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Dysregulation of maternal and placental vitamin D metabolism in preeclampsia

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39 **Abstract**

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

44
45 **Methods:** In a cross sectional analysis of normal pregnant women at 1st (n=25) and 3rd
46 trimester (n=21), pregnant women with PET (n=22), and non-pregnant female controls (n=20)
47 vitamin D metabolites were quantified in paired maternal serum, placental, and decidual tissue.

48
49 **Results:** Serum 25(OH)D3 was not significantly different in sera across all four groups. In
50 normal 3rd trimester pregnant women serum active 1,25-dihydroxyvitamin D3 (1,25(OH)₂D3)
51 was significantly higher than non-pregnant, normal 1st trimester pregnant, and PET women.
52 Conversely, PET sera showed highest levels of the catabolites 3-epi-25(OH)D3 and 24,25-
53 dihydroxyvitamin D3 (24,25(OH)₂D3). Serum albumin was significantly lower in normal 3rd
54 trimester pregnant women and PET relative to normal 1st trimester pregnant women, but there
55 was no change in free/bioavailable 25(OH)D3. In PET placental tissue, 25(OH)D3 and 3-epi-
56 25(OH)D3 were lower than normal 3rd trimester tissue, whilst placental 24,25(OH)₂D3 was
57 highest in PET. Tissue 1,25(OH)₂D3 was detectable in 1st trimester decidua, which also showed
58 10-fold higher 25(OH)D3 relative to paired placentae. 3-epi-25(OH)D3 and 24,25(OH)₂D3 were
59 not different for decidua and placenta. In normal 3rd trimester pregnant women, total, free and
60 bioavailable maternal 25(OH)D3 correlated with placental 25(OH)D3, but this was not
61 conserved for PET.

62
63 **Discussion:** These data indicate that PET is associated with decreased activation, increased
64 catabolism, and impaired placental uptake of 25(OH)D3.

1 Introduction

2 Human pregnancy is associated with important changes in vitamin D physiology. Maternal
3 circulating concentrations of the active form of vitamin D, 1,25-dihydroxyvitamin D₃
4 (1,25(OH)₂D₃), increase significantly during early gestation [1]. This appears to be due to
5 increased renal activity of the enzyme 25-hydroxyvitamin D-1 α -hydroxylase (1 α -hydroxylase),
6 which converts inactive 25-hydroxyvitamin D (25(OH)D₃) to 1,25(OH)₂D₃ [2]. 1 α -hydroxylase
7 expression and activity has also been described in human decidua and fetal trophoblast [3-5].
8 Placental 1 α -hydroxylase does not appear to make a major contribution to the elevated
9 maternal 1,25(OH)₂D₃ associated with pregnancy [2]. Instead co-expression of the nuclear
10 vitamin D receptor (VDR) in maternal and fetal placental tissues suggests a more localised role
11 for 1,25(OH)₂D₃. Albeit less well understood, recent studies suggest this extends far beyond the
12 known classical calciotropic effects of vitamin D, and includes key roles in normal decidual
13 immune function and placental implantation [6-9]

14
15 Vitamin D-insufficiency is prevalent in pregnant women [10, 11]. Maternal 25(OH)D is the
16 principal determinant of neonatal circulating 25(OH)D₃, thus infants of vitamin D-deficient
17 mothers are also at risk of vitamin D-deficiency [12]. Maternal 25(OH)D₃-deficiency has also
18 been linked to adverse pregnancy outcomes associated with malplacentalation, including
19 preeclampsia (PET), small-for-gestational age and preterm birth [13-17]. Mothers receiving
20 vitamin D supplementation from early pregnancy (two trials, 219 women) have been reported to
21 have a lower risk of PET and a positive association between serum 25(OH)D₃ and reduced
22 PET risk has been reported [18]. A recent systematic review and meta-analysis, which included
23 11 observational studies, found a significant inverse relationship between maternal 25(OH)D₃
24 and risk of PET in 5 of the studies. Meta-analyses similarly suggested an inverse relationship
25 between maternal 25(OH)D₃ and PET risk, but could not infer causality due to the insufficient
26 quality of evidence [19, 20].

