

1 **Title**

2 The differential regulation of placenta trophoblast bisphosphoglycerate mutase in fetal growth  
3 restriction: preclinical study in mice and observational histological study of human placenta.

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6 **Authors**

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12 **Abstract**

13 **Background** Fetal growth restriction (FGR) is a pregnancy complication in which a newborn  
14 fails to achieve its growth potential, increasing the risk of perinatal morbidity and mortality.  
15 Chronic maternal gestational hypoxia, as well as placental insufficiency are associated with  
16 increased FGR incidence; however, the molecular mechanisms underlying FGR remain unknown.

17 **Methods** In a case control study of murine and human control and FGR placentae, we  
18 implied MR imaging, IHC and metabolomics to assess the levels of BPGM and 2,3 BPG to  
19 elucidate the impact of maternal gestational hypoxia, and the molecular mechanisms underlying  
20 human FGR.

21 **Results** We show that murine acute and chronic gestational hypoxia recapitulates FGR  
22 phenotype and affects placental structure and morphology. Gestational hypoxia decreased  
23 labyrinth area, increased the incidence of red blood cells (RBCs) in the labyrinth while expanding  
24 the placental spiral arteries (SpA) diameter. Hypoxic placentae exhibited higher hemoglobin-  
25 oxygen affinity compared to the control. Placental abundance of bisphosphoglycerate mutase  
26 (BPGM) was upregulated in the syncytiotrophoblast and spiral artery trophoblast cells (SpA  
27 TGCs) in the murine gestational hypoxia groups compared to the control. In contrast, human  
28 FGR placentae exhibited reduced BPGM levels in the syncytiotrophoblast layer compared to  
29 placentae from healthy uncomplicated pregnancies. Levels of 2,3 BPG, the product of BPGM,  
30 were lower in cord serum of human FGR placentae compared to control. Polar expression of

31 BPGM, was found in both human and mouse placenta syncytiotrophoblast, with higher  
32 expression facing the maternal circulation. Moreover, in the murine SpA TGCs expression of  
33 BPGM was concentrated exclusively in the apical cell side, in direct proximity to the maternal  
34 circulation.

35 **Conclusions** This study suggests a possible involvement of placental BPGM in maternal-fetal  
36 oxygen transfer, and in the pathophysiology of FGR.

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39 Foundation KillCorona grant 3777/19 (to MN, MK).

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## 42 **Introduction**

43 The placenta is a transient organ, crucial for the growth and development of the fetus during  
44 gestation<sup>12</sup>. The placenta provides the interface between the maternal and fetal circulation,  
45 mediating gas and metabolic exchange along with fetal waste disposal<sup>3</sup>. Abnormalities in  
46 placental growth, structure, and function are associated with gestational complications such as  
47 fetal growth restriction (FGR)<sup>45</sup>, which is defined as the failure of the fetus to reach its growth  
48 potential<sup>6</sup>. The clinical definition of FGR is fetal weight below the 10<sup>th</sup> percentile of predicted  
49 fetal weight for gestational age<sup>7</sup>. FGR affects approximately 10-15% of pregnancies, increasing  
50 the risk of perinatal morbidity and mortality<sup>6</sup>. Long-term complications of FGR include poor  
51 postnatal development and are associated with multiple adverse health outcomes including  
52 respiratory, metabolic and cardiovascular deficits<sup>89</sup>.

53 There are numerous etiologies for FGR, some of which are related to fetal genetic aberrations or  
54 malformations, others related to placental or umbilical malformation, or also to maternal  
55 infections or diseases. Maternal anemia, smoking, high altitude residency, as well as placental  
56 and umbilical cord anomalies, are all associated with restricted placental and fetal oxygen  
57 availability<sup>10</sup>. Interestingly, about 40 percent of all FGR cases are idiopathic<sup>11</sup>, with no  
58 identifiable cause, which might hint on possible biological pre disposition factors that contribute  
59 to FGR development by creating an hypoxic placental or embryo environment. However, the

60 molecular mechanisms that provoke and contribute to this pregnancy complication have yet to  
61 be elucidated.

62 One of the key placental functions is the transfer of oxygen from the mother to the fetus<sup>12</sup>, and  
63 inefficient oxygen transport and availability is detrimental for placental and embryonic  
64 development<sup>13,14</sup>. Late-gestation hypoxia results in utero-placental vascular adaptations, such as  
65 capillary expansion, thinning of the inter-haemal membrane and increased radial artery  
66 diameters<sup>15</sup>. Moreover, there is substantial evidence that late-gestation exposure to hypoxic  
67 environment alters placental structure and functionality<sup>16,17</sup>. *In-vitro* studies on human placental  
68 samples under acute reduction of oxygen tension induced direct placental vasoconstriction<sup>18</sup>.  
69 Placental oxygen transport depends on Hemoglobin (Hb), which is responsible for carrying and  
70 mediating oxygen transfer in mammalian organisms<sup>19</sup>. BOLD contrast MR imaging is a powerful  
71 tool that utilizes hemoglobin as an endogenous reporter molecule to assess oxygen-hemoglobin  
72 affinity<sup>20</sup>. Previous MR studies have shown altered placental oxygen-Hb affinity following  
73 exposure to hypoxia<sup>21</sup>. However, limited information is available on how placental structure and  
74 function is altered in chronic gestational hypoxia that commences at the onset of gestation.

75 The most significant allosteric effectors of Hb are organic phosphates, specifically 2,3 BPG,  
76 which is produced by the BPGM enzyme in a unique side reaction of glycolysis, known as the  
77 Luebering-Rapoport pathway<sup>22</sup>. 2,3 BPG plays a key role in delivering O<sub>2</sub> to tissues by binding to  
78 and stabilizing deoxy-hemoglobin, thus leading to the release of oxygen from the Hb unit<sup>23,24</sup>.  
79 During gestation, fetal hemoglobin (HbF) is the dominant form of Hb present in the fetus,  
80 comprised of  $\alpha$  and  $\gamma$  subunits<sup>25</sup>. During late gestation, the  $\gamma$  subunit is gradually replaced by the  
81 adult  $\beta$  subunit<sup>25</sup>. HbF has a higher affinity to oxygen compared to the adult Hb, caused by a  
82 structural difference, which leads to a weakened ability to bind 2,3 BPG<sup>26,27,28</sup>. The transfer of  
83 oxygen from maternal to fetal Hb is facilitated by the higher affinity of maternal Hb to 2,3 BPG<sup>24</sup>.  
84 Remarkably, BPGM expression is specifically restricted to erythrocytes and the  
85 syncytiotrophoblast of the placenta, a multinucleated layer that mediates transport of oxygen  
86 and nutrients from the mother to the fetus<sup>29</sup>. In a study that used *igf2*<sup>+/-</sup> knockout mice as a  
87 model of FGR, BPGM expression in the placental labyrinth was lower compared to wild type  
88 placentae<sup>30</sup>. However, scarce information is available on the role of this enzyme during  
89 gestation.

90 We report here that placental BPGM expression pattern is consistent with a role in adaptation  
91 of the placenta to gestational hypoxia, facilitating the transfer of oxygen from maternal to fetal  
92 circulation. Here we show that gestational hypoxia augments placental BPGM expression in  
93 mice, while in human FGR placentae of unknown etiology BPGM expression is suppressed.

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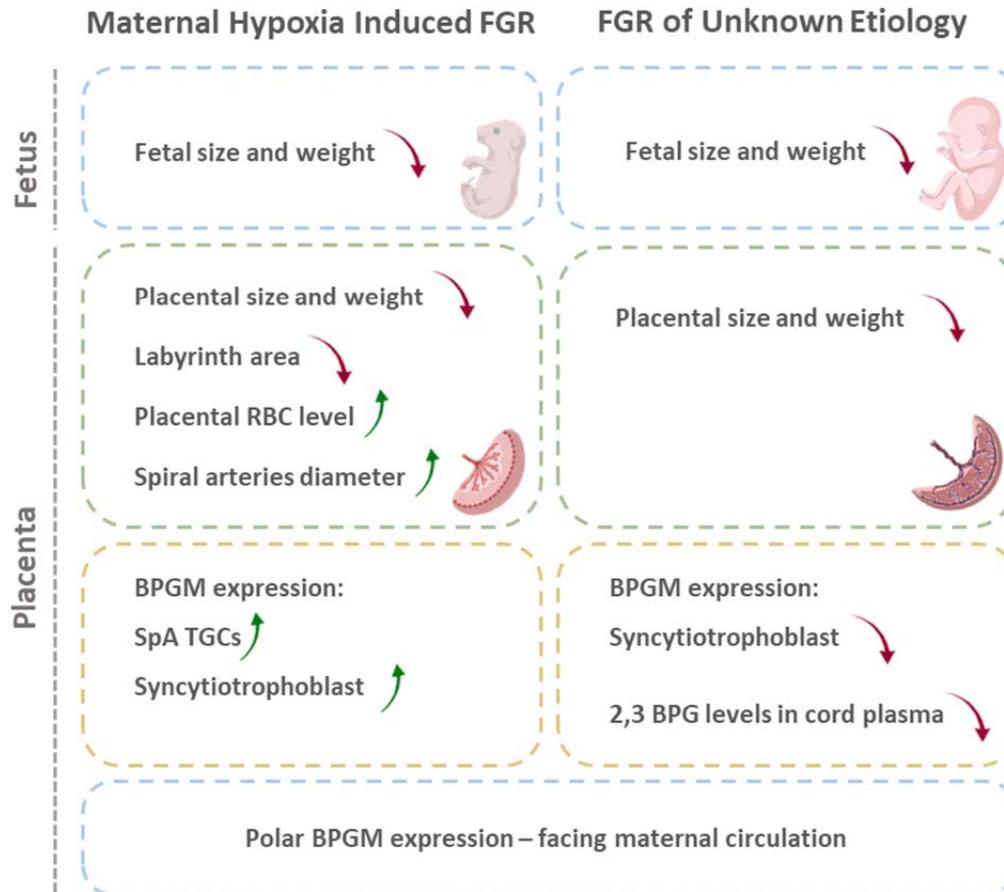
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102 **Graphical abstract**



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108 **Results**

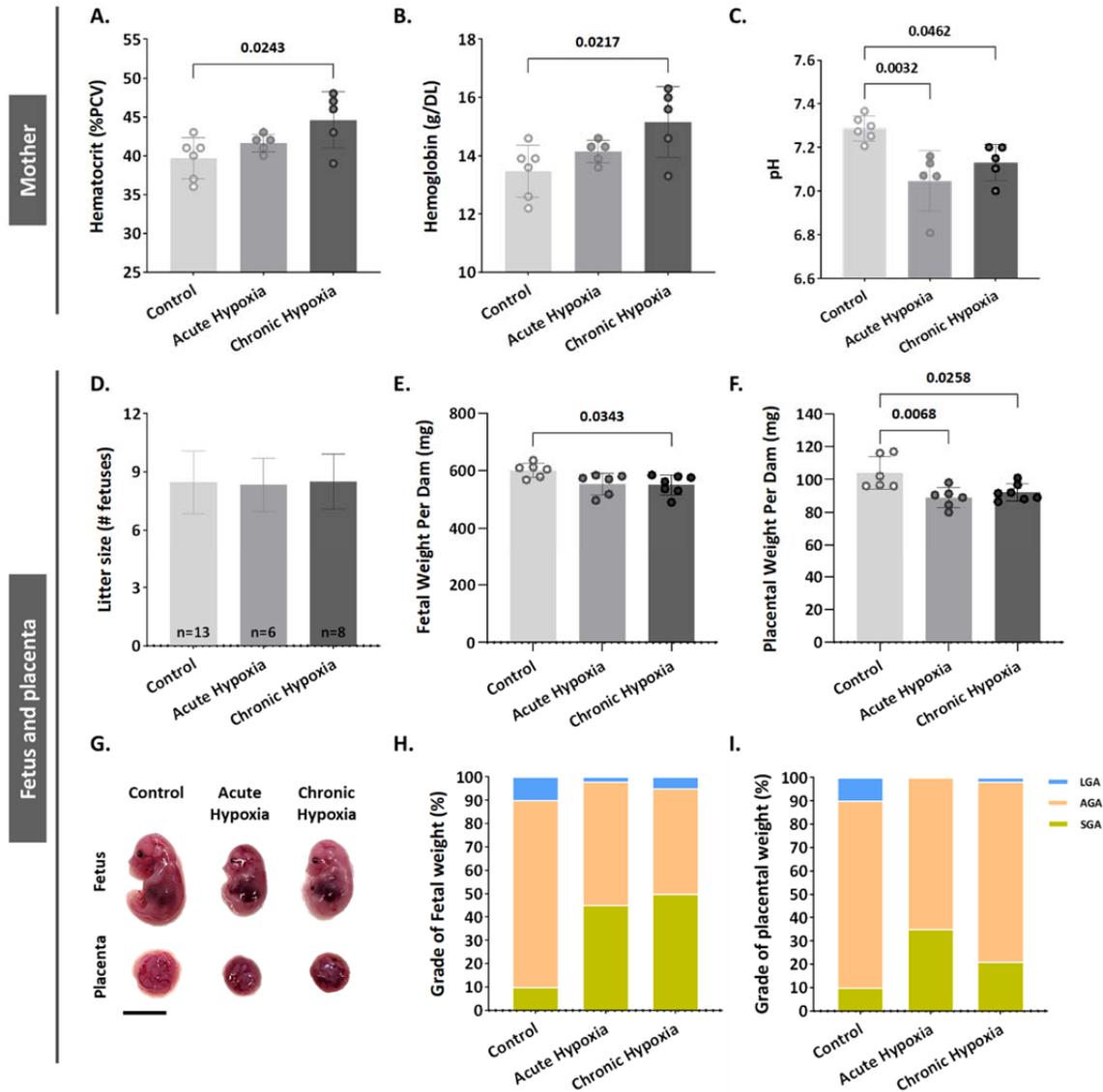
## 109 **Gestational Hypoxia Affects Maternal Hematological Parameters and Recapitulates FGR**

### 110 **Phenotype**

111 Maternal hypoxia during pregnancy increases the risk of FGR<sup>31,32</sup>. To gain an understanding of  
112 BPGM contribution to placental development and functionality following maternal hypoxia, we  
113 established a murine model of acute and chronic gestational hypoxia. Increased erythropoiesis is  
114 the best-known physiological response to chronic hypoxia<sup>33</sup>. Exposure to chronic hypoxia during  
115 gestation significantly elevated maternal blood hematocrit and Hb levels (by  $4.9 \pm 1.62$  %PCV,  
116  $P=0.0243$  and by  $1.693 \pm 0.54$  g/DL,  $P=0.0217$  respectively, Figure 1 A, B) relative to the control  
117 group. Both acute and chronic gestational hypoxia resulted in a significant increase in blood  
118 acidity, presented by a decrease in pH values ( $P=0.0032$  acute hypoxia versus control,  $P=0.0462$   
119 chronic hypoxia versus control, Figure 1 C).

