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1 **The placental renin-angiotensin system and oxidative stress in pre-eclampsia**

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20 **Short Tital:** Placental RAS in Pre-eclampsia

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24

25 **Abstract**

26 There is an inverse correlation between human birthweight and umbilical venous angiotensin
27 II (AngII) concentrations. Oxidative stress and increased prorenin receptor (PRR) both
28 enhance the cleavage of angiotensin I from angiotensinogen (AGT). Pre-eclampsia, a
29 hypertensive disorder of pregnancy, manifests as high blood pressure and proteinuria, and is a
30 state of increased oxidative stress.

31 **Objectives, study design and main outcome measures:** Hypothesis: Pre-eclampsia will be
32 associated with increased placental expression of components of the renin-angiotensin
33 system, which could result in reduced infant birthweight. Biopsies were taken 1cm from the
34 placental edge from 27 normotensive controls and 23 pre-eclamptic White European women.
35 Immunohistochemistry was performed for AGT, PRR, glutathione peroxidase 3 (GPx3) and
36 the AT1R and AT2R AngII receptors. Protein expression was semi-quantitatively assessed
37 (H-score).

38 **Results:** AT1R expression was significantly increased in pre-eclamptic placentae, and
39 negatively correlated with birthweight ($r = -0.529$, $P = 0.009$). AT1R expression was also
40 negatively correlated with GPx3 expression overall ($r = -0.647$; $P = 0.005$). AT2R expression
41 positively correlated with AGT ($r = 0.615$, $P = 0.002$) in the pre-eclamptic placentae only.

42 **Conclusions:** The raised AT1R expression in pre-eclampsia, together with inadequate
43 antioxidant protection, possibly through lower GPx activity, might enhance the
44 vasoconstrictor effect of locally-generated AngII, contributing to the restricted fetal growth
45 characteristic of pre-eclampsia. Conversely, the AT2R:AGT association within the pre-
46 eclamptic placenta may provide a compensatory mechanism.

47

48 **Keywords:** Pre-eclampsia, Renin-angiotensin-system, angiotensin receptors, angiotensinogen,
49 pro-renin receptor, placenta.

50 **Introduction**

51 Pre-eclampsia is a hypertensive disorder of pregnancy that occurs in ~3% of all pregnancies.
52 This disease, which manifests as high blood pressure and proteinuria, is probably mediated
53 by endothelial damage and may affect multiple systems of the body and contribute to adverse
54 pregnancy outcomes including maternal death, preterm birth, intrauterine growth restriction
55 (IUGR) and fetal death [1]. Together with other hypertensive disorders of pregnancy, pre-
56 eclampsia is responsible for at least 60,000 maternal deaths each year [2] and increases
57 perinatal mortality 5-fold [3]. Placental and maternal systemic oxidative stress are
58 components of the syndrome [4, 5] and contribute to a generalised maternal systemic
59 inflammatory activation [6]. Placental ischemia-reperfusion injury has been implicated in
60 excessive production of reactive oxygen species (ROS), which could cause release of
61 placental factors that mediate the inflammatory responses [7]. We have recently shown that
62 placental antioxidant glutathione peroxidase (GPx) activity and expression are reduced in pre-
63 eclampsia [8].

64
65 The renin–angiotensin system (RAS) is known to be an important regulator of blood pressure,
66 sodium and fluid homeostasis. In non-pregnant models, enhanced RAS activity causes
67 hypertension [9], salt retention [10], and hyperaldosteronism [11]. Although the exact
68 mechanism of pre-eclampsia remains unclear, it appears that angiotensinogen (AGT) could
69 play critical roles in its development [12]. AGT is the only renin substrate, and is thus a
70 major molecule in the RAS. Prorenin (PR) is the biosynthetic precursor of renin, the structure
71 of which includes a peptide which obstructs the access of AGT to the active site of renin,
72 which binds to its receptor, prorenin receptor (PRR or ATPase H⁺ transporting lysosomal
73 accessory protein 2; ATP6AP2) [13]. Oxidative stress and increased PRR expression both
74 enhance the cleavage of angiotensin I (Ang I) from AGT. Hypoxaemia in the chronically-
75 cannulated fetal lamb was associated with a doubling of circulating angiotensin II (AngII)

