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Vitamin D, the placenta and early pregnancy

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1 **Vitamin D, the placenta and early pregnancy: effects on trophoblast**

2 **function**

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34   **Abstract**

35   Pregnancy is associated with significant changes in vitamin D metabolism, notably increased

36   maternal serum levels of active vitamin D, 1,25-dihydroxyvitamin ($1,25(OH)_2D$). This appears

37   to be due primarily to increased renal activity of the enzyme 25-hydroxyvitamin D-1α-

38   hydroxylase (CYP27B1) that catalyzes synthesis of $1,25(OH)_2D$, but CYP27B1 expression is

39   also prominent in both the maternal decidua and fetal trophoblast components of the

40   placenta. The precise function of placental synthesis of $1,25(OH)_2D$ remains unclear, but is

41   likely to involve localised tissue-specific responses with both decidua and trophoblast also

42   expressing the vitamin D receptor (VDR) for $1,25(OH)_2D$. We have previously described

43   immunomodulatory responses to $1,25(OH)_2D$ by diverse populations of VDR-expressing

44   cells within the decidua. The aim of the current review is to detail the role of vitamin D in

45   pregnancy from a trophoblast perspective, with particular emphasis on the potential role of

46    $1,25(OH)_2D$ as a regulator of trophoblast invasion in early pregnancy. Vitamin D-deficiency is

47   common in pregnant women, and a wide range of studies have linked low vitamin D status to

48   adverse events in pregnancy. To date most of these studies have focused on adverse

49   events later in pregnancy, but the current review will explore the potential impact of vitamin

50   D on early pregnancy, and how this may influence implantation and miscarriage.

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58 **Introduction**

59 The human placenta is a vital organ without which the mammalian fetus cannot survive. It

60 forms the interface between the mother and fetus, supplying the fetus with oxygen, nutrients,

61 excreting waste products, whilst protecting against maternal immunologic attack. The main

62 functions of the placenta can be broadly categorised into transport and metabolism,

63 protection and endocrine (Gude, et al. 2004). The complex architecture of the placenta,

64 bounded by the maternal aspect (basal plate) and the fetal aspect (chorionic plate), houses

65 an abundance of the fundamental functional unit of the placenta, the chorionic villus, where

66 all nutritional-waste exchange between the maternal blood and the fetal circulation occurs.

67 As well as facilitating a good maternal blood supply for nutrition-waste exchange, and

68 orchestrating endocrine mediators of pregnancy to maintain maternal physiological changes

69 for an optimal environment for fetal development, the placenta also acts to protect the fetus

70 from xenobiotic materials and infectious agents (Gude et al. 2004; Moore, et al. 1999;

71 Rudge, et al. 2009; Yang 1997). Successful development of the placenta involves two

72 distinct mechanisms: implantation of the blastocyst, initiated by attachment of the embryo to

73 the maternal endometrial epithelium, and invasion of fetal trophoblast cells into the maternal

74 endometrium to facilitate maternal-fetal exchange of nutrients, gases and waste. The diverse

75 mechanisms associated with the regulation of trophoblast invasion have been well

76 documented (Menkhorst, et al. 2016). The aim of the current review is to provide an

77 overview of these early events in placental development, with particular emphasis on the

78 potential role of vitamin D as a determinant of early placental development through effects

79 on trophoblast cells, particularly via effects of vitamin D on trophoblast invasion.

80

81 **Vitamin D and pregnancy**

82 Despite its long-standing association with rickets and osteoporosis, vitamin D has become

83 increasingly recognized as a pluripotent regulator of biological functions above and beyond

84 its classical effects on bone and calcium homeostasis. Expression of vitamin D receptor

3

85 (VDR) for the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), as well as the

86 1α-hydroxylase enzyme that synthesizes 1,25(OH)$_2$D (CYP27B1), has been reported for

87 various tissues that can be broadly termed 'barrier sites' (Jones, et al. 1998; Townsend, et

88 al. 2005), indicating that localized responses to vitamin D may be a key feature of these

89 tissues. Prominent amongst these barrier sites is the placenta, acting as the interface

