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Transfer and Metabolism of Cortisol by the Isolated Perfused Human Placenta

*Laura I Stirrat[1], *Bram G Sengers[2,3], Jane E Norman[1], Natalie Z.M Homer[4,5], Ruth Andrew[4,5], **Rohan M Lewis[3] and **Rebecca M Reynolds[1,5]

1. Tommy's Centre for Maternal and Fetal Health, MRC Centre for Reproductive Health, University of Edinburgh
2. Bioengineering Science Research Group, Faculty of Engineering and the Environment, University of Southampton, UK
3. Institute for Life Sciences, University of Southampton, UK.
4. Mass Spectrometry Core, Edinburgh Clinical Research Facility, University of Edinburgh, UK
5. University/BHF Centre for Cardiovascular Science, University of Edinburgh, UK

*joint first author

**joint last author

Precis: Placental cortisol metabolism and transfer was studied using tracers and computational modelling. This indicated that the placenta presents both metabolic and physical barriers to cortisol transfer.

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Corresponding author and person to whom reprint requests should be addressed:

Professor Rebecca Reynolds

University/BHF Centre for Cardiovascular Science

Queen's Medical Research Institute

47 Little France Crescent

Edinburgh EH16 4TJ

25 Phone: +44 131 2426762
26 Fax: +44 131 2426779
27 Email: R.Reynolds@ed.ac.uk
28
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48 **ABSTRACT**

49 **Context:** Fetal overexposure to glucocorticoids *in utero* is associated with fetal growth
50 restriction and is postulated to be a key mechanism linking suboptimal fetal growth with
51 cardiovascular disease in later life.

52 **Objective:** To develop a model to predict maternal-fetal glucocorticoid transfer. We
53 hypothesised placental 11β-HSD2 would be the major rate-limiting step in maternal cortisol
54 transfer to the fetus.

55 **Design:** We used a deuterated cortisol tracer in the *ex vivo* placental perfusion model, in
56 combination with computational modelling, to investigate the role of interconversion of cortisol
57 and its inactive metabolite cortisone on transfer of cortisol from mother to fetus.

58 **Participants:** Term placentas were collected from five women with uncomplicated
59 pregnancies, at elective caesarean delivery.

60 **Intervention**: Maternal artery of the isolated perfused placenta was perfused with D4-cortisol.

61 **Main Outcome Measures:** D4-cortisol, D3-cortisone and D3-cortisol were measured in
62 maternal and fetal venous outflows.

63 **Results:** D4-cortisol, D3-cortisone and D3-cortisol were detected and increased in maternal
64 and fetal veins as the concentration of D4-cortisol perfusion increased. D3-cortisone synthesis
65 was inhibited when 11β-HSD activity was inhibited. At the highest inlet concentration only
66 3.0% of the maternal cortisol was transferred to the fetal circulation, while 26.5% was
67 metabolised and 70.5% exited via the maternal vein. Inhibiting 11β-HSD activity increased the
68 transfer to the fetus to 7.3% of the maternal input, while 92.7% exited via the maternal vein.

69 **Conclusions:** Our findings challenge the concept that maternal cortisol diffuses freely across
70 the placenta and confirm that 11β-HSD2 acts as a major 'barrier' to cortisol transfer to the
71 fetus.

72 **Keywords:** Cortisol; placenta; 11β-HSD2; cortisone; tracer

73 **Word count: 3564**

74 # 1. Introduction

75 Cortisol, the principal circulating glucocorticoid hormone in humans, is essential for normal
76 fetal development and tissue maturation. Fetal overexposure to glucocorticoids *in utero* is
77 associated with intrauterine growth restriction, [1] and is postulated to be a key mechanism
78 linking suboptimal fetal growth with increased risk of cardiovascular disease in later life. [2]
79 Better knowledge of the factors regulating cortisol transfer to the fetus is essential to
80 understand the pathophysiology of fetal growth restriction and is also relevant for prescribing
81 of antenatal steroids which are widely used in clinical management of women at threat of pre-
82 term birth.

83 Maternal circulating cortisol levels rise 3-fold during pregnancy. [3] Although glucocorticoids
84 are lipophilic and thus are believed to freely cross the placenta, fetal cortisol levels are 5 to
85 10-fold lower than maternal levels [4] due to the activity of the placental enzyme 11-beta-
86 hydroxysteroid dehydrogenase-type 2 (11β-HSD2) [5-7] which catalyses the conversion of
87 active cortisol into inactive cortisone. In human placenta 11β-HSD2 is localized to the
88 syncytiotrophoblast, [7] which is the primary barrier between the mother and the fetus and
89 thus prevents glucocorticoids accessing placental cells and the fetal compartment. [8] Indeed
90 placental 11β-HSD2 has been suggested to inactivate the majority of maternal glucocorticoids
91 passing to the fetus in rodents [9] and in humans. [10] 11-beta-hydroxysteroid dehydrogenase-
92 type 1 (11β-HSD1), which regenerates cortisol from inactive cortisone, is undetectable in the
93 syncytiotrophoblast, but is localized in the extravillous trophoblasts (situated near maternal
94 circulation) and endothelial cells lining fetal capillaries in terminal villi. [11] Whether or not the
95 activity of placental 11β-HSD1 regenerates a substantial amount of cortisol or contributes
96 significantly to maternal or fetal circulations is not well understood. With a number of studies
97 demonstrating links between placental glucocorticoid transfer, sensitivity and metabolism and
98 adverse outcomes in infancy, childhood and adolescence, [12,13] understanding of the
99 regulatory mechanisms and rate-limiting steps of maternal-fetal cortisol transfer is essential in

100 order to identify whether there are any options for targeted intervention to improve pregnancy
101 outcomes.

102 Studies using the *ex vivo* dual perfused placental perfusion model together with computational
103 modelling have generated new mechanistic insights into placental amino acid and lipid transfer
104 from mother to fetus. [14-16] In the current study we used this combined experimental and
105 computational modelling approach to develop a model to explore placental cortisol metabolism
106 and transfer and its regulation. We hypothesised that activity of placental 11β-HSD2 would be
107 the major rate limiting step in maternal cortisol transfer to the fetus.