76 concentrations [14], and umbilical venous Ang II concentrations are higher in smaller babies
77 [15, 16]. AngII exerts part of its vasoconstrictor effect through the generation of ROS [17].
78
79 Ang II exerts a majority of its effects through two major angiotensin receptors: AT1R and
80 AT2R. These highly conserved seven-transmembrane G-protein–coupled receptors share a
81 34% sequence identity and have comparable affinities for Ang II [18]. Most of the actions of
82 Ang II, including vasoconstriction and stimulation of aldosterone synthesis, angiogenesis and
83 cell growth are mediated by the AT1R [17]. The AT2R is implicated in apoptosis, reduction
84 in endothelial cell growth and migration, and vasodilation in the adult, the reduction in
85 neointima formation after vascular injury [19-23], although it is usually expressed at low
86 density. However, expression is much higher during fetal life, where it may counterbalance
87 the effects of the AT1Rs during fetal development. Endothelial cell growth, migration,
88 angiogenesis and apoptosis allowing spiral artery remodelling are all central to placentation.
89
90 In the placenta, an intrinsic angiotensin-generating system has been well documented based
91 on the presence of major RAS components including renin, AGT, angiotensin-converting
92 enzyme (ACE), and Ang II as well as its receptor subtypes [24-26]. The conversion of Ang I
93 to Ang II by ACE in the fetoplacental bed leads to a potent vasoconstrictor activity in the
94 fetoplacental circulation [27, 28]. Placental trophoblasts are particularly AT1R rich [29]. A
95 functioning placental RAS appears necessary for an uncomplicated pregnancy [30]. There is
96 an inverse correlation between human birthweight and umbilical venous AngII concentrations
97 [31]. An elevated fetal plasma renin activity [32] and fetal Ang II concentration [16] have
98 also been reported in IUGR. However, there is controversy about the expression of placental
99 angiotensin receptors in pre-eclampsia.

100 These early studies did not examine the placental RAS in detail. A recent paper has reported
101 on placental expression of PRR and AGT in normal term pregnancy [33], but these have not
102 been described in relation to pre-eclampsia. We thus hypothesised that pre-eclampsia, a state
103 of increased oxidative stress, will be associated with increased placental expression of
104 components of the RAS, which could result in reduced infant birthweight.

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125 **Methods**

126 *Subjects:*

127 The study population consisted of White European women who had either a normotensive or
128 pre-eclamptic pregnancy as previously described [4]. The investigations were approved by
129 the Nottingham Hospital Ethics Committee and written, informed consent was obtained from
130 each subject. Cases were defined on admission with a clinical diagnosis of pre-eclampsia,
131 defined using the International Study for the Study of Hypertension in Pregnancy guidelines
132 [34]. Medical and obstetric histories, including delivery data, were obtained for each woman.
133 The birthweight centile for each baby was computed, correcting for gestation age, sex,
134 maternal parity and body mass index (BMI) [35].

135

136 *Sample collection:*

137 Full depth placental biopsies were taken 1cm from the placental edge from 27 normotensive
138 and 23 pre-eclamptic women, avoiding placental infarcts. Tissues were taken within 10 min
139 of delivery, membranes removed and tissue washed in ice cold $1 \times$ PBS to remove maternal
140 blood contamination. Biopsies were formalin fixed and wax-embedded for
141 immunohistochemical analysis.

142

143 *Immunohistochemistry:*

144 Immunohistochemical staining was performed on serial sections as previously described [8]
145 using the Dako Envision™ visualization system (Dako, Ely, UK). Table 1 provides further
146 details on antibody dilutions used, which were optimised by performing a dilution series for
147 each antibody. The heat-induced epitope retrieval was achieved by heating in a citrate buffer
148 (pH 6.0) using a microwave oven for 15 min. A negative control was performed for each test
149 section by incubation with mouse or rabbit IgG as appropriate. Sections were dehydrated and

150 cleaned in ascending concentrations of alcohol and xylene before coverslips were mounted
151 (DPX mountant, BDH). Protein expression was semi-quantitatively assessed (H-score) at
152 x200 magnification (Nikon Eclipse II microscope) by two blinded observers as described
153 previously [36]. Between-observer agreement for H-scoring was excellent (kappa 0.97).

154

155 Maternal plasma thiobarbituric acid reactive substances (TBARS) concentrations were
156 measured as a global measure of oxidative stress; plasma total GPx activity and placental
157 GPx3 protein expression were measured as indices of specific antioxidant activity. These data
158 have been previously reported [4].

159

160 *Statistical analysis*

161 All tests were performed using SPSS for Windows version 19.0. Summary data are presented
162 as median (interquartile range) as appropriate for their distribution after testing for normality
163 using the Kolmogorov-Smirnov test. Mann-Whitney *U*-tests were used to test differences
164 between normotensive and pre-eclampsia groups. Spearman's Rho correlation and Kendall's
165 Tau ranking tests were also conducted as appropriate. The null hypothesis was rejected where
166 $P < 0.05$.