120 Gestational acute and chronic hypoxia did not affect litter size (Figure 1 D). Thereafter, the  
121 effect of gestational hypoxia on placental and fetal weight was assessed. A significant decrease  
122 in placental weight was observed in both gestational hypoxia groups and in fetuses of the  
123 chronic hypoxia group (acute hypoxia placentae by  $15.03 \pm 4.2$  mg,  $P=0.0068$ ,; chronic hypoxia  
124 placentae by  $11.84 \pm 4.06$  mg,  $P=0.0258$  and fetuses by  $50.24 \pm 18.11$  mg,  $P=0.0343$ , Figure 1 E- G)  
125 when compared to the control group. To further examine the weight differences, the percent of  
126 small, average or large for gestational age (SGA, AGA and LGA respectively) fetuses and  
127 placentae were compared to the control group. The results show that in the acute hypoxia  
128 group 45% of the fetuses are SGA and only 2% LGA, whereas in the chronic hypoxia group 50%  
129 of the fetuses are SGA and only 5% LGA (Figure 1 H). Furthermore, the placentae exhibited a  
130 similar phenotype, where in the acute hypoxia group 35% of the placentae are SGA and none  
131 were LGA, whereas in the chronic hypoxia group 21% of the placentae were SGA and only 1.6%  
132 LGA (Figure 2 I).

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135 **Figure 1. Gestational hypoxia elevates maternal hemoglobin, hematocrit and blood acidity,**  
 136 **and recapitulates FGR phenotype. (A-B)** Graphs showing hematocrit and hemoglobin levels in  
 137 maternal venous blood. **(C)** Graph shows pH levels in maternal venous blood. **(D-F)** Graphs  
 138 showing litter size, fetal weight and placental weight. **(G)** Representative picture of fetuses and  
 139 placentae (E16.5) from control and gestational hypoxia groups. **(H-I)** Analysis of the percentage  
 140 of small for gestational age (SGA, weight less than the 10<sup>th</sup> percentile) fetuses and placentae,  
 141 large for gestational age (LGA, weight greater than the 90<sup>th</sup> percentile) fetuses and placentae,  
 142 and appropriate for gestational age (AGA, weight between the 10<sup>th</sup> and 90<sup>th</sup> percentiles) fetuses  
 143 and placentae at E16.5. Scale bars: 1 cm. Data displayed as mean  $\pm$  SD and are from 49-62  
 144 fetuses and placentae from 6-7 dams per group (8-9 conceptuses per litter used). Ordinary one-  
 145 way ANOVA test was used for statistical analysis.

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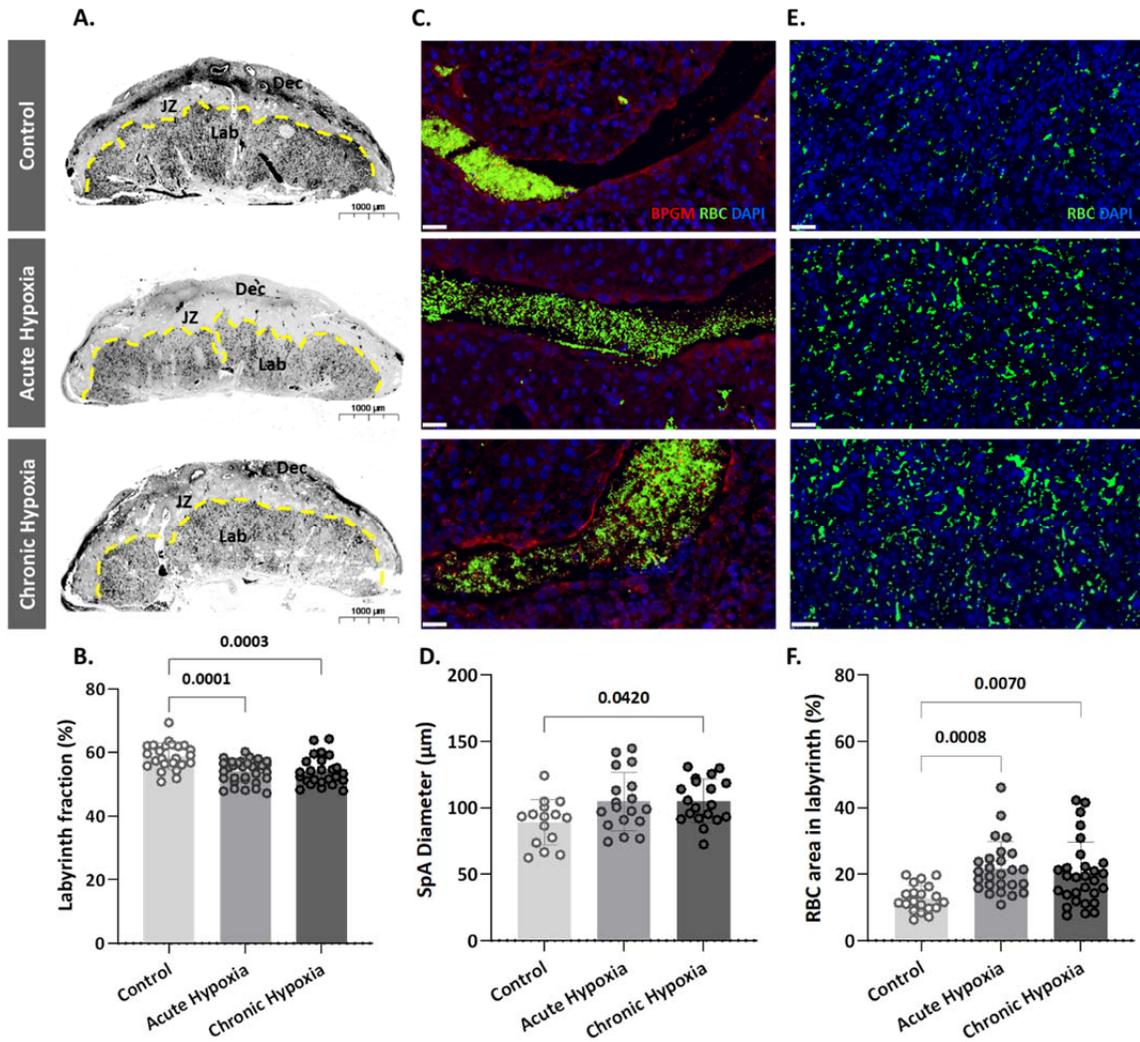
147 **Gestational Hypoxia Alters Placental Morphology**

148 To determine whether the gestational hypoxia leads to structural changes of the placenta, the  
149 placental morphology, and particularly the labyrinth area were examined. The labyrinth area of  
150 the chronic and gestational hypoxia-exposed mice was significantly smaller ( $P=0.0001$  for the  
151 acute and  $P=0.0003$  for the chronic hypoxia groups, Figure 2 A, B) compared to the control  
152 group. Furthermore, the diameter of the placental spiral arteries (SpA) was enlarged in the  
153 chronic hypoxia group (Figure 2 C, D,  $P=0.0420$ ) as compared to the control. In addition, in both  
154 acute and chronic hypoxia groups the density of RBCs in the labyrinth were significantly higher  
155 ( $P=0.0008$  for the acute and  $P=0.007$  for the chronic hypoxia groups, Figure 2 E, F) compared to  
156 the control.

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161 **Figure 2. Maternal hypoxia during gestation results in enlarged spiral arteries, increased RBC**  
162 **levels and decreased placental labyrinth area.** (A, B) Placentae of hypoxic chamber groups have  
163 significantly smaller labyrinth area in comparison to the control group.(C ,D, E, F) Placentae of  
164 hypoxic chamber groups display enlarged spiral arteries and increased RBC levels in the  
165 labyrinth. Scale bars: 40 µm. Data are from 3 control, 4 chronic hypoxia and 4 acute hypoxia  
166 dams, 5-7 placentae per dam and presented as mean ± SD values. Ordinary one-way ANOVA test  
167 was used for statistical analysis.

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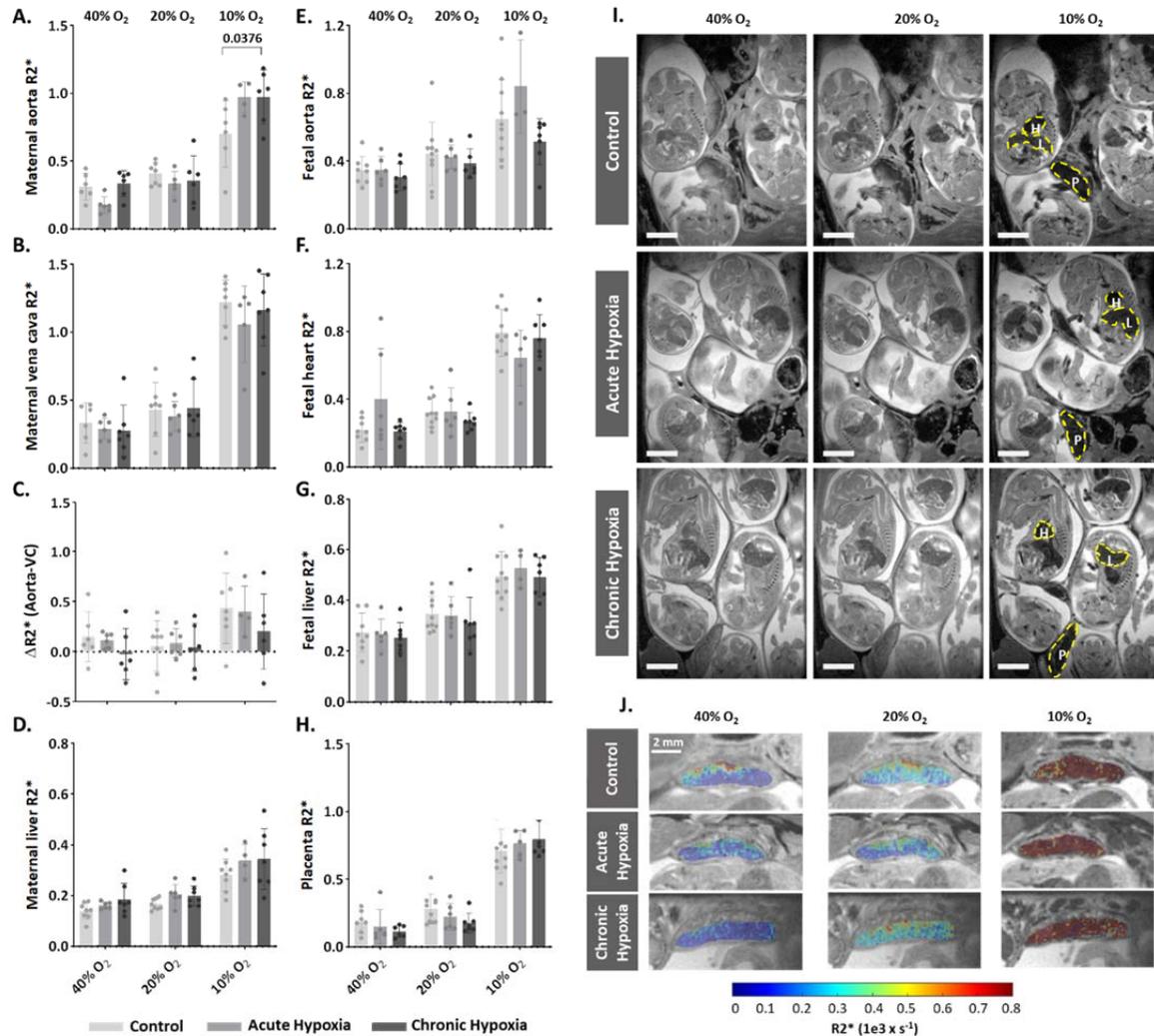
173 **R2\* Maps Reveals Maternal, But Not Placental or Fetal changes in deoxygenated hemoglobin**  
174 **concentration**

175 As shown above, gestational hypoxia alters placental structure. To determine whether and how  
176 gestational hypoxia affects placental functionality, the pregnant dams (E16.5) were subjected to  
177 hyperoxia-hypoxia challenge during ultra-high field (15.2T) MR imaging (Appendix, video 1, 2, 3).  
178 R2\* values were calculated at each oxygen challenge for the maternal aorta, vena cava and liver  
179 (Figure 3 A-D, Appendix Figure S1), and for the placenta, embryo heart, liver and aorta (Figure 3  
180 E-H). The maternal aorta R2\* levels from the chronic hypoxia group were significantly higher  
181 (P=0.0376, Figure 3 A) than in the control group, when subjected to 10% O<sub>2</sub>. However, no  
182 differences were observed in maternal liver and vena cava when compared to that of the  
183 control group (Figure 4 B, D). Similarly, no differences were observed in the R2\* of embryonic  
184 tissues (aorta, heart and liver), nor in the placenta, when comparing the hypoxic groups to the  
185 control (Figure 4 E-H). To better understand the signal distribution in the different placental  
186 regions, the R2\* maps of the placentae were further analyzed. Interestingly no significant  
187 differences in the spatial distribution of R2\* were observed in the placentae of hypoxic and  
188 control groups (Figure 3 J).

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193 **Figure 3. Effects of maternal hypoxia during gestation on R2\* values following hyperoxia-**  
 194 **hypoxia challenge. (A-H)** Graphs show that hypoxic challenge results in elevation in R2\* values  
 195 in maternal aortas of chronic hypoxia chamber group, while no differences are observed in the  
 196 respective placentae and fetuses. **(I)** Representative R2\* images of control and hypoxic chamber  
 197 group show several fetuses and their placenta (P), heart (H) and liver (L). Scale bars: 0.5 cm. **(J)**  
 198 Representative R2\* maps inside the placenta of control, acute hypoxia (AH) and chronic hypoxia  
 199 (CH) chamber groups at E16.5 show distribution of R2\* values following hyperoxia-hypoxia  
 200 challenge. Data are from 8 control, 6 acute hypoxia and 7 chronic hypoxia per dams presented  
 201 as mean  $\pm$  SD values. R2\* values of embryonic tissues and placentae are calculated as the  
 202 median per mother, 5-8 embryos per each mother. Ordinary one-way ANOVA test was used for  
 203 statistical analysis.