90 between mother and fetus. Historically, the placenta was one of the first extra-renal tissues

91 shown to be capable of synthesizing 1,25(OH)$_2$D, with CYP27B1 activity detectable in both

92 maternal decidua and fetal trophoblast (Gray, et al. 1979; Weisman, et al. 1979). Initially,

93 this was linked to the rise in maternal serum 1,25(OH)$_2$D that occurs at the end of the first

94 trimester of pregnancy. However, studies of CYP27B1-deficient animals and an anephric

95 pregnant woman indicated that this is not likely to be the case (Kovacs and Kronenberg

96 1997). Instead, the presence of VDR in the placenta suggests that vitamin D functions in

97 tissue-specific fashion at the fetal-maternal interface (Bruns and Bruns 1983). One possible

98 explanation is that 1,25(OH)$_2$D acts as a regulator of placental calcium transport (Bruns and

99 Bruns 1983), but a placental immunomodulatory function has also been proposed (Liu and

100 Hewison 2012). Moreover, the rapid induction of VDR and CYP27B1 early in pregnancy

101 (Zehnder, et al. 2002) suggests that vitamin D may play a more fundamental role in the

102 process of conception, implantation and development of the placenta itself.

103

Vitamin D and implantation

105 To date the precise role of vitamin D in the process of implantation remains unclear.

106 Nevertheless, vitamin D has a biologically plausible role in female reproduction and

107 implantation process. 1,25(OH)$_2$D has been shown to regulate expression of the homeobox

108 gene HOXA10 in human endometrial stromal cells (Du, et al. 2005b). HOXA10 is important

109 for the development of the uterus during fetal life and, later in adulthood, is essential for

110 endometrial development, allowing uterine receptivity to implantation (Bagot, et al. 2000).

111 Interestingly, animal studies have shown that vitamin D-deficiency reduces mating success

112 and fertility in female rats. Female rats fed with a vitamin D-deficient diet are capable of

113 reproduction, but overall fertility is reduced including the failure of implantation (Halloran and

114 DeLuca 1980). This was shown to be corrected by administration of $1,25(OH)_2D$ (Kwiecinksi,

115 et al. 1989), but also by use of diets high in calcium, phosphate and lactose (Johnson and

116 DeLuca 2002), suggesting that the fertility effects of vitamin D may be due to indirect effects

117 on mineral homeostasis. Other studies using knockout mouse models have further

118 highlighted the importance of the vitamin D metabolic and signalling system in the process of

119 implantation, with *Vdr* -/- and *Cyp27b1* -/- female mice both presenting with uterine

120 hypoplasia and infertility (Panda, et al. 2001; Yoshizawa, et al. 1997). Conversely, injection

121 of $1,25(OH)_2D$ has been shown to increase uterine weight and promote endometrial to

122 decidual differentiation (Halhali, et al. 1991).

123

124 As well as regulating uterine and decidual development, vitamin D may also influence

125 implantation indirectly via its well-known immunomodulatory actions. Regulation of immune

126 function at the maternal-fetal interface involves a heterogeneous population of innate and

127 adaptive immune cell subsets. Thus throughout pregnancy, decidual synthesis of

128 $1,25(OH)_2D$ has the potential to influence uterine natural killer cells, dendritic cells,

129 macrophages, and T-cells (Evans, et al. 2004; Tamblyn, et al. 2015). Notable effects include

130 inhibition of Th1 cytokines and promotion of Th2 cytokines (Gregori, et al. 2001), which are

131 known to play a significant role in the process of implantation (Piccinni, et al. 2000; Zehnder

132 et al. 2002). Purification of decidual cells into non-adherent stromal cells and adherent cells,

133 which include decidual macrophages and uterine natural killer cells, has shown that

134 adherent cells demonstrate a greater capacity for $1,25(OH)_2D$ production (Kachkache, et al.