108

109 # 2. Methods

110 Five term placentas from women with uncomplicated pregnancies were collected on ice
111 immediately after delivery by elective caesarean section at the Royal Infirmary of Edinburgh,
112 with ethical approval (REC09/S0704/3) and written informed consent. Elective caesarean
113 sections were performed between 39-40 weeks of gestation.

114 ## A. Placental Perfusions

115 Placentas were perfused using the methodology of Schneider [17] as adapted in a previous
116 study. [18] Non-recirculating maternal and fetal circulations were established in an isolated
117 cotyledon within 30 minutes of delivery. The fetal circulation and maternal intervillous space
118 were perfused with a modified Earle's bicarbonate buffer (EBB: 5 mmol L^{-1} glucose, 1.8 mmol
119 L^{-1} $CaCl_2$, 0.4 mmol L^{-1} $MgSO_4$, 116.4 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 26.2 mmol L^{-1},
120 $NaHCO_3$, 0.9 mmol L^{-1} NaH_2PO_4), with Heparin (25,000 units/L; Fannin, Northamptonshire,
121 UK) and bovine serum albumin (BSA [Fraction V; 98 %], 2 g/L, Sigma, UK) added. Maternal
122 perfusate was equilibrated with 95% air and 5% CO_2, and fetal perfusate with 95% N_2 and 5%
123 CO_2 (BOC, UK). Maternal circulation was at 14 mL/min and fetal circulation at 6 mL/min using
124 a peristaltic pump (Watson-Marlow, UK).

125 Approximately 2 mL of venous perfusate was collected from the maternal and fetal venous
126 outflows, at 5-minute intervals. Fetal artery pressure was maintained between 40 – 70 mmHg
127 and fetal venous return was > 95%. At the end of the experiments, the perfused mass was
128 identified on the 'maternal side' by slight blanching. The perfused placental cotyledon was
129 weighed. Cotyledon volume was calculated on the basis of 1 mL per g tissue. Samples of
130 maternal and fetal perfusate fluid, un-perfused tissue and perfused tissue were stored at -80
131 °C until analysis.

132 ## B. Use of deuterated tracers to investigate cortisol metabolism

133 Cortisol metabolism by 11β-HSD enzymes and transport between the maternal and fetal
134 circulations was investigated using the stable isotope deuterium (D)-labelled tracer,
135 [9,11,12,12 $^{2}H_{4}$]-cortisol "D4-cortisol" [19] which is converted to [9,12,12 $^{2}H_{3}$]-cortisone "D3-
136 cortisone" by 11β-HSD2. Measurement of [9,12,12 $^{2}H_{3}$]-cortisol "D3-cortisol", which is
137 regenerated from D3-cortisone can be used to assess activity of 11β-HSD1 (Figure 1). After
138 an initial 'washout' period of 30 minutes, D4-cortisol (Steraloids, USA) was perfused into the
139 maternal circulation with stepped increases in concentrations of 20 nM, 200 nM and 800 nM
140 every 30 minutes. The 800 nM D4-cortisol concentration was considered to be representative
141 of circulating maternal cortisol levels in the third trimester [20]. The HSD inhibitor
142 carbenoxolone (Sigma, UK) was added to the perfusion solution in addition to 800 nM D4-
143 cortisol in the final 30 minutes at a concentration of 1000 nM, as informed by a previous study.
144 [10]

145 C. LC-MS/MS quantification

146 Endogenous (cortisol, cortisone) and deuterated (D4-cortisol, D3-cortisone and D3-cortisol)
147 glucocorticoids were measured simultaneously by liquid chromatography tandem mass
148 spectrometry (LC-MS/MS) using a Waters Acquity™ UPLC (Manchester, UK) liquid
149 chromatography system followed by mass spectral detection on an ABSciex QTRAP® 5500
150 (Warrington, UK) operated in positive electrospray ionization mode. Mass spectral conditions
151 are described in Supplementary Table 1 in conjunction with ion spray voltage (5500 V) and
152 source temperature (700 °C).

153 D. Perfusate fluid extraction

154 Following enrichment of perfusate (500 μL) with the internal standard epi-cortisol (10 ng;
155 Steraloids, USA) and dilution with water (500 μL) analytes were extracted using a Sep-Pak
156 C18 40 mg 96-well plate (Waters, Manchester, UK). Plates were primed with methanol (1 mL),
157 then EBB (1 mL) then samples (500 μL) were loaded and plates washed with water (1 mL).

158 Analytes were eluted from the plate using acetonitrile (1 mL) directly into a 2 mL deep well
159 collection plate (Waters, UK). Eluants were dried under oxygen-free nitrogen (60 °C) using a
160 96-well Dry down apparatus, and reconstituted in mobile phase (30:70 methanol: water; 100
161 µL).

162 ## E. Tissue Extraction

163 Placental tissue (200 mg) was homogenized in 3 mL 7:2 methanol: water and enriched with
164 internal standard epi-cortisol (10 ng, as above) before being centrifuged at 3200 g for 45
165 minutes at 4 °C. Supernatant was transferred to a clean glass vial and dried under oxygen-
166 free nitrogen (60 °C) and reconstituted in water (5 mL). Analytes were extracted using Sep-
167 Pak C18 360 mg Classic Cartridges (Waters). Cartridges were primed with 100% methanol (5
168 mL) followed by water (5 mL). Samples were added to cartridges and allowed to flow through
169 with gravity. Cartridges were washed with water (5 mL), and analytes were eluted with 100%
170 methanol (2 mL) into a 3.5 mL glass vial. Eluants were dried down under oxygen-free nitrogen
171 (60 °C) and reconstituted in 100 µL mobile phase.

