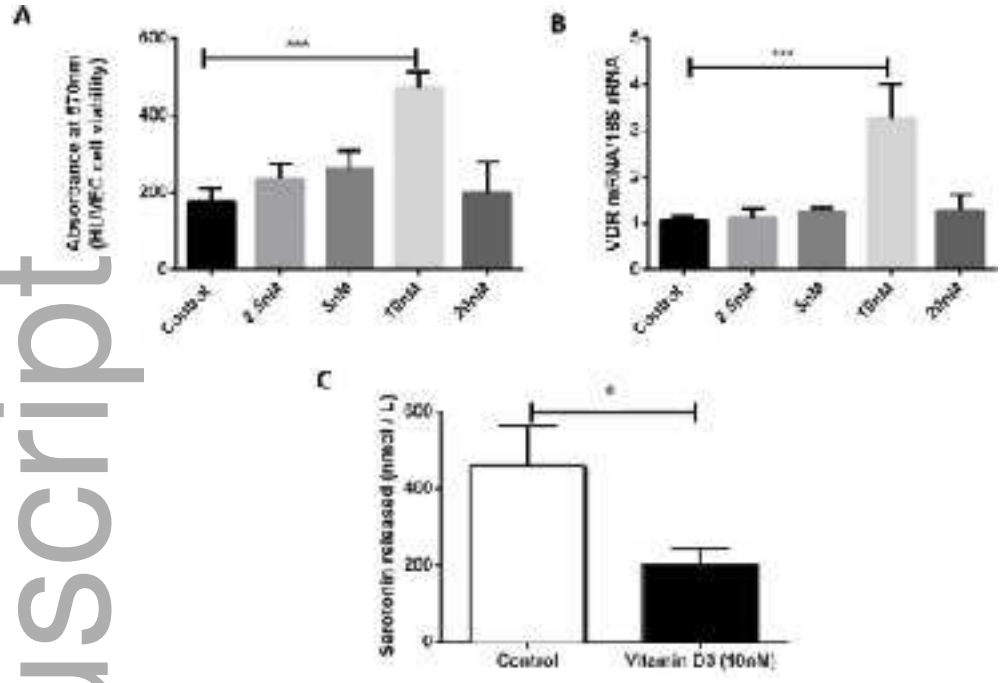

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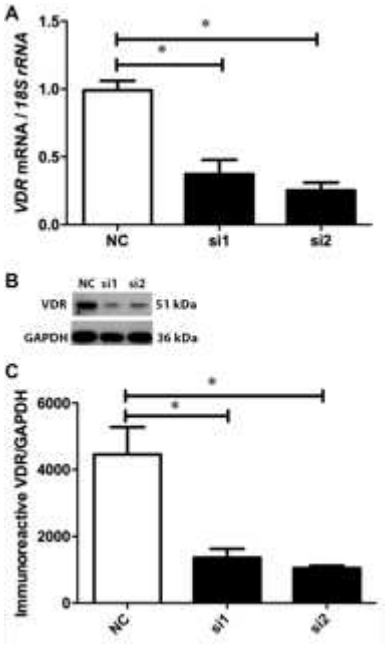

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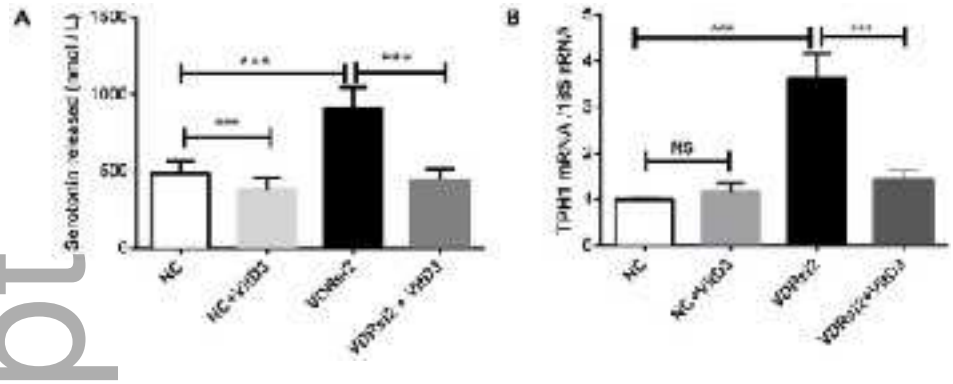

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