135 1993). Furthermore, first-trimester decidual cells treated with either 25OHD- or $1,25(OH)_2D$

136 demonstrate significant induction of antibacterial protein cathelicidin and β-defensins (Evans,

137 et al. 2006; Liu, et al. 2009). Since similar effects of vitamin D are observed in peripheral

138 monocytes, an equivalent innate antimicrobial responsivity is postulated to exist at the

139 maternal-fetal interface (Liu and Hewison 2012).

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Vitamin D metabolism and function in trophoblast cells

142   The organisation of maternal and fetal cells within the developing placenta has been well

143   documented elsewhere (Oreshkova, et al. 2012; Vigano, et al. 2003), and is represented

144   schematically in **Figure 1**. Both the maternal decidua and fetal trophoblast components of

145   the placenta (including syncytiotrophoblast and invasive extravillous trophoblast [EVT])

146   express CYP27B1 (Zehnder et al. 2002), and are able to produce detectable levels of

147    $1,25(OH)_2D$ (Gray et al. 1979; Weisman et al. 1979). The resulting tissue concentrations of

148    $1,25(OH)_2D$ appear to be significantly higher in the decidua (Tamblyn, et al. 2017), but the

149   coincident expression of VDR in trophoblast as well as decidua (Evans et al. 2004) means

150   that multiple cell types within the placenta are capable of responding to the locally

151   synthesized $1,25(OH)_2D$, either in an autocrine or paracrine fashion.

152

153   To date, studies of the physiological impact of decidual-trophoblast $1,25(OH)_2D$ production

154   have focused primarily on trophoblast cells, using both primary cultures of EVT and

155   trophoblast cells lines. Primary cultures of human syncytiotrophoblast express CYP27B1 and

156   are able to synthesize $1,25(OH)_2D$ (Diaz, et al. 2000), and also express VDR (Pospechova,

157   et al. 2009). However, in choriocarcinoma trophoblast cell lines such as BeWo and JEG-3,

158   expression of VDR is low, with analysis of the effects of chromatin remodelling agents

159   suggesting that this may be due to epigenetic suppression of VDR in these cells

160   (Pospechova et al. 2009). Further studies to assess the impact of differentiation of cultured

161   trophoblast cells have been carried out using cyclic AMP (cAMP) to mimic the process of

162   syncytialisation (Keryer, et al. 1998). Expression of hCG is elevated by cAMP in trophoblast

163   cells, and this was associated with decreased expression of CYP27B1, with VDR expression

164   being unaffected (Avila, et al. 2007), suggesting that presence of the vitamin D metabolic

165   and signalling pathways in the placenta is differentiation-sensitive. The JEG-3 trophoblast

166   cell line has also been reported to express CYP27B1, but synthesis of $1,25(OH)_2D$ by these

6

167   cells appears to be significantly less than observed with primary trophoblast cells and

168   unaffected by cAMP (Pospechova et al. 2009). In addition to cAMP, inflammatory cytokines

169   (Noyola-Martinez, et al. 2014), and insulin-like growth factor I (Halhali, et al. 1999) also

170   stimulate trophoblast expression of CYP27B1 and synthesis of $1,25(OH)_2D$.

171

172   The vitamin D catabolic enzyme CYP24A1 has been reported to be undetectable in

173   trophoblast cells, consistent with methylation epigenetic silencing of this gene in the human

174   placenta (Novakovic, et al. 2009). This suggests that synthesis of $1,25(OH)_2D$ by trophoblast

175   cells is not subject to the same catabolic feedback control observed in other VDR-expressing

176   tissues. However, other studies have shown that trophoblast expression of CYP24A1 is

177   increased following treatment with cAMP (Avila et al. 2007). In addition, studies using the

178   *Hyp* mouse model, which has elevated circulating levels of the positive regulator of 24-

179   hydroxylase fibroblast growth factor 23 (FGF23), showed elevated placental expression of

180   CYP24A1 mRNA in these mice (Ma, et al. 2014; Ohata, et al. 2014). Likewise, direct

181   injection of FGF23 into normal placentas from wild type mice also induced expression of

182   CYP24A1 (Ohata et al. 2014). This appears to be mediated via trophoblast expression of

183   fibroblast growth factor receptor 1 and its co-receptor α-klotho by trophoblast, suggesting

184   that catabolism via CYP24A1 plays an as yet undefined role in mediating trophoblast effects

185   of vitamin D.

