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1   **Thyroid hormones and their placental deiodination in normal and pre-eclamptic**

2   **pregnancy**

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22   **Short title:** Thyroid hormones & IDs in pre-eclampsia

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Abstract

Pre-eclampsia is associated with lower serum selenium concentrations and glutathione peroxidase expression/activity; total thyroid hormones are also lower. ***Objectives, study design and main outcome measures:*** We hypothesised that the placental selenoprotein deiodinase (D3) will be protected in pre-eclampsia due to the hierarchy of selenoprotein biosynthesis in selenium deficiency. Venous blood and tissue from three standardised placental sites were obtained at delivery from 27 normotensive and 23 pre-eclamptic women. mRNA expression and enzyme activity were assessed for both deiodinases (D2 and D3); protein expression/localisation was also measured for D3. FT_4, FT_3 and TSH concentrations were measured in maternal and umbilical cord blood. ***Results:*** No significant differences in D3 mRNA or protein expression between normotensive and pre-eclamptic pregnancies. There was a significant effect of sampling site on placental D3 activity only in pre-eclamptic women ($P=0.034$; highest activity nearest the cord). A strong correlation between D3 mRNA expression and enzyme activity existed only in the pre-eclamptic group; further strengthened when controlling for maternal selenium ($P<0.002$). No significant differences were observed between groups for any of the maternal thyroid hormones; umbilical TSH concentrations were significantly higher in the pre-eclamptic samples ($P<0.001$). ***Conclusions:*** D3 mRNA and protein expression appear to be independent of selenium status. Nevertheless, the positive correlation between D3 mRNA expression and activity evident only in pre-eclampsia, suggests that in normotensive controls, where selenium is higher, translation is not affected, but in pre-eclampsia, where selenium is low, enzyme regulation may be altered. The raised umbilical TSH concentrations in pre-eclampsia may be an adaptive fetal response to maximise iodide uptake.

Keywords: Placenta, deiodinases, pre-eclampsia, thyroid hormones

Introduction

The availability and integration of the trace element selenium into the selenocysteine amino acid (Sec) is crucial to the enzymatic function of deiodinases (D1, D2 and D3). The regulation of selenoprotein synthesis is highly selenium-dependent; it has been shown that a hierarchy exists for the synthesis of different selenoproteins, both via differential mRNA translation and sensitivity to nonsense-mediated decay with D3 being prioritised [1]. The placenta is a key site for the activity of many selenoproteins such as the antioxidant glutathione peroxidase (GPx), iodothyronine deiodinase, and redox signalling thioredoxin reductase families [2]. Many of these roles appear to be particularly relevant to the aetiology of the pregnancy-specific condition of pre-eclampsia, a hypertensive disorder of pregnancy that occurs in ~3% of all pregnancies (*de novo* proteinuric hypertension), a leading cause of maternal and perinatal mortality and morbidity worldwide [3]. Placental and maternal systemic oxidative stress are components of the syndrome [4] and contribute to a generalised maternal systemic inflammatory activation [5]. Placental ischemia-reperfusion injury has been implicated in excessive production of reactive oxygen species, which could cause release of placental factors that mediate the inflammatory responses [6]. We have recently shown increased maternal and fetal plasma thiobarbituric acid reactive substances (TBARS) concentrations which were measured as a global marker of oxidative stress in pre-eclampsia [4].

There are three iodothyronine deiodinases, which all utilise Sec at their active site. Deiodinase types 1 and 2 (D1 and D2) primarily catalyse the removal of an iodine from the outer (phenolic) ring and in so doing convert inactive T_4 to T_3. Deiodinase type 3 (D3) catalyses the deiodination of the inner (tyrosyl) ring of both T_4 and T_3 to produce the inactive products

76 reverse T_3 (rT_3) and 3, 3'-diiodothyronine (T_2), respectively [7, 8]. D2 and D3 mRNA and

77 activity have both been identified in homogenates of human placenta from near the cord

