

## Induction of hyperandrogenism and insulin resistance differentially modulates ferroptosis in uterine and placental tissues of pregnant rats

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35 **Abstract**

36 Ferroptosis, a form of regulated necrotic cell death, plays roles in diverse physiological processes  
37 and diseases. Women with polycystic ovary syndrome (PCOS) have hyperandrogenism and insulin  
38 resistance (HAIR) and an increased risk of miscarriage and placental dysfunction during pregnancy.  
39 However, whether maternal HAIR alters mechanisms leading to ferroptosis in the gravid uterus and  
40 placenta remains unknown. Previous studies in rats showed that maternal exposure to 5α-  
41 dihydrotestosterone (DHT) and insulin (INS) from gestational day 7.5 to 13.5 induces HAIR and  
42 subsequently leads to placental insufficiency and fetal loss. We therefore hypothesized that maternal  
43 HAIR triggers ferroptosis in the uterus and placenta in association with fetal loss in pregnant rats.  
44 Compared with controls, we found that co-exposure to DHT and INS led to decreased levels of Gpx4  
45 and glutathione (GSH), increased GSH+glutathione disulfide (GSSG) and malondialdehyde (MDA),  
46 aberrant expression of ferroptosis-associated genes (*Acsl4*, *Tfrc*, *Slc7a11*, and *Gclc*), increased iron  
47 deposition, and activated ERK/p38/JNK phosphorylation in the gravid uterus. However, in the  
48 placenta, DHT and INS exposure only partially altered the expression of ferroptosis-related markers  
49 (e.g., region-dependent Gpx4, GSH+GSSG, MDA, *Gls2* and *Slc7a11* mRNAs, and phosphorylated  
50 p38 levels). In the uteri co-exposed to DHT and INS, we also observed shrunken mitochondria with  
51 electron-dense cristae, which are key features of ferroptosis-related mitochondrial morphology, as  
52 well as increased expression of *Dpp4*, a mitochondria-encoded gene responsible for ferroptosis  
53 induction. In contrast, in placentas co-exposed to DHT and INS we found decreased expression of  
54 *Dpp4* mRNA and increased expression of *Cisd1* mRNA (a mitochondria-encoded iron-export factor).  
55 Further, DHT+INS-exposed pregnant rats exhibited decreased apoptosis in the uterus and increased  
56 necroptosis in the placenta. Our findings suggest that maternal HAIR causes the activation of  
57 ferroptosis in the gravid uterus and placenta, although this is mediated via different mechanisms  
58 operating at the molecular and cellular levels. Furthermore, our data suggest other cell death  
59 pathways may play a role in coordinating or compensating for HAIR-induced ferroptosis when the  
60 gravid uterus and placenta are dysfunctional.

61 **Introduction**

62 Polycystic ovary syndrome (PCOS) is a complex and heterogeneous hormone-imbalance  
63 gynecological disorder that is influenced by genetic, environmental, and metabolic factors (1). This  
64 disorder affects approximately 4%–21% of all adolescent and reproductive-aged women and has a  
65 significant impact on their reproduction (2). Women with PCOS often suffer from hyperandrogenism  
66 (androgen excess) and insulin resistance (HAIR), and they are at high risk for miscarriage and  
67 obstetric complications during pregnancy (3, 4). Therapeutic interventions for different phenotypes  
68 and disease-related pregnancy complications in women with PCOS present a significant unmet  
69 medical need (5). Although it is thought that maternal, placental, and fetal defects all contribute to the  
70 onset and progression of miscarriage in PCOS patients, the pathogenesis of the pregnancy loss  
71 induced by HAIR and its precise regulatory mechanisms are still significant issues to be solved.

