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# Accepted Manuscript



Evidence that Fetal Death is Associated with Placental Aging

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**Title: Evidence that Fetal Death is Associated with Placental Aging**

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27 **Condensation:**

28 Fetal death is associated with features of placental aging.

29

30 **Short title:** Fetal Death and Placental Aging

31

32

**33 Abstract****34 Background:**

35 The risk of unexplained fetal death or stillbirth increases late in pregnancy suggesting that  
36 placental aging is an etiological factor. Aging is associated with oxidative damage to DNA,  
37 RNA and lipids. We hypothesized that placentas at more than 41 completed weeks of  
38 gestation (late-term) would show changes consistent with aging that would also be present in  
39 placentas associated with stillbirths.

**40 Objective:**

41 We sought to determine whether placentas from late-term pregnancies and unexplained  
42 stillbirth show oxidative damage and other biochemical signs of aging. We also aimed to  
43 develop an *in vitro* term placental explant culture model to test the aging pathways.

**44 Study design:**

45 We collected placentas from women at 37-39 weeks gestation (early-term and term), late-term  
46 and with unexplained stillbirth. We used immunohistochemistry to compare the three groups  
47 for: DNA/RNA oxidation (8-hydroxy-deoxyguanosine, 8OHdG), lysosomal distribution  
48 (Lysosome-associated membrane protein 2, LAMP2), lipid oxidation (4-hydroxynonenal,  
49 4HNE), and autophagosome size (Microtubule-associated proteins 1A/1B light chain 3B,  
50 LC3B). The expression of aldehyde oxidase 1 (AOX1) was measured by real-time PCR.  
51 Using a placental explant culture model, we tested the hypothesis that AOX1 mediates  
52 oxidative damage to lipids in the placenta.

**53 Results:**

54 Placentas from late-term pregnancies show increased AOX1 expression, oxidation of  
55 DNA/RNA and lipid, perinuclear location of lysosomes and larger autophagosomes compared  
56 to placentas from women delivered at 37-39 weeks. Stillbirth associated placentas showed  
57 similar changes in oxidation of DNA/RNA and lipid, lysosomal location and autophagosome

58 size to placentas from late-term. Placental explants from term deliveries cultured in serum  
59 free medium also showed evidence of oxidation of lipid, perinuclear lysosomes and larger  
60 autophagosomes, changes that were blocked by the G protein-coupled estrogen receptor 1  
61 (GPER1) agonist G1, while the oxidation of lipid was blocked by the AOX1 inhibitor  
62 raloxifene.

### 63 **Conclusions:**

64 Our data are consistent with a role for AOX1 and GPER1 in mediating aging of the placenta  
65 that may contribute to stillbirth. The placenta is a tractable model of aging in human tissue.

66

67 **Key words:** placenta; aging; stillbirth; fetal death; autophagosome; DNA / RNA oxidation;  
68 lipid oxidation; AOX1; GPER1; raloxifene; placental explant culture

69

### 70 **Glossary of Terms**

71 Aldehyde Oxidase 1(AOX1) — an oxidizing enzyme with a wide range of substrates, that  
72 generates peroxides

73 Autophagosome — an intracellular organelle that collects damaged proteins and old  
74 mitochondria

75 G protein-coupled estrogen receptor 1 (GPER1) — a cell surface estrogen receptor distinct  
76 from nuclear estrogen receptors

77 8-hydroxy-deoxyguanosine (8OHdG) — a product of DNA oxidation

78 4-hydroxynonenal (4HNE) — a product of lipid peroxidation

79 Lipid peroxidation — the oxidative degradation of lipids

80 Lysosome — an intracellular organelle that contains proteolytic enzymes in an acid  
81 environment

## 82 **Introduction**

83 Unexplained fetal death is a common complication of pregnancy occurring in approximately 1  
84 in 200 pregnancies in developed countries<sup>1</sup> and more frequently in the developing world.  
85 While no cause has been established, the rate of fetal death rises rapidly as gestation  
86 progresses beyond 38 weeks<sup>2</sup>. Johnson *et al.*<sup>3</sup> have proposed the operational definition of  
87 aging as an increase in risk of mortality with time, which is consistent with a role for aging in  
88 the etiology of stillbirth<sup>4</sup>. Supporting this view a histopathological study of placentas  
89 associated with cases of unexplained intrauterine death at term revealed that 91% showed  
90 thickening of the maternal spiral artery walls, 54% contained placental infarcts, 10% had  
91 calcified areas and 13% demonstrated vascular occlusion<sup>5</sup>, another reported increased  
92 atherosclerosis<sup>6</sup>; changes that are associated with aging in other organs. Supporting a link  
93 between placental aging and stillbirth, Ferrari *et al.*, have recently reported that telomere  
94 length is reduced in placentas associated with stillbirth<sup>7</sup>. Fetal growth restriction is also  
95 associated with both stillbirth and telomere shortening<sup>8</sup>. We therefore sought to determine  
96 whether placentas from women who delivered after 41 completed weeks (late-term) or had  
97 stillbirth had biochemical evidence of aging. As markers of aging we chose to measure 8-  
98 hydroxy-deoxyguanosine (a marker of DNA oxidation) and 4-hydroxynonenal (a marker of  
99 lipid oxidation) as both have been described to increase in the brain with aging, and the  
100 enzyme aldehyde oxidase which is known to generate oxidative damage in the kidney. Aging  
101 is also known to affect the effectiveness of the intracellular recycling process that involves  
102 fusion of acidic hydrolase containing lysosomes with autophagosomes, we therefore sought  
103 changes in these intracellular organelles in the late-term placentas and those associated with  
104 stillbirth.

