Dysregulation of maternal and placental vitamin D metabolism in preeclampsia

Tamblyn, Jennifer; Susarla, Radhika; Jenkinson, Carl; Jeffery, Louisa; Ohizua, O.; Chun, Rene; Chan, Shiao Y; Kilby, Mark; Hewison, Martin

DOI:
10.1016/j.placenta.2016.12.019

Accepted Manuscript

Dysregulation of maternal and placental vitamin D metabolism in preeclampsia

J.A. Tamblyn, R. Susarla, C. Jenkinson, L.E. Jeffery, O. Ohizua, R.F. Chun, S.Y. Chan, M.D. Kilby, M. Hewison

PII: S0143-4004(16)30673-7

DOI: 10.1016/j.placenta.2016.12.019

Reference: YPLAC 3532

To appear in: *Placenta*

Received Date: 12 October 2016

Revised Date: 27 November 2016

Accepted Date: 17 December 2016

Please cite this article as: Tamblyn JA, Susarla R, Jenkinson C, Jeffery LE, Ohizua O, Chun RF, Chan SY, Kilby MD, Hewison M, Dysregulation of maternal and placental vitamin D metabolism in preeclampsia, *Placenta* (2017), doi: 10.1016/j.placenta.2016.12.019.

Dysregulation of maternal and placental vitamin D metabolism in preeclampsia

Tamblyn JA[1,5,6], Susarla R[1], Jenkinson C[1], Jeffery LE[1], Ohizua O[2], Chun RF[3], Chan SY[1,4], Kilby MD[1,5,6,7], Hewison M[1,6,7]

[1]Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, B15 2TT, UK

[2]Women, Children and Sexual Health Directorate, Walsall Hospitals NHS Trust, Walsall, WS2 9PS, UK

[3]Department of Orthopaedic Surgery, University of California Los Angeles, Los Angeles, CA 90095, USA

[4]Department of Obstetrics and Gynecology, National University of Singapore, Singapore 119228

[5]Fetal Medicine Centre, Birmingham Women's Foundation Trust, Edgbaston, Birmingham, B15 2TG, UK

[6]Centre for Women's & Newborn Health, Birmingham Health Partners, Birmingham B15 2TH, UK.

[7]Centre for Endocrinology Diabetes and Metabolism, Birmingham Health Partners, Birmingham B15 2TH, UK

Abbreviated title: Vitamin D metabolome in pregnancy

Key words: Vitamin D, pregnancy, placenta, decidua, preeclampsia

Number of words: 3180 (Max 3,000)

Number of figures: 4; Number of tables: 0

Corresponding author:
Address for correspondence:
Martin Hewison PhD
Institute of Metabolism & Systems Research
Level 2, IBR, Rm 225
The University of Birmingham
Birmingham
B15 2TT
UK

Email: m.hewison@bham.ac.uk
Tel: 44 (0)121 414 6908
Fax: 44(0)121 415 8712

Disclosure statement: The authors have nothing to disclose

Abstract

Introduction: Epidemiology has linked preeclampsia (PET) to decreased maternal serum 25-hydroxyvitamin D3 (25(OH)D3). However, alterations in systemic and placental/decidual transport and metabolism of 25(OH)D3 during pregnancy suggest that other forms of vitamin D may also contribute to the pathophysiology of PET.

Methods: In a cross sectional analysis of normal pregnant women at 1st (n=25) and 3rd trimester (n=21), pregnant women with PET (n=22), and non-pregnant female controls (n=20) vitamin D metabolites were quantified in paired maternal serum, placental, and decidual tissue.

Results: Serum 25(OH)D3 was not significantly different in sera across all four groups. In normal 3rd trimester pregnant women serum active 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) was significantly higher than non-pregnant, normal 1st trimester pregnant, and PET women. Conversely, PET sera showed highest levels of the catabolites 3-epi-25(OH)D3 and 24,25-dihydroxyvitamin D3 (24,25$(OH)_2$D3). Serum albumin was significantly lower in normal 3rd trimester pregnant women and PET relative to normal 1st trimester pregnant women, but there was no change in free/bioavailable 25(OH)D3. In PET placental tissue, 25(OH)D3 and 3-epi-25(OH)D3 were lower than normal 3rd trimester tissue, whilst placental 24,25$(OH)_2$D3 was highest in PET. Tissue 1,25$(OH)_2$D3 was detectable in 1st trimester decidua, which also showed 10-fold higher 25(OH)D3 relative to paired placentae. 3-epi-25(OH)D3 and 24,25$(OH)_2$D3 were not different for decidua and placenta. In normal 3rd trimester pregnant women, total, free and bioavailable maternal 25(OH)D3 correlated with placental 25(OH)D3, but this was not conserved for PET.

Discussion: These data indicate that PET is associated with decreased activation, increased catabolism, and impaired placental uptake of 25(OH)D3.

Introduction

Human pregnancy is associated with important changes in vitamin D physiology. Maternal circulating concentrations of the active form of vitamin D, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$), increase significantly during early gestation [1]. This appears to be due to increased renal activity of the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), which converts inactive 25-hydroxyvitamin D (25(OH)D3) to $1,25(OH)_2D3$ [2]. 1α-hydroxylase expression and activity has also been described in human decidua and fetal trophoblast [3-5]. Placental 1α-hydroxylase does not appear to make a major contribution to the elevated maternal $1,25(OH)_2D3$ associated with pregnancy [2]. Instead co-expression of the nuclear vitamin D receptor (VDR) in maternal and fetal placental tissues suggests a more localised role for $1,25(OH)_2D3$. Albeit less well understood, recent studies suggest this extends far beyond the known classical calciotropic effects of vitamin D, and includes key roles in normal decidual immune function and placental implantation [6-9]

Vitamin D-insufficiency is prevalent in pregnant women [10, 11]. Maternal 25(OH)D is the principal determinant of neonatal circulating 25(OH)D3, thus infants of vitamin D–deficient mothers are also at risk of vitamin D-deficiency [12]. Maternal 25(OH)D3-deficiency has also been linked to adverse pregnancy outcomes associated with malplacentation, including preeclampsia (PET), small-for-gestational age and preterm birth [13-17]. Mothers receiving vitamin D supplementation from early pregnancy (two trials, 219 women) have been reported to have a lower risk of PET and a positive association between serum 25(OH)D3 and reduced PET risk has been reported [18]. A recent systematic review and meta-analysis, which included 11 observational studies, found a significant inverse relationship between maternal 25(OH)D3 and risk of PET in 5 of the studies. Meta-analyses similarly suggested an inverse relationship between maternal 25(OH)D3 and PET risk, but could not infer causality due to the insufficient quality of evidence [19, 20].