172 ## F. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

173 Samples in the auto-sampler were maintained at 10 °C. Analytes were separated at 40 °C on
174 an ACE Excel C18-AR column (100 x 2.1 mm, 1.7 um; Hichrom Limited®, Berkshire, UK) at a
175 flow rate of 0.5 mL/min. Samples in the auto-sampler and sample manager were maintained
176 at 10 °C. Starting with 70% water with 0.1% formic acid (FA) (solution A) and 30% acetonitrile
177 with 0.1% FA (solution B), maintained for 4 minutes followed by a 1-minute linear rise to 60%
178 solution B, a subsequent rise to 90% solution B, before restoring to 30% solution B at 6.1
179 minutes. This condition was sustained for 1-minute to re-equilibrate.

180 The inter-assay precision of D4-Cortisol in perfusate fluid was 3.6% - 11.6%, and inter-assay
181 accuracy was 93% - 103%. Inter-assay precision of D3-Cortisol in perfusate fluid was 8.8% -
182 17.3% and inter-assay accuracy was 98% - 101%. For placental tissue samples (which were

183 all analysed on the same day), intra-assay precision was 7.0% for D4-Cortisol, and 6.4% for
184 D3-Cortisol.

185 The peak areas of deuterated steroids were corrected for the abundances of naturally
186 occurring isotopomers at baseline. In addition, the peak area of D4-cortisol was corrected for
187 interference from the M+4 isotopologue of cortisol and the M+1 isotopologue of D3-cortisol.
188 There was no available standard for D3-cortisone, so concentrations were estimated using
189 the calibration curve for cortisone and the 'fold-change' or 'units / mL' rather than concentration
190 calculated. The peak area of D3-cortisol was corrected for interference from the M+3
191 isotopologue of cortisol.

192 G. Data analysis

193 Deuterated hormone levels were adjusted for flow rate and were normalised to tissue weight
194 of the perfused cotyledon. D4-cortisol and D3-cortisol were reported in ng, and in the absence
195 of a standard for accurate quantification, D3-cortisone was measured in arbitrary units.

196 H. Computational model for placental transfer

197 A compartmental modelling framework was adopted to model the placental transfer of cortisol
198 and cortisone in the *ex-vivo* perfusion experiments, based on our previous work. [14,15,21]
199 The model distinguishes three separate physiological compartments associated with the
200 maternal, syncytiotrophoblast and fetal capillary volumes (Figure 1a). Each compartment is
201 described as well mixed. Transfer between compartments is determined by the fluxes across
202 the apical and basal membranes and assumed to occur by simple diffusion for both cortisol
203 and cortisone. Metabolic conversion from cortisol to cortisone within the syncytiotrophoblast
204 is described as unidirectional using Michaelis-Menten kinetics. Model equations were
205 implemented in Matlab (R2016a) as outlined previously [14,15,21]. Details of the equations
206 that resulted and model parameters are described in Supplementary Methods.

207 A sensitivity analysis was carried out in which the model parameters were varied with respect
208 to the values for the reference fit. The reported changes in placental transfer predicted by the
209 model were based on the steady state results at the highest maternal input concentration.

210

211 # 3. Results

212 *Characterisation of subjects*

213 The mean (sd) maternal age was 36.4 ± 6.3 years, mean gestational length was 277 ± 2 days
214 (39+4 weeks ± 2 days), and mean birthweight was 3721 ± 223 g.

215

216 *D4-cortisol, D3-cortisone and D3-cortisol levels*

217 Figure 2 shows the levels of D4-cortisol, D3-cortisone and D3-cortisol (plotted data with error
218 bars) in maternal and fetal veins increased as the concentration of D4-cortisol in the maternal
219 artery perfusion increased. D4-cortisol (Figure 2a-b) and D3-cortisone (Figure 2c-d) were
220 detected in maternal and fetal vein 5-minutes after commencement of D4-cortisol perfusion
221 (20 nM) in the maternal artery. D3-cortisol (Figure 2e-f) was detected at 95-minutes into the
222 experiment in the maternal vein (perfusion phase: 800 nM D4-cortisol), and at 75-minutes in
223 the fetal vein (perfusion phase: 200 nM D4-cortisol). Variation in the D3-cortisol levels reflects
224 both the fact that D3-cortisol levels were near the limit of detection, and technical
225 considerations when collecting maternal side samples in the perfusion system where variation
226 tends to be higher. The biggest increase in D4-cortisol and D3-cortisone levels occurred when
227 maternal artery D4-cortisol perfusion increased from 200 nM to 800 nM. Levels of D3-cortisone
228 in the maternal circulation were approximately 5-fold higher than in the fetal circulation. When
229 carbenoxolone was added to the maternal artery perfusion, D4-cortisol levels further
230 increased in maternal and fetal veins, and D3-cortisone synthesis was completely inhibited.
231 D3-cortisol levels were around 300-fold lower than D4-cortisol in both maternal and fetal
232 circulations, and were close to the assay limit of detection. Levels of D3-cortisol in the maternal
233 circulation were approximately 2-3-fold higher than levels in the fetal circulation.
234 Proportionately more of the produced D3-cortisol was released into the fetal circulation than
235 maternal circulation, when compared with the proportion of D3-cortisol released into maternal

236 and fetal circulations. Samples of buffer obtained on completion of the 'washout' phase of the
237 experiment confirmed that there were no remaining endogenous or labelled glucocorticoids
238 within the tubing used for the circuit.