27

28 This heterogeneity of data for vitamin D and PET in part reflects our limited understanding of the
29 effects of vitamin D during pregnancy. Moreover, almost all studies to date have relied on
30 maternal serum concentrations of 25(OH)D₃ as the determinant of vitamin D status and
31 function, despite the potential importance of other vitamin D metabolites such as 1,25(OH)₂D₃
32 [21], 3-epi-25(OH)D₃ [22], and 24-hydroxylated vitamin D metabolites (24,25(OH)₂D₃) [23].
33 Furthermore, placental expression of 1 α -hydroxylase suggests that tissue-specific
34 concentrations of 25(OH)D₃ and other vitamin D metabolites are likely to be potential
35 determinants of local vitamin D function across gestation [6]. Finally, vitamin D binding protein
36 (DBP) and albumin are known to act as serum transporters of vitamin D metabolites, but also
37 define tissue bioavailability and function by modulating the balance of bound and free forms of
38 vitamin D [24]. The aim of the current study was therefore to characterise the relative impact of
39 each of these facets of vitamin D metabolism and transport on normal and PET pregnancies.

40

41 **Materials and Methods**

42 ***Ethical approval***

43 Written informed consent was obtained from all women recruited into the study. Matched human
44 sera, placenta and decidua samples were collected with the approval of Health Research
45 Authority - West Midlands, Edgbaston Research Ethics Committee (NHS REC 06/Q2707/12
46 [2006 approval]) (RG_14-194 [10.2014 approval]).

47

48 ***Sample collection***

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

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

58

59 Normal uncomplicated 3rd trimester (>37 weeks) ($n=21$) and PET (>34 weeks) ($n=22$) sera and
60 placental samples were collected from pregnant women consented prior to delivery at
61 Birmingham Women's Foundation Hospital Trust (BWFHT). All PET cases were prospectively
62 diagnosed according to current International definitions (ISSHP, 2014)[25]; new hypertension
63 presenting after 20 weeks, with one or more of the following new onset conditions: 1. proteinuria
64 (urinary protein: creatinine ratio > 30 mg/mmol or a validated 24-hour urine collection > 300 mg
65 protein); 2. other maternal organ dysfunction (renal insufficiency, liver involvement, neurological
66 and/ or haematological complications); 3. utero-placental dysfunction (fetal growth restriction).
67 PET severity was categorised as; mild - diastolic ≥ 90 – 99 mmHg, systolic ≥ 140 – 149 mmHg
68 ($n=9$), moderate - diastolic ≥ 100 – 109 mmHg ($n=7$), systolic ≥ 150 – 159 mmHg, severe - diastolic
69 ≥ 110 mmHg, systolic ≥ 160 mmHg ($n=6$). Maternal mean arterial blood pressure (MABP) was
70 significantly elevated comparative to the normal pregnant control group. A healthy non-pregnant
71 female 'control' group ($n=20$) was also recruited.

72

73 ***Sample preparation for LC-MS/MS Analysis***

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

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

80

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

82 Vitamin D metabolites were extracted from donor serum (0.2 mL) or placental tissue
83 homogenates as described previously [26]. Resulting samples were reconstituted in 125 μ L
84 water/methanol (50/50%) for LC-MS/MS analysis as previously described [26] using a Waters
85 ACQUITY ultra performance liquid chromatography [uPLC] coupled to a Waters Xevo TQ-S
86 mass spectrometer [Waters, Manchester, UK]. Analysis was carried out in multiple reaction
87 monitoring (MRM) mode, with optimised MRM transitions for each analyte as described
88 previously [26]. External quality control (QC) samples (LGC Standards, Teddington, UK) for
89 25(OH)D3 and 25(OH)D2 were used to assess accuracy and precision within batch runs. QCs
90 across different concentration ranges to determine inter- and intra- day accuracy and precision
91 for each analyte were as described previously [26]. Data analysis was performed with Waters
92 Target Lynx.

93

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

95 Human vitamin D binding protein (DBP) (R&D Biosystems, Abingdon, UK), and human albumin
96 (Abcam, Cambridge, UK) were measured using ELISA analyses as per manufacturer's
97 instructions. Serum concentrations of free (total minus DBP and albumin-bound) and
98 bioavailable (total minus DBP bound) serum 25(OH)D3 were calculated based on total
99 25(OH)D3 and DBP/albumin values using equations described previously [27].