This heterogeneity of data for vitamin D and PET in part reflects our limited understanding of the effects of vitamin D during pregnancy. Moreover, almost all studies to date have relied on maternal serum concentrations of 25(OH)D3 as the determinant of vitamin D status and function, despite the potential importance of other vitamin D metabolites such as $1,25(OH)_2D3$ [21], 3-epi-25(OH)D3 [22], and 24-hydroxylated vitamin D metabolites ($24,25(OH)_2D3$) [23]. Furthermore, placental expression of 1α-hydroxylase suggests that tissue-specific concentrations of 25(OH)D3 and other vitamin D metabolites are likely to be potential determinants of local vitamin D function across gestation [6]. Finally, vitamin D binding protein (DBP) and albumin are known to act as serum transporters of vitamin D metabolites, but also define tissue bioavailability and function by modulating the balance of bound and free forms of vitamin D [24]. The aim of the current study was therefore to characterise the relative impact of each of these facets of vitamin D metabolism and transport on normal and PET pregnancies.

Materials and Methods

Ethical approval

Written informed consent was obtained from all women recruited into the study. Matched human sera, placenta and decidua samples were collected with the approval of Health Research Authority - West Midlands, Edgbaston Research Ethics Committee (NHS REC 06/Q2707/12 [2006 approval]) (RG_14-194 [10.2014 approval]).

Sample collection

All samples were obtained from women in the West Midlands area of the UK (n=88). Patient demographics and baseline clinical data are summarised in **Supplemental Table 1**. Importantly, no significant difference in maternal age or BMI was measured. As anticipated, in the PET group mean arterial blood pressure was significantly raised ($p<0.0001$) and fetal

birthweight reduced ($p<0.01$) comparative to NP3. There was however no significant difference in gestational age at delivery. 1st trimester sera, placental and decidual samples were obtained from women with uncomplicated pregnancies undergoing surgical termination of pregnancy between 8-13 weeks gestation (n=25), as determined by ultrasound measurement of crown rump length (Walsall Manor NHS Trust).

Normal uncomplicated 3rd trimester (>37 weeks) (n=21) and PET (>34 weeks) (n=22) sera and placental samples were collected from pregnant women consented prior to delivery at Birmingham Women's Foundation Hospital Trust (BWFHT). All PET cases were prospectively diagnosed according to current International definitions (ISSHP, 2014)[25]; new hypertension presenting after 20 weeks, with one or more of the following new onset conditions: 1. proteinuria (urinary protein: creatinine ratio > 30 mg/mmol or a validated 24-hour urine collection > 300 mg protein); 2. other maternal organ dysfunction (renal insufficiency, liver involvement, neurological and/ or haematological complications); 3. utero-placental dysfunction (fetal growth restriction). PET severity was categorised as; mild - diastolic ≥ 90–99 mmHg, systolic ≥140–149 mmHg (n=9), moderate - diastolic ≥ 100–109 mmHg (n=7), systolic ≥150–159 mmHg, severe - diastolic ≥ 110 mmHg, systolic ≥160 mmHg (n=6). Maternal mean arterial blood pressure (MABP) was significantly elevated comparative to the normal pregnant control group. A healthy non-pregnant female 'control' group (n=20) was also recruited.

Sample preparation for LC-MS/MS Analysis

Placental biopsies (approximately 1g weight) were defrosted on ice and homogenised in 700µl ice-cold PBS using a gentle MACS tissue dissociator (Miltenyi Biotec, Woking, UK,) with M tubes using pre-set programs developed for total RNA or mRNA isolation from fresh or frozen samples. Homogenates were centrifuged at 10,000g for 5 minutes and the clear homogenate

was transferred to a separate Eppendorf tube. Total protein content in the homogenate was immediately measured (ThermoFisher, Waltham, MA, USA).

Extraction of serum and tissues samples for LC-MS/MS analysis

Vitamin D metabolites were extracted from donor serum (0.2 mL) or placental tissue homogenates as described previously [26]. Resulting samples were reconstituted in 125 µL water/methanol (50/50%) for LC-MS/MS analysis as previously described [26] using a Waters ACQUITY ultra performance liquid chromatography [uPLC] coupled to a Waters Xevo TQ-S mass spectrometer [Waters, Manchester, UK]). Analysis was carried out in multiple reaction monitoring (MRM) mode, with optimised MRM transitions for each analyte as described previously [26]. External quality control (QC) samples (LGC Standards, Teddington, UK) for 25(OH)D3 and 25(OH)D2 were used to assess accuracy and precision within batch runs. QCs across different concentration ranges to determine inter- and intra- day accuracy and precision for each analyte were as described previously [26]. Data analysis was performed with Waters Target Lynx.

Analysis of DBP and albumin and estimation of free vitamin D metabolites

Human vitamin D binding protein (DBP) (R&D Biosystems, Abingdon, UK), and human albumin (Abcam, Cambridge, UK) were measured using ELISA analyses as per manufacturer's instructions. Serum concentrations of free (total minus DBP and albumin-bound) and bioavailable (total minus DBP bound) serum 25(OH)D3 were calculated based on total 25(OH)D3 and DBP/albumin values using equations described previously [27].