105

## 106 **Materials and Methods**

**107 Ethics, Collection and Processing of Tissues**

108 This study was approved by the human research ethics committee of the Hunter New England  
109 Health Services and the University of Newcastle, NSW, Australia. Human placentas were  
110 collected after written informed consent was obtained from the patients by midwives.  
111 Placentas were collected from women at 37-39 weeks gestation undergoing caesarean section  
112 for previous caesarean section or normal vaginal delivery, women at 41<sup>+</sup> weeks gestation  
113 undergoing caesarean section or normal vaginal delivery, and women who had stillborn  
114 infants undergoing vaginal delivery. Placentas were collected immediately after delivery and  
115 processed without further delay. Villous tissues were sampled from multiple sites and  
116 prepared for histology and RNA extraction. For each placenta, tissues were obtained from at  
117 least 5 different regions of the placenta and 4-5 mm beneath the chorionic plate. Samples  
118 from each individual placenta were immediately frozen under liquid nitrogen and stored at -  
119 80° C until subsequent experiments. For histology experiments, tissues were fixed in 2%  
120 formaldehyde for 24 h, stored in 50% ethanol at room temperature (RT) and embedded in  
121 paraffin. To create a placental roll a 2 cm strip of chorioamniotic membrane was cut from the  
122 periphery of the placenta keeping a small amount of placenta attached to the membrane. The  
123 strip was rolled around forceps leaving residual placenta at the centre of the cylindrical roll.  
124 The cylindrical roll was then cut perpendicular to the cylindrical axis to obtain 4 mm thick  
125 sections and fixed in formalin. Placentas from patients with infection, diabetes, pre-eclampsia,  
126 placenta praevia, intra-uterine growth restriction or abruption were excluded.

127

**128 Reagents and Antibodies**

129 Antibodies against LAMP2 and AOX1 were obtained from BD Biosciences (North Ryde,  
130 Australia) and Proteintech (Rosemont, USA), respectively. Antibody against LC3B and  
131 GPER1 were obtained from Novus Biologicals (Littleton, USA). Antibodies against 8OHdG

132 and 4HNE were purchased from Abcam (Melbourne, Australia). Dulbecco's modified Eagle's  
133 medium (DMEM), antibiotic-antimycotic (anti-anti), Nupage precast 12 well protein gel and  
134 prolong gold antifade mounting media with DAPI, Alexa conjugated secondary antibodies  
135 were obtained from Thermo Fisher Scientific Australia Pty (Scoresby, Australia). The horse  
136 radish peroxidase (HRP) conjugated secondary antibodies were purchased from Cell Signalling  
137 Technologies (Beverly, MA, USA). Fetal bovine serum was obtained from Bovogen  
138 Biologicals Pty Ltd (VIC, Australia). Protease inhibitor and phosphatase inhibitor were  
139 supplied by Roche (Castle Hill, Australia). Raloxifene was purchased from Sigma-Aldrich  
140 (Sydney, Australia) and G1 was supplied by Tocris-bioscience (Bristol, UK). The BCA  
141 protein assay kit was obtained from Thermo Fisher Scientific (Scoresby, Australia). All other  
142 chemicals were purchased from either Ajax Finechem Pty Ltd or Sigma-Aldrich (Sydney,  
143 Australia).

144

#### 145 *Placental Explant Culture*

146 For *in vitro* experiments, human term placentas (all at 39 weeks of gestation) were obtained  
147 from women with normal singleton pregnancies without any symptoms of labour after an  
148 elective (a scheduled repeat) caesarean section. Placentas were collected immediately after  
149 delivery and prepared for explant culture. Villous tissues of placentas were randomly sampled  
150 from different regions of the placenta 4-5 mm beneath the chorionic plate. Tissues were  
151 washed several times with Dulbecco's phosphate-buffered saline (PBS) under sterile  
152 conditions to remove excess blood. Villous explants of  $\sim 2 \text{ mm}^3$  were dissected and placed into  
153 100 mm culture dishes (30 pieces/dish) containing 25 ml of DMEM supplemented with 2 mM  
154 L-glutamine, 1% Na-pyruvate, 1% penicillin/streptomycin (100X) solution with the addition  
155 of 10% (v/v) fetal bovine serum (FBS) and cultured in a cell culture chamber at 37 °C  
156 temperature under 95% air (20% oxygen) and 5% CO<sub>2</sub> for 24 h. At day 2, villous explants

157 were transferred to fresh 30 ml growth medium and incubated in a cell culture chamber for 90  
158 minutes and washed in DMEM without FBS (referred to as 'serum-free medium' or 'growth  
159 factor deficient medium'). Next 6-7 pieces of villous tissue weighing approximately 400 mg  
160 were transferred to a culture dish (60 mm) containing 6 ml serum-free medium with or  
161 without the addition of pharmacological agents, for example, raloxifene (1 nM) or the GPER1  
162 agonist G1 (1 nM), for subsequent incubation for 24 h. At the end of 24 h some tissues were  
163 fixed in 2% formaldehyde, subjected to routine histological processing and embedded in  
164 paraffin wax, and some tissues were immediately frozen in liquid nitrogen and stored at -80  
165 °C until subsequent experiments. For each placental explant culture, samples were also  
166 collected at time '0 (zero)' h i.e., before incubation in serum free medium, and were formalin  
167 fixed and stored frozen at -80 °C until further experiments.