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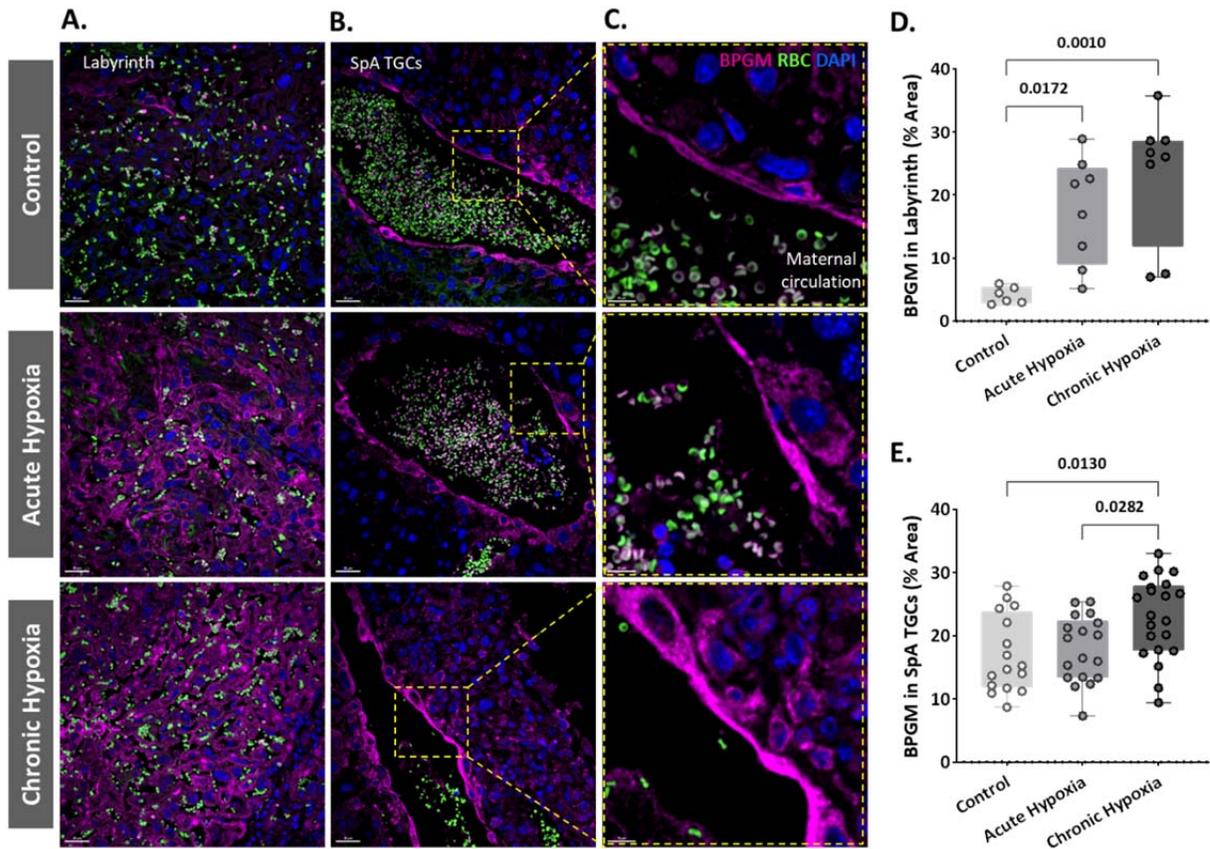
208 **BPGM is Upregulated in Placental Cells Following Gestational Hypoxia**

209 Our present findings revealed structural changes in placentae from hypoxic mothers, however  
210 functional MRI experiments demonstrated that placental deoxyhemoglobin concentrations are  
211 similar to the control group. BPGM expression was previously observed in human placental  
212 syncytiotrophoblast cells from healthy pregnancies<sup>29</sup>. Therefore, we inspected the expression of  
213 BPGM in the labyrinth of the gestational hypoxia FGR murine model compared to the control.  
214 Significant differences were observed in the syncytiotrophoblast BPGM expression between the  
215 hypoxic and control placentae (Figure 4 A, C). Although BPGM expression has only been  
216 reported in the syncytiotrophoblast, we also inspected the BPGM expression in other placental  
217 cells that come in direct contact with maternal blood. BPGM expression was found also in the  
218 spiral artery trophoblast cells (SpA TGCs), an expression that is upregulated following acute and  
219 chronic maternal hypoxia (Figure 4 D); moreover, SpA TGCs BPGM expression was found to be  
220 polar and concentrated in the apical cell side facing the arterial lumen (Figure 4 B).

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225 **Figure 4. Maternal hypoxia during gestation results in elevated placental BPGM expression**  
 226 **levels. (A,D)** Representative images and quantification of BPGM expression in the placental  
 227 labyrinth at E16.5 of control and hypoxic chamber groups. Scale bars: 30  $\mu$ m (B,C,E) Trophoblast  
 228 cells lining the arteries show an increase of BPGM expression in chronic hypoxia group. The  
 229 expression of BPGM is restricted to the apical trophoblast cell side facing the arterial lumen.  
 230 Scale bars: 30  $\mu$ m (B), 10  $\mu$ m (C). Data are from 3 control, 4 chronic hypoxia and 4 acute hypoxia  
 231 dams, 2-3 placentae per group and presented as mean  $\pm$  SD values. Ordinary one-way ANOVA  
 232 test was used for statistical analysis.

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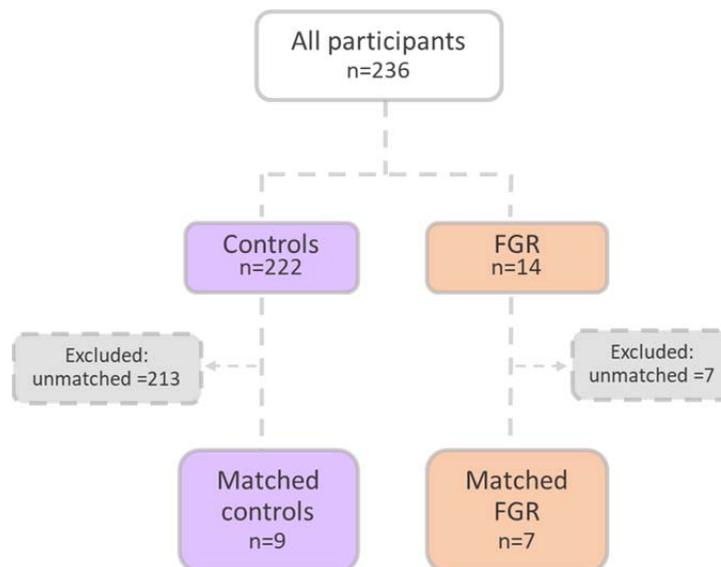
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238 **BPGM expression is Downregulated in Human FGR placentae**

239 An upregulation of syncytiotrophoblast and SpA TGCs BPGM levels was detected in the murine  
240 gestational hypoxia placentae. Therefore, to determine whether BPGM expression is also  
241 altered in human placental syncytiotrophoblast cells of pregnancies complicated by FGR, human  
242 placentae from healthy and FGR-complicated third-trimester pregnancies were examined.  
243 Seventeen samples collected from Meir and Wolfson Medical Centers were selected from 236  
244 deliveries ,following childbirth and classified into two groups: FGR complicated pregnancies and  
245 matched control deliveries (Table 1 and Figure 5). Clinical characteristics and neonatal outcomes  
246 are provided in Table 1. Clinical parameters did not differ among the groups, except for  
247 birthweight, which was significantly lower in the FGR group, as compared with the control  
248 (Unpaired t-test;  $P = 0.0004$ ). A downregulation of syncytiotrophoblast cells BPGM levels was  
249 observed in the FGR placentae (Figure 6 C). No differences were observed in 2,3 BPG levels in  
250 maternal plasma analyzed by mass spectrometry (Figure 6 D, E). However, the results  
251 demonstrated a significant reduction of 2,3 BPG levels in cord plasma from FGR complicated  
252 pregnancies (Figure 6 D, F).

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256 **Figure 5. Patient selection flow chart.** 16 Pregnant women were recruited from the Meir and  
257 Wolfson Medical Centers.

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Parameter	Control <i>n</i> =9	FGR <i>n</i> =7	<i>P</i> value
Maternal age, mean ± SD, years	30.2 ± 5.6	29.14 ± 5.6	0.7291
Gestational age, mean ± SD, weeks	38.2 ± 1	37.5 ± 0.6	0.1644
Preterm delivery (<37), <i>n</i> (%)	0	0	
Pregravid BMI (kg/m <sup>2</sup> ), mean ± SD	22.8 ± 4.5	27.1 ± 3.6	0.2598
Gravidity, median (IQR)	2.3 (1.5)	2. (2)	
Parity, median (IQR)	1.2 (1.5)	1 (2)	
Maternal comorbidities, <i>n</i> (%)			
Hypertensive disorders	0	0	
Diabetes or gestational diabetes	1 (11)	1 (14)	
Asthma	0	0	
Thyroid disease	0	0	
Smoker	5	3	
Infant sex, <i>n</i> (%)			
Male	7 (77)	4 (57)	
Female	2 (23)	3 (43)	
Birthweight, mean ± SD, grams	3167 ± 494	2189.4 ± 189	***0.0004
NICU, <i>n</i> (%)	0	1 (14)	

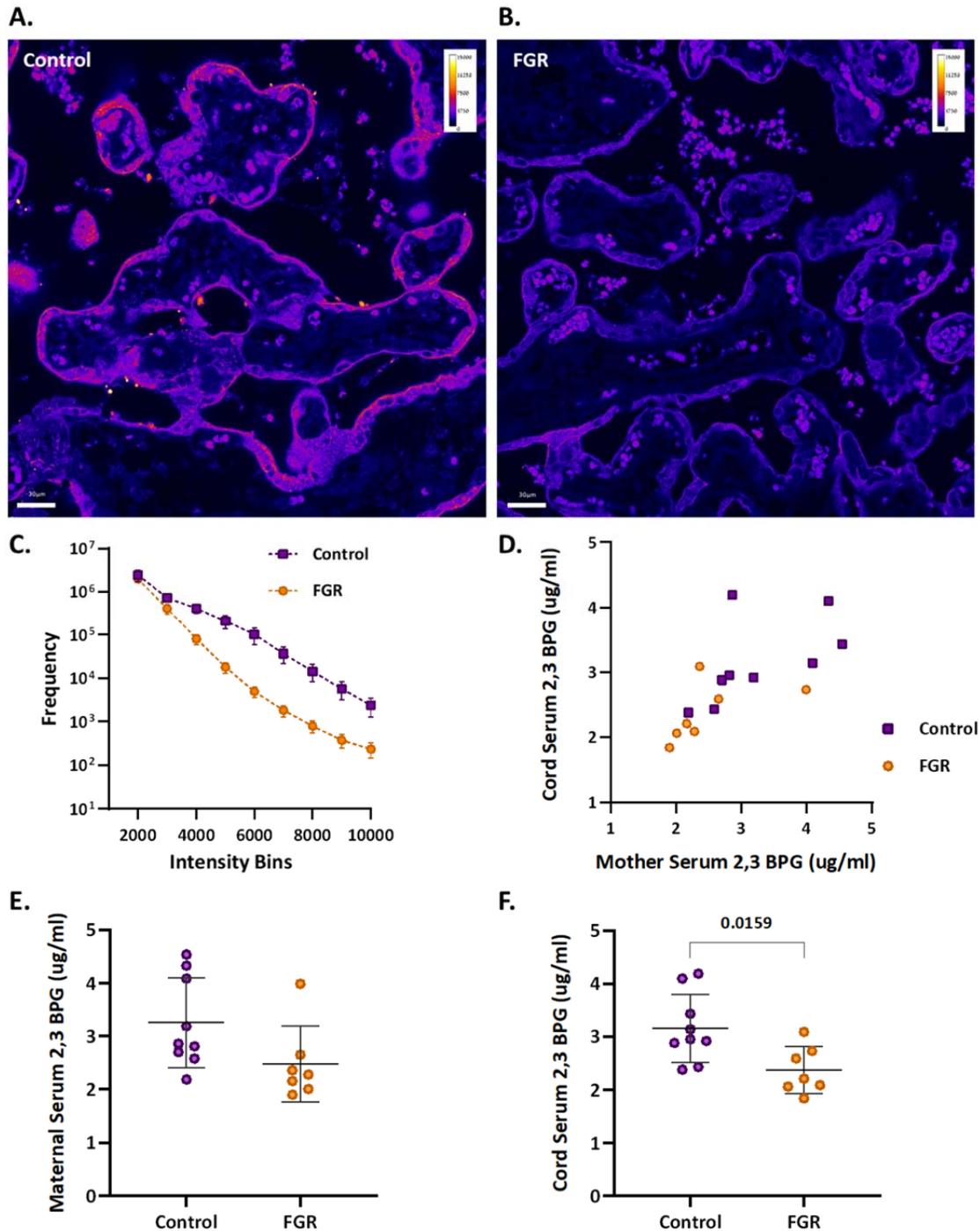
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261 **Table 1. Clinical parameters of women included in the study.** Clinical parameters did not differ  
 262 among the groups, except for birthweight, which was significantly lower in the FGR group  
 263 (Unpaired *t*-test, *P*=0.0004).

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269 **Figure 6. Human FGR placentae exhibit lower BPGM and 2,3 BPG levels.** (A, B) Representative

270 images of BPGM expression in control and FGR placentae. Scale bars: 30  $\mu$ m. (C) Graph

271 representing intensity of BPGM expression in control and FGR placentae. (D-F) Levels of 2,3 BPG

272 in maternal and cord serum of control and FGR placentae. Data are from 9 control and 7 FGR

273 women and presented as mean  $\pm$  SD values. Unpaired *t* test was used for statistical analysis.

## Discussion

274 Proper placental and fetal oxygenation is essential for a healthy pregnancy. Accordingly,  
275 maternal gestational hypoxia constitutes a risk factor for FGR incidence<sup>34</sup>. However, the etiology  
276 and molecular mechanism underlying idiopathic as well as maternal gestational hypoxia induced  
277 FGR remains unclear. In order to elucidate on the mechanisms leading to FGR, this study  
278 employed a murine FGR model based on maternal acute and chronic gestational hypoxia.  
279 Hypoxia-induced FGR placentae displayed smaller labyrinth fraction, higher RBC content and  
280 enlarged spiral arteries. However, *in vivo* functional MRI experiments in response to hypoxia-  
281 hyperoxia challenge are consistent with similar deoxyhemoglobin content in all groups. Oxygen  
282 release under hypoxia might be regulated by 2,3BPG, as suggested by the BPGM expression in  
283 the murine hypoxic placentae which was upregulated and concentrated in the cell side facing  
284 the maternal circulation. Conversely, human FGR placentae of unknown etiology exhibited an  
285 opposite phenotype, presenting lower BPGM expression and reduced level of 2,3 BPG in the  
286 chord serum. This suggests that induction of placenta BPGM may be part of the hypoxic  
287 adaptation response in the murine placenta; while suppression of BPGM may contribute to  
288 placenta deficiency in the human FGR.