78 insertion site [9-12]; their activity decreases with gestational age from the end of the first

79 trimester [10, 11]. D2 is an integral membrane protein found mainly in the endoplasmic

80 reticulum [13], while D3 is localised in the plasma membrane of the intra-placental cells; the

81 highest levels of D3 are found in the placenta [12]. In the human feto-placental unit, D3

82 metabolizes T_4 to rT_3 throughout pregnancy [14]; only later in pregnancy there is an increase

83 in T_4 to T3 conversion by D1 and D2. Fetal thyroxine-binding globulin (TBG) concentrations

84 rise to non-pregnant levels by the late 3rd trimester, although remaining lower than

85 maternal[15]; the fetal T_4:TBG ratio is, however, higher at term.

86

87 Placental D3 enzyme activity is 100-400 fold greater than D2 activity and the D3/D2 mRNA

88 ratio varies from 0.5-50 [10]. Placental D2 mRNA concentrations correlate with neither

89 protein nor activity rates [10]. Placental D3 activity is unaffected by plasma T_4 concentrations

90 [9, 16] and is controlled by post-transcriptional and post-translational regulation [17] such as

91 the TGF-β via Smad-dependent pathway [18].

92

93 Total T_3 and T_4, as well as TBG concentrations in women with pre-eclampsia have been

94 reported to be lower compared to normotensive pregnant women but TSH concentrations are

95 higher [19-21]; these changes have also been observed in fetal samples from pre-eclamptic

96 pregnancies [22]. We have also shown maternal and umbilical venous serum selenium

97 concentrations to be decreased in pregnancy and to be further reduced in pre-eclamptic

98 pregnancy [4]. A strong positive relationship exists between GPx activity and serum selenium

99 concentrations in both maternal plasma and placental tissue and we have reported significant

100   reductions in maternal and fetal GPx protein expression and activity in both plasma and

101   placental tissue [4, 23]. The hierarchal control of selenoproteins appears to exist in selenium

102   deficient conditions and ranks deiodinases higher than GPxs [24]. Systematic investigation of

103   the placental deiodinases in relation to pre-eclampsia appears not to have been undertaken.

104

105   We hypothesised that D3 would be preserved in placentae from pre-eclamptic women despite

106   their lower serum selenium. We also hypothesised that decreased selenium would be

107   associated with increased TSH due to the role of deiodinases in extrathyroidal production of

108   T_3, to maintain FT_3 and FT_4 concentrations.

109

110   **Methods**

111   *Subjects:* The investigations were approved by the Nottingham Hospital Ethics Committee;

112   written, informed consent was obtained from each participant. Pre-eclampsia was defined as a

113   systolic blood pressure of 140 mm Hg or more and diastolic pressure (Korotkoff V) of 90 mm

114   Hg or more on 2 occasions after 20 weeks gestation in a previously normotensive woman

115   together with proteinuria ≥ 300 mg/L, ≥ 500 mg/day or $\geq 2+$ on dipstick analysis of midstream

116   urine (MSU) if 24-hour collection result was not available [25]. The study population

117   consisted of White European women who had either a normotensive (n=27) or pre-eclamptic

118   (n=23) pregnancy (Table 1) [4]. Umbilical venous blood samples were obtained from babies

119   from 24 of the normotensive and 14 pre-eclamptic women. Medical and obstetric histories,

120   including delivery data, were obtained for each woman. The birthweight centile for each baby

121   was computed, correcting for gestation age, sex, maternal parity and body mass index (BMI)

122   [26].

5

123   *Sample collection:* Venous blood samples were taken from mothers before delivery; where

124   possible, umbilical venous samples were also taken, immediately after placental delivery.