168

### 169 ***Western Blotting***

170 The western blotting was performed as previously described<sup>9</sup>. Samples of placenta (1gm)  
171 were crushed under liquid nitrogen. Aliquots of 100 mg of placental tissues were  
172 homogenised in 1 ml of lysis buffer (PBS, 1% Triton-X-100, 0.1 % Brij-35, 1 X protease  
173 inhibitor, 1 X phosphatase inhibitor, pH 7.4). The protein concentration of each placental  
174 extract was measured using a BCA protein assay kit and 40 µg of placental extract was  
175 separated by electrophoresis in NuPage bis-tris precast 12 well gels for 50 mins at a constant  
176 200 V. Separated proteins were then transferred to nitrocellulose membrane using a Novex  
177 transfer system for 70 mins and blocked overnight at 4 °C with 1% bovine serum albumin  
178 (BSA) in tris buffered saline with 0.1 % tween-20 (TBST). The membranes were then  
179 incubated with primary antibody in 1% BSA in TBST for 2 hours at RT, then washed three  
180 times with TBST, followed by incubating with HRP conjugated secondary antibodies in 1%  
181 BSA in TBST for an hour. After three further washes with TBST, the immuno-reactive bands

182 were developed in Luminata reagent (Merck Millipore) and detected using an Intelligent Dark  
183 Box LAS-3000 Imager (Fuji Photo Film, Tokyo, Japan).

184

### 185 *Immunohistochemistry*

186 Fluorescent immunohistochemistry (IHC) was performed according to previously published  
187 methods<sup>9</sup>. Six  $\mu\text{m}$  paraffin placental sections were deparaffinised and hydrated, then heated  
188 with tris-EDTA buffer (pH 9) in a microwave oven for antigen retrieval. The sections were  
189 blocked with 1 % BSA in TBST for an hour at RT. The sections were incubated with primary  
190 antibodies overnight and washed three times with TBST, before incubation with Alexa-  
191 conjugated secondary antibodies for 90 mins. The sections were mounted with prolong gold  
192 antifade mounting media with DAPI. The fluorescent photographs for Figures 2, 3, 4, 5, 6, 7,  
193 S1, S2 and S3 were taken on a Nikon eclipse 90i confocal microscope (Nikon Instruments  
194 Inc.). The fluorescent photographs for Figure 8 were taken on Nikon eclipse Ti fluorescence  
195 microscope (Nikon Instruments Inc.).

196

### 197 *RNA isolation and Real time PCR*

198 Placental tissues were crushed under liquid nitrogen. Approximately 100 mg of crushed  
199 placental tissues were homogenised in 2 ml of Trizol reagent (Life Technologies) with an  
200 Ultra Turrax homogenizer. Total RNA was extracted from the Trizol-extract by Direct-zol™  
201 RNA MiniPrep (Zymo Research). The RNA was treated with DNase and purified with a  
202 RNA Clean & Concentrator™-5 kit (Zymo Research). The RNA quality was checked by  
203 running the DNase treated sample in agarose gel with ethidium bromide in 1X TAE buffer.  
204 The purified RNA was used to make cDNA using a SuperScript® III First-Strand Synthesis  
205 System kit (Life Technologies). The cDNA was used to run real time PCR by Taqman  
206 primers for aldehyde oxidase 1 (AOX1) (Life Technologies, Assay ID: Hs00154079\_m1) and

207 Taqman gene expression master mix (Life Technologies) with an internal control of 18s  
208 ribosomal RNA (Life Technologies) to quantify mRNA for AOX1. We used a SyBr green  
209 master mix to quantify mRNA for G-protein coupled receptor 1 (GPER1) (Invitrogen,  
210 Forward primer 5'-CGTCCTGTGCACCTTCATGT-3' Backward primer 5'-  
211 AGCTCATCCAGGTGAGGAAGAA-3') with respect to beta-actin as an internal control  
212 using an Applied Biosystem 7500 PCR system.

213

### 214 *Statistical analysis*

215 Sample numbers are shown in the legends to individual figures. The data in Figures 2, 4, 5, 6  
216 and 8 were analysed using the Mann-Whitney test (two way) and results are presented as  
217 scatter plots showing the median. The data in Figure 7, S2 and S3 were analysed using the  
218 Wilcoxon matched-pairs signed rank test and results are presented as mean showing the  
219 standard error of the mean (S.E.M.). All the *p*-values were calculated using the Graphpad  
220 Prism software (Version 7, Graph Pad Software Inc., San Diego, California). A *p*-value of  
221  $\leq 0.05$  was considered statistically significant.

222

## 223 **Results**

### 224 *Subject characteristics*

225 Demographic and clinical characteristics of the study participants are reported in table 1.

226

### 227 *Relationship between stillbirth risk and length of gestation*

228 To illustrate the relationship between stillbirth risk and length of gestation we created a  
229 Kaplan Myer plot of the data on human gestational length in a population with relatively low  
230 levels of medical intervention from Omigbodun and Adewuyi<sup>10</sup> and combined it with the data  
231 on risk of stillbirth per 1000 continuing pregnancies from Sutan *et al.*<sup>2</sup> (Figure 1 reproduced

232 with permission<sup>4</sup>). The data illustrate that stillbirth is consistent with an aging etiology as  
233 defined by Johnson *et al.*<sup>3</sup>.

234

#### 235 ***DNA/RNA Oxidation***

236 We sought evidence of placental DNA/RNA oxidation as measured by 8-hydroxy-  
237 deoxyguanosine (8OHdG), as a marker of DNA/RNA oxidation that has previously been  
238 observed in aging tissues<sup>11</sup> such as the brain in Alzheimer's disease<sup>12</sup>. Immunohistochemistry  
239 (IHC) was performed for 8OHdG and the average intensity of 8OHdG staining in  
240 nuclei/frame demonstrated a significant increase in DNA/RNA oxidation in late-term and  
241 stillbirth associated placentas (Figure 2).