289 Intra-uterine hypoxia has adverse effects on placental and embryonic development. This study  
290 shows a decreased placental and embryonal weight, and a reduction in the percent of AGA and  
291 LGA placentae and fetuses in the gestational hypoxia groups, with no difference in litter size  
292 between hypoxic and control groups. Moreover, the labyrinth area of hypoxic placentae was  
293 significantly smaller, implying an improper placental development. Previous studies showed that  
294 intermittent hypoxia increased placental weight and labyrinth size, while chronic gestational  
295 hypoxia in mice leads to reduced litter size and had no effect on the labyrinth zone<sup>3536</sup>. These  
296 contradictory results may be due to the different experimental setups employed in the  
297 intermittent hypoxia model, and the differences in litter size of the chronic hypoxia model,  
298 which might in turn affect placental size and development. Furthermore, the current study  
299 demonstrated an increase in the diameter of placental SpA following gestational hypoxia. This  
300 enlargement might serve as a compensational mechanism for the placental and labyrinthine size  
301 reduction, by supplying higher volumes of blood to the placenta thereby increasing oxygen  
302 content, tissue oxygenation and oxygen supply to the fetus. Previous studies have shown that  
303 gestational hypoxia from mid-late gestation increased the diameter of radial arteries compared  
304 to control<sup>15</sup>; however, no significant difference was observed in the spiral arteries, possibly due  
305 to the late exposure to hypoxia. However, this study mimics adaptation to early gestational

306 hypoxia and early onset placental dysfunction leading to severe FGR and therefore, might serve  
307 as a better model for the human hypoxic-induced FGR.

308 MRI is an important tool for imaging changes in deoxyhemoglobin concentration *in vivo*.  
309 Previous *in vivo* studies on non-treated pregnant mice obtained oxygen-hemoglobin dissociation  
310 curves in mid-late gestation placentae under hyperoxia - hypoxia challenge<sup>37</sup>. Interestingly, in  
311 the present study no significant differences were found in the R2\* values between the hypoxic  
312 and control placentae under hyperoxic, normoxic and hypoxic conditions. This result is  
313 consistent with similar deoxyhemoglobin levels in the hypoxic and control placentae, despite the  
314 upregulation of RBC levels in the hypoxic placentae. These results indicate that the partial  
315 amount of HbO<sub>2</sub> is higher in the hypoxic placentae compared to the control, implying on the  
316 ability of the placenta to maintain its oxygen levels albeit the maternal hypoxia.

317 In RBCs, the BPGM enzyme is responsible for the synthesis of 2,3 BPG, which induces the release  
318 of oxygen from Hb in the mammalian organism. Remarkably, the expression of BPGM has been  
319 reported in the human placental labyrinth<sup>29</sup>, suggesting on its role in placental oxygen transfer.  
320 This study shows for the first time the polar pattern of BPGM expression in both the murine and  
321 human placental cells, amassing at the apical lumen, facing the maternal circulation. This polar  
322 expression might increase the efficiency of oxygen sequestering from maternal blood by  
323 reducing the distance between 2,3 BPG molecule and the maternal RBCs. Moreover, following  
324 maternal intra-uterine hypoxia, the expression of murine placental BPGM is further upregulated,  
325 suggesting a physiological role for placenta BPGM in the placental acclimatization to low oxygen  
326 availability. Strikingly, attenuation in the expression of BPGM in FGR human placentae was  
327 found when compared to the control. Moreover, 2,3 BPG levels in the cord serum of FGR  
328 placentae were also decreased compared to control. This suggests that failure in induction of  
329 placental BPGM and subsequently lower 2,3 BPG levels may contribute to the pathophysiology  
330 of FGR. Remarkably, the same phenotype was observed in a murine FGR model of *igf2+/-*  
331 knockout mice, where labyrinthine BPGM expression was lower compared to control dams<sup>30</sup>.  
332 This study demonstrates opposite BPGM expression patterns in mouse and human FGR,  
333 suggesting that the murine FGR in our model originates in low maternal oxygen concentrations,  
334 which are compensated by the placenta *via* upregulation of BPGM levels, while human FGR of  
335 unknown etiology is related to a placental pathology that might include inadequate BPGM  
336 expression. During human gestation, the  $\gamma$  hemoglobin subunit starts to decline around week 32

337 and  $\beta$  hemoglobin rises, switching from fetal to adult hemoglobin. Following this increase in HbA  
338 in the fetus, it might be possible that placental BPGM and 2,3 BPG are also used by the fetus at  
339 that stage, to mediate the release of oxygen to its organs. However, the question of how  
340 placental 2,3 BPG might be transported to the nearby maternal RBCs needs to be addressed,  
341 while a possible explanation would be a specific transport system.

342 In summary, we propose that placental BPGM provides an important mechanism for placental  
343 adaptation to oxygen transfer during the course of gestation. We suggest that placental BPGM  
344 sequesters oxygen from the maternal Hb, and facilitates oxygen diffusion from the maternal to  
345 the fetal circulation. These results offer a possible causative link between the expression of this  
346 enzyme and the development of an FGR. This novel molecular mechanism for the regulation of  
347 oxygen availability by the placenta might provide a better understanding of the FGR pathology  
348 and possibly pave the way toward development of novel therapies for FGR complications.

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## 350 **Materials and Methods**

### 351 **Animals**

352 Female C57BL/6J0laHsd mice (8-12 weeks old; Envigo, Jerusalem; n=28) were mated with  
353 C57BL/6J0laHsd male mice (Envigo, Jerusalem; n=8). Detection of a vaginal plug the following  
354 day was considered embryonic day 0.5 (E0.5). At E0.5 or E11.5, the pregnant females were  
355 randomly allocated to control (21% O<sub>2</sub>, n = 15) or hypoxia group (12.5% O<sub>2</sub>, acute hypoxia; n=6,  
356 chronic hypoxia; n=7). Throughout the experiments, the animals were maintained in a  
357 temperature-controlled room (22 ± 1°C) on a 12h:12h light–dark cycle. Food and water was  
358 provided *ad libitum* and animal well-being was monitored daily. At E16.5 the pregnant females  
359 were analyzed using high-field MRI under a respiration challenge of hyperoxia-to-hypoxia (40%  
360 O<sub>2</sub>, 20% O<sub>2</sub>, 10% O<sub>2</sub>). After MR imaging, the animals were sacrificed for tissue collection. All  
361 experimental protocols were approved by the Institutional Animal Care and Use Committee  
362 (IACUC) of the Weizmann Institute of Science, Protocol number: 07341021-2.

### 363 **Establishment of Maternal Hypoxia Models**

364 We applied two models of maternal hypoxia – acute and chronic. The pregnant mice were  
365 housed in a hypoxic chamber (VeIO2x, Baker Ruskinn, Sanford, Maine, USA) from E11.5 (acute

366 hypoxia; n=6) or E0.5 (chronic hypoxia; n=7) until E16.5. On the first day in the hypoxic chamber,  
367 maternal oxygen supply was gradually reduced from 21%O<sub>2</sub> to 12.5 ± 0.2% O<sub>2</sub> by continuous  
368 infusion of a nitrogen gas. The water contained in the expired gas was trapped using silica gel  
369 beads (Merck, CAS #: 7631-86-9). A portable oxygen analyzer (PO<sub>2</sub>-250, Lutron, Coopersburg,  
370 Pennsylvania, United States) was used to monitor the oxygen concentration in the chamber.  
371 Pregnant control females were housed in an identical chamber supplied with a constant 21% ±  
372 0.2% O<sub>2</sub> concentration.

### 373 ***In Vivo* MR Imaging**

374 MR imaging examinations were performed at a 15.2T with an MR spectrometer (BioSpec 152/11  
375 US/R; Bruker, Karlsruhe, Germany) equipped with a gradient-coil system capable of producing  
376 pulsed gradients of 10 mT/cm in each of the three orthogonal directions. A quadrature volume  
377 coil with a 35-mm inner diameter and an homogeneous radiofrequency field of 30 mm along the  
378 axis of the magnetic field was used for both transmission and reception. Immediately prior to  
379 MR imaging, the pregnant females were anesthetized with isoflurane (3% for induction; Piramal,  
380 Mumbai, India) mixed with 2 L/min of 40% O<sub>2</sub> and 60% N<sub>2</sub> delivered into a closed induction  
381 chamber. Once anesthetized, the animals were placed in a prone position in a head holder with  
382 breathing gas mixed with isoflurane delivered through a tooth bar. Respiration rate and rectal  
383 temperature were monitored using a monitoring and gating system (Model 1030-S-50; SA  
384 Instruments, Stony Brook, NY). Respiration rate was maintained throughout the experimental  
385 period at approximately 20-30 breaths per minute by adjusting the isoflurane level (1%–2% for  
386 maintenance). Body temperature was maintained at 30±1°C (to reduce fetal movement) by  
387 adjusting the temperature of a circulating water heating blanket placed above the animal.

### 388 **MR Imaging Data Acquisition**

389 Anatomic data to determine optimal animal positioning was acquired by using a short Gradient  
390 Recalled Echo (GRE) sequence with imaging slices acquired in three orthogonal planes. The  
391 animals were positioned to maximize the number of fetuses that could be viewed while still  
392 observing maternal liver. The duration of the MRI measurements at each oxygen level was  
393 approximately 20 min. After the O<sub>2</sub> concentration was reduced, a 2 minute interval was given  
394 before acquiring the next set of MRI images, allowing R2\* stabilization. At each oxygen phase,  
395 the nitrogen level was adjusted to maintain a constant flow of inhaled gas. To determine R2\*

396 values three Gradient Recalled Echo (GRE) acquisitions were performed with TE= 1.6 ms, 2.6 ms  
397 and 3.6 ms. The parameters for these GRE measurements were as follows: 48 slices with slice  
398 thickness of 0.4 mm with 0.1 mm inter-slice gap, field of view 4.2 X 3.3 cm<sup>2</sup>, pulse flip angle 40°,  
399 matrix size 280 x 220 (150 x 150 um<sup>2</sup> pixel size), 2 averages (motion averaging). Images were  
400 acquired with fat suppression and RF spoiling. The excitation pulse was 0.5 ms (6400 Hz  
401 bandwidth) and the acquisition bandwidth was 200 kHz. The slice order was interleaved. The  
402 sequence was respiration triggered (per slice) with an approximate TR of 800 ms.

### 403 **MR Imaging Data Analysis**

404 Images were reconstructed by Paravision 6.0 (Bruker, Karlsruhe, Germany). The GRE images  
405 used for calculating R2\*s were interpolated in Matlab (MathWorks, Natick, Massachusetts, USA)  
406 to 75X75 um<sup>2</sup> pixel size. Regions of Interest (ROIs) were manually marked with ImageJ (U. S.  
407 National Institutes of Health, Bethesda, Maryland, USA). Subsequently, using custom written  
408 scripts all ROIs and images were imported into Matlab and the R2\* for each O<sub>2</sub> level was  
409 determined by fitting the changes in the median signal intensity of each ROI to a single  
410 exponential decay [Eq 1]:

$$411 \quad Int = Int_0 \cdot e^{-R_2^* \cdot TE} \quad [\text{Equation 1}]$$

### 412 **Tissue collection**

413 Mouse placenta samples: After MR Imaging of the animals, maternal blood was collected from  
414 the submandibular vein, followed by cervical dislocation. Maternal hematocrit and Hb levels  
415 were determined using i-STAT CG8+ cartridge (Abbott, Cat. No. ABAXIS-600-9001-10, Chicago,  
416 Illinois, USA). Uterine tissues were immersed in PBS to count the number of fetuses and  
417 resorptions. Fetuses and placenta were immediately removed and weighed, following by  
418 fixation in 4% paraformaldehyde. Grade of embryonic and placental weight was classified as SGA  
419 (weight less than the 10<sup>th</sup> percentile), large for gestational age (LGA, weight greater than the  
420 90<sup>th</sup> percentile), and appropriate for gestational age (AGA, weight between the 10<sup>th</sup> and 90<sup>th</sup>  
421 percentiles).

422 Human placenta samples: The study was approved by the Meir and Wolfson Medical Center  
423 IRB Local Committee (Protocols: # 0147-20 MMC and #185-19-WOMC). Written informed  
424 consent was obtained from all participants prior to delivery. Placenta from 9 healthy  
425 uncomplicated pregnancies and from 7 pregnancies complicated by fetal growth restriction

426 (FGR) were collected immediately after elective cesarean deliveries. Two biopsies were taken  
427 from each placenta, one from a peripheral and one from a central lobule. The biopsied material  
428 (~ 1 cm<sup>3</sup>) was immediately fixed in formalin. FGR birth weight standards were based on the  
429 Dollberg curve.

430 Human Serum: Maternal and cord serum samples were collected from the enrolled patients  
431 prior to delivery, and from the umbilical cord just following delivery. The umbilical cord was  
432 wiped clean and blood was drawn from the vein. Blood samples were centrifuged (1000g, 10  
433 minutes at room temperature), and serum aliquots were stored at -80°C in dedicated tubes for  
434 analyses at the Weizmann Institute.

### 435 **Immunohistochemistry and Microscopy**

436 Fixed murine and human placentae were processed and embedded in paraffin. Representative 5  
437 µm sections were taken from each tissue and used for immunohistochemistry (IHC).

438 All slides were dewaxed and rehydrated in xylene and a series of ethanol washes. IHC staining  
439 involved antigen retrieval in a pressure cooker using citrate buffer (pH=6) and blocking of non-  
440 specific binding with 20% NHS and 0.2% Triton in PBS. Slides were incubated with polyclonal  
441 rabbit primary anti-BPGM antibody (1:200, Sigma-Aldrich, Cat. No. HPA016493,  
442 RRID:AB\_1845414), followed by incubation with an HRP anti-Rabbit secondary antibody (1:100,  
443 Jackson ImmunoResearch Labs, Cat# 111-035-003, RRID:AB\_2313567) followed by Opal 690  
444 (1:500, Akoya Biosciences, Cat. No. FP1497001KT). Negative controls for each immunostaining  
445 were incubated with secondary antibody only.

446 Images were captured using Nikon Eclipse Ti2\_E microscope, Yokogawa CSU W1 spinning disk,  
447 photometrics Prime 25B camera with NIS elements AR 5.11.01 64bit software.