72 Ferroptosis is a recently described, iron-dependent form of regulated necrosis induced by oxidative  
73 stress, and it is distinct from other established forms of cell death such as apoptosis and necroptosis  
74 due to its unique morphological and biochemical features (6, 7). Growing evidence indicates that  
75 excessive or impaired ferroptosis plays a causative role in a variety of pathological conditions and  
76 diseases (8-11). It appears that the outcome of ferroptosis is programmed cell death, but which  
77 specific physiological processes or pathological conditions and disorders lead to ferroptosis activation  
78 remain poorly explored. The major molecular mechanisms and signaling pathways that are involved in  
79 the regulation of ferroptosis have been demonstrated in *in vivo* and *in vitro* studies (12). For example,  
80 suppression of glutathione (GSH) biosynthesis and subsequent inhibition or degradation of  
81 glutathione peroxidase 4 (Gpx4) activity, disturbed balance of iron homeostasis, and activation of the  
82 mitogen-activated protein kinase (MAPK) signaling pathways all contribute to regulate the initiation  
83 and execution of ferroptosis (6, 7, 10, 11). However, little is known about the role of ferroptosis (13) in  
84 comparison with other forms of programmed cell death such as apoptosis (14, 15) in female  
85 reproduction.

86 Given the significant association of PCOS with miscarriage during pregnancy (16, 17), we have  
87 recently demonstrated that the effects of HAIR in causing fetal loss is the consequence of uterine and  
88 placental defects (18, 19). To this end, we exposed pregnant rats to 5 $\alpha$ -dihydrotestosterone (DHT)  
89 and insulin (INS) from gestational day (GD) 7.5 to 13.5 and found that this triggered many features of  
90 pregnant PCOS patients (including HAIR), as well as increased fetal loss. Moreover, the fetal loss

91 was related to disrupted reactive oxygen species (ROS) production in the uterus and placenta of rat  
92 dams with induced HAIR. In particular, our previous animal experiments have shown that maternal  
93 HAIR-induced fetal loss is also associated with the inactivation of antioxidative proteins in the gravid  
94 uterus and placenta, namely nuclear factor erythroid 2-related factor 2 (Nrf2) and superoxide  
95 dismutase 1 (18, 19), which play an inhibitory role in the ferroptosis pathway (6, 10). Moreover, the  
96 expression of several other negative regulators of ferroptosis such as *Ho1* (heme oxygenase 1) (6)  
97 and *Mt1g* (metallothionein 1G) (20) are downregulated in the gravid uterus after combined maternal  
98 exposure to DHT and INS (18). Increased circulating ROS levels have been observed in both non-  
99 pregnant and pregnant rodents in which PCOS features have been induced (19, 21). Elevated ROS  
100 production and decreased anti-oxidative capacity has been observed in the ovarian granulosa cells  
101 and leukocytes of PCOS patients (21-23), and oxidative stress is proposed to contribute to  
102 miscarriage and infertility in women with PCOS (24, 25). It is therefore likely that the promotion of  
103 pathologic oxidative stress and activation of ferroptosis in the gravid uterus and placenta contribute to  
104 HAIR-induced fetal loss in both animal models and humans.

105 Mitochondria play a protective role in the regulation of exhausted GSH-induced ferroptosis (26,  
106 27). In women with PCOS and miscarriage (24, 28), as well as in pregnant PCOS-like rodents with  
107 fetal loss (18, 19, 29, 30), there is mounting evidence for mitochondrial abnormalities and oxidative  
108 damage. For instance, decreased mitochondrial DNA copy number is associated with the  
109 developmental clinical phenotype and severity of PCOS, and several mitochondria-tRNA mutations  
110 are seen in PCOS patients. In addition, aberrant expression of mitochondrial biogenesis genes,  
111 oxidative phosphorylation and anti-oxidative proteins are found in PCOS patients who suffer from  
112 recurrent miscarriage, as well as in PCOS-like rodents. On the basis of these preclinical and clinical  
113 studies, we hypothesized that maternal HAIR triggers impairments in Gpx4/GSH-regulated lipid  
114 peroxidation and iron-associated and mitochondria-mediated ferroptosis in the gravid uterus and  
115 placenta resulting in increased fetal loss during pregnancy.

116 The aim of this study was to determine whether exposure to DHT and INS in pregnant rats (which  
117 induces HAIR/PCOS (18, 19)) leads to activation of the ferroptosis cascade and malondialdehyde  
118 (MDA, a marker of oxidative stress), iron accumulation, and perturbed mitochondrial function in the  
119 uterus and placenta. Further, we conducted a parallel analysis of the expression of genes and  
120 proteins that are involved in necroptosis and apoptosis, two other programmed cell death pathways

121 that might contribute to defects in the gravid uterus and the placenta. This study is the first to report  
122 an association between HAIR and different forms of regulated cell death in the gravid uterus and  
123 placenta *in vivo*. Our findings indicate that ferroptosis is one of the potential mechanisms by which  
124 maternal HAIR leads to uterine and placental dysfunction and at least partially explains the resultant  
125 fetal loss observed.