239 *Placental model results*

240 The results of the model fit of the average maternal and fetal D4-cortisol measurements
241 demonstrated an excellent overall ability of the computational model to represent the
242 experimental data (Figure 2). From the model the estimated effective membrane permeability
243 constant k_{MVM} = 0.011 L/min for the maternal facing MVM and k_{BM} = 0.0015 L/min for the
244 fetal facing BM. Thus the permeability of the MVM was estimated to be 7.4 times higher than
245 that of the BM. The estimated maximum rate capacity for the conversion of cortisol into
246 cortisone $V_{4F\to3E}^{max}$ = 5.0 nmol/min per cotyledon. At the highest inlet concentration only 3.0 %
247 of the maternal cortisol input was transferred to the fetal circulation, while 26.5% was
248 metabolised and the remaining 70.5% exited via the maternal vein. Inhibiting 11β-HSD activity
249 increased the transfer to the fetus to 7.3% of the maternal input, while 92.7% exited via the
250 maternal vein. Based on these results it can also be seen that enzyme metabolism reduced
251 transfer to the fetus by 59%. Note that if there were no placental barrier and no metabolism
252 then the maternal and fetal vein would have an output of respectively 70% and 30% of the
253 maternal inlet, based on the difference in flow rates alone (i.e. if concentrations within the
254 placenta were perfectly mixed). The comparison between the predicted D3-cortisone and the
255 scaled experimental data is shown in figure 2. It can be observed that the relative steady state
256 levels correspond well for the fetal D3-cortisone, while the maternal D3-cortisone shows some
257 larger discrepancies. In addition, the model responds much more rapidly to changes in input
258 conditions. In this respect, the sharp peak at t = 150 min predicted by the model is due to the
259 absence of blocker in the washout buffer, which is assumed to take immediate effect in the
260 model.

261 The results of the sensitivity analysis in figure 3 show that when varying single
262 parameters the placental transfer of cortisol was affected most by changes in k_{BM}, the
263 membrane permeability of the BM, and the metabolic conversion rate of cortisol into cortisone
264 $V_{4F \to 3E}^{max}$. In addition, placental transfer was predicted to be moderately sensitive to k_{MVM}, the
265 permeability of the MVM, and the maternal flow rate used in the experiment Q_m. Variations in
266 K_m only had a small impact as the metabolism continued to operate in the saturated regime,
267 while increasing the fetal flow rate Q_f used in the experiment was predicted to only have a
268 minor effect on transfer. Steady state transfer was not sensitive to any of the compartment
269 volumes, as expected. To evaluate the impact of the overall membrane permeability, an
270 additional study was done in which k_{BM} and k_{MVM} were both varied simultaneously,
271 demonstrating a considerably larger effect than for the permeability of each membrane
272 separately (figure 3).

273

274

275 ## 4. Discussion

276 The experiments performed in this study using a deuterated cortisol tracer in the *ex vivo*
277 placental perfusion model allowed investigation of the role of interconversion of cortisol and
278 its inactive metabolite cortisone on transfer of cortisol from mother to fetus at term. The
279 application of computational modelling enabled interpretation of the transfer mechanisms that
280 underlie these processes. Our findings challenge the concept that maternal cortisol diffuses
281 freely across the placenta, confirm that 11β-HSD2 acts as a major 'barrier' to cortisol transfer
282 to the fetus and show preliminary evidence of local cortisol production within the placenta.

283 Addition of carbenoxolone (a potent HSD inhibitor) to the maternal artery perfusion, resulted
284 in no further production of D3-cortisone. This supports the role of 11β-HSD2 as a key player
285 in the maternal barrier to fetal glucocorticoid exposure. The activity (but not mRNA) of 11β-
286 HSD2 has been shown to decrease in the last two weeks before parturition. [22] The placentas
287 used in the experiments were obtained from elective caesarean sections at between 39-40
288 weeks gestation, so it is not known when parturition would have occurred in these
289 pregnancies. The model allowed an estimation of the maximum capacity of 11β-HSD2 for
290 conversion of cortisol to cortisone as 5.0 nmol/min per cotyledon. It is not known what the
291 capacity of 11β-HSD2 would be if exposed to high levels of maternal glucocorticoids for more
292 prolonged periods, but studies have demonstrated that 11β-HSD2 mRNA and activity is down-
293 regulated by maternal stress [23] and inflammatory diseases. [22] Further, inhibition of 11β-
294 HSD2 by maternal liquorice consumption has adverse consequences on child development
295 [24,25]. Our study supports the premise that the adverse offspring outcomes are due to
296 increased fetal glucocorticoid exposure as when 11β-HSD2 was inhibited by carbenoxolone,
297 transplacental passage of maternal cortisol to the fetal circulation was more than doubled.

298 Yet, even when 11β-HSD activity was inhibited using carbenoxolone, less than 10% of
299 maternal D4-cortisol crossed the placenta in our experiments. This observation challenges the
300 concept that cortisol freely diffuses across the placenta, and suggests alternate mechanisms

301 to protect the fetus from high maternal cortisol levels in addition to the well described
302 inactivation of cortisol by 11β-HSD2. Three ABC-transporters; multidrug-resistant protein
303 (MRP1, encoded by *ABCC1*), p-glycoprotein (P-gp, encoded by *ABCB1*) and breast-cancer-
304 resistant protein (BCRP, encoded by *ABCG2*) are localised to placental syncytiotrophoblast,
305 and the fetal vessel endothelium [26,27] consistent with the potential for active transport of
306 cortisol in and out of the placenta. Further studies are needed to investigate the contribution
307 of ABC transporters, levels of which are known to alter across gestation, [28-31] in regulating
308 maternal cortisol transfer to the fetus and in particular to understand the kinetics of efflux
309 transporters, which our preliminary observations suggest may also protect the fetus.

310 Further we observed approximately a 5-fold higher D3-cortisone release to the maternal
311 circulation compared with the fetal circulation. It also needs to be considered that the physical
312 process of cortisol diffusion across tissues may be more challenging than has been thought
313 previously. In particular, in the placenta diffusion across the water filled villous stroma may
314 prove a barrier to cortisol diffusion. This is consistent with the observation that cortisone was
315 preferentially released into the maternal circulation (2:1 maternal:fetal circulation), and the
316 lower placental to fetal permeability calculated within the model.