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175 **Results**

176 All women conceived naturally and carried singleton pregnancies. The demographic,
177 obstetric and pregnancy data of the 50 women (27 normotensive, 23 pre-eclampsia) who
178 participated in the study are shown in Table 2. The complete and detailed data have been
179 previously published [4]. In summary, women in the normotensive group gave birth without
180 developing hypertension or proteinuria, to infants weighing >2500 g, delivered at 37 weeks
181 or later. Overall, the pre-eclamptic women all had moderate to severe disease and had lower
182 gestational ages at delivery than the control group ($P < 0.05$; Table 2). No pre-eclamptic
183 woman had Haemolysis Elevated Liver enzymes, and Low Platelet count (HELLP). All
184 neonates from both pregnancy groups survived.

185

186 All components of the RAS that were measured in this study were found to be expressed in
187 placental tissue, mainly localised to the villous syncytiotrophoblast, with some stromal
188 staining (Figure 1). Table 3 summarises the staining intensity of AGT, PRR, AT1R, AT2R
189 and GPx3 in placentae from normotensive and pre-eclamptic women. AT1R expression was
190 significantly increased in pre-eclamptic compared to normotensive placentae ($P = 0.032$;
191 Table 3); all other RAS components were similarly expressed in both groups. Placental GPx3
192 expression (Table 3) and maternal plasma total GPx activity [4, 8] were significantly reduced
193 in pre-eclampsia while TBARS were highly significantly increased (1.2 [0.6, 1.6] compared
194 to 0.45 [0.2, 0.8] $\mu\text{mol/l}$ ($P < 0.001$)[4].

195

196 A negative association was observed between AT1R and birthweight in the pre-eclamptic
197 group (Figure 2; $r = -0.529$, $P=0.009$) but not in the normotensive group ($P > 0.5$). A
198 significant inverse correlation was also found overall between placental AT1R and GPx3
199 protein expression (Figure 3; $r = 0.634$; $P = 0.006$). AT2R expression was found to be

200 positively related to AGT in pre-eclampsia ($r = 0.615$, $P = 0.002$), but not control, placentae
201 ($r = 0.064$, $P > 0.05$; Figure 4).

202

203 Although staining density was diffuse throughout the villous syncytiotrophoblasts, there was
204 also dense staining localised specifically around fetal vessels in 18 (PRR) and 8 (AGT) of the
205 50 placenta (Figure 1). The presence of perivascular staining for PRR and AGT was found to
206 be associated with increased maternal TBARS (Chi-squared $P = 0.001$ and $P = 0.033$).

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225 **Discussion**

226 It has been known for nearly 40 years [37] that there is a tissue-based RAS in the
227 uteroplacental unit, suggesting a locally acting RAS. Our localised staining of the RAS
228 components to the syncytiotrophoblast is consistent with other very recent results [38]. Our
229 study demonstrates that the placental RAS may be involved in pre-eclampsia, with raised
230 AT1R expression, together with the raised AngII concentrations previously reported [15, 16],
231 contributing to the restricted fetal growth characteristically observed in this syndrome. The
232 pressor response to AngII is well known to be enhanced before pre-eclampsia is clinically
233 detectable [39] and is associated with an increased density of AT1Rs [40]. Agonist
234 autoantibodies to the AT1R are also increased in pre-eclampsia [41].

235

236 The inverse correlation between placental AT1R and GPx3 expression (Figure 3) is
237 particularly interesting in light of the stimulatory effect of AngII on ROS generation [17].
238 The absence of adequate antioxidant protection in pre-eclampsia might enhance the
239 vasoconstrictor effect of locally-generated AngII. The specific clustering of both the PRR and
240 AGT around the vessels, in conjunction with elevated measures of oxidative stress (TBARS),
241 suggests that the RAS may be contributing to the heightened state of oxidative stress in
242 placental tissue of pre-eclamptic pregnancies. ROS superoxide anion (O_2^-) has been
243 implicated in Ang II-mediated hypertension [42] and experimental data in the non-pregnant
244 state support the concept that Ang II-mediated hypertension in pregnancy may also be due, in
245 part, to effects on the oxidative state in vascular-endothelial tissue [43].