125   Samples were taken into chilled tubes with no anticoagulant and the serum fraction stored at −

126   80°C until required. Two full depth placental tissue samples were collected from three

127   standardised locations between the cord insertion and placental periphery (1 cm from the cord

128   insertion (Near), 1 cm from the periphery (Outer), and midway between the two (Middle)),

129   avoiding placental infarcts. The placental samples were taken within 10 minutes of delivery,

130   membranes removed and the tissue washed in ice cold 1x PBS to remove maternal blood

131   contamination. One set of samples was snap frozen in liquid nitrogen and stored at -80 °C for

132   measurement of deiodinase activity and RNA assessment; the other was formalin fixed and

133   wax-embedded for immunohistochemical analysis.

134

135   *Quantitative real-time PCR:* Total RNA was extracted from a known amount of placental

136   tissue (100 mg) using QIAzol lysis reagent (Qiagen, Crawley, UK). RNA concentration and

137   quality were verified spectrophotometrically, using the Nanodrop ND-1000 (Nanodrop

138   Technologies, Labtech, Ringmer, UK); all samples had an A_{260}/A_{280} ratio greater than 1.96

139   and were stored at −80 °C. RNA (1 µg) was then reverse transcribed using the QuantiTect

140   Reverse Transcription Kit containing a mix of random primers and Oligo dT (Qiagen,

141   Crawley, UK) in a Primus 96 advanced gradient thermocycler (Peqlab Ltd, Fareham, UK).

142   Quantitative real time PCR (7500 FAST thermocycler; Applied Biosystems) was used to

143   examine the expression of D2 and D3 relative to stably expressed beta -2-microglobulin

144   (B2M) [27, 28]. Reactions set up in duplicate were carried out in total volume of 20 µl

145   comprising 10 µl FAST SYBR Green Master Mix (Applied Biosystems), 500nM forward

146   primer, 500nM reverse primer, nuclease-free water and 1µl cDNA. The PCR programme ran

147   at 95°C (20s) followed by 40 cycles of 95°C (3s), 60°C (30 s). Melt-curve analysis was

148   performed at 95°C - 60°C to confirm the presence of one single product. Two negative

149   controls were included with each set of samples: (1) no RNA template; (2) RNA provided but

150   no reverse transcription. The crossing point (CP) values were used for analysis, using a

151   mathematical model for relative quantification developed by Pfaffl[29]. The relative

152   expression ratio (R) of the target gene is calculated based on efficiency (E) and the *CP*

153   deviation of an unknown sample versus a calibrator, and is expressed in comparison to a

154   housekeeping gene [29, 30]. Primer sequences for D2 and D3 and for the housekeeping genes

155   were as previously reported [31].

156

157   *Immunohistochemistry:* Immunohistochemical analysis was performed using the Dako

158   Envision™ visualization system (Dako, Ely, UK) as previously described [23, 32]. D3

159   antibody (Abcam) was used at 0.5 µg/ml respectively, after determination of optimal

160   dilutions (data not shown). Rabbit IgG was used in place of the specific antibodies as a

161   negative control. Cerebral cortex was used as the positive control for the D3 antibody to

162   verify specificity. A specific antibody for D2 in placentae could not be found and therefore

163   not assayed.

164

165   *D2 and D3 activity assays:* The activities of specific deiodinase subtypes were estimated

166   using methods previously described [33]. Briefly, the placental samples were homogenized in

167   10 vol 0.1 M phosphate (pH 7.2), 2 mM EDTA and 10 mM dithiothreitol (P100E2D1 buffer).

168   Protein concentrations were estimated using the Bradford method [34]. D2 activity was

169   determined by HPLC analysis of the production of radioactive iodide and T3 outer ring-

170   labelled T_4, and D3 activity by HPLC analysis of the formation of radioactive T_2 and 3'-

171   iodothyronine from outer ring-labeled T_3. Deiodination in the presence of placental

172 homogenate (∼1 mg protein/ml) was corrected for non-enzymatic deiodination in the absence

173 of homogenate.

174

175 D2 activity assay: Incubations were carried out for 120 min at 37°C with 1 nM (10^5 cpm)

176 $[3',5'-^{125}I]T_4$ in the presence of 1 μM T_3 to block D3 and in the absence or presence of 100

177 nM T_4 to saturate D2, in 0.1 ml P100E2D10 buffer. Deiodinase activity was ascribed to D2 if

178 inhibited by excess unlabeled T_4.