### 448 **Placental Morphological Analysis**

449 For the assessment of placental labyrinth size, fractional area expressing both BPGM and  
450 containing fetal RBCs of each placenta was computed *via* use of the color thresholding and area  
451 fraction tools in ImageJ. Approximately 10 measurements were made per each placenta. Spiral  
452 arteries diameter was measured manually using ImageJ, namely, for each spiral artery 5-6  
453 measurements were made. For the assessment of RBC levels in the labyrinth, thresholding of  
454 the RBC auto fluorescence signal was employed. Quantification of mouse placental BPGM in the

455 labyrinth was performed using color thresholding in ImageJ, 10 identical measurements were  
456 done for each placenta, 500x500  $\mu\text{m}$  each. For the assessment of BPGM in the SpA TGCs, regions  
457 of interest were drawn manually implying the same thickness from the inner vessel border  
458 followed by color thresholding in ImageJ. We quantified human BPGM expression level by  
459 creating a binned intensity histogram of all the pixels expressing BPGM signal above a minimal  
460 background value (of 1000), in a single slice of each sample using Fiji Macro<sup>38</sup>. As red blood cells  
461 (RBC) have high auto fluorescence in all channels, we discarded RBC regions them prior BPGM  
462 quantification. This is done in Imaris (Oxford company) by creating Surface object for RBC  
463 (default parameters, automated absolute intensity threshold), and using it to create new PBGM  
464 channel in which the values in the RBC regions are set to zero.

#### 465 **LC–MS/MS measurement of 2,3-BPG**

466 Ten- $\mu\text{L}$  aliquots of plasma were extracted with 80 $\mu\text{L}$  of extraction buffer (10mM ammonium  
467 acetate/5mM ammonium bicarbonate, pH 7.7 and methanol in ratio 1:3 by volume), and 10 $\mu\text{L}$   
468 of methionine sulfone (1 $\mu\text{g}/\text{mL}$  in water) was added as internal standard. The mixture was  
469 vortexed, incubated at 10°C for 10min, then centrifuged (21,000g for 10min). The supernatant  
470 was collected for consequent LC–MS/MS analysis. The LC–MS/MS instrument consisting of an  
471 Acquity I-class UPLC system (Waters) and Xevo TQ-S triple quadrupole mass spectrometer  
472 (Waters), equipped with an electrospray ion source, was used for analysis of 2,3-BPG. MassLynx  
473 and TargetLynx software (v.4.1, Waters) were applied for the acquisition and analysis of data.  
474 Chromatographic separation was performed on a 150mm  $\times$  2.1mm internal diameter, 1.7- $\mu\text{m}$   
475 BEH Z-HILIC column (Waters Atlantis Premier) with mobile phases A (20mM ammonium  
476 carbonate, pH 9.25/acetonitrile, 80/20 by volume) and B (acetonitrile) at a flow rate of  
477 0.4ml  $\text{min}^{-1}$  and column temperature of 25°C. A gradient was used as follows: for 0–0.8min a  
478 linear decrease from 80 to 35%B, for 0.8–5.6min further decrease to 25%B, for 5.6–6.0min hold  
479 on 25%B, then for 6.0-6.4min back to 80%B, and equilibration at 80%B for 2.6min. Samples kept  
480 at 8°C were automatically injected in a volume of 5 $\mu\text{L}$ . 2,3-BPG concentration was calculated  
481 using a standard curve, ranging from 0.1–100 $\mu\text{g ml}^{-1}$ . For MS detection MRM transitions  
482 265.0>78.8, 265.0>167.0 m/z (ESI -) were applied in case of 2,3-BPG, with collision energies 31  
483 and 12eV, respectively. Internal standard was detected using MRM 182.1>56.0 m/z (ESI +), with  
484 collision energy 18eV.

#### 485 **Statistical Analysis**

486 Ordinary one-way Anova test was applied for the comparison between the three pregnant  
487 females groups (control, acute and chronic hypoxia). Litter means were used for statistical  
488 analysis of fetal and placental weights. Unpaired *t*-test was used for the analysis of the IF  
489 images of FGR and control human placentae. The data were considered to indicate a significant  
490 difference when *P* values were less than 0.05. All results are represented as the mean  $\pm$  SD.  
491 Statistical analysis was performed using Graphpad Prism 6 (GraphPad Software, San Diego, USA)  
492 for Windows.

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### 497 **Author contributions and disclosures**

498 S.S., N.D., M.N. designed the research, S.S., T.H., L.F.A, L.B.M., A.B., T.M., performed the  
499 research, L.F.A., R.P.M., M.K., T.B.S, contributed vital new reagents or analytical tools, S.S, T.H.,  
500 O.G., analyzed the data, and S.S., N.D. and M.N. wrote the paper.

### 501 **Conflict-of-Interest**

502 The authors declare no conflicts of interest.

### 503 **Data availability**

504 All data are available, without restriction, upon request.

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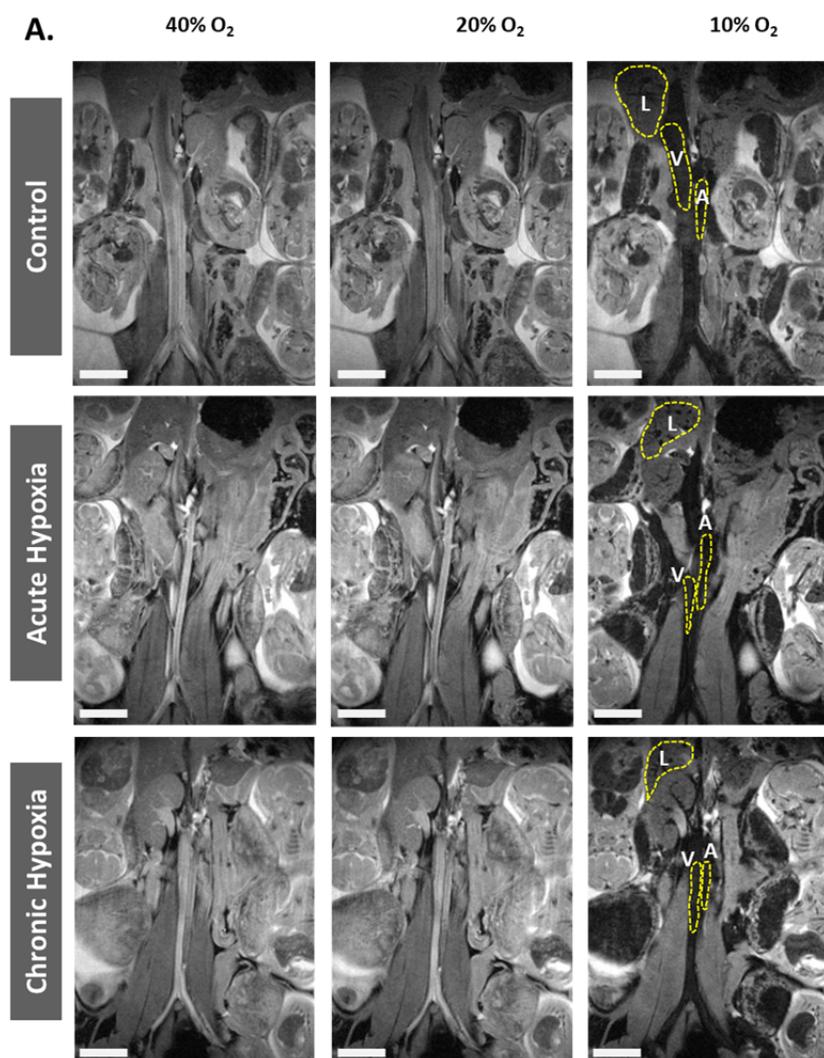
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625 **Supplementary**