## 126 **Materials and Methods**

### 127 ***Ethics approval***

128 All experiments were conducted in compliance with all relevant local ethical regulations. Animal  
129 experiments were approved and authorized by the Animal Care and Use Committee of the  
130 Heilongjiang University of Chinese Medicine, China (HUCM 2015-0112), and followed the National  
131 Institutes of Health guidelines on the care and use of laboratory animals. The human study protocol  
132 conformed to the principles outlined in the Declaration of Helsinki under approval from the institutional  
133 Ethical Review Committee of the Obstetrics and Gynecology Hospital of Fudan University, Shanghai,  
134 China (OGHFU 2013-23), and informed consent was obtained from each patient in written form.

### 135 ***Animals, experimental setting, and tissue collection***

136 Adult Sprague–Dawley female and male rats were obtained from the Laboratory Animal Centre of  
137 Harbin Medical University, Harbin, China. All animals were health checked daily throughout the  
138 experiment and were maintained in an environmentally controlled and pathogen-free barrier facility on  
139 a standard 12 h light/dark cycle at  $22 \pm 2^\circ\text{C}$  and 55–65% humidity and with free access to normal  
140 diet and water. Before the experiment, female rats were allowed to acclimatize for a minimum of 7  
141 days and then were monitored daily by vaginal lavage to determine the stage of the estrous cycle (31,  
142 32). Pregnancy was achieved by housing female rats on the night of proestrus with fertile males of the  
143 same strain at a 2:1 ratio. Confirmation of mating was defined by the presence of a vaginal plug, and  
144 this was considered as GD 0.5. The rats were sacrificed between 0800 and 0900 hours on GD 14.5.  
145 All animal procedures in this study were performed as described in our previous publications (18, 19).

146 To induce HAIR, pregnant rats were randomly assigned to be intraperitoneally injected with DHT  
147 (1.66 mg/kg/day, suspended in sesame oil, Sigma-Aldrich, St. Louis, MO, USA) and/or human  
148 recombinant INS (6.0 IU/day, diluted in sterile saline, Eli Lilly Pharmaceuticals, Giza, Egypt) or an  
149 equal volume of saline and sesame oil as controls on GD 7.5 as previously described (18, 19). This  
150 therefore generated the following four study groups: Control, DHT+INS, DHT, and INS. All animals

151 were treated for 7 consecutive days. The dose of DHT used in our rats was chosen to mimic the  
152 hyperandrogenic state in PCOS patients who have approximately 1.7-fold higher circulating DHT  
153 concentrations compared to healthy controls (33, 34). The dose of INS was chosen because it  
154 induces metabolic disturbances including peripheral and uterine insulin resistance in rats (32, 35).  
155 The body weight, oral glucose tolerance test and circulating levels of androgens (testosterone,  
156 dehydroepiandrosterone, and DHT) were measured to confirm that HAIR was induced by exposure to  
157 DHT and INS, as reported previously (18, 19). Pregnant rats co-exposed to DHT and INS had  
158 metabolic and endocrine aberrations at GD 14.5, thus replicating the changes observed in pregnant  
159 PCOS patients (36, 37). On GD 14.5, tissues, including the maternal uterus and placenta, as well as  
160 fetuses were dissected. These were then either fixed for morphological and immunohistochemical  
161 analyses or immediately frozen in liquid nitrogen and stored at -70°C for quantitative real-time  
162 polymerase chain reaction (qPCR) (38) and Western blot analyses. Only viable conceptuses (fetuses  
163 and placentas) were analyzed further.