242

#### 243 ***Movement and clustering of lysosomes in late-term and stillbirth placentas***

244 Misfolded proteins and damaged mitochondria are normally recycled in autophagosomes in a  
245 process that involves autophagosome fusion with proteolytic enzyme containing lysosomes.  
246 Accumulation of abnormal protein is thought to play a role in aging particularly in the brain,  
247 for instance the accumulation of tau and amyloid protein in Alzheimer's disease<sup>13, 14</sup> and  
248 mutant huntin in Huntington's disease<sup>15</sup>. In Huntington's disease, the distribution of the  
249 lysosomes within neurones is altered with increased perinuclear accumulation of lysosomes<sup>16</sup>.  
250 We used a lysosomal marker, lysosome-associated membrane protein-2 (LAMP2) to analyse  
251 the distribution of lysosomes in the placenta by IHC. IHC showed lysosomes positioned on  
252 the apical surface of early-term placental syncytiotrophoblast (Figures 3A, 3D and 3E),  
253 whereas lysosomes relocated to the perinuclear and the basal surface in late-term and stillbirth  
254 placentas (Figures 3B, 3C, 3F and 3G).

255

#### 256 ***Lipid oxidation in placental tissue***

257 The increase in DNA oxidation which we had demonstrated suggested free radical damage  
258 that might also lead to lipid peroxidation. Lipid peroxidation has been observed to increase in  
259 Alzheimer's disease as measured by the formation of 4-hydroxynonenal (4HNE)<sup>17</sup>. We  
260 therefore performed IHC for 4HNE in late-term, stillbirth and 37-39 weeks placental tissue.  
261 This revealed a marked, statistically significant increase in 4HNE staining in late-term  
262 syncytiotrophoblast that we also observed in placentas associated with stillbirth shown in  
263 Figure 4 (A-D).

264

265 ***Larger autophagosomes containing 4HNE occur in late-term and stillbirth associated***  
266 ***placentas***

267 Inhibition of autophagosome function with failure of fusion with lysosomes leads to an  
268 increase in autophagosome size<sup>18, 19</sup>. This process leads to inhibition of overall autophagic  
269 function that is seen in Alzheimer's disease<sup>18</sup>, Danon's disease<sup>19</sup> and neurodegeneration<sup>20</sup>. We  
270 detected autophagosomes using IHC with an antibody against LC3B. We observed a  
271 significant increase in the size of autophagosomes (Figure 5D) in both late-term (Figure 5B)  
272 and stillbirth (Figure 5C) associated placentas compared to 37-39 week placentas (Figure 5A).  
273 Dual labelled fluorescence immunostaining showed that the larger autophagosomes of late-  
274 term and stillbirth placentas contained 4HNE, a product of lipid peroxidation (Supplementary  
275 Figure S1).

276

277 ***Role of aldehyde oxidase 1 (AOX1) in placental oxidative damage***

278 Aldehyde oxidase 1 (AOX1) is a molybdoflavoenzyme, which oxidises a range of aldehydes  
279 including 4HNE into corresponding acids and peroxides<sup>21</sup>. We provide evidence that AOX1 is  
280 involved in the generation of the increased 4HNE observed in late-term and stillbirth  
281 associated placentas using co-localisation. Dual labelled fluorescence IHC showed that AOX1

282 co-localises to 4HNE positive particles in late-term (Figure 6A-C) and stillbirth placentas  
283 (Figure 6D-F). Additionally real-time qPCR showed that late-term and stillbirth placentas  
284 expressed significantly higher mRNA for AOX1 than 37-39 week placentas (6G). These data  
285 support the concept that AOX1 plays a role in the oxidative damage that occurs in the late-  
286 term and stillbirth associated placentas.

287

### 288 *Pharmacological inhibition of AOX1 using placental explant culture*

289 Our data provide clear evidence for increased lipid oxidation, disordered lysosome-  
290 autophagosome interactions and increased AOX1 expression in the late-term and stillbirth  
291 placental syncytiotrophoblast. To determine if these events were causally linked we developed  
292 a placental explant culture system using term placental tissue cultured in serum-free (growth  
293 factor deficient) medium. IHC showed that serum deprivation significantly increased  
294 production of 4HNE at 24 h after incubation (Figure 7A-C, F and G). We also found a  
295 significant increase in the size of autophagosomes (Supplementary Figure S2) and a change  
296 in lysosomal distribution to a perinuclear location after 24 h incubation in serum-free medium  
297 (Supplementary Figure S3). We sought to determine cause and effect relationships between  
298 the development of lipid oxidation observed when placental explants were cultured in the  
299 absence of serum, and AOX1. To achieve this we used a potent AOX1 inhibitor, raloxifene<sup>22</sup>  
300 and a GPER1 agonist, G1. We used the GPER1 agonist G1 as we had detected GPER1  
301 expression on the apical surface of syncytiotrophoblast (Figure 8A and B) and the GPER1  
302 agonist has been shown to inhibit production of 4HNE in the kidney<sup>23</sup>. Both raloxifene and  
303 G1 inhibited the production of 4HNE in the serum starved placental explants after 24 h of  
304 treatment (Figure 7D, E, F and G). G1 also prevented the changes in lysosomal distribution  
305 within the syncytiotrophoblast (Supplementary Figure S3).