Statistics

Unless otherwise stated, data are shown as median values with interquartile ranges (IQR). All statistical analyses were carried out using GraphPad PRISM Version 6.07 software (San Diego,

CA, USA). Normality was assessed using D'Agostino-Pearson omnibus normality test, with Student's t-test (parametric), or Mann-Whitney (non-parametric) test utilised to compare two data sets. Multifactorial data were compared using either one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric) based on ranks, with Tukey or Dunn's method used for post hoc multiple-comparison procedures.

Results

Dysregulation of serum vitamin D metabolism in PET

Four serum vitamin D metabolites were consistently quantifiable in both pregnant and non-pregnant women; 25(OH)D3, 1,25$(OH)_2$D3, 24,25$(OH)_2$D3, 3-epi-25(OH)D3. In non-pregnant women 25(OH)D3 concentrations (median 33.4, IQR 20.8–44.3 nmol/L), were similar to healthy 1st trimester (NP1, 28.8, 20.3–46.9nmol/L) and 3rd trimester (NP3, 45.2, 32.5–59.2nmol/L) pregnancies, as well as women diagnosed with PET (35.3, 17.7–54.7 nmol/L) (**Figure 1A**). By contrast, serum 1,25$(OH)_2$D3 concentrations in non-pregnant women (34.2, 29.3–55.0 pmol/L), were significantly lower than in pregnant women, including NP1 (113.7, 82.7–198.3 pmol/L, p<0.0001), NP3 (254.7, 195.7–310.1 pmol/L, P<0.0001), and PET (171.2, 113.0–236.3 pmol/L, p<0.0001) groups (**Figure 1B**). Consistent with previous studies [28], NP3 levels of 1,25$(OH)_2$D3 were more than two-fold higher than NP1 (p<0.0001), and significantly lower concentrations of 1,25$(OH)_2$D3 (p<0.01) were observed in the PET cohort compared to NP3 (**Figure 1B**). Linear regression analysis confirmed gestational age was not a significant determinant of any serum vitamin D metabolite (data not shown).

Serum concentrations of 24,25$(OH)_2$D3 in non-pregnant women (3.3, 1.6–4.7 nmol/L) were higher than NP1 (1.8, 0.8-3.7 nmol/L), but lower than NP3 (7.6, 5.6-10.0 nmol/L, p<0.05) and PET (10.9, 7.3-22.5 nmol/L, p<0.001) (**Figure 1C**). Both NP3 and PET samples showed significantly higher 24,25$(OH)_2$D3 concentrations than NP1 (both p<0.0001). Concentrations of

3-epi-25(OH)D3 were lowest in non-pregnant women (5.1, 3.9-6.4 nmol/L). Both NP1 (7.6, 6.0-9.2 nmol/L) and NP3 (7.5, 5.9-8.6 nmol/L) had higher levels of 3-epi-25(OH)D3 but this was not significant. Highest 3-epi-25(OH)D3 levels were observed with PET (8.8, 5.9-11.8 nmol/L), with significant differences compared to non-pregnant ($p<0.001$), NP1 ($p<0.05$) and NP3 groups ($p<0.05$) (**Figure 1D**). In non-pregnant women serum 25(OH)D3 was strongly correlated with 1,25$(OH)_2$D3 ($p=0.013$), 24,25$(OH)_2$D3 ($p<0.0001$) and 3-epi-25(OH)D3 ($p=0.012$), but similar correlations were not consistently observed in pregnancy (**Supplemental Figure 1**).

Serum DBP, albumin and free/bioavailable 25(OH)D3

Data in **Figure 2A** showed a trend towards increased serum DBP in NP1 and NP3 pregnancies relative to non-pregnant women, but there was no significant difference in DBP between NP3 and PET. Serum albumin was significantly lower in NP3 and PET pregnancies relative to non-pregnant women ($p<0.001$ and $p<0.05$ respectively) and NP1 pregnancies ($p<0.001$ and $p<0.05$ respectively) (**Figure 2B**). DBP and albumin values, together with total serum 25(OH)D3 levels, were used to calculate bioavailable (**Figure 2C**), and free serum 25(OH)D3 (**Figure 2D**) but these showed no significant change across pregnancy or with PET.

Ratios of DBP-bound 25(OH)D3 to total 25(OH)D3 were unaffected by pregnancy or PET (**Supplemental Figure 2A**). However, the suppression of serum albumin with pregnancy significantly decreased the ratio of 'bioavailable' 25(OH)D3 (25(OH)D3 bound to albumin but not DBP) to 'total' serum 25(OH)D3 across normal pregnancy and PET (**Supplemental Figure 2B**). In a similar fashion, elevation of DBP levels in pregnant women resulted in decrease ratios of 'free' 25(OH)D3 to total 25(OH)D3, with this effect being more pronounced in PET pregnancies (**Supplemental Figure 2C**).

Decreased placental tissue concentrations of 25(OH)D3 in PET pregnancies

In contrast to the placenta, 1,25$(OH)_2$D3 was quantifiable in decidual tissue (17.6, 11.0–23.4 pmol/mg protein), and this paralleled increased decidual concentrations of 25(OH)D3 (21.0, 9.3–60.5 nmol/mg protein) relative to paired NP1 placentae (1.2, 0.7-2.2 nmol/mg protein, p<0.001) **(Figure 3A)**. By contrast no difference in tissue levels of 24,25$(OH)_2$D3 were observed between decidua (0.3, 0.2–0.4 nmol/mg) and placenta (0.2, 0.1–0.3 nmol/mg). Similarly, decidual concentrations of 3-epi-25(OH)D3 (0.1, 0.1–0.3 nmol/mg) were not significantly different to NP1 placental 3-epi-25(OH)D3 (0.2, 0.1–0.3 nmol/mg) (**Figure 3A**).