164 ***Gene expression analysis by qPCR***

165 The isolation and quantification of the RNA and the qPCR assay were performed as previously  
166 described (32, 39). The PCR amplifications were performed with SYBR green qPCR master mix  
167 (#K0252, Thermo Scientific, Rockford, IL). Total RNA was prepared from the frozen whole uterine and  
168 placental tissues, and single-stranded cDNA was synthesized from each sample (2 µg) with M-MLV  
169 reverse transcriptase (#0000113467, Promega Corporation, Fitchburg, WI) and RNase inhibitor (40  
170 U) (#00314959, Thermo Scientific). Uterine and placental RNA purities (A260/A280 ratios) were  
171 evaluated with a NanoDrop 1000 spectrometer (Thermo Fisher Scientific). Only samples presenting a  
172 ratio greater than 1.8 were kept for further analyses. The integrity of the extracted RNA samples was  
173 additionally determined by using an Experion RNA StdSens Analysis Kit (Bio-Rad). Any samples  
174 showing poor RNA quality were also excluded from further analysis. cDNA (1 µl) was added to a  
175 reaction master mix (10 µl) containing 2× SYBR green qPCR reaction mix (Thermo Scientific) and  
176 gene-specific primers (5 µM of forward and reverse primers). All reactions were performed at least  
177 twice, and each reaction included a non-template control, and specific sample sizes are denoted in  
178 the figure legends. Fold changes in mRNA expression were calculated by the  $\Delta\Delta CT$  method using  
179 *Gapdh* (18) as the reference gene, which was stably expressed between the groups (38). Results are  
180 expressed as fold changes after normalizing to the control group. The qPCR primers used in this

181 study are listed in **Table 1**. All sets of primers were validated for qPCR prior to analysis. This involved  
182 determining that the efficiency of amplification using a standard curve of cDNA was above 85% and  
183 not different from the *Gapdh* reference gene, and there were no non-specific PCR products seen in a  
184 melt curve analysis immediately after the amplification or in parallel reactions with un-transcribed RNA  
185 or in reactions without templates (the negative controls). Further, in order to avoid introducing  
186 variability, all uterine and placental samples for a given target gene were analysed on a single plate.

187 ***Protein isolation and Western blot analysis***

188 A detailed explanation of the tissue lysate preparation and the Western blot analysis protocol has  
189 been published (32, 39). Tissue proteins were isolated by homogenization in RIPA buffer (Sigma-  
190 Aldrich) supplemented with cOmplete Mini protease inhibitor cocktail tablets (Roche Diagnostics,  
191 Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostics).  
192 After determining the total protein concentration by Bradford protein assay (Thermo Fisher Scientific),  
193 equal amounts (30 µg) of protein were resolved on 4–20% TGX stain-free gels (Bio-Rad Laboratories  
194 GmbH, Munich, Germany) and transferred onto PVDF membranes. The membranes were probed  
195 with anti-Gpx4 antibody (ab125066, Abcam, Cambridge, UK), anti-ERK1/2 antibody (#4695, Cell  
196 Signaling Technology, Danver, MA, USA), anti-phospho-ERK1/2 antibody (#9911, Cell Signaling  
197 Technology), anti-p38 MAPK antibody (#8690, Cell Signaling Technology), anti-phospho-p38 MAPK  
198 antibody (#4511, Cell Signaling Technology), anti-JNK antibody (#9252, Cell Signaling Technology),  
199 anti-phospho-JNK antibody (#4668, Cell Signaling Technology), and anti-cleaved caspase-3 antibody  
200 (#9664, Cell Signaling Technology) all diluted 1:1,000 in 0.01 M Tris-buffered saline supplemented  
201 with Triton X-100 (TBST) containing 5% w/v non-fat dry milk followed by anti-rabbit IgG HRP-  
202 conjugated goat secondary antibody (A0545, Sigma-Aldrich). Signal was detected using the  
203 SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and captured using a  
204 ChemiDoc MP Imaging System (Bio-Rad). Initial experiments were performed to verify the  
205 identification of cytosolic, mitochondrial and nuclear Gpx4 by Western blot analysis using rat testis  
206 (which has high expression compared to other tissues (40, 41)), epididymis and ovary (**Supplemental**  
207 **Fig. 1**). For each Western blot, ultraviolet activation of the Criterion stain-free gel was used to assess  
208 total protein loading for each sample (32). Band densitometry was performed using Image Laboratory  
209 (Version 5.0, Bio-Rad) and the intensity of each protein band was normalized to the total protein in the  
210 individual sample. Due to the number of samples per group, multiple gels were run per group, each

211 containing three replicates per group. For quantification and to ensure standardisation across blots,  
212 the expression of the target protein was normalised to the mean value for the control group on the blot  
213 and then all the normalised values were statistically compared to assess the effect of the treatment  
214 groups. This ensured that we could accurately compare protein abundance across groups with the  
215 one tissue.