186

187   Despite a wide range of studies showing regulation and activity of vitamin D metabolic

188   enzymes in primary trophoblast cells and trophoblast cell lines, the principal functional

189   analysis of vitamin D in these cells has centered on responses to $1,25(OH)_2D$. Initial

190   experiments using JEG-3 cells described stimulation of calcium uptake (Tuan, et al. 1991),

191   and the regulation of the cytosolic calcium binding protein calbindin-D28K (Belkacemi, et al.

192   2005) by $1,25(OH)_2D$, consistent with a role for vitamin D in the endocrinology of placental

193   calcium homeostasis. However, subsequent investigations of trophoblast cells and

194    $1,25(OH)_2D$ have explored other mechanisms associated with placental endocrine function.

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195   These reports include the stimulation of human placental lactogen synthesis and release

196   (Stephanou, et al. 1994), hCG expression (Barrera, et al. 2008), and the regulation of

197   estradiol and progesterone synthesis (Barrera, et al. 2007).

198

199   In recent years, our perspective on vitamin D and trophoblast function has been expanded to

200   include studies of immunomodulatory function. In primary trophoblast cells and trophoblast

201   cell lines, $1,25(OH)_2D$ has been shown to potently stimulate expression of the antibacterial

202   protein cathelicidin (Liu et al. 2009), whilst also suppressing inflammatory responses to

203   tumor necrosis factor α (TNFα) (Diaz, et al. 2009). Similar anti-inflammatory responses to

204    $1,25(OH)_2D$ have also been reported using trophoblasts from women with the inflammatory

205   disorders of pregnancy, preeclampsia (Noyola-Martinez, et al. 2013), and antiphospholipid

206   syndrome (APS) (Gysler, et al. 2015). In recent studies the anti-inflammatory effects of

207    $1,25(OH)_2D$ on trophoblasts have been reported to include attenuation of oxidative stress-

208   induced microparticle release from preeclampsia trophoblastic cells (Xu, et al. 2017), further

209   underlining the importance of this facet of vitamin D function within the placenta. *In vivo*,

210   studies using *Cyp27b1-/-* and *Vdr-/-* mice have shown that loss of both alleles for either of

211   these genes on the fetal side of the placenta alone was sufficient to dramatically exacerbate

212   anti-inflammatory responses to lipopolysaccharide (LPS) immune challenge (Liu, et al.

213   2011). Thus, in addition to the active immune cell function classically observed in the

214   maternal decidua, trophoblast cells also appear to make a major contribution to the

215   regulation of placental inflammation.

216

217   **A role for vitamin D in EVT invasion?**

218   Controlled invasion of fetal cytotrophoblast and differentiated EVT cells into the maternal

219   decidua and myometrium in the first trimester of pregnancy is a key process in placentation,

220   and is essential for successful pregnancy. A complex network of communications among

221   trophoblast, decidual stromal, and immune cells is reported to facilitate implantation and

222   maintenance of pregnancy, with key roles in tissue remodelling, cell trafficking, and immune

223   tolerance being evident (Oreshkova et al. 2012). The mechanisms underpinning these

224   processes have received increasing attention since abnormal placentation due to shallow

225   invasion of EVT can cause important pregnancy disorders such as miscarriage (Ball, et al.

226   2006), pre-eclampsia (Caniggia, et al. 2000), fetal growth restriction, pre-term birth, and

227   stillbirth (Reddy, et al. 2006) (Goldman-Wohl and Yagel 2002; Kadyrov, et al. 2006;

228   Kaufmann, et al. 2003). By contrast, unrestricted invasion resulting from a failure to restrain

229   the invading cytotrophoblast is associated with premalignant conditions such as malignant

230   choriocarcinomas and invasive mole (Caniggia et al. 2000; Ringertz 1970), and can lead to

231   aberrant placentation such as pathological adhesion to the myometrium (placenta accreta),

232   extension into the myometrium (placenta increta), or invasion through the myometrium into

233   adjacent organs (placenta percreta) (Khong 2008).