179

180 D3 activity assay: Incubations were carried out for 60 min at 37°C with 1 nM (2×10^5 cpm)

181 $[3'-^{125}I]T_3$ in the absence or presence of 100 nM T_3 to saturate D3 in 0.1 ml P100D2D10

182 buffer. Deiodinase activity was ascribed to D3 if inhibited by excess unlabeled T_3. The

183 minimum detectable activity for deiodinase assays is (< 0.1fmol/min/mg protein) using this

184 methodology.

185

186 *Thyroid hormone assays:* Competitive immunoassays, using direct chemiluminescent

187 technology were used to measure FT3 and FT4 concentrations; a two-site sandwich

188 immunoassay for TSH concentrations in serum was used in the ADVIA Centaur system. All

189 serum samples were analysed in triplicate, with the inter- and intra-assays being less than 5%

190 and 10%, respectively. The pregnancy-specific reference ranges using this methodology for

191 the third trimester have been established in a recent study and are as follows, TSH: 0.5-4

192 mU/L; FT4: 8-14.5 pmol/L and FT3: 2.5-5.5 pmol/L [35].

193

194 *Selenium measurements:* Maternal and umbilical cord serum selenium concentrations on

195 these samples were determined by a Varian SpectrAA graphite furnace atomic absorption

196 spectrophotometer. These data have been previously reported [4].

197   *Statistical analysis:* All analyses were performed using SPSS for Windows, PASW18.0.

198   Summary data are presented as means ± SD or median [interquartile range] as appropriate for

199   their distribution, as determined by the Kolmogorov-Smirnov test. Within subject

200   comparisons were made using Friedman repeated measures ANOVA, between group analysis

201   using Mann-Whitney U or Student's t tests depending on the distribution and Spearman's

202   Rank tests were used for correlation analysis. The null hypothesis was rejected where $P <$

203   0.05.

204

Results

Subjects: Table 1 describes the demographic, obstetric, and pregnancy data of the 50
participants. Both pregnancy groups conceived spontaneously and carried singleton
pregnancies. The normotensive pregnant women gave birth to infants weighing > 2500 g,
delivered at 37 weeks or later.

<mark>*Selenium concentrations:* As previously reported [4], both maternal and umbilical cord serum
selenium concentrations were significantly reduced in the pre-eclamptic compared with the
normotensive group (Table 3).</mark>

Expression of mRNA for D2 and D3: Placental mRNA expression normalised to stably
expressed B2M is reported as median value [interquartiles] and values are given for the
middle sampling location; there was no effect of sampling site (P>0.3). D2 mRNA expression
in the normotensive group was 0.23 [0.1-0.77] and in the pre-eclamptic placentae 0.38 [0.19-
1.48]. There was no significant difference in expression between the two pregnancy groups
(P=0.14). Placental D3 mRNA expression was also similarly expressed in both study groups
(P=0.50); normotensive pregnancy, 2.8 [0.9-3.9] and pre-eclamptic pregnancy, 1.6 [0.6-4.2].
However, D3 expression was higher than D2 expression (Figure 1).

D3 immunohistochemistry: D3 immunostaining was localised to the syncytiotrophoblast with
no difference in expression between the two groups (Figure 2).

D2 and D3 enzyme activities: D3 enzyme activity was identified in all placentae. Overall,
when comparing groups, the enzyme activity did not differ between normotensive and pre-
eclamptic women (P>0.05; Table 2). Placentae from pre-eclamptic women showed a

228 significant positive correlation between D3 activity and mRNA expression for all locations

229 sampled (Figure 3; P<0.05); this correlation did not exist in the normotensive samples

230 (P>0.05). However, a significant gradient in activity across the placental bed was evident

231 only in the normotensive placentae; the highest activity was demonstrated nearest the cord

232 (Friedman-Repeated Measures; P=0.034; Table 2). Mode of delivery or birthweights had no

233 influence on D3 activities. To ensure observed differences in D3 activity and expression were

234 not related to gestational age at delivery, we also compared these data with normotensive

235 controls only for the 11 pre-eclamptic pregnancies who were delivered at ≥ 37 weeks'

236 gestation. The comparison remained statistically significantly different (*P*>0.1 for both D3

237 activity and expression). D2 activity was undetectable in these samples.