317 A novel finding is the observation of *de novo* placental cortisol synthesis, as evidenced by the
318 detection of D3-cortisol in both maternal and fetal circulations. Though the absolute levels of
319 D3-cortisol were low, this regeneration of cortisol may have local paracrine roles and
320 increased placental 11β-HSD1 mRNA levels have been associated with maternal depression
321 and with altered infant regulatory behaviours. [12,13] Further, proportionately more D3-cortisol
322 was transferred to the fetus than D3-cortisone, which is in line with localisation of 11β-HSD1
323 to the endothelium. [11] The computational model provided a good overall representation of
324 the experimental data under different experimental conditions. In general, the compartmental
325 model showed a faster response due to the well-mixed assumption, but this did not affect the
326 steady state levels. The model predicted that changing membrane permeability of the BM
327 would affect placental transfer of cortisol. Placental transfer of lipids has been reported to be

increased in pre-eclampsia. [32] Further studies are required to investigate whether inflammatory conditions such as pre-eclampsia and preterm labour alter the permeability of the BM, and thus alter placental cortisol transfer.

Our study has several limitations. This study focused on cortisol concentrations in the maternal and fetal plasma, as these determine the gradient driving transfer. However, from a whole body perspective, it needs to be realised that the maternal plasma compartment is larger than the fetal compartment and this would need to be taken into account for more broadly focused models. Our experiments were conducted using EBB buffer and albumin. The findings may be altered *in vivo* with the presence of corticosteroid binding globulin (CBG), the primary binding protein for cortisol [33] and this should be considered in future studies. Including such binding effects would not affect the overall modelling results if the unbound fraction is constant in the concentration range used, but would become important if binding differs between compartments. We were also unable to accurately quantify D3-cortisone concentrations, as there are no available standards. Nevertheless, we were able to estimate fold-changes in D3-cortisone concentrations so this should not limit interpretation of the results. A caveat of the model is that it does not account for further interconversion of D3-cortisol to D3-cortisone, although the net values of D3-cortisol quantified were very low. We did not study other pathways of cortisol metabolism such as the A-ring reductase enzymes, although Benediktsson et al., 1997 found that the products of 5β-reductase or 20α/β-hydroxysteroid dehydrogenase did not co-elute with cortisol or cortisone in placental perfusion studies, suggesting that these pathways may not metabolise cortisol or cortisone in the placenta. The contribution of other potential metabolism pathway, such as via carbonyl reductase 1 [34] which is located in placenta, is also unknown. Direct measurement of arterial input concentrations would also have provided additional confidence to this analysis.

Further studies using this model could investigate in more detail the contribution of the fetal circulation to maternal cortisol levels. Regeneration of cortisol from cortisone could be studied by perfusing the fetal circuit with D2-cortisone [35], and measuring the regenerated cortisol in

the maternal or fetal circuits. The potential for free placental passage of cortisol from the fetal to maternal circuit could be studied by perfusing the fetal circuit with D4-cortisol and measuring D4-cortisol, D3-cortisone and D3-cortisol in the maternal circulation. Future studies utilising inhibitors of ABC transporters are also needed to assess their contribution to placental cortisol transport. While technically challenging, functional studies using early and mid-gestation tissue would be of value as cortisol exposure at earlier gestations is thought to influence fetal growth. [36,37] Our model may also be a helpful tool in predicting fetal effects of synthetic glucocorticoids such as dexamethasone and betamethasone, used clinically to promote fetal lung maturation when preterm delivery is anticipated.

To conclude, we have developed a model to predict maternal-fetal cortisol transfer, which can now be used in future experimental design. Further studies are now needed to refine and develop the model in order to improve understanding of the mechanisms underlying maternal-fetal cortisol transfer and the pathways to normal fetal growth.

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Author Contributions

LS designed the study, conducted the placental perfusion experiments and laboratory analysis, interpreted data and wrote the manuscript. BG designed the study, conducted the computational modelling, interpreted data and wrote the manuscript. JN interpreted data. NH and RA advised with laboratory assay development and data interpretation. RL designed the study, conducted computational modelling, interpreted data and wrote the manuscript. RR designed the study, interpreted data and wrote the manuscript. All authors reviewed the manuscript.

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501 **Figure legends**

502

503 **Figure 1 a-b** Model Schematic and Metabolism of deuterium-labelled glucocorticoids.

504 Model schematic showing the three compartments (maternal, syncytiotrophoblast and fetal;
505 1a) distinguished in the model. It is assumed that transfer between compartments is by simple
506 diffusion, while metabolic conversion between cortisol and cortisone takes place in the
507 syncytiotrophoblast (Equations. 1-6, see methods section). The input concentration of D4-
508 cortisol in the maternal compartment varies over time according to the experimental protocol,
509 while the input concentration in the fetal compartment is zero at all times. The output
510 concentrations of the maternal and fetal compartments from the model can be compared to
511 the experimental data.

512 D4-Cortisol is inactivated by 11β-HSD2 to D3-cortisone, with the loss of the deuterium on C11.
513 11β-HSD1 regenerates D3-cortisol from D3-cortisone, with the addition of an unlabeled
514 hydrogen (1b).

515

516

517 **Figure 2 a-f** Model fit of experimental data.

518 In maternal circulation was 0-30 minutes EBB alone, 30-60 minutes EBB + 20nM D4-Cortisol,
519 60-90 minutes EBB + 200nM D4-Cortisol, 90-120 minutes EBB + 800nM D4-Cortisol, 120-150
520 minutes EBB + 800nM D_4F + 0.001M Carbenoxolone, 150-170 minutes EBB alone. The
521 appearance of D4-cortisol in the fetal circulation is consistent with free transplacental passage
522 of D4-cortisol. Inactivation of D4-cortisol by 11β-HSD2 is indicated by the appearance of D3-
523 cortisone in the maternal or fetal circulations, and cortisol regeneration from D3-cortisone is
524 indicated by the appearance of D3-cortisol.

525 Model fit of the experimental data for D4-cortisol in the maternal (2a) and fetal (2b)
526 compartments, with a single set of parameters. Results show an excellent correspondence
527 between model (straight line) and experiments (plotted data and error bars) ($R^2 = 0.99$). Model
528 prediction of D3-cortisone in comparison with the scaled experimental data (2c-d). Note the
529 experimental units for D3-cortisone could not be directly related to concentration and have
530 been scaled here to allow comparison of the relative changes predicted by the model. The
531 same conversion factor was applied to both maternal and fetal D3-cortisone based on the
532 average ratio between experimental units and computed concentrations at the highest input
533 level (time points t = 110, 115 and 120 min). Experimental data for D3-cortisol (2e-f). Values
534 were comparatively low and were not modelled as they do not contribute significantly to the
535 overall mass balance. All experimental results are the average of 5 placentas, expressed as
536 mean and SEM (n = 5).