234

235   In recent studies we have shown that human EVT isolated from first trimester pregnancies

236   are a target for both 25(OH)D and 1,25(OH)$_2$D (Chan, et al. 2015). In *ex vivo* experiments

237   both vitamin D metabolites promoted the invasion of EVT through Matrigel, with zymographic

238   analysis showing that this effect involves enhanced expression of the matrix

239   metalloproteinases pro-MMP2 and pro-MMP9 (Chan et al. 2015). These observations are in

240   direct contrast to previously published studies describing 1,25(OH)$_2$D inhibition of matrix

241   invasion by tumor cells (Bao, et al. 2006). In this case the primary mode of action for

242   1,25(OH)$_2$D was indirect suppression of MMPs via enhanced tissue inhibitor of

243   metalloproteinase-1 (TIMP-1) expression. However, in other reports, low vitamin D status

244   has been shown to be associated with elevated circulating MMP2 and MMP9 (Timms, et al.

245   2002). Suppression of a variety of MMPs, including MMP2 and MMP9, by 1,25(OH)$_2$D has

246   also been described for primary cultures of human uterine fibroid cells and uterine fibroid cell

247   lines (Halder, et al. 2013). Thus, the pro-invasive effects of vitamin D on EVTs appear to be

248   quite distinct to pregnancy and the placenta.

249

250   The concept of vitamin D as a regulator of cellular motility and invasion is not novel and has

251   been extensively reported in cancer states (Krishnan, et al. 2012; Leyssens, et al. 2014; Ma,

252   et al. 2016), where effects of vitamin D have been related to modulation of epithelial

253   mesenchymal transition (EMT) (Chen, et al. 2015; Fischer and Agrawal 2014; Hou, et al.

254   2016). Interestingly, this effect of vitamin D has not been observed in non-pathophysiological

255   states or during embryogenesis. For example, vitamin D is known to inhibit invasion and

256   motility of ovarian cancer and teratocarcinoma cell lines, but does not affect these cellular

257   characteristics in the non-neoplastic ESD3 murine embryonic cell line (Abdelbaset-Ismail, et

258   al. 2016). The precise molecular mechanisms that mediate migration and invasion regulation

259   by vitamin D remain unclear, although several different pathways have been studied.

260   Notably, vitamin D has been shown to regulate the actin cytoskeleton in numerous cell

261   types. In osteoblast-like cells, vitamin D promotes actin polymerisation as part of its

262   transcriptional induction of fibroblast growth factor 23 (Fajol, et al. 2016). In endometrial

263   cells, vitamin D treatment has also been shown to induce changes in actin architecture,

264   through regulation of the RAc1/Pak1 axis (Zeng, et al. 2016). It is not clear if such responses

265   are also seen in trophoblast cells during placental development, but vitamin D has been

266   shown to rescue motility defects in fetal endothelial colony forming cell function of umbilical

267   vein endothelial cells derived from pregnancies complicated by preeclampsia (von Versen-

268   Hoynck, et al. 2014) and gestational diabetes (Gui, et al. 2015).

269

270   Effects of vitamin D on EVT invasion and migration may also be mediated indirectly via

271   effects on other known EVT regulators. $1,25(OH)_2D$ has been shown to abolish S1P

272   mediated inhibition of migration via suppression of S1PR2 in trophoblast cell lines Swan-71

273   and JEG-3 (Westwood 2017). $1,25(OH)_2D$ has also been shown to stimulate

274   hCG expression and secretion via a cAMP/PKA-mediated signalling pathway (Barrera et al.

275   2008). Although hCG is a potent regulator of trophoblast motility and invasion (Chen, et al.