306

307 *Presence of the cell surface estrogen receptor GPER1 on the apical surface of the*  
308 *syncytiotrophoblast*

309 As the GPER1 agonist had evident effects in placental explant cultures we undertook  
310 characterisation of GPER1 expression in placental tissue. The expression of GPER1 in a  
311 section of placenta roll (described in the Method section) detected by fluorescent IHC showed  
312 that GPER1 is expressed in placental villi (Figure 8A), which at higher magnification (100X),  
313 was localised to the apical surface of placental villi (Figure 8B). Real time PCR for GPER1  
314 showed that placental villi have significantly higher expression of GPER1 than amnion,  
315 chorion or decidua (Figure 8C). Western-blot for GPER1 also confirmed higher protein levels  
316 of GPER1 in placental villous tissue than amnion, chorion or decidua (Figure 8D). The  
317 demonstration of GPER1 localisation on the apical surface of the syncytiotrophoblast  
318 indicates the plausibility of estrogen inhibition of AOX1 activity in the placenta.

319

320 **Comment**

321 Our data indicate that between 37-39 and 41 weeks of gestation dramatic changes occur in the  
322 biochemistry and physiology of the placenta. In particular there is increased oxidative damage  
323 to DNA/RNA and lipid, a change in position of lysosomes which accumulate at the  
324 perinuclear and basal surface of the syncytiotrophoblast, the formation of larger  
325 autophagosomes which are associated with oxidised lipid, and there is increased expression of  
326 the enzyme AOX1. The same changes are observed in placentas associated with stillbirth.  
327 Some of our results are semi-quantitative as this is the nature of western analysis, nevertheless  
328 the robustness of our results is supported by the use of multiple end points for aging, and the  
329 biological plausibility of the reported links. Further supporting our hypothesis, similar  
330 changes in oxidation of lipid, localisation of lysosomes and size of autophagosomes occurred

331 in placental explants deprived of growth factors, and these changes were blocked by  
332 inhibition of AOX1.

333

334 Stillbirth occurs in approximately 1 in 200 pregnancies in developed countries<sup>1</sup>. The Lancet<sup>1</sup>  
335 and the BMJ<sup>24</sup> have recently highlighted gaps in our knowledge of this condition. Stillbirth  
336 frequently occurs in the setting of fetal growth restriction and in this setting telomere  
337 shortening and oxidative damage have been observed in associated placentas<sup>25</sup>. The risk of  
338 stillbirth per 1000 continuing pregnancies rises dramatically after 38 weeks of gestation. We  
339 have suggested<sup>4</sup> that stillbirths in late gestation are a consequence of placental aging. More  
340 than 90% of pregnancies have delivered by the end of the 40th week of gestation<sup>10</sup>,  
341 consequently changes that occur in the placenta in pregnancies that have gone past the usual  
342 term have little effect on population level infant survival, since most have already delivered.  
343 Such late gestation changes may exist in a kind of Medawar's Shadow<sup>26</sup> that allows  
344 deleterious genes to persist in the population if their damaging effects occur after  
345 reproduction, especially if the same genes exert positive actions earlier in pregnancy. This  
346 Medawar's Shadow effect has been proposed to explain the high prevalence of Huntington's  
347 disease that is associated with increased fertility in early life but disastrous neurological  
348 deterioration after reproduction has occurred<sup>27</sup>. Our immunofluorescence data show high  
349 levels of 8OHdG and 4HNE in late-term and stillbirth placentas supporting this postulated  
350 pathway to placental aging. Increases in oxidative damages to DNA and lipid have also been  
351 reported in Alzheimer's disease<sup>17, 28</sup>.

352

353 We have also seen marked accumulation of particles positive for the lysosomal marker  
354 LAMP2 in the perinuclear and basal side of the syncytiotrophoblast of late-term placentas and  
355 placentas associated with stillbirth. This phenomenon closely resembles 'lysosomal

356 positioning' which occurs in cells under nutritional stress<sup>29</sup>. Autophagy is an important  
357 cellular recycling process that involves fusion of acidic lysosomes with the autophagosomes.  
358 Our data show that stillbirth and late-term placentas contain larger autophagosomes than 37-  
359 39 week placentas indicating inhibition of the autophagic process in these placentas. Our data  
360 further indicate that the autophagosomes are coated with oxidised lipid in the form of 4HNE  
361 which may play a role in the failure of lysosomal-autophagosome fusion. Such disturbances in  
362 the function of autophagosomes may lead to the accumulation of abnormal protein and  
363 deterioration in the function of the syncytiotrophoblast.

364

365 Stillbirth is not restricted to the late-term setting and is known to be associated with cigarette  
366 smoking and growth restriction. It seems likely that smoking accelerates aging related  
367 pathways. Evidence for this is the finding that telomere length is reduced in the fetuses of  
368 women who actively smoke during pregnancy<sup>30</sup>, and similar changes are to be expected in the  
369 placentas of smokers. Down's syndrome is associated with advanced aging or progeria<sup>31, 32</sup>  
370 and also with increased rates of stillbirth<sup>33, 34</sup>, raising the possibility that accelerated placental  
371 aging may play a part in stillbirth related to Down's and some other congenital anomalies.  
372 Similarly placental abruption is associated with growth restriction, maternal smoking and  
373 stillbirth, and placental aging may play a part in this condition<sup>35, 36</sup>.