In placenta tissue, concentrations of 25(OH)D3 increased significantly from NP1 (1.2, 0.7-2.2 nmol/mg protein) to NP3 (5.0, 4.0-6.5 nmol/mg protein, p<0.0001), but this effect was not observed for PET placenta levels of 25(OH)D3 (2.5, 1.4-3.5 nmol/mg protein) which were significantly lower than NP3 values (p<0.01) **(Figure 3B)**. Consistent with maternal serum data, placental 24,25$(OH)_2$D3 values were highest for PET (0.4, 0.3–0.6 nmol/ mg) relative to NP3 (0.3, 0.3–0.5 nmol/mg) and NP1 (0.2, 0.1-0.4 nmol/mg, p<0.01) (**Figure 3B**). Placental 3-epi-25OHD3 values were also higher for PET (0.4, 0.3–0.7 nmol/mg) relative to both NP3 (0.3, 0.2–0.4 nmol/mg, p<0.05) and NP1 pregnancies (0.2, 0.1–0.3 nmol/mg, p<0.001) (**Figure 3B**). Placental concentrations of 1,25$(OH)_2$D3 were below the lower limit of quantification. In NP1 (data not shown) and NP3 pregnancies (**Figure 4A**) placental concentrations of 25(OH)D3 correlated with maternal serum total, DBP-bound, bioavailable and free 25(OH)D3. By contrast, placental concentrations of 25(OH)D3 in PET pregnancies showed no association with any form of maternal serum 25(OH)D3 (**Figure 4B**).

Discussion

PET is a pregnancy-specific hypertensive, multisystem syndrome which complicates up to 8% of pregnancies [25], and is associated with significantly increased maternal and perinatal mortality and morbidity [29]. Although pathogenesis is not fully understood, PET is characterised

182 by abnormal decidual maternal spiral artery remodelling by invading fetal extravillous
183 trophoblast (EVT) cells. [30]. Importantly, this critical placentation process appears sensitive to
184 local vitamin D metabolites within both decidua and placental tissues [9]. In contrast to previous
185 reports describing decreased serum 25(OH)D3 in PET [13, 14, 31], vitamin D-deficiency was
186 observed for most of the women in the current study, despite this being a predominantly white
187 Caucasian cohort. This may be due to the smaller size and non-matched cohort used [14], or
188 the fact that some studies quantified serum 25(OH)D3 using ELISA technology which cannot
189 distinguish between 25(OH)D3 and 3-epi-25(OH)D3 and may therefore over-estimate serum
190 vitamin D 'status' [13, 31]. Nevertheless, the over-arching conclusion from data presented here
191 is that simple measurement of serum 25(OH)D3 provides a very limited perspective of vitamin D
192 in pregnancy.
193
194 Previous studies have reported PET-associated declines in serum $1,25(OH)_2D3$ [31, 32], similar
195 to those reported in the current study. This may be due to decreased serum levels of insulin-like
196 growth factor 1 [32], a stimulator of renal 1α-hydroxylase, or lower expression of 1α-hydroxylase
197 in the placenta [33], but other PET studies have reported increased whole human placental
198 tissue 1α-hydroxylase expression [34]. Data presented here suggest that metabolism of
199 25(OH)D3 to $24,25(OH)_2D3$ may indirectly lower $1,25(OH)_2D3$ in PET. Enhanced 'catabolism' of
200 25(OH)D3 to $24,25(OH)_2D3$ in PET may be due to increased placental (trophoblast) expression
201 of the enzyme 24-hydroxylase [34], although the underlying basis for this remains unclear [35].
202 Alternative metabolism of vitamin D may also occur via epimerisation of 25(OH)D3. The
203 resulting 3-epi-25(OH)D3 can be converted to 3-epi-$1,25(OH)_2D3$, and then bind to VDR to
204 activate target gene transcription [36]. However, 3-epi-$1,25(OH)_2D3$ is a much less potent VDR
205 agonist than $1,25(OH)_2D3$, suggesting that epimerisation of 25(OH)D3 acts to dial-down VDR
206 activity by generating a less effective ligand for the receptor [37]. It is notable that 3-epi-

25(OH)D3 concentrations were significantly higher in the PET cohort, so this metabolic pathway may also play a key role in the dysregulation of vitamin D function in PET.

This is the first study to use paired placental, decidual and serum samples to assess the relationship between circulating and tissue-specific levels of vitamin D metabolites. The relatively high levels of 25(OH)D3 in decidua enabled quantification of $1,25(OH)_2D3$, but determinants of both decidual 25(OH)D3 and $1,25(OH)_2D3$ remain unclear (**Supplemental Figure 2**). The most likely determinant of decidual $1,25(OH)_2D3$ is local tissue expression of 1α-hydroxylase. In unpublished studies we have shown that decidual 1α-hydroxylase mRNA correlates with mRNA for inflammatory cytokines such as interleukin-6 and interferon-γ, suggesting that immune activity could be a key driver of decidual $1,25(OH)_2D3$. Less clear is what determines decidual levels of the substrate for 1α-hydroxylase, 25(OH)D3. It was interesting that neither maternal nor placental 25(OH)D3 showed any correlation with decidual 25(OH)D3, despite the proximity of these tissues (**Supplemental Figure 2**), suggesting that the the decidua has an autonomously regulated vitamin D system.

In contrast to the decidua, data for placental tissue support the general assumption that variations in circulating 25(OH)D3 are manifested by equivalent tissue changes in 25(OH)D3. In NP3, placental 25(OH)D3 was closely correlated with maternal 25(OH)D well beyond levels of sufficiency (>75 nmoL/L). This was not observed in placentas from PET pregnancies, irrespective of serum 25(OH)D3 concentration (**Figure 4B**). This may, in part, reflect dysregulation of tissue catabolism of 25(OH)D3, as placental $24,25(OH)_2D3$ was highest for PET. However, tissue 3-epi-25(OH)D3 was significantly lower in PET placentas, suggesting that this catabolic pathway does not contribute to suppression of 25(OH)D3 in the placenta.