276   2011; Evans 2016), it is unclear whether changes in hCG expression are specifically

277   required for effects of vitamin D on trophoblast invasion. In a similar fashion, $1,25(OH)_2D_3$

278   has been shown to positively regulate progesterone synthesis by human trophoblast cells

279   from term placenta (Barrera et al. 2007). In HTR8/SVneo trophoblast cells, which have been

280   reported to consist of a mixed population of cells, progesterone appears to suppress

281   trophoblast motility and invasion (Chen et al. 2011). Thus, $1,25(OH)_2D$ may exert indirect

282   effects on trophoblast invasion, although it is still not clear whether these effects are pro-

283   migratory. Indirect actions of vitamin D on EVT function may also stem from effects on

284   placental cell differentiation. Recent studies have shown that inactivation of VDR in

285   trophoblastic BeWo cells resulted in increased trophoblast differentiation and syncytium

286   formation (Nguyen, et al. 2015). In a similar fashion vitamin D may also influence EVT

287   invasion and motility indirectly by targeting the development of cells on the maternal side of

288   the placenta. Endometrial stromal cells treated with $1,25(OH)_2D$ have elevated expression of

289   specific genes, including HOXA10 (Du, et al. 2005a), which are known to be involved in the

290   regional development of uterine decidualization and embryo implantation by controlling

291   downstream target genes. The complex circuitry of vitamin D metabolism and function

292   involved in mediating direct or indirect effects on EVT invasion and migration has still to be

293   fully elucidated and is likely to be a key component of future studies of vitamin D in

294   pregnancy.

295

296   **Vitamin D and trophoblast function: clinical implications**

297   Irrespective of proposed functional targets, vitamin D-dysregulation during pregnancy has

298   been linked to adverse effects on placental function and pregnancy in general. In 2010 the

299   Institute of Medicine (IOM) defined vitamin deficiency as serum concentrations of 25(OH)D

300   less than 20 ng/ml (50 nM) (Holick, et al. 2011a). Subsequently the Endocrine Society

301   issued slightly different guidelines, defining vitamin D-insufficiency as being serum 25(OH)D

302   levels below 30 ng/ml (75 nM) (Holick, et al. 2011b). Against this backdrop, several recent

303   publications have highlighted the prevalence of low serum concentrations of 25(OH)D (less

304   than 25 nM) in pregnant women: 20% of pregnant women in the UK (Javaid, et al. 2006),

305   25% in the UAE (Dawodu, et al. 1997), 80% in Iran (Bassir, et al. 2001), 45% in northern

306   India (Sachan, et al. 2005), 60% in New Zealand (Eagleton and Judkins 2006) and 60–84%

307   of pregnant non-Western women in the Netherlands (van der Meer, et al. 2006). It remains

308   unclear if this reflects simply a normal physiological drop in vitamin D concentrations during

309   pregnancy or if pregnancy is a stress test which can exacerbate and unmask pathological

310   vitamin D deficiency.

311

312   Vitamin D deficiency in pregnant women has been shown to be associated with increased

313   risk for pregnancy complications (Lewis, et al. 2010). These include preeclampsia (Bodnar,

314   et al. 2007b), fetal growth restriction, small for gestational age fetus (Bodnar, et al. 2010),

315   bacterial vaginosis (Bodnar, et al. 2009), and gestational diabetes mellitus (Maghbooli, et al.

316   2008; Zhang, et al. 2008). Maternal vitamin D-deficiency has also been linked to adverse

317   effects in offspring, including reduced bone density (Javaid et al. 2006) and childhood rickets

318   (Wagner and Greer 2008), as well as increased risk of asthma (Camargo, et al. 2007), and

319   schizophrenia (McGrath 2001).

320

321   The impact of vitamin D status on early events in pregnancy has also been studied. In

322   northern countries, where there is a strong seasonal contrast in light exposure and UVB-

323   induced vitamin D production in skin, conception rates are decreased during winter months,

324   with rates rising during summer and an increased birth rate in spring (Rojansky, et al. 1992).