537 **Key:** D4F (D4-Cortisol), EBB (Earle's Bicarbonate Buffer), CBX (carbenoxolone).

538

539 **Figure 3** Sensitivity analysis for D4-Cortisol transfer to the fetus as a function of variations in
540 the model parameters.

541 The model parameters were varied with respect to the values for the reference fit. The reported
542 changes in placental transfer predicted by the model were based on the steady state results
543 at the highest maternal input concentration.

544 **Key:** k_{MVM} (MVM permeability constant), k_{BM} (BM permeability constant), V^{max} (maximum rate
545 of reaction), K_m (Michaelis-Menton constant), V_m (maternal compartment volume), V_s
546 (syncytiotrophoblast compartment volume), V_f (fetal compartment volume), Q_m (maternal flow
547 rate, L/min), Q_f (fetal flow rate, L/min).

548

549

Transfer and Metabolism of Cortisol by the Isolated Perfused Human Placenta

*Laura I Stirrat[1], *Bram G Sengers[2,3], Jane E Norman[1], Natalie Z.M Homer[4,5], Ruth Andrew[4,5], **Rohan M Lewis[3] and **Rebecca M Reynolds[1,5]

1. Tommy's Centre for Maternal and Fetal Health, MRC Centre for Reproductive Health, University of Edinburgh
2. Bioengineering Science Research Group, Faculty of Engineering and the Environment, University of Southampton, UK
3. Institute for Life Sciences, University of Southampton, UK.
4. Mass Spectrometry Core, Edinburgh Clinical Research Facility, University of Edinburgh, UK
5. University/BHF Centre for Cardiovascular Science, University of Edinburgh, UK

*joint first author

**joint last author

Supplementary Method

Details of the model equations and model parameters are described below.

1. Model equations

$$\frac{dC_A^m}{dt} = \frac{1}{v_m}\left(Q_m\left(C_A^{in,m} - C_A^m\right) - k_{MVM}\left(C_A^m - C_A^s\right)\right) \quad [1]$$

$$\frac{dC_A^s}{dt} = \frac{1}{v_s}\left(k_{MVM}\left(C_A^m - C_A^s\right) - k_{BM}\left(C_A^s - C_A^f\right) + J_A^{metab}\right) \quad [2]$$

$$\frac{dC_A^f}{dt} = \frac{1}{v_f}\left(k_{BM}\left(C_A^s - C_A^f\right) - Q_f C_A^f\right) \quad [3]$$

where C_A^m, C_A^s and C_A^f are the concentrations (mol/L) of solute A which can be either D4-cortisol (D4F), D3-cortisone (D3E) or D3-cortisol (D3F) in the maternal "*m*", syncytiotrophoblast "*s*" and fetal "*f*" compartment respectively. Similarly, the volumes v (L) of the different compartments are indicated with subscripts using the same notation. Q_m and Q_f (L/min) are the fluid flow rates in the maternal and fetal circulation. $C_A^{in,m}$ is the maternal inlet concentration, which is zero for all solute species except D4-cortisol. Note that the fetal inlet concentration is zero for all species and therefore has not been included. k_{MVM} and k_{BM} denote the effective overall permeability constants (L/min) for the microvillous membrane (MVM) and basal membrane (BM) including surface area. These diffusive permeability constants were assumed to be the same for all solute species. The metabolic conversion rate J_A^{metab} (mol/min) depends on the solute species as follows:

$$J_{D4F}^{metab} = -\frac{V_{4F\to3E}^{max}\, C_{D4F}^s}{K_m + C_{D4F}^s} \qquad [4]$$

$$J_{D3E}^{metab} = \frac{V_{4F\to3E}^{max}\, C_{D4F}^s}{K_m + C_{D4F}^s} - \frac{V_{3E\to3F}^{max}\, C_{D3E}^s}{K_m + C_{D3E}^s} \qquad [5]$$

$$J_{D3F}^{metab} = \frac{V_{3E\to3F}^{max}\, C_{D3E}^s}{K_m + C_{D3E}^s} \qquad [6]$$

where V^{max} (mol/min) is the maximum overall metabolic conversion rate and K_m (mol/L) is the Michaelis-Menten constant, i.e. the concentration at which half the maximum rate occurs.

2. Model parameters

The total cotyledon volume was based on the average cotyledon weight from the experiments (30.8 × 10^{-3} kg, n = 5), which was directly equated to the volume in L. The volume fractions of the maternal, syncytiotrophoblast and fetal compartments distinguished in the model were set to 34%, 15% and 7.4% respectively, as in our previous work. [14,22] The flow rates in the maternal and fetal circulations Q_m= 14 × 10^{-3} L/min and Q_f= 6 × 10^{-3} L/min were directly based on the experimental settings. To account for any discrepancies between nominal and actual values, the D4-cortisol input concentrations C_A^{in} used in the model were calculated based on the combined maternal and fetal steady state output during the blocking phase. The Michaelis-Menten constant K_m was set to 44 × 10^{-9} mol/L, based on the value for the enzyme 11β-HSD2 for cortisol. [23] In first instance the same value was adopted for both metabolic conversion steps in Equations 4-6.

2. Parameter estimation

The remaining parameters in the model were determined by fitting the experimental data. The following error criterion was defined for a certain species A and compartment j in general:

$$R_A^j = \frac{1}{\left(\bar{C}_A^{exp,j}\right)^2} \sum_{i \in T} \left(C_{A,i}^j - C_{A,i}^{exp,j}\right)^2 \quad [7]$$

where $C_{A,i}^j$ and $C_{A,i}^{exp,j}$ are the computed and experimental concentrations at time point i, respectively, while $\bar{C}_A^{exp,j}$ is the mean of the experimental time points considered. The model was fitted to the steady state values after each change in maternal input concentration, including the blocking phase, therefore the set of time points T consisted of the last 4 time points for each different input phase (16 time points in total).