374

375 We have used cultured term placental explants to interrogate the pathways leading to the lipid  
376 oxidation and disturbed autophagosome function. We measured production of 4HNE and the  
377 diameter of autophagosomes following serum depletion. We observed a significant increase  
378 in 4HNE and a significant increase in autophagosome size suggesting inhibition of autophagy  
379 by oxidative damage as we had previously observed in the stillbirth and late-term placentas.  
380 Raloxifene a potent inhibitor of AOX1 has been shown to reduce oxidative damage in

381 endothelial cells<sup>37</sup>. We have demonstrated that the AOX1 inhibitor raloxifene is also able to  
382 block the oxidative damage to the lipid in placental explants. The role of AOX1 was  
383 confirmed using the GPER1 agonist G1 that has been shown to block AOX1 activation and  
384 reduce 4HNE in renal tissue<sup>23</sup>. The G1 also blocked the changes in lysosomal positioning  
385 within the explants. We report the novel finding of the presence of the cell surface estrogen  
386 receptor GPER1 on the syncytiotrophoblast apical membrane, suggesting that this receptor  
387 may play a role in modulating oxidative damage within the placenta. It has been shown that  
388 urine from pregnant women carrying a fetus with post-maturity syndrome have lower  
389 estrogen:creatinine ratios than women carrying normal foetuses<sup>38</sup>. These data support the  
390 possibility that low estrogen concentrations may lead to loss of the cell surface estrogen  
391 receptor (GPER1) mediated inhibition of AOX1 and consequently placental oxidative damage  
392 and impaired function.

393  
394 The changes in the late-term placenta occur as the fetus continues to grow and to require  
395 additional supplies of nutrients. Post-maturity syndrome is a condition seen in post-dates  
396 infants who show evidence of late gestation failure of nutrition<sup>39</sup>. Normal human infants born  
397 at term have 12-14% body fat whereas post maturity syndrome is associated with the birth of  
398 a baby with severe wrinkling of the skin due to loss of subcutaneous fat. Post-maturity  
399 syndrome is rarely seen in modern obstetric practice where delivery is usually effected before  
400 42 weeks of gestation using induction of labour or caesarean section if labour has not  
401 occurred spontaneously. While none of the infants born to mothers in our study exhibited  
402 post-maturity syndrome, our data suggest that the physiological function of the placenta after  
403 41 completed weeks is showing evidence of decline that has many features in common with  
404 aging in other tissues. The known exponential increase in unexplained intrauterine death that  
405 occurs after 38 weeks of gestation may therefore be a consequence of aging of the placenta

406 and decreasing ability to adequately supply the increasing needs of the growing fetus. This  
407 knowledge may impact on obstetric practice to ensure infants are born before the placenta  
408 ages to the point of critical failure<sup>40</sup>. However, it is notable that not all placentas in our late-  
409 term cohort exhibited evidence of aging and it is known that infants born later in gestation  
410 have lower rates of special school needs, with those born at 41 weeks having the lowest  
411 rates<sup>41</sup>. The conflicting pressures of late gestation increases in stillbirth and falling rates of  
412 intellectual disability make obstetric care at this time very challenging, diagnostics that can  
413 predict pregnancies at increased risk of stillbirth would be useful and some progress in their  
414 development has been made<sup>42</sup>. Our data also indicate that the placenta may provide a tractable  
415 model of aging in a human tissue that uniquely ages in a 9 month period of time. The results  
416 suggest that the rate of aging of the placenta varies in different pregnancies and raises the  
417 possibility that the rate of aging of the placenta may parallel the rate of aging of the associated  
418 fetus carrying the same genome. Our work identifies potential therapeutic targets such as  
419 AOX1, that may arrest the oxidative damage to placentas in pregnancies identified at high  
420 risk of stillbirth when extreme prematurity precludes delivery. Finally, our data raise the  
421 possibility that markers of placental oxidative damage and AOX1 mRNA may be released  
422 into maternal blood where they may have diagnostic value in predicting the fetus at risk for  
423 stillbirth.

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429

430 **Author Contributions:**

431 K.M. developed the biochemical concept of the project, designed and performed experiments,  
432 and analysed the data. Z.S. designed and performed the *in vitro* culture experiments and  
433 analysed the data. R.S. developed the clinical concept of the project. J.A. was involved in  
434 developing the biochemical concepts of the study. J.M., F.P. and B.A. were involved in the  
435 clinical aspects of the project. S.R was involved in determining the level of mRNA for  
436 GPER1 in gestational tissues. The manuscript was written by K.M., R.S. and Z.S. and  
437 approved by all authors.

438

439 **Footnote:** \* Figure 1 reprinted from “Smith R, Maiti K, Aitken R. Unexplained antepartum  
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442

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546

547 **List of Tables and Figures**

548

549 **Table 1:** Demographic and clinical characteristics of the study subjects.

550

551 **Figure Legends:**

552

553 **Figure 1: Relationship between stillbirth and number of continuing pregnancies.** Kaplan  
554 Myer plot of number of continuing pregnancies as a function of gestational age and plot of  
555 unexplained stillbirth per 1000 continuing pregnancies, data from Omigbodun and Adewuyi<sup>10</sup>  
556 and Sutan et al.<sup>2</sup>. Plot shows the increase in risk of stillbirth with time consistent with the  
557 operational definition of aging proposed by Johnson et al.<sup>3</sup> and the relatively small number of  
558 pregnancies at risk of stillbirth by 41 weeks because of prior delivery. Reproduced with  
559 permission from Smith et al.,<sup>4\*</sup>