325   Interestingly, ovulation rates and endometrial receptivity also appear to be reduced during

326   long dark winters in northern countries (Rojansky, et al. 2000), which may be explained in

327   part by seasonal variations in vitamin D levels. With this in mind, several observational

328   studies have investigated the potential impact of vitamin D on *in vitro* fertilisation (IVF), albeit

329   with largely conflicting outcomes. In a study of infertile women undergoing IVF, those with

330   higher levels of 25(OH)D in serum and follicular fluid, were more likely to achieve pregnancy

331   following IVF, and high vitamin D levels were also shown to improve the parameters of

332   controlled ovarian hyperstimulation (Ozkan, et al. 2010). Aleyasin et al. found no significant

333   association between 25(OH)D levels in serum and follicular fluid with IVF outcomes

334   (Aleyasin, et al. 2011). However, this did not include any women with a serum vitamin D

335   level >50nmol/L. In another study of 100 women undergoing IVF, serum concentrations of

336   25(OH)D were positively associated with fertilization rate (Abadia, et al. 2016). However,

337   serum 25(OH)D was unrelated to the probability of pregnancy or live birth after IVF (Abadia

338   et al. 2016). Anifandis et al. investigated 101 women who received IVF-intracytoplasmic

339   sperm injection (ICSI) ovarian stimulation cycles. In this study, women with vitamin D-

340   sufficiency (25(OH)D level >30 ng/ml in follicular fluid) had a lower quality of embryos and

341   were less likely to achieve clinical pregnancy, compared with women with insufficient

342   (follicular fluid 25(OH)D level 20.10 to 30 ng/ml) or deficient vitamin D status (follicular fluid

343   25(OH)D level <20 ng/ml) (Anifandis, et al. 2010).

344

345   Elucidation of the immunomodulatory effects of $1,25(OH)_2D$ has led to the suggestion that

346   vitamin D might have a role in protecting against spontaneous abortion (Bubanovic 2004).

347   This was supported by *ex vivo* analyses showing that $1,25(OH)_2D$ is able to suppress

348   inflammatory cytokine production by endometrial cells from women with unexplained

349   recurrent spontaneous abortions (Tavakoli, et al. 2011). More recently, $1,25(OH)_2D$ has

350   been shown to potently regulate natural killer cells from women with recurrent miscarriage

351   (Ota, et al. 2015). Considering these observations, the impact of maternal vitamin D status

352   on pregnancy outcome has been studied in several cohorts. In a large prospective cohort

353   study of 1683 pregnant women donating serum before gestational week 22, serum

354   concentrations of 25(OH)D less than 50 nM were associated with a >2-fold increase in first

355   miscarriage rate, although no significant effect was observed for second trimester

356   miscarriage (Andersen, et al. 2015). In a prospective study of pre-conceptual vitamin D,

357   maternal serum 25(OH)D levels were not found to be associated with chances of conceiving

358   or overall risk of miscarriage (Moller, et al. 2012). However, women with miscarriage in the

359   second trimester had lower first trimester serum concentrations of 25(OH)D than those

360   women who did not miscarry (Moller et al. 2012). In a much larger, nested case-control study

361   of over 5,000 women did not reveal any adverse effects of low serum 25(OH)D on

362   pregnancy outcomes (Schneuer, et al. 2014). A recent meta-analysis and systematic review

363   concluded that vitamin D-deficiency is not associated with increased risk of spontaneous

364   recurrent abortion (Amegah, et al. 2017). Thus, the possible impact of sub-optimal vitamin D

365   on implantation and adverse pregnancy outcomes such as miscarriage still remains unclear.

366   Interestingly, in endometrial tissue from women with unexplained recurrent spontaneous

367   abortion, expression of key components in the vitamin D metabolic (CYP27B1/CYP24A1)

368   and signalling (VDR) systems was found to be comparable to endometrial tissue from

369   healthy fertile women (Tavakoli, et al. 2015). By contrast, recent studies of women with

370   recurrent miscarriage showed that expression of mRNA and protein for CYP27B1 in villous

371   and decidual tissue was lower than in control tissues from normal healthy pregnancies

372   (Wang, et al. 2016). In future studies it will be important to clarify how variations in the

373   vitamin D system within the placenta and fetal trophoblast cells affect implantation and the

374   maintenance of a successful healthy pregnancy.