The D3-cortisol concentrations measured experimentally were 300 times smaller compared to D4-cortisol and did not contribute significantly to the overall mass balance. Therefore the conversion to D3-cortisol was neglected in the parameter estimation by setting $V_{3E \to 3F}^{max}$ to zero. In addition, the measured D3-cortisone values could not be directly related to concentration. Therefore D3-cortisone was not fitted, but instead the experimental values for D3-cortisone were scaled to allow comparison of the relative changes predicted by the model. Thus, only the D4-cortisol values in the maternal and fetal compartments (averaged over 5 placentas) were fitted according to the following overall error criterion:

$$R_{tot} = R_{D4F}^{m} + R_{D4F}^{f} \qquad [8]$$

In total 3 parameters were fitted, the membrane permeability constants k_{MVM} and k_{BM} and the maximum rate of conversion from cortisol to cortisone $V_{4F \to 3E}^{max}$. Time integration of Equations 1-3 was performed in Matlab (R2016a) using the *ode45* function (Runge-Kutta (4, 5) method). Parameter estimation by minimising Eq. 8 was implemented using the *fminsearch* function (Nelder-Mead method). Initial parameter estimates were varied to verify that the algorithm converged to a unique solution.

Supplementary Tables

Supplementary Table 1 Mass spectral conditions for analysis of analytes and internal standards by positive ion electrospray ionisation

	Molecular Weight (amu)	Precursor ion (*m/z*)	Product ion (*m/z*) Quan; Qual	Declustering Potential (V)	Collision energy (V) Quan; Qual	Cell exit potential (V) Quan; Qual
ANALYTES						
D4-cortisol	367.0	367.0	121; only one	121	25	20
D3-cortisol	366.0	366.0	121.1; only one	121	25	20
D3-cortisone	363.2	364.2	164.0; only one	166	31	14
INTERNAL STANDARDS						
Epi-cortisol	363.2	363.2	121.0; 77.0	131	29; 101	14; 14

Abbreviations : Atomic mass units (amu) Quan (quantifier ion), Qual (qualifier ion), V (volts)

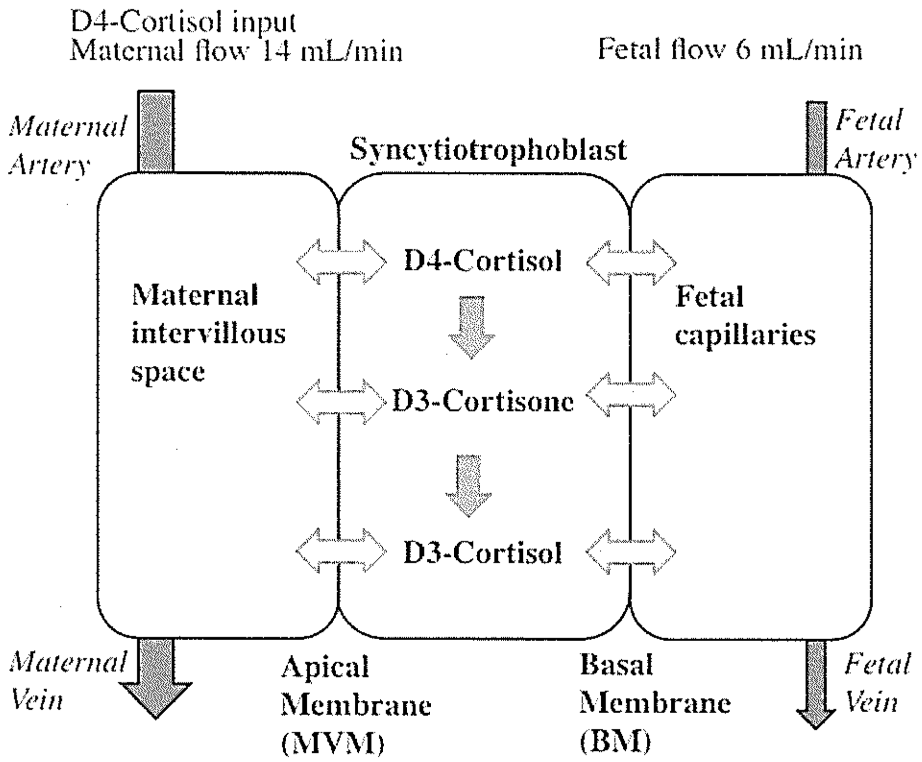
Supplementary Table 2 Inter-assay precision and accuracy

Concentrations of cortisol, cortisone, D4-Cortisol and D3-Cortisol were determined using calibration curves. Fourteen standards were prepared in 500 µL EBB (range of concentrations 0.1 ng – 400 ng) enriched with internal standards (10 ng) along with blank samples were diluted in 500 µL of water and processed using the same extraction method and analysis conditions as perfusate samples. Standard curves were plotted by calculating the peak area (analyte peak area / internal standard peak area). Weighting of 1/x and was applied to form standard curves of best fit with a regression coefficient above 0.99. The ion ratio (quantitative ion/qualitative ion) of the analytes was calculated using MultiQuant software and results were not considered acceptable if the ratio was greater than 20% of the ratio of the standards. Inter-assay fourteen point standard curve validation (n=6 different day respectively) was used to assess the limits of quantification of accuracy and precision for each analyte. Precision was based on the percentage relative standard deviation (%RSD), which was calculated using peak area ratios. Tissue sample* is intra-assay (amount, ng for tissue replicates (n=6). Inter-assay was not performed for tissue samples, as all tissue samples were analysed on the same day. Low values are the limit of quantification for each analyte.