In recent years there has been increasing interest in the potential role of serum DBP not only as a transporter of vitamin D metabolites, but also as a determinant of 25(OH)D3 tissue access either as unbound or 'free' 25(OH)D3, or through receptor-mediated uptake of DBP-bound 25(OH)D3 [24]. In the current study variations in serum DBP and albumin across pregnancy (**Figure 2**) resulted in subtle changes in the relative proportions of bioavailable and free 25(OH)D3 (**Supplemental Figure 3**). It has been recognised for many years that serum albumin decreases with pregnancy, due to increased maternal blood volume [38]. This may be exacerbated in PET, although the extent to which this occurs varies with disease severity [38]. Previous studies using 1st trimester serum did not demonstrate any significant variation in DBP or 25(OH)D3 between pregnancies that went on to normal term or PET delivery [39].

For both NP1 and NP3, serum DBP correlated with placental DBP, but this was not observed in PET placentas, or NP1 decidua (**Supplemental Figure 4A**). PET may therefore involve dysregulated endocytic uptake of DBP via the membrane receptor megalin which is expressed in the placenta [40]. Although DBP uptake by the placenta appears to be dysregulated in PET, other data do not support a major role for DBP as a determinant of placental or decidual 25(OH)D3. Firstly, placental DBP was higher in NP1 than NP3, whereas placental 25(OH)D3 was higher in NP3 (**Supplemental Figure 4A**). Most importantly there was no correlation between placental or decidual DBP and the levels of 25(OH)D3 in these tissues for NP1 (**Supplemental Figure 4C** and **4D**) or NP3 (data not shown). Collectively these data suggest that the close association between maternal serum 25(OH)D3 and levels of this metabolite in placental tissue involves placental uptake of DBP, but other mechanisms determine the final tissue-specific concentrations of 25(OH)D3. Dysregulation of this process in PET may reflect aberrant spiral artery development and placental blood flow, both of which are associated with malplacentation and may alter DBP uptake and 25(OH)D3 metabolism. We have shown previously that 25(OH)D3 and 1,25$(OH)_2$D3 promote matrix invasion by human trophoblastic

cells [9], and similarly demonstrated dysregulated placental vascularisation and elevated blood pressure in vitamin D-deficient pregnant mice [41]. Thus further studies are required to determine whether decreased placental 25(OH)D3 is a cause or consequence of PET.

The validity of the monoclonal antibody to DBP used in the R&D assay employed in the current study has been subject to recent debate, specifically reported differential immunoreactivity against epitopes on major DBP isoforms [42, 43]. This potential limitation of the DBP assay is specifically relevant to black populations, and it is important to recognise that the cohort in the current study was predominantly white. Nevertheless, future studies will benefit from direct measurement method for 'free' 25(OH)D3 and using newly established assays [44].

The over-arching conclusions from this study are: 1) that PET is characterised by changes in multiple vitamin D metabolic pathways, emphasising the limited information to be gained from measurement of maternal 25(OH)D3; 2) changes in maternal DBP and albumin do not appear to have a major impact on the bioavailability and placental/decidual accumulation of vitamin D; 3) in normal healthy pregnancies, maternal serum 25(OH)D3 is closely correlated with placental 25(OH)D3, underlining the potential benefits of vitamin D supplementation in pregnancy; 4) this effect is lost in PET pregnancies, and the potential impact of this on resulting offspring will be a target for future studies; 5) in contrast to the placenta, the decidua can synthesise detectable levels of $1,25(OH)_2D3$. However, the underlying mechanistic basis for regulation of this metabolism in the decidua is still unclear, and requires further investigation. An important limitation of the present study is the large inter-group variability in all vitamin D metabolites measured and most likely reflects the small sample size and non-matched study design. Validation of these findings in a high-powered, matched cohort study including pregnant women with PET versus normotensive pregnant and non-pregnant controls is required to inform any future vitamin D supplementation trial targeting correction of the vitamin D metabolome.

Acknowledgements. This study was supported by funding from NIH (AR063910, MH), Action Medical Research (#1949 to MDK, SYC), and Wellbeing of Women (RTF401, JAT).

References

[1] R. Kumar, W.R. Cohen, P. Silva, F.H. Epstein, Elevated 1,25-dihydroxyvitamin D plasma levels in normal human pregnancy and lactation, The Journal of clinical investigation 63(2) (1979) 342-4.

[2] M. Turner, P.E. Barre, A. Benjamin, D. Goltzman, M. Gascon-Barre, Does the maternal kidney contribute to the increased circulating 1,25-dihydroxyvitamin D concentrations during pregnancy?, Mineral and electrolyte metabolism 14(4) (1988) 246-52.

[3] T.K. Gray, G.E. Lester, R.S. Lorenc, Evidence for extra-renal 1 alpha-hydroxylation of 25-hydroxyvitamin D3 in pregnancy, Science 204(4399) (1979) 1311-3.

[4] Y. Weisman, A. Harell, S. Edelstein, M. David, Z. Spirer, A. Golander, 1 alpha, 25-Dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 in vitro synthesis by human decidua and placenta, Nature 281(5729) (1979) 317-9.

[5] D. Zehnder, K.N. Evans, M.D. Kilby, J.N. Bulmer, B.A. Innes, P.M. Stewart, M. Hewison, The ontogeny of 25-hydroxyvitamin D(3) 1alpha-hydroxylase expression in human placenta and decidua, The American journal of pathology 161(1) (2002) 105-14.

[6] N.Q. Liu, M. Hewison, Vitamin D, the placenta and pregnancy, Arch Biochem Biophys 523(1) (2012) 37-47.

[7] N.Q. Liu, A.T. Kaplan, V. Lagishetty, Y.B. Ouyang, Y. Ouyang, C.F. Simmons, O. Equils, M. Hewison, Vitamin D and the regulation of placental inflammation, J Immunol 186(10) (2011) 5968-74.