375

376   A major contributing factor to vitamin D status in pregnant women is obesity, with lower

377   circulating levels of 25(OH)D being reported in in pregnant women with high body mass

378   index (BMI), relative to pregnant women with a normal BMI (Bodnar, et al. 2007a; Karlsson,

379   et al. 2015). Maternal obesity is associated with adverse health effects for both mother and

380   child, with increased inflammation has been proposed as an important pathological

381   mechanism for the detrimental effects of obesity during pregnancy (Denison, et al. 2010;

382   Pantham, et al. 2015). A role of vitamin D in the process is still unclear. However, given the

383   established anti-inflammatory effects of vitamin D at the fetal-maternal interface (Tamblyn et

384   al. 2015) it is possible that some pregnancy effects of obesity are mediated via low

385   circulating maternal vitamin D.

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Conclusions

Expression of placental CYP27B1 and VDR at early stages of pregnancy suggests an important role for vitamin D in placental physiology. In previous studies we have hypothesized that placental vitamin D may function, at least in part, to promote anti-microbial and anti-inflammatory immune activity, with both the maternal decidua and fetal trophoblast contributing to these actions. However, analysis of trophoblast cells *ex vivo* and *in vitro* indicates that vitamin D may have a much broader role in placental function, including the regulation of trophoblast differentiation and EVT invasion of the decidua and myometrium (**Figure 1**). Thus, effects of vitamin D may occur earlier in pregnancy than previously appreciated, underlining the requirement for adequate vitamin D status across gestation. To date, studies of vitamin D status (maternal serum 25(OH)D) in pregnancy have tended to focus on later stages of pregnancy, and associated adverse events such as preterm birth, gestational diabetes and preeclampsia. Likewise, supplementation trials for vitamin D in pregnancy have focused on women between 10 and 18 weeks of pregnancy. However, the responsiveness of trophoblast cells to 1,25(OH)$_2$D, notably effects on EVT invasion, suggests that further studies of vitamin D and adverse events in early pregnancy are required. To date there have been a limited number of reports of vitamin D-deficiency and miscarriage, but these need to be expanded to include more rigorous supplementation trials. The review we present is supportive of early, pre-conceptual, supplementation with vitamin D.

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411 **Declaration of Interests**

412 The authors declare that there is no conflict of interest that could be perceived as prejudicing

413 the impartiality of the research reported.

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Legend to figure

Figure 1. Vitamin D pathway components at the maternal-fetal interface associated with implantation. Schematic showing key cell types involved in implantation and associated expression of components of the vitamin D system: vitamin D binding protein (DBP); vitamin D receptor (VDR); retinoid X receptor (RXR); vitamin D-25-hydroxylase (CYP2R1); 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1); vitamin D-24-hydroxylase (CYP24A1); human chorionic gonadotropin (hCG); human prolactin (hPL).

Decidua basalis (including inflammatory cells)
Expresses CYP27B1, VDR, CYP24A1
Vitamin D regulates key target genes associated
with implantation, promotes immunosuppression,
and induces decidualisation

Myometrium (including inflammatory cells)
Expresses VDR
Vitamin D regulates myometrial contractile profile,
and myometrial cell proliferation

**Intervillous space –
maternal blood**

**Invasive extra-villous
trophoblast (EVT)**
Expresses DBP, CYP24A1,
VDR, extracellular fibronectin.
Vitamin D promotes EVT
invasion and anti-bacterial/
anti-inflammatory responses

Syncytiotrophoblast
Expresses CYP27B1,
VDR, CYP24A1
Vitamin D promotes anti-
inflammatory and anti-migratory
function, and regulates
production of hCG, hPL,
estradiol and progesterone

**Monocellular layer of
cytotrophoblast**
Expresses DBP, VDR, RXRα.
Vitamin D promotes
anti-bacterial/anti-inflammatory
responses

Trophoblastic basement membrane
Produces fibronectin, laminin,
type 4 collagen, hCG
Response to vitamin D unknown

Villous core fetal vessels
Express CYP27B1, CYP2R1
Response to vitamin D unknown