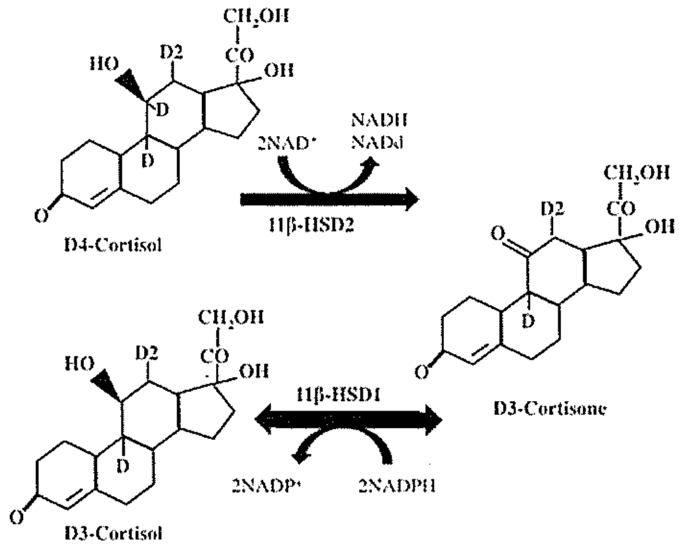
		Concentration (ng/200 µL perfusate or mg tissue*): mean (SD)	**Precision (% RSD)**	**Accuracy (%)**
D4-Cortisol	Low (0.2)	0.21 (0.02)	10.5	103
	Mid (50)	46.7 (1.7)	3.6	93
	High (400)	396.6 (43.4)	11.6	93
	Tissue Sample*	8.3 (0.6)	7.0	
D3-Cortisol	Low (0.1)	0.1 (0.02)	17.3	98
	Mid (10)	10.4 (0.9)	8.8	104
	High (20)	20.2 (2.0)	10.1	101
	Tissue Sample*	0.6 (0.04)	6.4	

Abbreviations: EBB (Earle's Bicarbonate Buffer), SD (Standard Deviation), RSD (Relative Standard Deviation)

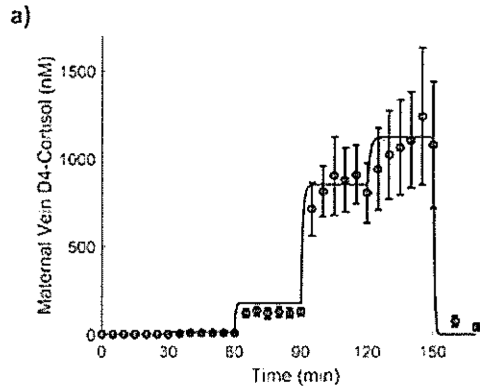
1.a



1.b

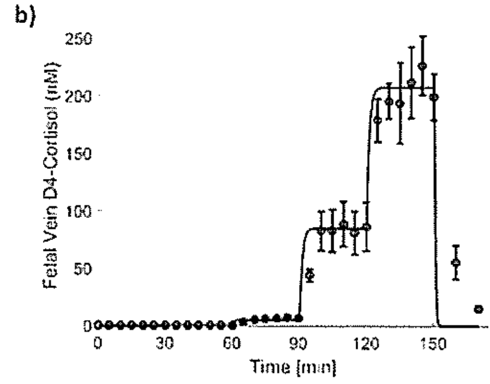


a)



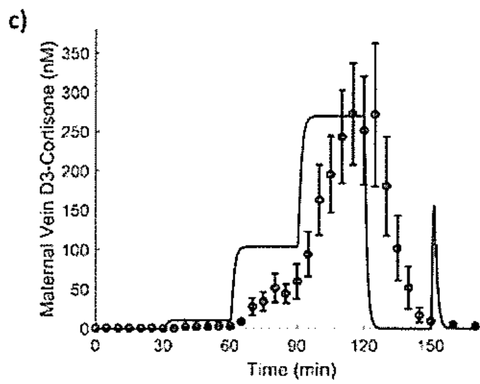
Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX

b)



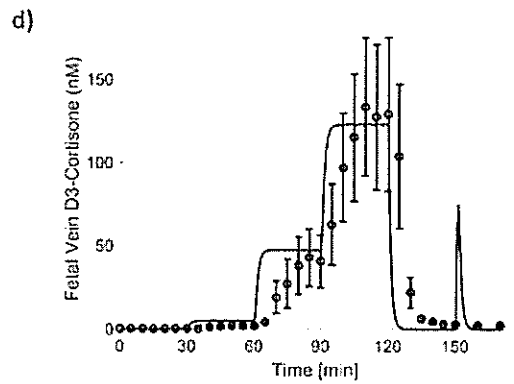
Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX

c)



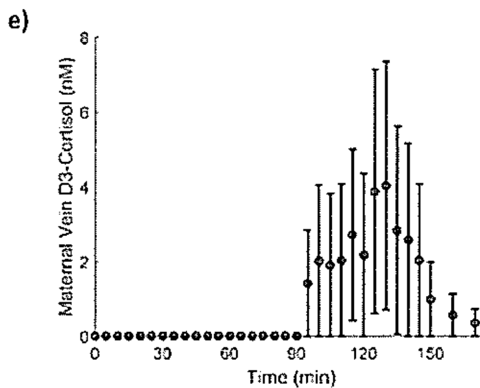
Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX

d)



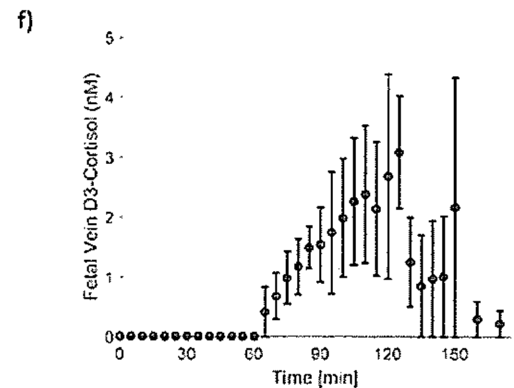
Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX

e)



Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX

f)



Maternal Perfusion	Washout	20 nM D4F	200 nM D4F	800 nM D4F	800 nM D4F + CBX