560

561 **Figure 2: DNA/RNA oxidation in late-term and stillbirth placentas.** Confocal microscopy  
562 showed increased 8OHdG staining (red) in nuclei from late-term (B) and stillbirth placentas  
563 (C) compared to 37-39 week placentas (A). DAPI (blue) staining identifies the nuclei. The  
564 graph (D) illustrates that late-term and stillbirth placentas have increased intensity of nuclear  
565 8OHdG staining ( $p < 0.0001$  for late-term placentas,  $p = 0.0005$  for stillbirth placentas, Mann  
566 Whitney test) compared to 37-39 week placentas. In Figure 2D open circles and filled circles  
567 represent 37-39 week caesarean non-labouring placentas ( $n = 10$ ) and vaginal delivery  
568 labouring placentas ( $n = 8$ ) respectively, and open squares and filled squares represent late-term  
569 labouring caesarean placentas ( $n = 5$ ) and labouring vaginal delivery placentas ( $n = 13$ )  
570 respectively, and filled triangles represent third trimester labouring vaginal delivery  
571 unexplained stillbirth placentas ( $n = 4$ ). Each point in the graph represents the average intensity

572 of 8OHdG of 60 nuclei in 6 images per placenta photographed at 100X magnification and 1.4  
573 optical resolution. Scale bar, 20  $\mu$ m. The microscopy also indicates increased staining in the  
574 cytosol of late-term and stillbirth placentas representing oxidised RNA (8-hydroxyguanosine)  
575 that is also detected by the antibody.

576

577 **Figure 3: Changes in lysosomal distribution in late-term and stillbirth placentas.** IHC of  
578 LAMP2 (red), a lysosomal marker showed that lysosomes predominantly localise to the  
579 apical surface of 37-39 week placentas (A), whereas lysosome distribution extends to the  
580 perinuclear and basal surface of syncytiotrophoblast in late-term (B) and stillbirth placentas  
581 (C). Intensity calculation across the syncytiotrophoblast showed that the distribution of  
582 LAMP2 in late-term (n=5, Figure 3F) and unexplained stillbirth placentas (n=4, Figure 3G)  
583 shifts to the perinuclear and basal surface whereas lysosome distribution in 37-39 week  
584 caesarean placentas (n=5, Figure 3D) and vaginal delivery placentas (n=5, Figure 3E)  
585 remained in the apical region of the syncytiotrophoblast. DAPI (blue) staining identifies the  
586 nuclei. In Figures 3D to 3G each coloured line represents results on an individual placenta,  
587 and shows the mean intensity of LAMP2 across the syncytiotrophoblast at 5 random sites per  
588 image (example represented by light green line in 3A, 3B and 3C) for 6 separate images per  
589 placenta. Images were photographed at 100X magnification; scale bar, 20  $\mu$ m.

590

591 **Figure 4: Lipid peroxidation is increased in late-term and stillbirth placentas.** 4HNE  
592 (red) immunostaining in 37-39 week (A), late-term (B), and stillbirth (C) placentas. DAPI  
593 (blue) staining identifies nuclei. The intensity of 4HNE is significantly increased in late-term  
594 placentas (D) ( $p < 0.0001$ , Mann Whitney test) and stillbirth placentas ( $p = 0.0014$ , Mann  
595 Whitney test). In Figure 4D open circles and filled circles represent 37-39 week caesarean  
596 non-labouring placentas (n=20) and vaginal delivery labouring placentas (n=14) respectively,

597 and open squares and filled squares represent late-term labouring caesarean placentas (n=10)  
598 and vaginal delivery placentas (n=18) respectively, while filled triangles represent third  
599 trimester labouring vaginal delivery unexplained stillbirth placentas (n=4). Each point in 4D  
600 represents the mean intensity per unit area for 6 images taken for each individual placenta.  
601 Images were photographed at 100X magnification; scale bar, 20  $\mu$ m.

602

603 **Figure 5: Larger autophagosomes occur in late-term and stillbirth placentas.**

604 Immunofluorescence staining of LC3B (green) in the 37-39 week (A), late-term (B), and  
605 unexplained stillbirth (C) placentas. DAPI (blue) staining indicates the nuclei.  
606 Autophagosome size was quantified using NIS element software and the diameter was  
607 measured at an arbitrary intensity range of 1000-3000, diameter range 0.2-1  $\mu$ m and  
608 circularity range 0.5-1. Analysis (D) showed that late-term and stillbirth placentas have  
609 significantly larger (p=0.012 and p=0.0019, respectively, Mann Whitney test)  
610 autophagosomes than 37-39 week placentas. In 'D' open circles and filled circles represent  
611 37-39 week caesarean non-labouring placentas (n=11) and vaginal delivery labouring  
612 placentas (n=10) respectively, and open squares and filled squares represent late-term  
613 labouring caesarean placentas (n=8) and labouring vaginal delivery placentas (n=15)  
614 respectively, while filled triangles represents unexplained stillbirth placentas (n=4). Each  
615 point in the graph represents the average diameter of LC3B particles in six images taken for  
616 each placenta. Original magnification, 100X; scale bar, 20  $\mu$ m. Arrow heads indicate  
617 autophagosomes (LC3B positive particles).

618

619 **Figure 6: Co-localisation of aldehyde oxidase (AOX1) and 4HNE, and increased**  
620 **expression of AOX1 mRNA in late-term and stillbirth placentas.** Representative dual  
621 labelled fluorescence immunostaining in late-term (A-C) and stillbirth (D-F) placentas

622 showed that AOX1 positive particles (green) are co-localized with 4HNE (red). Orange dots  
623 (pointed by arrow heads in C and F) indicate co-localization. Nuclei are stained with DAPI  
624 (blue). Real-time PCR showed that expression of AOX1 mRNA is increased in late-term  
625 ( $p=0.0097$ ) and stillbirth ( $p=0.012$ ) placentas compared to early-term placentas (G). Original  
626 magnification 100X; scale bar 20  $\mu\text{m}$ .