100

101 ***Statistics***

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

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

109

110 **Results**

111 ***Dysregulation of serum vitamin D metabolism in PET***

112 Four serum vitamin D metabolites were consistently quantifiable in both pregnant and non-
113 pregnant women; 25(OH)D3, 1,25(OH)₂D3, 24,25(OH)₂D3, 3-epi-25(OH)D3. In non-pregnant
114 women 25(OH)D3 concentrations (median 33.4, IQR 20.8–44.3 nmol/L), were similar to healthy
115 1st trimester (NP1, 28.8, 20.3–46.9nmol/L) and 3rd trimester (NP3, 45.2, 32.5–59.2nmol/L)
116 pregnancies, as well as women diagnosed with PET (35.3, 17.7–54.7 nmol/L) (**Figure 1A**). By
117 contrast, serum 1,25(OH)₂D3 concentrations in non-pregnant women (34.2, 29.3–55.0 pmol/L),
118 were significantly lower than in pregnant women, including NP1 (113.7, 82.7–198.3 pmol/L,
119 $p<0.0001$), NP3 (254.7, 195.7–310.1 pmol/L, $P<0.0001$), and PET (171.2, 113.0–236.3 pmol/L,
120 $p<0.0001$) groups (**Figure 1B**). Consistent with previous studies [28], NP3 levels of
121 1,25(OH)₂D3 were more than two-fold higher than NP1 ($p<0.0001$), and significantly lower
122 concentrations of 1,25(OH)₂D3 ($p<0.01$) were observed in the PET cohort compared to NP3
123 (**Figure 1B**). Linear regression analysis confirmed gestational age was not a significant
124 determinant of any serum vitamin D metabolite (data not shown).

125

126 Serum concentrations of 24,25(OH)₂D3 in non-pregnant women (3.3, 1.6–4.7 nmol/L) were
127 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
128 PET (10.9, 7.3-22.5 nmol/L, $p<0.001$) (**Figure 1C**). Both NP3 and PET samples showed
129 significantly higher 24,25(OH)₂D3 concentrations than NP1 (both $p<0.0001$). Concentrations of

130 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-
131 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
132 significant. Highest 3-epi-25(OH)D3 levels were observed with PET (8.8, 5.9-11.8 nmol/L), with
133 significant differences compared to non-pregnant ($p < 0.001$), NP1 ($p < 0.05$) and NP3 groups
134 ($p < 0.05$) (**Figure 1D**). In non-pregnant women serum 25(OH)D3 was strongly correlated with
135 1,25(OH)₂D3 ($p = 0.013$), 24,25(OH)₂D3 ($p < 0.0001$) and 3-epi-25(OH)D3 ($p = 0.012$), but similar
136 correlations were not consistently observed in pregnancy (**Supplemental Figure 1**).

137

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

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

146

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

154

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

156 In contrast to the placenta, 1,25(OH)₂D₃ was quantifiable in decidual tissue (17.6, 11.0–23.4
157 pmol/mg protein), and this paralleled increased decidual concentrations of 25(OH)D₃ (21.0,
158 9.3–60.5 nmol/mg protein) relative to paired NP1 placentae (1.2, 0.7-2.2 nmol/mg protein,
159 $p < 0.001$) (**Figure 3A**). By contrast no difference in tissue levels of 24,25(OH)₂D₃ were observed
160 between decidua (0.3, 0.2–0.4 nmol/mg) and placenta (0.2, 0.1–0.3 nmol/mg). Similarly,
161 decidual concentrations of 3-epi-25(OH)D₃ (0.1, 0.1–0.3 nmol/mg) were not significantly
162 different to NP1 placental 3-epi-25(OH)D₃ (0.2, 0.1–0.3 nmol/mg) (**Figure 3A**).