[8] J. Tamblyn, M. Hewison, C. Wagner, J. Bulmer, M. Kilby, Immunological role of vitamin D at the maternal–fetal interface, Journal of Endocrinology 224(3) (2015) R107-R121.

[9] S.Y. Chan, R. Susarla, D. Canovas, E. Vasilopoulou, O. Ohizua, C.J. McCabe, M. Hewison, M.D. Kilby, Vitamin D promotes human extravillous trophoblast invasion in vitro, Placenta 36(4) (2015) 403-9.

[10] M.K. Javaid, S.R. Crozier, N.C. Harvey, C.R. Gale, E.M. Dennison, B.J. Boucher, N.K. Arden, K.M. Godfrey, C. Cooper, Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: a longitudinal study, Lancet 367(9504) (2006) 36-43.

[11] L.M. Bodnar, H.N. Simhan, R.W. Powers, M.P. Frank, E. Cooperstein, J.M. Roberts, High prevalence of vitamin D insufficiency in black and white pregnant women residing in the northern United States and their neonates, The Journal of nutrition 137(2) (2007) 447-52.

[12] S.N. Karras, I. Shah, A. Petroczi, D.G. Goulis, H. Bili, F. Papadopoulou, V. Harizopoulou, B.C. Tarlatzis, D.P. Naughton, An observational study reveals that neonatal vitamin D is primarily determined by maternal contributions: implications of a new assay on the roles of vitamin D forms, Nutr J 12 (2013) 77.

[13] L.M. Bodnar, J.M. Catov, H.N. Simhan, M.F. Holick, R.W. Powers, J.M. Roberts, Maternal vitamin D deficiency increases the risk of preeclampsia, J Clin Endocrinol Metab 92(9) (2007) 3517-22.

[14] M. Djekic-Ivankovic, H. Weiler, G. Jones, M. Kaufmann, J. Kaludjerovic, V. Aleksic-Velickovic, L.M. Mandic, M. Glibetic, Vitamin D status in mothers with pre-eclampsia and their infants: a case-control study from Serbia, a country without a vitamin D fortification policy, Public Health Nutr (2016) 1-11.

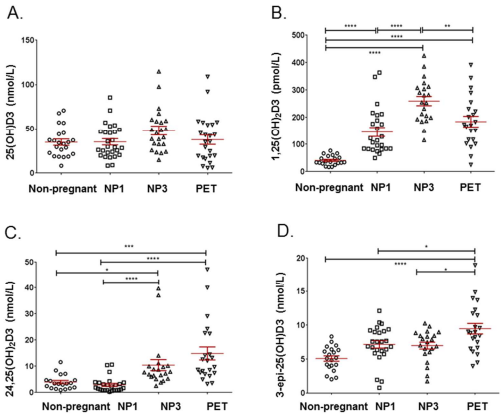
[15] H.H. Burris, S.L. Rifas-Shiman, K. Kleinman, A.A. Litonjua, S.Y. Huh, J.W. Rich-Edwards, C.A. Camargo, Jr., M.W. Gillman, Vitamin D deficiency in pregnancy and gestational diabetes mellitus, American journal of obstetrics and gynecology 207(3) (2012) 182 e1-8.

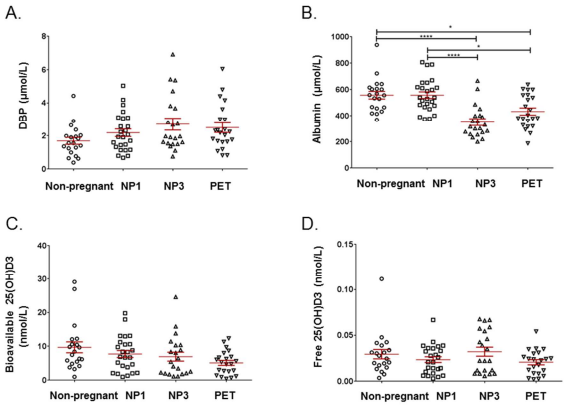
[16] L.M. Bodnar, J.M. Catov, J.M. Zmuda, M.E. Cooper, M.S. Parrott, J.M. Roberts, M.L. Marazita, H.N. Simhan, Maternal serum 25-hydroxyvitamin D concentrations are associated with small-for-gestational age births in white women, J Nutr 140(5) 999-1006.

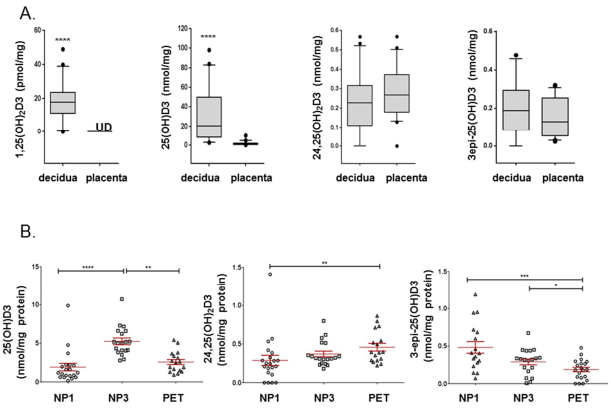
[17] C.L. Wagner, C. Baggerly, S. McDonnell, K.A. Baggerly, C.B. French, L. Baggerly, S.A. Hamilton, B.W. Hollis, Post-hoc analysis of vitamin D status and reduced risk of preterm birth in two vitamin D pregnancy cohorts compared with South Carolina March of Dimes 2009-2011 rates, The Journal of steroid biochemistry and molecular biology 155(Pt B) (2016) 245-51.