246

247 There is some controversy surrounding the regulation of placental AT1R in pre-eclampsia.
248 The increase in placental AT1R expression in pre-eclampsia we observed is in agreement
249 with previous studies in placental [44] and decidual [17] tissue. However, two earlier studies

250 reported that the capacity and affinity of AT1R were significantly lower in placentae from
251 pregnancies complicated by pre-eclampsia and IUGR [45] and in a previous study we also
252 found no significant increase in placental AT1R expression [26]. It is now well established
253 from several studies that gradients in gene expression [46] or enzyme activity [8] exist across
254 the placenta. In our original paper [26], results were reported from biopsies taken within 1cm
255 of the cord insertion. We have subsequently demonstrated an increase in placental ACE
256 activity at the periphery [47], where sensitivity of chorionic plate arteries to Ang II is greatest
257 [48]. In the current investigation, we therefore studied samples collected from 1cm from the
258 periphery of the placenta. This adds further weight to the requirement for structured, rather
259 than random, sampling across the placental disc, with clear reporting of sampling site.

260

261 We have, for the first time, identified a clear relationship between placental AT2R and AGT
262 expression in pre-eclamptic, but not normotensive pregnancy (Figure 4). The AT2R appears
263 to function as an ‘antagonist’ of AT1R [49], and is predominantly expressed in fetal tissue
264 [50]. Ang II binding to AT2Rs increases apoptosis, causes vasodilation and is thought to be
265 involved in fetal tissue development [51, 52]. Thus, the strong positive associations observed
266 in the pre-eclamptic placentae between AT2R and AGT may indicate a potential
267 compensatory mechanism, not activated in normotensive pregnancy.

268

269 The human placenta possesses an autonomous RAS, allowing the local generation of Ang II
270 and its fragments. Our data suggest that, at term, increased activity in this local system,
271 particularly evident in the syncytiotrophoblast, may contribute to both pre-eclampsia itself
272 and the IUGR which frequently accompanies it. We have not addressed the question of
273 distinguishing between the effects of placentally-generated AngII and fetal systemic AngII.

274

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439 **Figure Legends**

440 **Figure 1:** Immunohistochemical staining of AGT (A1 to A3), PRR (B1 to B3), AT1R (C1 to
441 C3) and AT2R (D1 to D3) at x200 magnification. Positive staining shown in brown, black
442 arrows indicate syncytiotrophoblast cells and red arrows show fetal vessels. AT1R expression
443 was increased in the pre-eclamptic compared to the normotensive control placentae ($P <$
444 0.05).

445 **Figure 2:** Scatter plot illustrating the inverse association between AT1R expression in the
446 placenta, and the birthweight in the pre-eclamptic group ($r = -0.529$; $P = 0.009$); not observed
447 in the controls ($r = -0.117$; $P > 0.05$).

448 **Figure 3:** Inverse association between the expression of GPx3 and AT1R in the placenta ($r =$
449 -0.634 ; $P = 0.006$). Placental GPx3 activity was only measured in 10 pre-eclampsia and 10
450 normotensive control samples.

451 **Figure 4:** Scatter plots showing the positive association between the placental AGT and
452 AT2R expression in pre-eclamptic ($r = 0.615$; $P = 0.002$) but not normotensive controls ($r =$
453 0.064 ; $P > 0.05$).

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460 **Table 1.** Details of antibody sources and dilutions.

Antigen	Supplier information	Concentration (mg/mL)
PRR	Sigma Prestige, rabbit polyclonal: HPA003156	1.8
AGT	Sigma Prestige, rabbit polyclonal: HPA0031557	0.26
AT1R	Abcam, mouse momoclonal: ab9391	80
AT2R	Abcam, rabbit polyclonal: ab19134	5.2

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471 **Table 2:** Obstetric data of subject groups. Data are presented as mean \pm SD or median [IQR]
472 as appropriate. * $P < 0.05$, ** $P < 0.001$; more detailed data has been previously published
473 (Mistry et al 2008).

Parameter	Normotensive control (n = 27)	Pre-eclampsia (n = 23)
Gestational age at delivery (weeks)	40 \pm 1.1	36.4 \pm 3.8*
Birthweight (kg)	3.55 [3.25, 3.86]	2.92 [1.92, 3.51]*
Birthweight centiles (%)	45 [23, 67]	35 [2, 87]

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488 **Table 3:** Placental protein expression (H-Score) of AGT, PRR, AT1R, ATT2R and GPx3. **P*
 489 < 0.05; #previously published (Mistry et al 2008) and placental GPx3 activity was only
 490 measured in 10 pre-eclampsia and 10 normotensive control samples.

Protein expression	H-Score (Median [interquartile range])	
	Normotensive control	Pre-eclampsia
AGT	34 [10,95]	30 [9,60]
PRR	117 [16,144]	139 [72,187]
AT1R	40 [10,70]	68 [30,112]*
AT2R	152 [120,180]	184 [74,220]
Placental GPx3 expression (AU) #	197 [124, 254]	110 [82, 124]*

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