163
164 In placenta tissue, concentrations of 25(OH)D₃ increased significantly from NP1 (1.2, 0.7-2.2
165 nmol/mg protein) to NP3 (5.0, 4.0-6.5 nmol/mg protein, $p < 0.0001$), but this effect was not
166 observed for PET placenta levels of 25(OH)D₃ (2.5, 1.4-3.5 nmol/mg protein) which were
167 significantly lower than NP3 values ($p < 0.01$) (**Figure 3B**). Consistent with maternal serum data,
168 placental 24,25(OH)₂D₃ values were highest for PET (0.4, 0.3–0.6 nmol/ mg) relative to NP3
169 (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-
170 25OHD₃ values were also higher for PET (0.4, 0.3–0.7 nmol/mg) relative to both NP3 (0.3, 0.2–
171 0.4 nmol/mg, $p < 0.05$) and NP1 pregnancies (0.2, 0.1–0.3 nmol/mg, $p < 0.001$) (**Figure 3B**).
172 Placental concentrations of 1,25(OH)₂D₃ were below the lower limit of quantification. In NP1
173 (data not shown) and NP3 pregnancies (**Figure 4A**) placental concentrations of 25(OH)D₃
174 correlated with maternal serum total, DBP-bound, bioavailable and free 25(OH)D₃. By contrast,
175 placental concentrations of 25(OH)D₃ in PET pregnancies showed no association with any form
176 of maternal serum 25(OH)D₃ (**Figure 4B**).

177

178 Discussion

179 PET is a pregnancy-specific hypertensive, multisystem syndrome which complicates up to 8%
180 of pregnancies [25], and is associated with significantly increased maternal and perinatal
181 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 1 α -hydroxylase, or lower expression of 1 α -hydroxylase
197 in the placenta [33], but other PET studies have reported increased whole human placental
198 tissue 1 α -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-

207 25(OH)D3 concentrations were significantly higher in the PET cohort, so this metabolic pathway
208 may also play a key role in the dysregulation of vitamin D function in PET.

209
210 This is the first study to use paired placental, decidual and serum samples to assess the
211 relationship between circulating and tissue-specific levels of vitamin D metabolites. The
212 relatively high levels of 25(OH)D3 in decidua enabled quantification of 1,25(OH)₂D3, but
213 determinants of both decidual 25(OH)D3 and 1,25(OH)₂D3 remain unclear (**Supplemental**
214 **Figure 2**). The most likely determinant of decidual 1,25(OH)₂D3 is local tissue expression of 1 α -
215 hydroxylase. In unpublished studies we have shown that decidual 1 α -hydroxylase mRNA
216 correlates with mRNA for inflammatory cytokines such as interleukin-6 and interferon- γ ,
217 suggesting that immune activity could be a key driver of decidual 1,25(OH)₂D3. Less clear is
218 what determines decidual levels of the substrate for 1 α -hydroxylase, 25(OH)D3. It was
219 interesting that neither maternal nor placental 25(OH)D3 showed any correlation with decidual
220 25(OH)D3, despite the proximity of these tissues (**Supplemental Figure 2**), suggesting that the
221 the decidua has an autonomously regulated vitamin D system.

222
223 In contrast to the decidua, data for placental tissue support the general assumption that
224 variations in circulating 25(OH)D3 are manifested by equivalent tissue changes in 25(OH)D3. In
225 NP3, placental 25(OH)D3 was closely correlated with maternal 25(OH)D well beyond levels of
226 sufficiency (>75 nmol/L). This was not observed in placentas from PET pregnancies,
227 irrespective of serum 25(OH)D3 concentration (**Figure 4B**). This may, in part, reflect
228 dysregulation of tissue catabolism of 25(OH)D3, as placental 24,25(OH)₂D3 was highest for
229 PET. However, tissue 3-epi-25(OH)D3 was significantly lower in PET placentas, suggesting that
230 this catabolic pathway does not contribute to suppression of 25(OH)D3 in the placenta.