238

239 *Thyroid hormone results:* One normotensive control woman had elevated maternal TSH

240 concentrations and elevated umbilical FT3; these anomalies were not associated with

241 clinically identified thyroid disorder and so were retained in the analysis.

242

243 No significant differences were seen between normotensive and pre-eclampsia samples for

244 any of maternal TSH, FT4 or FT3 concentrations (Table 3; P>0.1for all). Umbilical venous

245 TSH concentrations were significantly higher in the pre-eclamptic compared to the

246 normotensive samples (Table 3; P<0.001) but umbilical venous FT4 and FT3 concentrations

247 did not differ significantly. Mode of delivery had no influence on these levels. To ensure

248 observed differences in umbilical venous TSH concentrations were not related to gestational

249 age at delivery, we also compared these data with controls only for the 11 babies from pre-

250 eclamptic pregnancies who were delivered at ≥ 37 weeks' gestation. The comparison

251 remained statistically significantly different (*P* = 0.008). In addition, inverse relationships

252 were seen between umbilical venous TSH concentrations with our previously measured [4]

253 umbilical venous TBARS concentration (r = -0.60; R^2 = 0.40; P=0.03) and plasma GPx

254 activities (r = -0.57; R^2 = 0.23; P=0.04) in the pre-eclamptic samples only. Maternal TSH and

255 FT4 were significantly lower and FT3 significantly higher than in matched umbilical samples

256 (Table 3) in both normotensive and pre-eclamptic samples ($P < 0.05$ for all groups). There

257 was no association between maternal or umbilical selenium concentration and

258 simultaneously-measured TSH.

259 *Deiodinases and Selenium*: There was no direct association between mRNA and protein

260 expression or activity of either deiodinase enzyme and maternal or fetal serum selenium

261 concentrations.

262

Discussion

We have shown the presence of mRNA and protein D3 in term placenta, which is in agreement with previous studies [10-12]. Our original hypothesis was that there would be preferential utilisation of selenium by the iodothyronine deiodinases in pre-eclampsia. Our novel data support this hypothesis, since although there was no effect of pre-eclampsia on deiodinase mRNA or protein expression, there was a pre-eclampsia-related effect in relation to the selenoprotein D3 activity in the presence of significantly reduced serum selenium concentrations in both mother and fetus. Plasma FT_3 and FT_4 were similar in normotensive and pre-eclamptic women and their fetuses. The placental D2 activity, known to be at least 100 fold lower than D3 activity, was below the limits of detection in this study [9, 11].

Interestingly, a differential distribution of D3 activity was observed across the placental bed with highest activity near the cord insertion. However this gradient was seen only in placentae from normotensive pregnancies suggesting a possible blunting of D3 regulation in pre-eclampsia. We have previously reported gradients in enzyme activity across the placental bed in GPxs and Angiotensin converting enzyme [23, 36] as have others relating to gene expression [37] . This may relate to the lower tissue oxygenation at the periphery of the placenta, the central region being well-oxygenated owing to the direction of the maternal blood flow [38].

The regulation of D3 activity and expression is tightly controlled on a tissue-specific and even cellular level in a precise spatio-temporal manner [39]. D3 effects on thyroid hormone signalling occur via two routes according to oxygen availability [40]. When oxygen is adequate, D3 is moved from its site of synthesis in the endoplasmic reticulum to the Golgi

13

287   body and plasma membrane. However, when oxygen tension is low, D3 is redirected to the

288   nucleus to be physically closer to the thyroid hormone receptor-mediated gene transcriptional

289   control as demonstrated in human neuroblastoma cell line [40].