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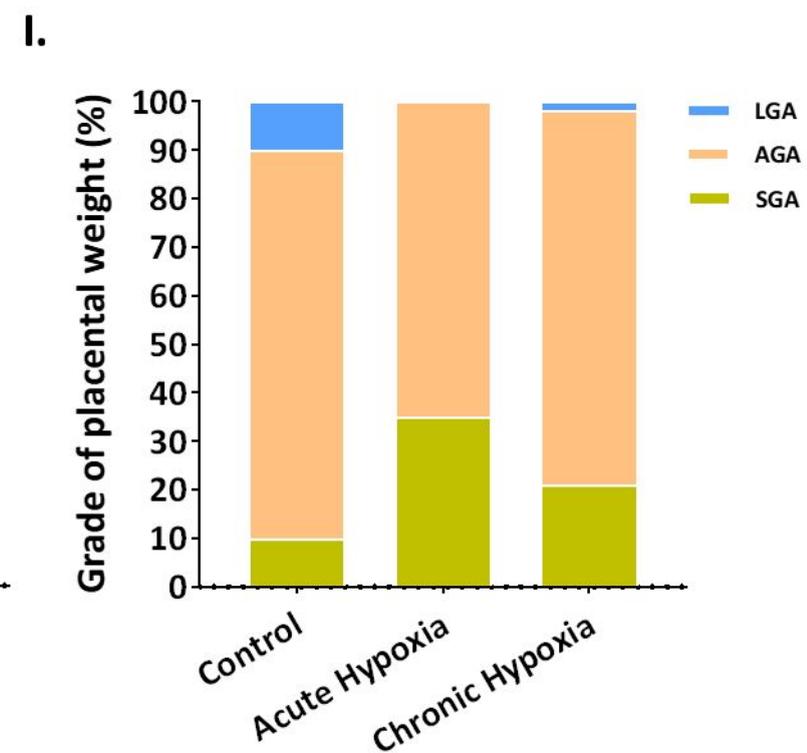
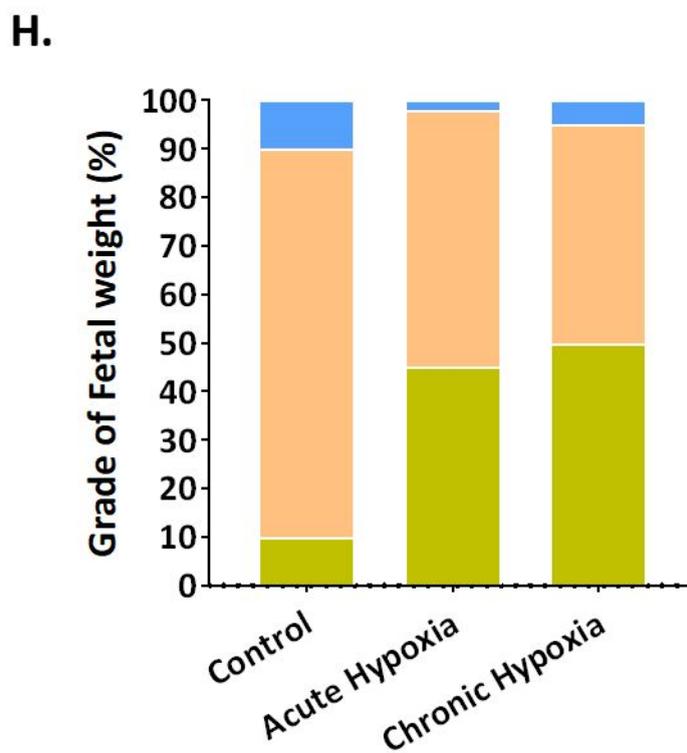
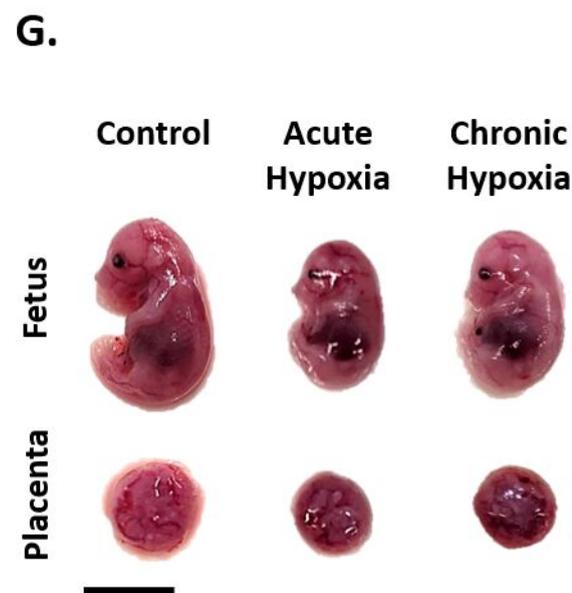
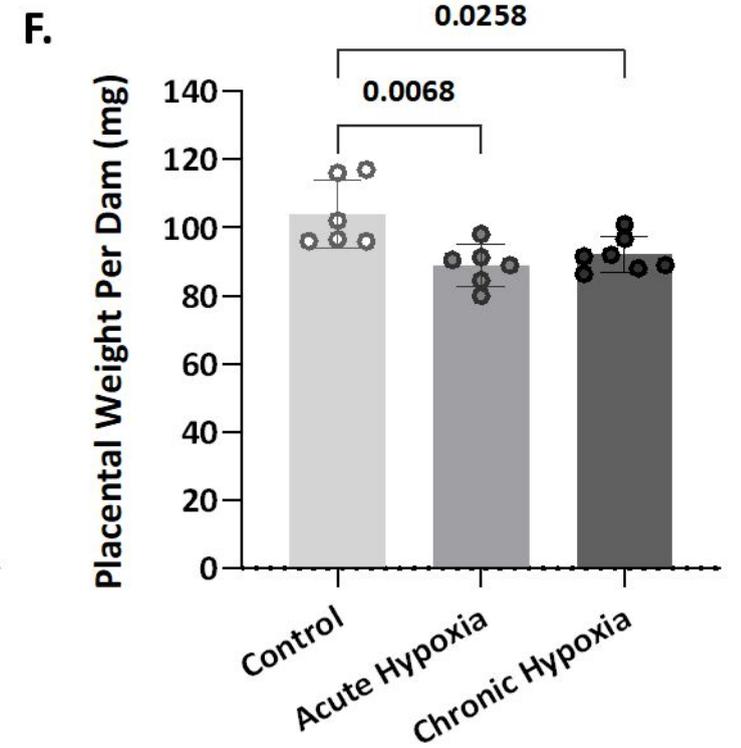
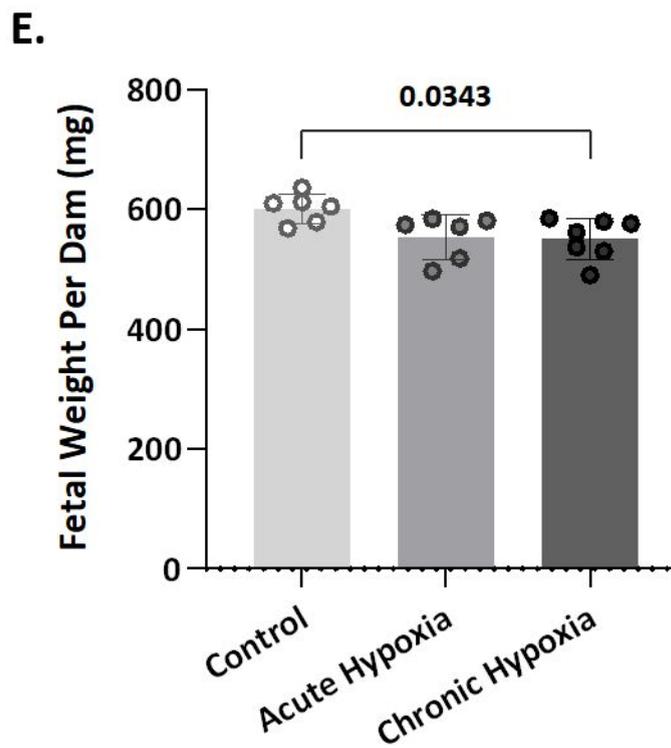
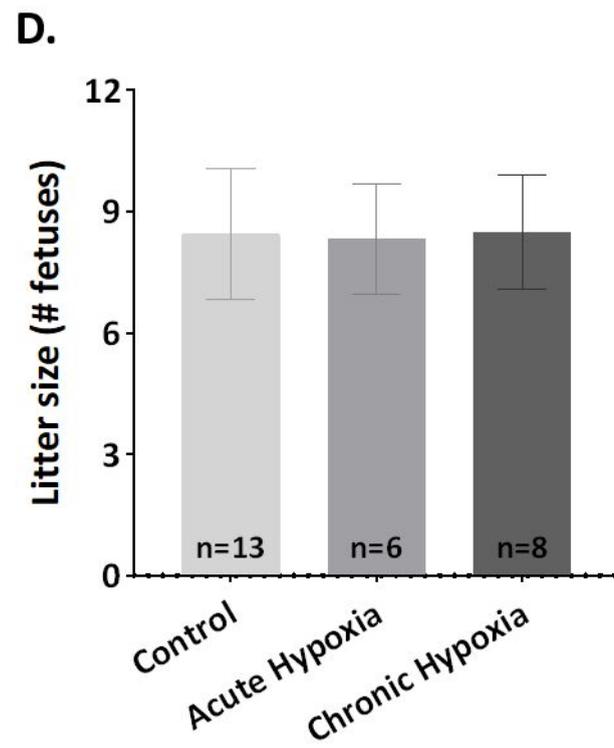
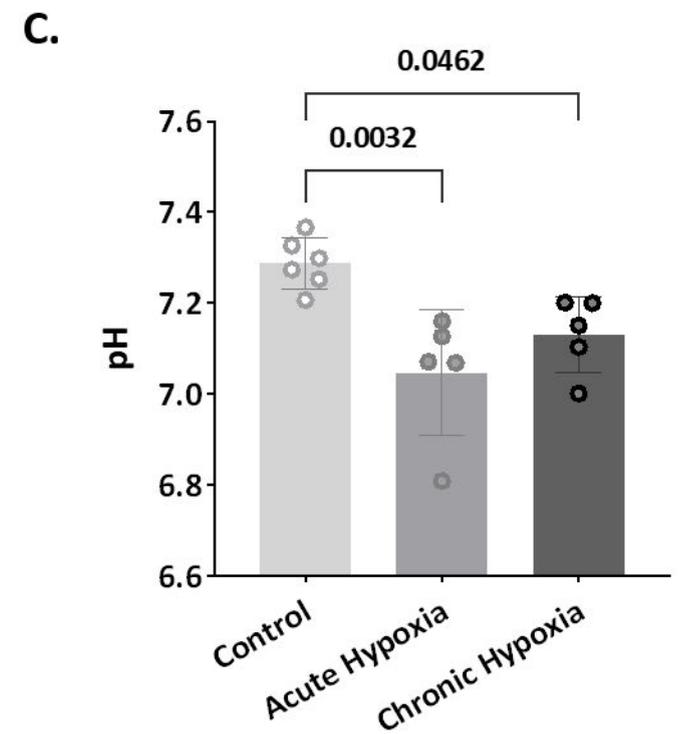
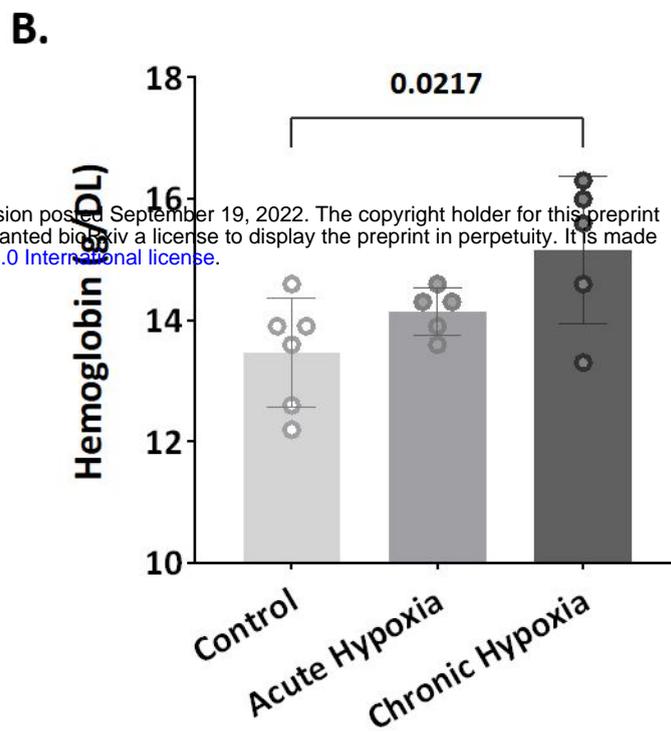
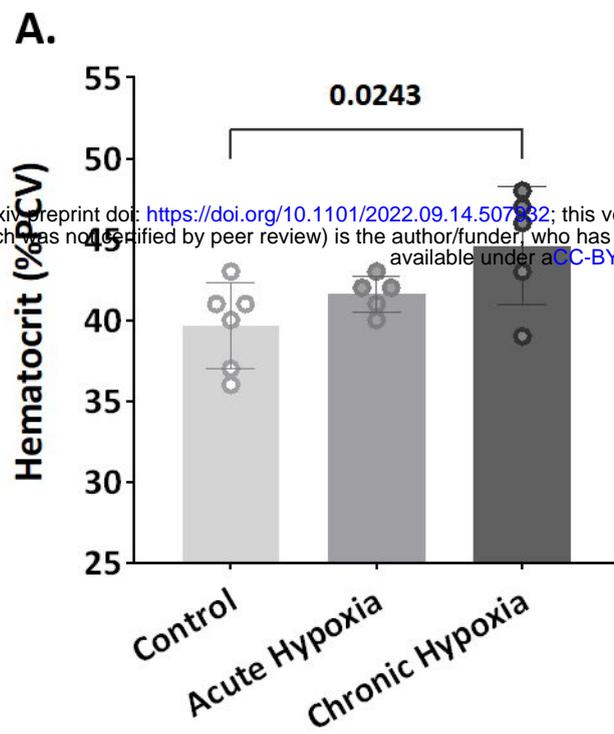
628 **Figure S1. Effects of maternal hypoxia during gestation on R2\* values following hyperoxia-**  
629 **hypoxia challenge. (A)** Representative R2\* images of control and hypoxic chamber group show  
630 several dams and their liver (L), aorta (A) and vena cava (V). Scale bars: 0.5 cm.

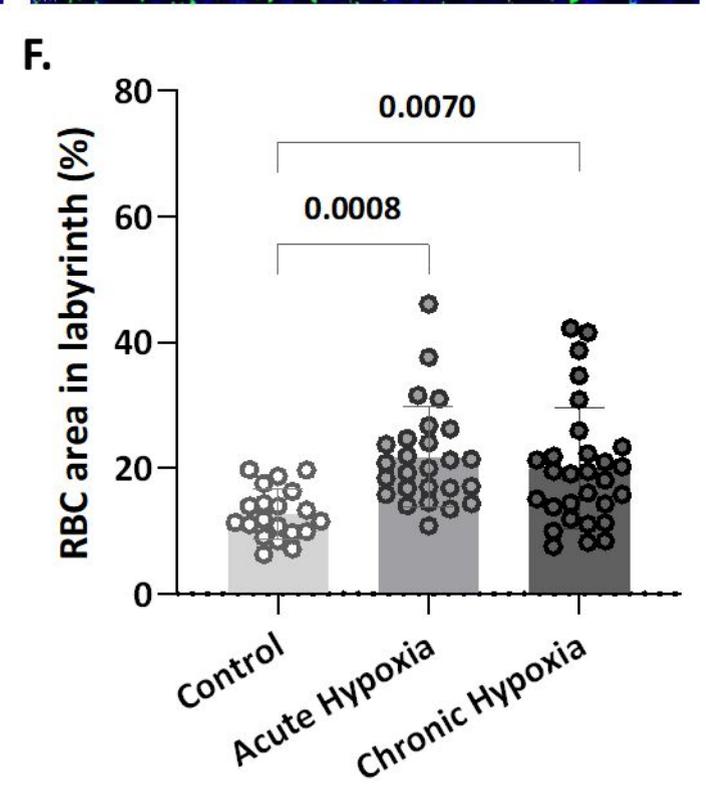
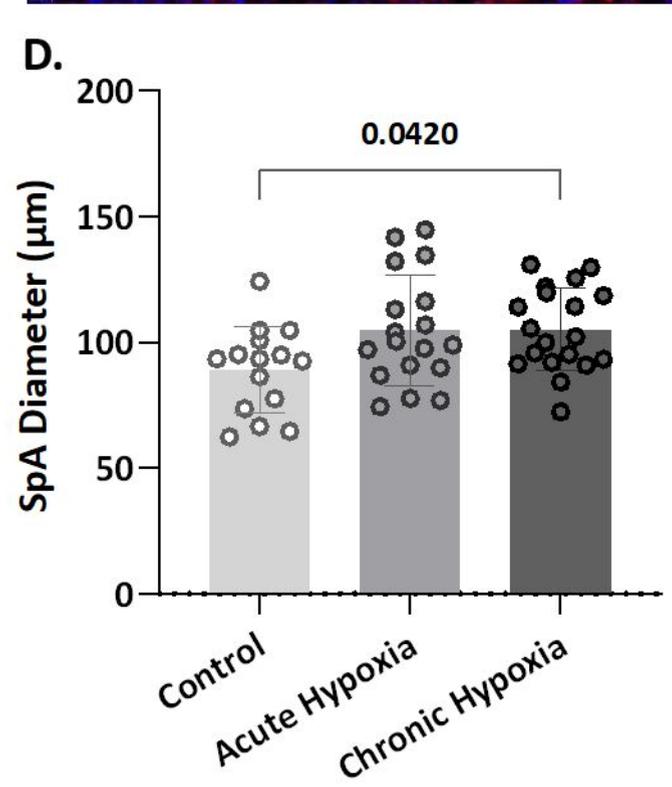
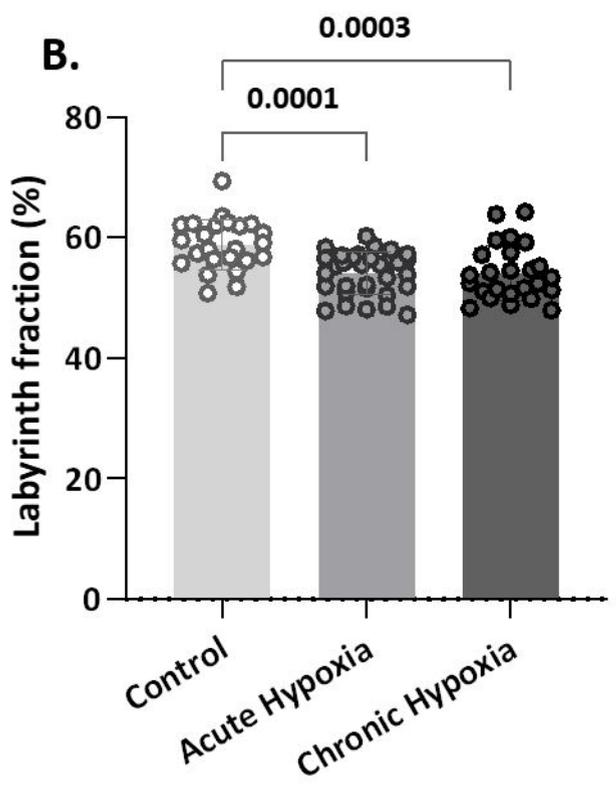
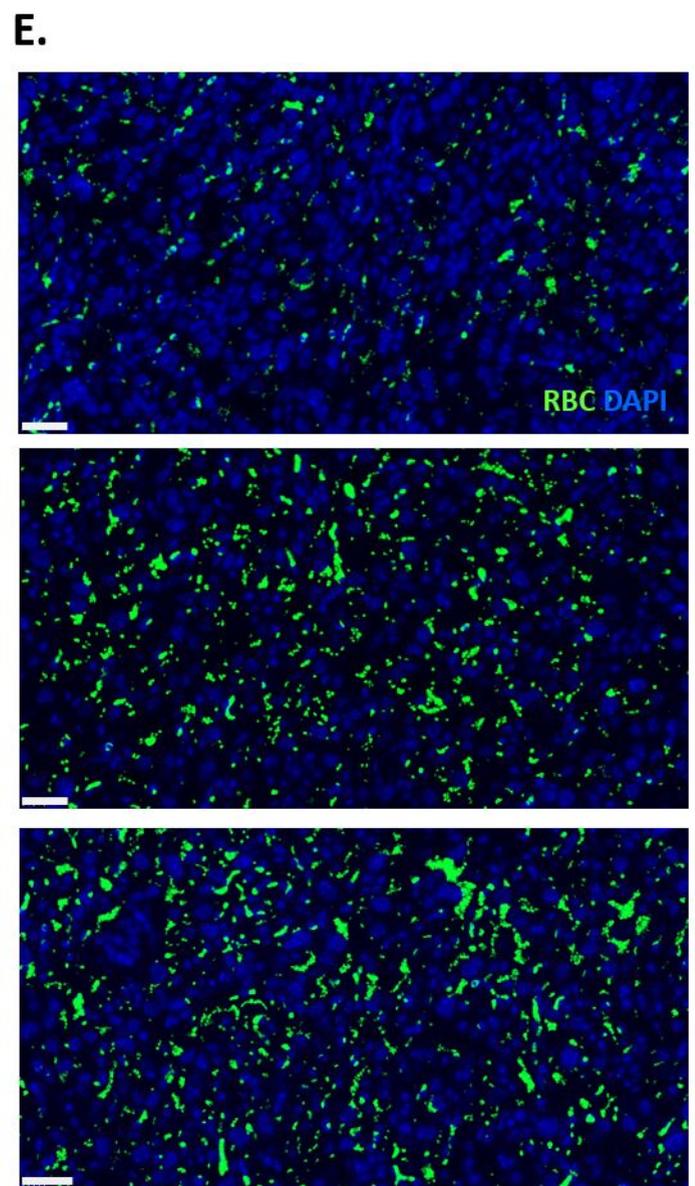
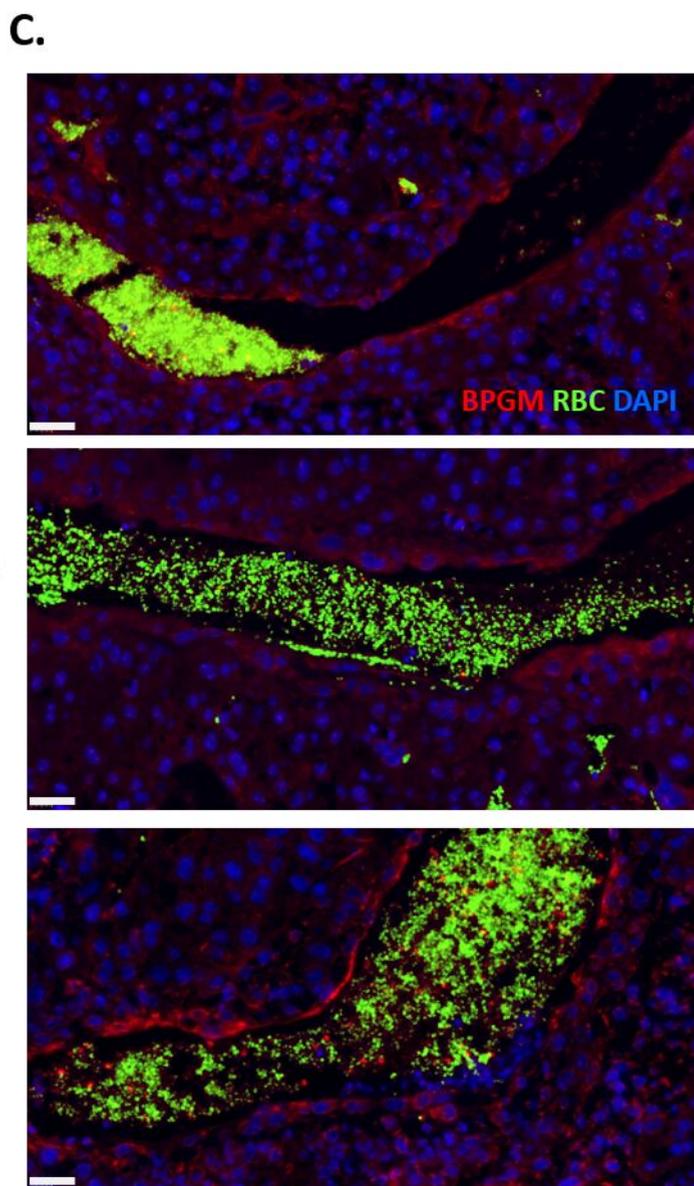
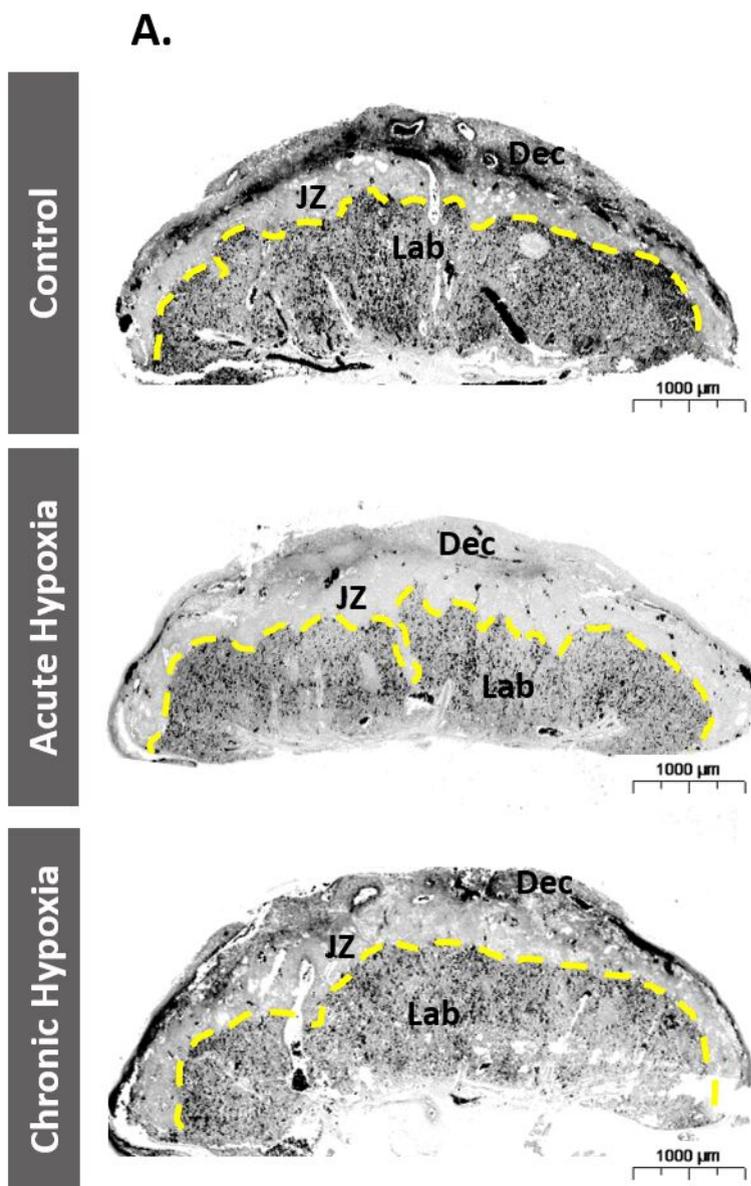
631