231

232 In recent years there has been increasing interest in the potential role of serum DBP not only as
233 a transporter of vitamin D metabolites, but also as a determinant of 25(OH)D3 tissue access
234 either as unbound or 'free' 25(OH)D3, or through receptor-mediated uptake of DBP-bound
235 25(OH)D3 [24]. In the current study variations in serum DBP and albumin across pregnancy
236 (**Figure 2**) resulted in subtle changes in the relative proportions of bioavailable and free
237 25(OH)D3 (**Supplemental Figure 3**). It has been recognised for many years that serum
238 albumin decreases with pregnancy, due to increased maternal blood volume [38]. This may be
239 exacerbated in PET, although the extent to which this occurs varies with disease severity [38].
240 Previous studies using 1st trimester serum did not demonstrate any significant variation in DBP
241 or 25(OH)D3 between pregnancies that went on to normal term or PET delivery [39].

242 For both NP1 and NP3, serum DBP correlated with placental DBP, but this was not observed in
243 PET placentas, or NP1 decidua (**Supplemental Figure 4A**). PET may therefore involve
244 dysregulated endocytic uptake of DBP via the membrane receptor megalin which is expressed
245 in the placenta [40]. Although DBP uptake by the placenta appears to be dysregulated in PET,
246 other data do not support a major role for DBP as a determinant of placental or decidual
247 25(OH)D3. Firstly, placental DBP was higher in NP1 than NP3, whereas placental 25(OH)D3
248 was higher in NP3 (**Supplemental Figure 4A**). Most importantly there was no correlation
249 between placental or decidual DBP and the levels of 25(OH)D3 in these tissues for NP1
250 (**Supplemental Figure 4C and 4D**) or NP3 (data not shown). Collectively these data suggest
251 that the close association between maternal serum 25(OH)D3 and levels of this metabolite in
252 placental tissue involves placental uptake of DBP, but other mechanisms determine the final
253 tissue-specific concentrations of 25(OH)D3. Dysregulation of this process in PET may reflect
254 aberrant spiral artery development and placental blood flow, both of which are associated with
255 malplacentation and may alter DBP uptake and 25(OH)D3 metabolism. We have shown
256 previously that 25(OH)D3 and 1,25(OH)₂D3 promote matrix invasion by human trophoblastic

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

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

266
267 The over-arching conclusions from this study are: 1) that PET is characterised by changes in
268 multiple vitamin D metabolic pathways, emphasising the limited information to be gained from
269 measurement of maternal 25(OH)D3; 2) changes in maternal DBP and albumin do not appear
270 to have a major impact on the bioavailability and placental/decidual accumulation of vitamin D;
271 3) in normal healthy pregnancies, maternal serum 25(OH)D3 is closely correlated with placental
272 25(OH)D3, underlining the potential benefits of vitamin D supplementation in pregnancy; 4) this
273 effect is lost in PET pregnancies, and the potential impact of this on resulting offspring will be a
274 target for future studies; 5) in contrast to the placenta, the decidua can synthesise detectable
275 levels of 1,25(OH)₂D3. However, the underlying mechanistic basis for regulation of this
276 metabolism in the decidua is still unclear, and requires further investigation. An important
277 limitation of the present study is the large inter-group variability in all vitamin D metabolites
278 measured and most likely reflects the small sample size and non-matched study design.
279 Validation of these findings in a high-powered, matched cohort study including pregnant women
280 with PET versus normotensive pregnant and non-pregnant controls is required to inform any
281 future vitamin D supplementation trial targeting correction of the vitamin D metabolome.