290

291   In colon cancer cells, D3 has been shown to be a direct transcriptional target for a complex

292   including β-catenin [41] a key molecule in the Wnt signalling pathway which interacts with

293   E-cadherin. Both mRNA and protein expression of β-catenin are down regulated in the term

294   placenta in a similar fashion to E-cadherin expression [42]. In pre-eclampsia however, E-

295   cadherin expression is elevated [43], whilst the expression of the zinc finger transcription

296   factor Snail, which controls E-cadherin, is reduced in the placental periphery [44]. The

297   perturbation of complex signalling networks within the placenta may contribute to the

298   blunting of D3 regulation in pre-eclampsia.

299

300   The lack of direct relationship between selenium deficiency and thyroid function concurs

301   with the current knowledge of the hierarchal control of selenoprotein expression in such

302   deficient conditions [24]. Endocrine tissues are well adapted to maintain selenoprotein

303   expression when selenium supply is limited and the deiodinases are maintained at the

304   expense of GPxs, which are quickly lost [45, 46].

305

306   Our results support that maternal thyroid function, based on maternal TSH, FT3 and FT4

307   concentrations, did not alter in pre-eclamptic women compared to normotensive pregnant

308   controls; this is consistent with others [47]. The thyroid hormones fell within the pregnancy

309   reference ranges for this methodology [35] and were comparable with other studies [19, 48].

310 No significant differences were observed in umbilical venous FT3 and FT4 concentrations

311 between the normotensive and pre-eclampsia groups; also consistent with other studies [49,

312 50]. However, a significant increase in TSH concentrations in the babies born to pre-

313 eclamptic mothers compared to those babies born to normotensive women was observed. It

314 has been suggested that the raised umbilical venous TSH concentrations in pre-eclampsia

315 may reflect an adaptive response by the fetus to maximise iodide uptake, thereby maintaining

316 normal levels of FT3 and FT4 [20]. Furthermore, the raised TSH concentrations may also be

317 a consequence of increased hypoxia placed on these babies due to the inadequate placentation

318 [49], although the exact mechanism has still to be elucidated. Interestingly, the novel inverse

319 relationships seen between umbilical venous TSH concentrations with our previously

320 measured umbilical venous TBARS concentration and plasma GPx activities in the pre-

321 eclamptic samples only indicate that higher TSH concentrations in the pre-eclamptic fetuses

322 are associated with increased the oxidative stress conditions.

323

324 This study illustrates that under the selenium deficiency seen in these pre-eclamptic women,

325 the thyroid hormone homeostasis remains largely unchanged though there appear to be subtle

326 differences in enzyme activity dependent on placental location, as well as in the translation

327 between mRNA expression and protein activity. Future studies with larger cohorts will focus

328 in the relationships of the fetal TSH with markers of both oxidative stress and the GPxs.

329

334

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466 **Figure Legends**

467 **Figure 1:** (A) D2 and (B) D3 mRNA expression across placental sampling sites normalised

468 to housekeeper gene B2M. The values of both deiodinases are shown to the same scale to

469 emphasise their relative differences in expression.

470 **Figure 2:** Placental D3 expression in (A) positive tissue control – cerebral cortex (B)

471 negative control – IgG – placenta (C) Normotensive placenta (D) pre-eclamptic placenta;

472 magnification x200 (arrows indicate syncytiotrophoblasts).

473 **Figure 3:** The relationship between the D3 mRNA expression and the D3 enzyme activity

474 with both parameters tested across the 3 locations between A) normotensive and B) pre-

475 eclamptic samples. Significant positive correlations were seen only in the pre-eclamptic

476 samples (normotensive R^2 - outer: 0.02; middle: 0.009; near: 0.003; P>0.05 for all, pre-

477 eclampsia R^2 – outer: 0.253, P=0.015; middle: 0.377, P=0.001; near: 0.253, P=0.034).