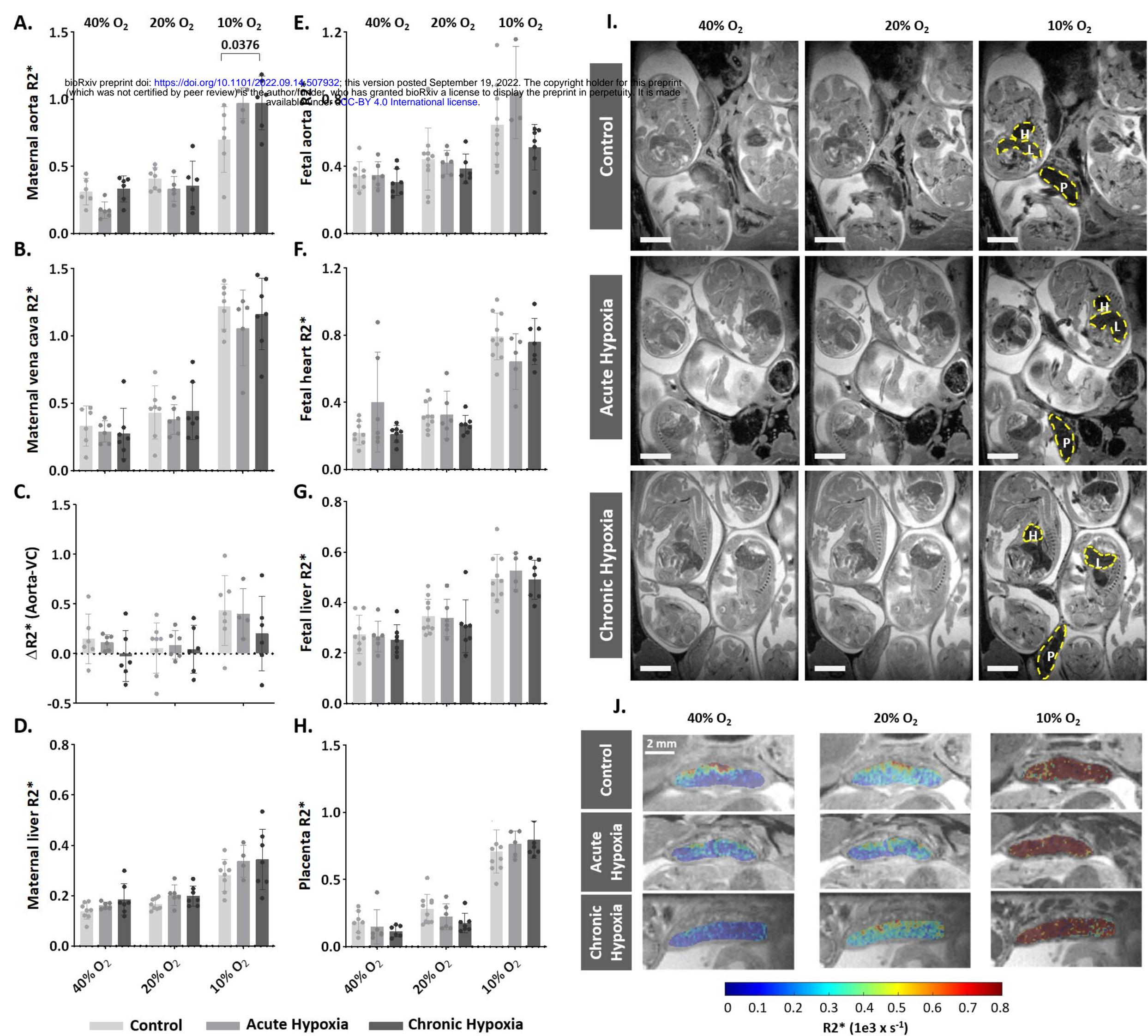
632 **MR imaging of mother, embryos and placentae**

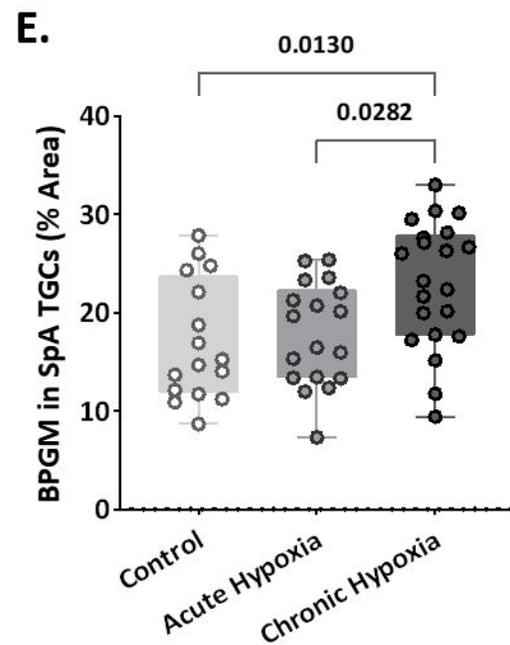
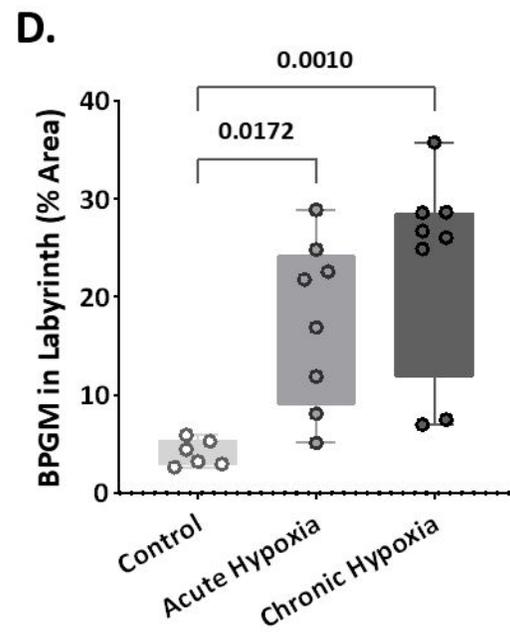
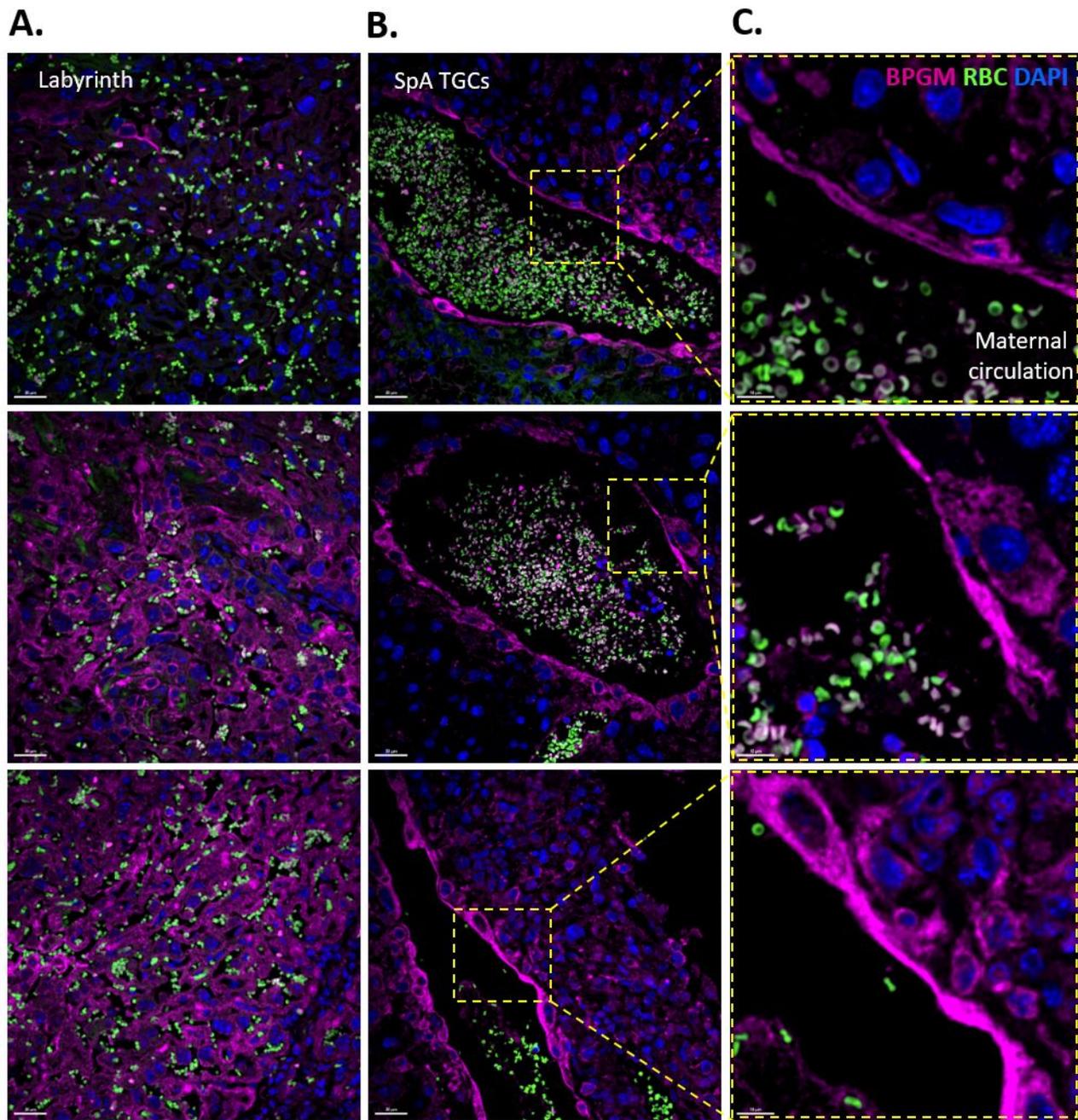
633 **Video 1, 2, 3:** Representative MRI scan videos of control, acute and chronic hypoxia dams  
634 respectively.