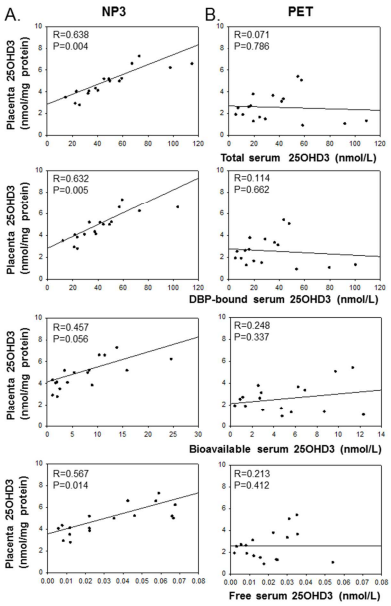
[18] L.M. De-Regil, C. Palacios, L.K. Lombardo, J.P. Pena-Rosas, Vitamin D supplementation for women during pregnancy, Cochrane Database Syst Rev 1 (2016) CD008873.
[19] E. Hypponen, A. Cavadino, D. Williams, A. Fraser, A. Vereczkey, W.D. Fraser, F. Banhidy, D. Lawlor, A.E. Czeizel, Vitamin D and pre-eclampsia: original data, systematic review and meta-analysis, Ann Nutr Metab 63(4) (2013) 331-40.
[20] N.C. Harvey, C. Holroyd, G. Ntani, K. Javaid, P. Cooper, R. Moon, Z. Cole, T. Tinati, K. Godfrey, E. Dennison, N.J. Bishop, J. Baird, C. Cooper, Vitamin D supplementation in pregnancy: a systematic review, Health technology assessment 18(45) (2014) 1-190.
[21] C. Thota, R. Menon, S.J. Fortunato, L. Brou, J.E. Lee, A. Al-Hendy, 1,25-Dihydroxyvitamin D deficiency is associated with preterm birth in African American and Caucasian women, Reprod Sci 21(2) (2014) 244-50.
[22] S.E. Bennett, C. Casey, J. McPeake, D.R. McCance, J.G. Manderson, A. McGinty, 3-Epi-25 hydroxyvitamin D in pregnancy, Pregnancy Hypertens 4(3) (2014) 236.
[23] A.D. Shah, E.C. Hsiao, B. O'Donnell, K. Salmeen, R. Nussbaum, M. Krebs, S. Baumgartner-Parzer, M. Kaufmann, G. Jones, D.D. Bikle, Y. Wang, A.S. Mathew, D. Shoback, I. Block-Kurbisch, Maternal Hypercalcemia Due to Failure of 1,25-Dihydroxyvitamin-D3 Catabolism in a Patient With CYP24A1 Mutations, The Journal of clinical endocrinology and metabolism 100(8) (2015) 2832-6.
[24] R.F. Chun, B.E. Peercy, E.S. Orwoll, C.M. Nielson, J.S. Adams, M. Hewison, Vitamin D and DBP: the free hormone hypothesis revisited, The Journal of steroid biochemistry and molecular biology 144 Pt A (2014) 132-7.
[25] A.L. Tranquilli, M.A. Brown, G.G. Zeeman, G. Dekker, B.M. Sibai, The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP), Pregnancy Hypertens 3(1) (2013) 44-7.
[26] C. Jenkinson, A.E. Taylor, Z.K. Hassan-Smith, J.S. Adams, P.M. Stewart, M. Hewison, B.G. Keevil, High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum, J Chromatogr B Analyt Technol Biomed Life Sci 1014 (2016) 56-63.
[27] R.F. Chun, B.E. Peercy, J.S. Adams, M. Hewison, Vitamin D binding protein and monocyte response to 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling, PloS one 7(1) (2012) e30773.
[28] S.N. Karras, P. Anagnostis, E. Bili, D. Naughton, A. Petroczi, F. Papadopoulou, D.G. Goulis, Maternal vitamin D status in pregnancy and offspring bone development: the unmet needs of vitamin D era, Osteoporos Int 25(3) (2014) 795-805.
[29] B.W. Mol, C.T. Roberts, S. Thangaratinam, L.A. Magee, C.J. de Groot, G.J. Hofmeyr, Pre-eclampsia, Lancet 387(10022) (2016) 999-1011.
[30] S.J. Fisher, Why is placentation abnormal in preeclampsia?, American journal of obstetrics and gynecology 213(4 Suppl) (2015) S115-22.
[31] C. Lechtermann, B.P. Hauffa, R. Herrmann, M.M. Schundeln, A. Gellhaus, M. Schmidt, C. Grasemann, Maternal vitamin D status in preeclampsia: seasonal changes are not influenced by placental gene expression of vitamin D metabolizing enzymes, PloS one 9(8) (2014) e105558.
[32] A. Halhali, L. Diaz, E. Avila, A.C. Ariza, M. Garabedian, F. Larrea, Decreased fractional urinary calcium excretion and serum 1,25-dihydroxyvitamin D and IGF-I levels in preeclampsia, The Journal of steroid biochemistry and molecular biology 103(3-5) (2007) 803-6.
[33] L. Diaz, C. Arranz, E. Avila, A. Halhali, F. Vilchis, F. Larrea, Expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase are restricted in cultures of human syncytiotrophoblast cells from preeclamptic pregnancies, The Journal of clinical endocrinology and metabolism 87(8) (2002) 3876-82.
[34] R. Ma, Y. Gu, S. Zhao, J. Sun, L.J. Groome, Y. Wang, Expressions of vitamin D metabolic components VDBP, CYP2R1, CYP27B1, CYP24A1, and VDR in placentas from normal and preeclamptic pregnancies, American journal of physiology. Endocrinology and metabolism 303(7) (2012) E928-35.