627

628 **Figure 7: Pharmacologic inhibition of 4HNE production.** Fluorescence immunostaining  
629 with antibody against 4HNE (red) in serum starved placental explant (A) at time 0 (just  
630 before starvation) (B) at 24 h after culturing in medium containing FBS (control treatment),  
631 (C) at 24 after starvation (culturing in medium without FBS), (D) 24 h after treatment with an  
632 AOX1 inhibitor, raloxifene (RLX) and (E) 24 h after treatment with a membrane estrogen  
633 receptor GPER1 agonist, G1. Intensity calculation showed that the production of 4HNE  
634 (induced by serum starvation) is significantly reduced after treating placental explants with  
635 raloxifene (F) and G1 (G). Data are mean  $\pm$  S.E.M.,  $*p<0.05$  (N=6). Original magnification,  
636 20X; scale bar, 100  $\mu\text{m}$ . DAPI (blue) staining indicates the nuclei.

637

638 **Figure 8: Expression of GPER1 in placenta and myometrium, but not in membranes by**  
639 **IHC, real-time PCR and western-blotting.** Fluorescence IHC showed that GPER1 (green)  
640 is localized predominantly in the placental in a section of a term placental roll photographed  
641 at 10X magnification (A). GPER1 (green) was shown to localize in the apical layer of  
642 syncytiotrophoblast of placental villi (B), when photographed at 100X magnification. Scale  
643 bar in 'A' and 'B' represent 100  $\mu\text{m}$  and 20  $\mu\text{m}$ , respectively. The real-time qPCR data  
644 showed that the mRNA for GPER1 is expressed in higher amounts in term placenta, whereas  
645 amnion, chorion and decidua show very low expression of GPER1 (C). The expression of  
646 mRNA for GPER1 follows the order: decidua<chorion<amnion<placenta. The western blot of

647 protein extract from the breast cancer cell line MCF-7, term placenta, myometrium, amnion,  
648 chorion and decidua are presented in 'D'. Placenta, myometrium and MCF-7 cell lines  
649 expressed higher amounts of GPER1 than amnion, chorion or decidua (D). Western-blotting  
650 data showed that all the tissues expressed glycosylated GPER1 (denoted by \*\* or by \*\*\*) and  
651 non-glycosylated nascent GPER1 (denoted by \*). The sypro-ruby stain of the same PVDF  
652 membrane is used as internal loading control (E).

653

#### 654 **Supplementary Figure Legends**

655

656 **Figure S1: Oxidised lipids within autophagosomes of late-term placentas.** Representative  
657 dual labelled fluorescence immunostaining showed that LC3B, an autophagosome marker  
658 (green) is co-localised with 4HNE, a marker of lipid peroxidation (red). Orange dots (pointed  
659 by arrow heads in C) indicate the co-localization. DAPI (blue) staining indicates the nuclei.  
660 Original magnifications 100X; scale bar 20  $\mu$ m.

661

662 **Figure S2: Changes in autophagosome size in placental explants cultured in serum**  
663 **deprived medium.** Fluorescence immunostaining with antibody against LC3B (green) in  
664 serum starved placental explant (A) at time 0 (just before starvation) and (B) at 24 h after  
665 starvation. DAPI (blue) staining indicates nuclei. (C) Immunohistochemical analysis showed  
666 that the size of autophagosomes (LC3B positive particles) increased 24 h after serum  
667 starvation compared to 0 h. Data presented as mean  $\pm$  S.E.M., \*\*\*p=0.0002 (N=13). Scale  
668 bar, 20  $\mu$ M.

669

670 **Figure S3: GPER1 regulates lysosomal distribution in placental explants cultured in**  
671 **serum deprived medium.** Fluorescence immunostaining with antibody against LAMP2

672 (red) in serum starved placental explant (A) at time 0 (just before starvation), (B) at 24 h after  
673 culturing in medium containing FBS, (C) at 24 after starvation (culturing in medium without)  
674 FBS, and (D) 24 h after treatment with GPER agonist, G1. DAPI (blue) staining indicates  
675 nuclei. Intensity calculation (E) across the syncytiotrophoblast showed that the distribution of  
676 LAMP2 at 24 h after starvation shifts to the perinuclear and basal surface compared to control  
677 treatment (N=7). Each coloured line in 'E' represents the mean intensity of LAMP2 across the  
678 syncytiotrophoblast at 5 random sites per image for 6 separate images per experiment. In 'F',  
679 each coloured bar indicates mean of the area under the curve (AUC) of the corresponding  
680 coloured line presented in 'E' and statistical differences were calculated. Original  
681 magnifications, 40X; scale bar, 20  $\mu$ m; error bar, S.E.M.; \* $p$ <0.05 (N=7).

Table 1: Demographic and clinical characteristics of the study subjects

Characteristic	37-39 Weeks	Late-term	Stillbirth			
Number of cases	34	28	4			
Gestational ages (weeks)	38.57 ± 0.15	41.46 ± 0.06	32	32.57	39	40.14
Fetal growth restriction (number of cases)	0	0	No	Yes	No	Yes
Maternal age (years)	31.03 ± 0.88	28.81 ± 1.15	30.21 ± 2.68			
Vaginal birth (%)	41.20 %	64.30 %	100.00 %			
BMI (kg/m <sup>2</sup> ) at second trimester or at birth	29.10 ± 1.50	28.52 ± 1.10	27.40 ± 2.40			
Ethnicity						
Caucasian (%)	82.35 %	96.42 %	75.00 %			
Smoker (%)	17.64 %	17.85 %	0.00 %			

Data are presented as (Mean ± SEM) or percentage. BMI, Body Mass Index