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All participants  
n=236

Controls  
n=222

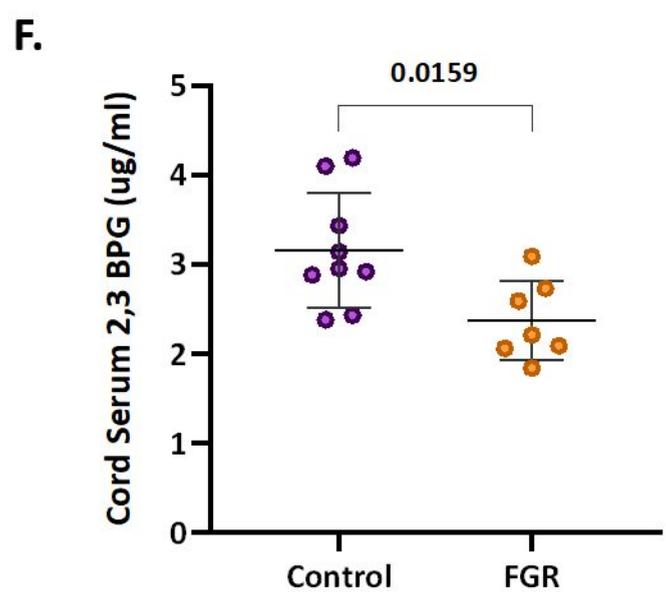
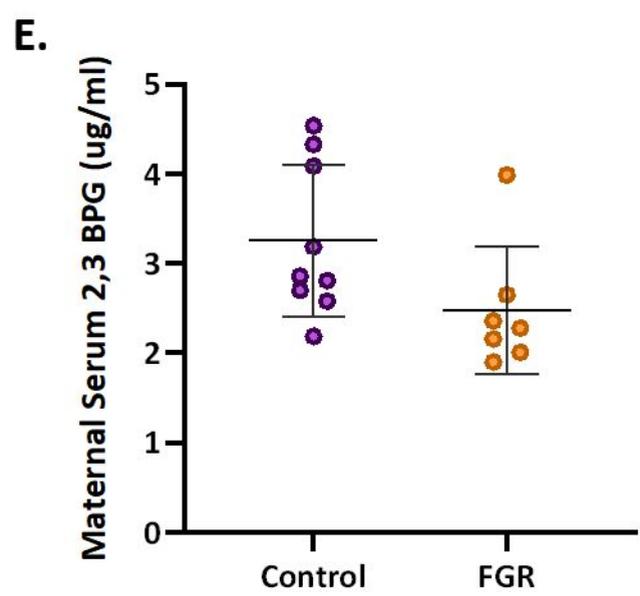
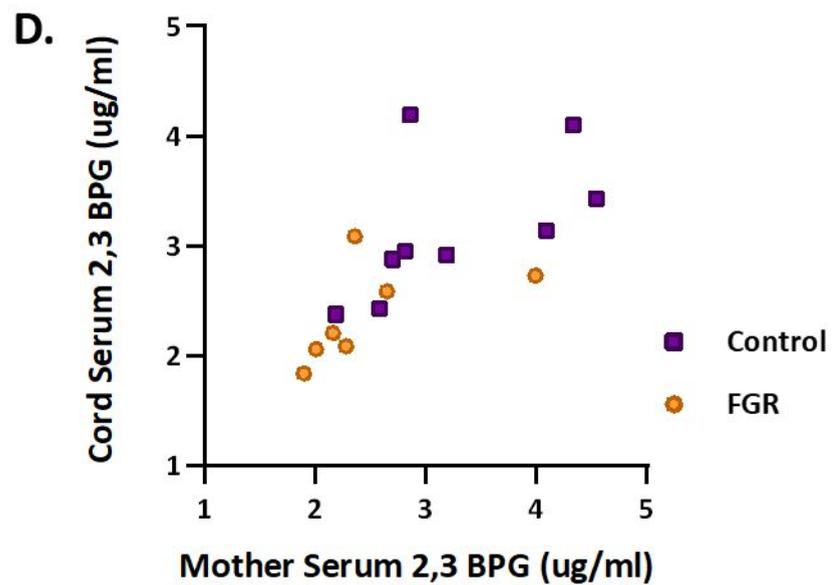
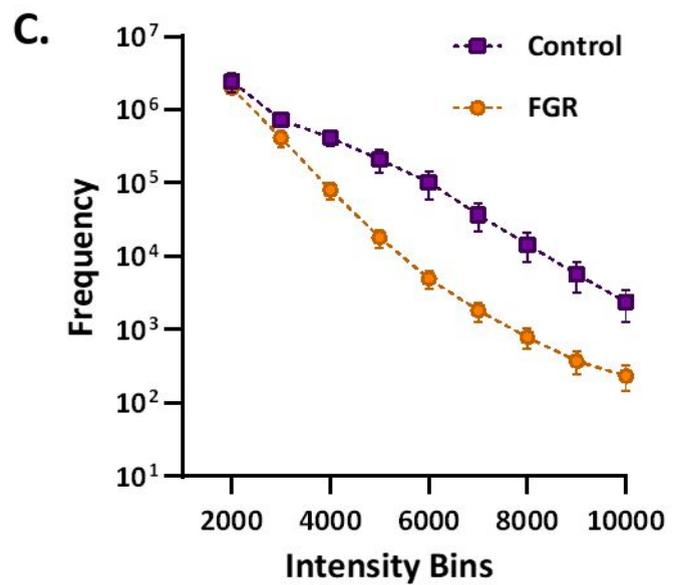
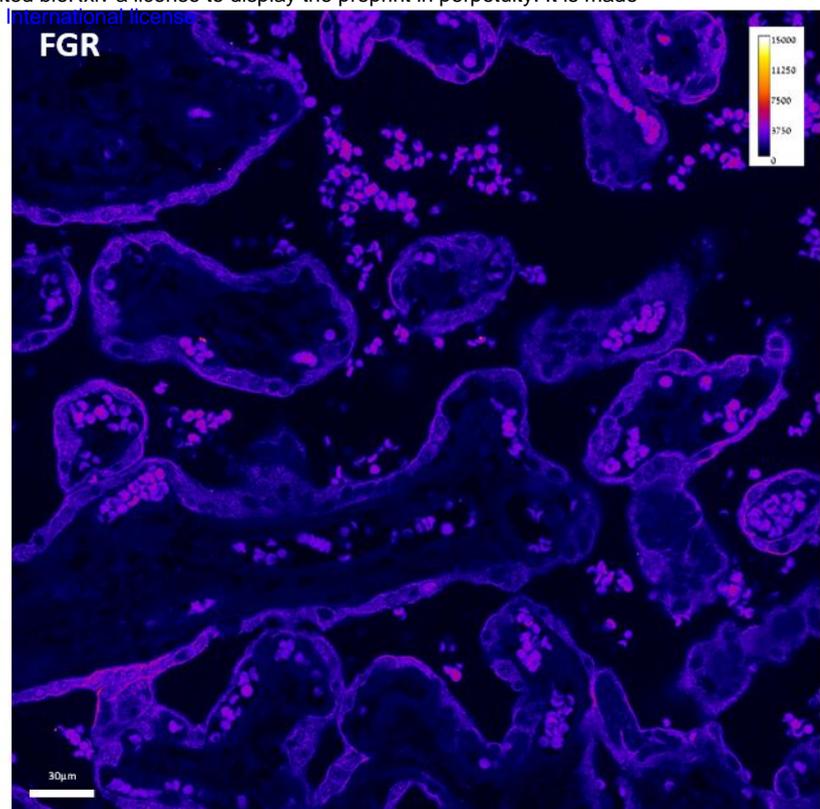
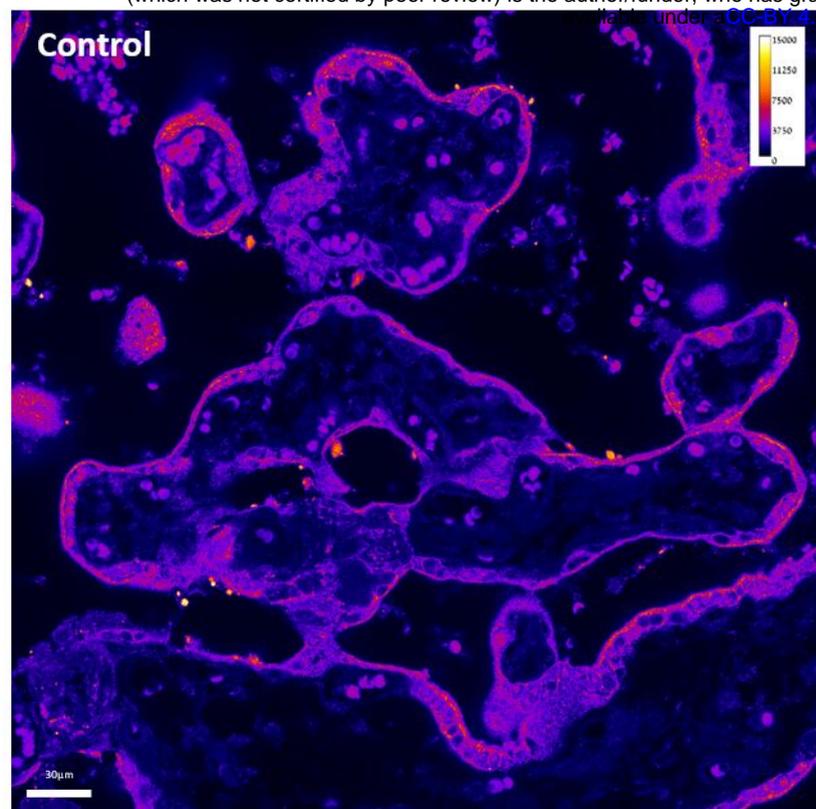
FGR  
n=14

Excluded:  
unmatched =213

Excluded:  
unmatched =7

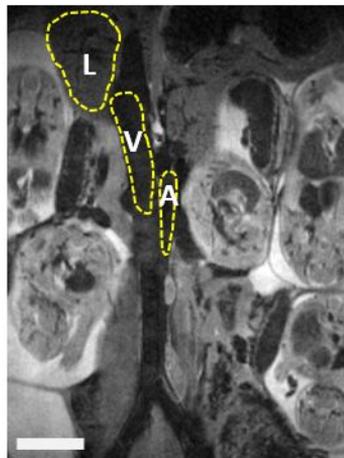
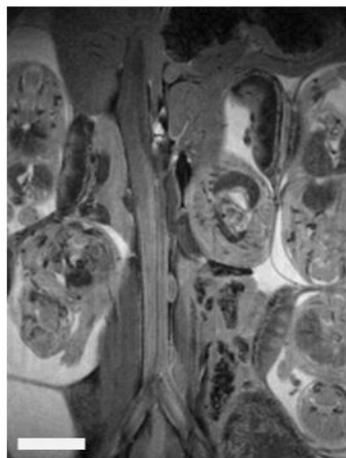
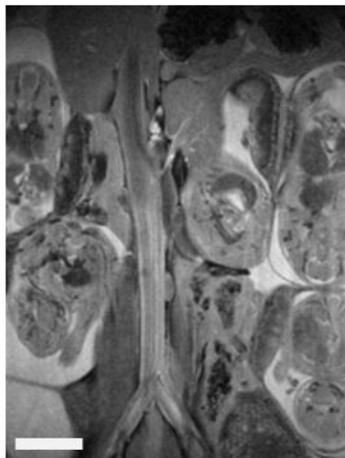
Matched  
controls  
n=9

Matched  
FGR  
n=7

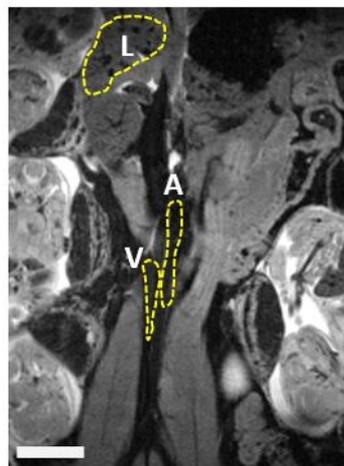
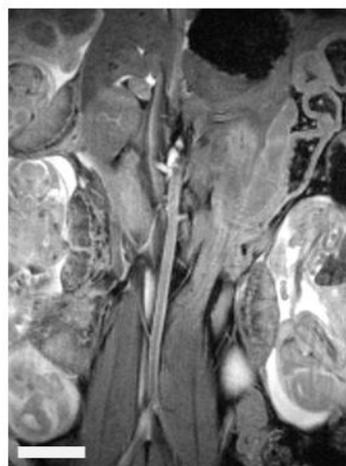
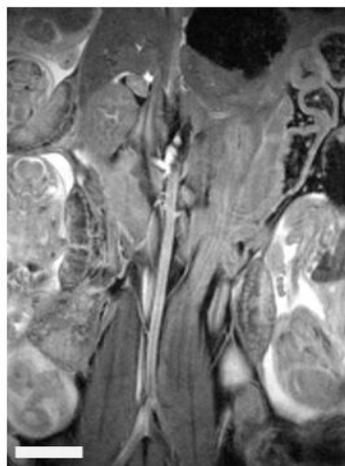


40% O<sub>2</sub>20% O<sub>2</sub>10% O<sub>2</sub>

Control



Acute Hypoxia



Chronic Hypoxia