[35] D. Fischer, A. Schroer, D. Ludders, T. Cordes, B. Bucker, J. Reichrath, M. Friedrich, Metabolism of vitamin D3 in the placental tissue of normal and preeclampsia complicated pregnancies and premature births, Clin Exp Obstet Gynecol 34(2) (2007) 80-4.
[36] S. Messerlian, X. Gao, R. St-Arnaud, The 3-epi- and 24-oxo-derivatives of 1alpha,25 dihydroxyvitamin D(3) stimulate transcription through the vitamin D receptor, J Steroid Biochem Mol Biol 72(1-2) (2000) 29-34.
[37] J.C. Fleet, J. Bradley, G.S. Reddy, R. Ray, R.J. Wood, 1 alpha,25-(OH)2-vitamin D3 analogs with minimal in vivo calcemic activity can stimulate significant transepithelial calcium transport and mRNA expression in vitro, Archives of biochemistry and biophysics 329(2) (1996) 228-34.
[38] C.H. Horne, P.W. Howie, R.B. Goudie, Serum-alpha2-macroglobulin, transferrin, albumin, and IgG levels in preeclampsia, Journal of clinical pathology 23(6) (1970) 514-6.
[39] C.E. Powe, E.W. Seely, S. Rana, I. Bhan, J. Ecker, S.A. Karumanchi, R. Thadhani, First trimester vitamin D, vitamin D binding protein, and subsequent preeclampsia, Hypertension 56(4) (2010) 758-63.
[40] S. Lundgren, T. Carling, G. Hjalm, C. Juhlin, J. Rastad, U. Pihlgren, L. Rask, G. Akerstrom, P. Hellman, Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein, J Histochem Cytochem 45(3) (1997) 383-92.
[41] N.Q. Liu, Y. Ouyang, Y. Bulut, V. Lagishetty, S.Y. Chan, B.W. Hollis, C. Wagner, O. Equils, M. Hewison, Dietary vitamin D restriction in pregnant female mice is associated with maternal hypertension and altered placental and fetal development, Endocrinology 154(7) (2013) 2270-80.
[42] C.E. Powe, M.K. Evans, J. Wenger, A.B. Zonderman, A.H. Berg, M. Nalls, H. Tamez, D. Zhang, I. Bhan, S.A. Karumanchi, N.R. Powe, R. Thadhani, Vitamin D-binding protein and vitamin D status of black Americans and white Americans, N Engl J Med 369(21) (2013) 1991-2000.
[43] C.M. Nielson, K.S. Jones, R.F. Chun, J.M. Jacobs, Y. Wang, M. Hewison, J.S. Adams, C.M. Swanson, C.G. Lee, D. Vanderschueren, S. Pauwels, A. Prentice, R.D. Smith, T. Shi, Y. Gao, A.A. Schepmoes, J.M. Zmuda, J. Lapidus, J.A. Cauley, R. Bouillon, I. Schoenmakers, E.S. Orwoll, Free 25-Hydroxyvitamin D: Impact of Vitamin D Binding Protein Assays on Racial-Genotypic Associations, The Journal of clinical endocrinology and metabolism 101(5) (2016) 2226-34.
[44] C.M. Nielson, K.S. Jones, R.F. Chun, J.M. Jacobs, Y. Wang, M. Hewison, J.S. Adams, C.M. Swanson, C.G. Lee, D. Vanderschueren, S. Pauwels, A. Prentice, R.D. Smith, T. Shi, Y. Gao, A.A. Schepmoes, J.M. Zmuda, J. Lapidus, J.A. Cauley, R. Bouillon, I. Schoenmakers, E.S. Orwoll, G. Osteoporotic Fractures in Men Research, Free 25-Hydroxyvitamin D: Impact of Vitamin D Binding Protein Assays on Racial-Genotypic Associations, The Journal of clinical endocrinology and metabolism 101(5) (2016) 2226-34.









Legends to figures

Figure 1. Serum vitamin D metabolites in pregnant and non-pregnant women. Serum concentrations of: A) 25-hydroxyvitamin D3 (25(OH)D3) nmol/L; B) 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) pmol/L; C) 24,25-dihydroxyvitamin D3 ($24,25(OH)_2D3$) nmol/L; D) 3-epi-25(OH)D3 nmol/L. Samples groups were: non-pregnant women; healthy 1st trimester (NP1); healthy 3rd trimester (NP3); preeclampsia 3rd trimester (PET). Statistically significant variations are indicated, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 2. DBP, albumin and 25(OH)D3 bioavailability in pregnant and non-pregnant women. Serum concentrations of: A) vitamin D binding protein (DBP) (µmol/L); B) albumin (µmol/L); C) DBP-bound 25-hydroxyvitamin D3 (25(OH)D3) (nmol/L); D) bioavailable 25(OH)D3; E) free 25(OH)D3. Samples groups were: non-pregnant women; healthy 1st trimester (NP1); healthy 3rd trimester (NP3); preeclampsia 3rd trimester (PET). Statistically significant variations are indicated, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p< 0.0001$.

Figure 3. Placental and decidual tissue vitamin D metabolites in pregnant women.
A) Comparison of decidual and placental concentrations of 1,25-dihydroxyvitamin D3 (1,25(OH)D3), 25-hydroxyvitamijn D3 (25(OH)D3), 24,25-dihydroxyvitamin D3 ($24,25(OH)_2D3$), and 3-epi-25(OH)D3 in NP1 pregnancies. All nmol/mg decidual protein. B) Placental concentrations of 25(OH)D3, $24,25(OH)_2D3$, and 3-epi-25(OH)D3 in: healthy 1st trimester (NP1); healthy 3rd trimester (NP3); pre-eclampsia 3rd trimester (PET) pregnancies. All nmol/mg placental protein. Statistically significant variations are indicated, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 4. Effect of maternal serum 25-hydroyxvitamin D3 (25(OH)D3) on placental concentrations of 25(OH)D3. Serum concentrations of total, DBP-bound, bioavailable and free

25-hydroxyvitamin D3 (25(OH)D3) (nmol/L) were correlated with placental tissue concentrations of 25(OH)D3 (nmol/g placental tissue) in healthy 1st trimester (NP1), healthy 3rd trimester (NP3) and pre-eclampsia 3rd trimester (PET) samples.

- Activation of vitamin D is decreased and its catabolism increased in preeclampsia

- Maternal and placental vitamin D are correlated but this does not occur with decidua

- Placental accumulation of vitamin D is impaired in preeclampsia

- Placental uptake of vitamin D binding protein is dysregulated in preeclampsia