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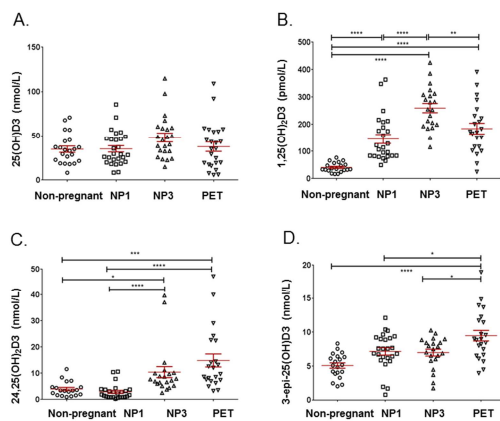
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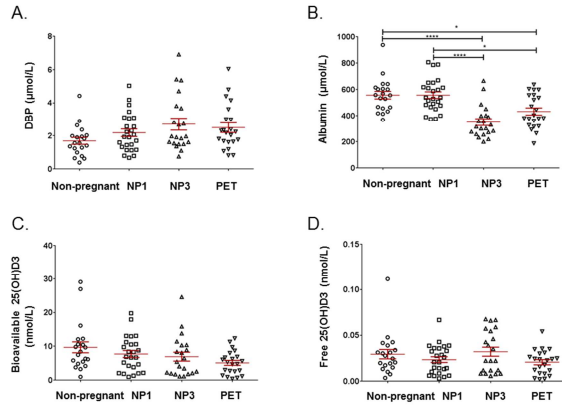
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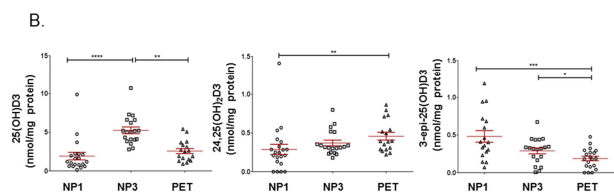
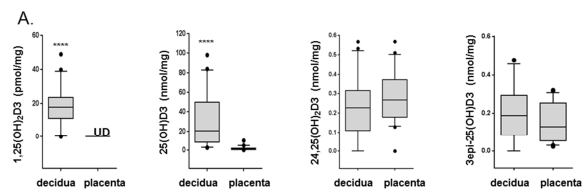
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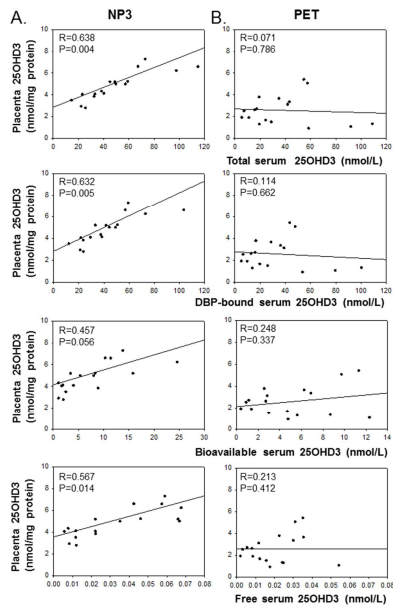
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1 **Legends to figures**

2 **Figure 1. Serum vitamin D metabolites in pregnant and non-pregnant women.** Serum
3 concentrations of: A) 25-hydroxyvitamin D3 (25(OH)D3) nmol/L; B) 1,25-dihydroxyvitamin D3
4 (1,25(OH)₂D3) pmol/L; C) 24,25-dihydroxyvitamin D3 (24,25(OH)₂D3) nmol/L; D) 3-epi-
5 25(OH)D3 nmol/L. Samples groups were: non-pregnant women; healthy 1st trimester (NP1);
6 healthy 3rd trimester (NP3); preeclampsia 3rd trimester (PET). Statistically significant variations
7 are indicated, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

8

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

15

16 **Figure 3. Placental and decidual tissue vitamin D metabolites in pregnant women.**

17 A) Comparison of decidual and placental concentrations of 1,25-dihydroxyvitamin D3
18 (1,25(OH)₂D3), 25-hydroxyvitamin D3 (25(OH)D3), 24,25-dihydroxyvitamin D3 (24,25(OH)₂D3),
19 and 3-epi-25(OH)D3 in NP1 pregnancies. All nmol/mg decidual protein. B) Placental
20 concentrations of 25(OH)D3, 24,25(OH)₂D3, and 3-epi-25(OH)D3 in: healthy 1st trimester (NP1);
21 healthy 3rd trimester (NP3); pre-eclampsia 3rd trimester (PET) pregnancies. All nmol/mg
22 placental protein. Statistically significant variations are indicated, * p<0.05, ** p<0.01, ***
23 p<0.001, **** p<0.0001.

24

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

27 25-hydroxyvitamin D3 (25(OH)D3) (nmol/L) were correlated with placental tissue concentrations
28 of 25(OH)D3 (nmol/g placental tissue) in healthy 1st trimester (NP1), healthy 3rd trimester (NP3)
29 and pre-eclampsia 3rd trimester (PET) samples.

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ACCEPTED MANUSCRIPT

- 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