216 ***Histological processing and Gpx4 immunohistochemistry***

217 Histological processing and immunohistochemistry were performed according to previously  
218 described methods (32, 42). Fresh tissues were dissected and immediately fixed in 4% formaldehyde  
219 in neutral buffered solution at 4°C for 24 h and then embedded in paraffin. Sections (5 µm) were  
220 deparaffinized and rehydrated in xylene and graded series of ethanol (99.99%, 80%, and 70% in  
221 distilled water, Sigma-Aldrich) for 10 min each. After incubation with the Gpx4 antibody (1:200  
222 dilution, Abcam) overnight at 4°C in a humidified chamber, the sections were stained using the avidin-  
223 biotinylated-peroxidase ABC kit followed by a 5-min treatment with 3,3'-diaminobenzidine (DAB, SK-  
224 4100, Vector Laboratories). Stained sections were observed and imaged on a Nikon E-1000  
225 microscope (Japan) under bright-field optics and photomicrographed using Easy Image 1 (Bergström  
226 Instrument AB, Sweden). A negative control was performed by using the same concentration of  
227 isotype-matched rabbit IgG instead of the primary antibody. Only minimal cytoplasmic background  
228 staining was observed (**Supplemental Fig. 2 right**).

229 ***Perls' histochemical reaction***

230 Iron deposition was detected using DAB-enhanced Perls' staining as previously described (43).  
231 After deparaffinization and rehydration, sections were immersed in a mixture of equal volumes of  
232 potassium ferrocyanide solution (HT201, Sigma-Aldrich) and hydrochloric acid solution (HT201,  
233 Sigma-Aldrich) for 1 h at room temperature. Sections were washed with PBS five times for 5 min each  
234 and incubated with DAB for 10 min and pararosaniline solution (HT203, Sigma-Aldrich) for 2 min.  
235 Images of excess iron deposits were captured on a Nikon E-1000 microscope (Japan) under bright-  
236 field optics and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden).

237 ***Mitochondria structure by transmission electron microscopy (TEM)***

238 TEM was performed according to a published method (30). Fresh uterine and placental tissues  
239 were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2–7.4) for 1 h at room  
240 temperature (RT) and further rinsed with 0.1 M PBS three times for 15 minutes each. Secondary

241 fixation with 1% osmium tetroxide in PBS was performed for 1 h prior to sequential dehydration with  
242 an acetone gradient (50%, 70%, and 90% for 15 min each and 100% three times for 30 min each time  
243 at RT). Samples were finally embedded in Epon epoxy resin. Random areas from uterine and  
244 placental tissues were oriented for ultrastructural analysis. The blocks were cut in 50–60 nm sections  
245 using a Reichert ultramicrotome (Leica, Germany), collected on 300 mesh copper grids, and stained  
246 with 3% uranyl acetate and counterstained with lead citrate before visualization. The post-stained  
247 sections were examined and imaged with a transmission electron microscope (H-7650, Hitachi,  
248 Japan) equipped with an electron imaging spectrometer. Image collection and parameter settings  
249 were identical for each of the different tissues/regions analyzed.

250 ***Quantification of GSH, MDA, and mitochondrial open reading frame of the 12S rRNA-c (MOTS-  
251 c)***

252 The intracellular GSH, MDA, and MOTS-c levels were assessed using a GSH/GSH+glutathione  
253 disulfide (GSSG) assay kit (ab239709, Abcam, Cambridge, UK), MDA assay kit (ab118970, Abcam),  
254 and MOTS-c ELISA kit (CEX132Ra, Cloud-Clone/USCNK, Oxfordshire, UK), respectively, according  
255 to the manufacturers' protocols. A standard curve for GSH, MDA, and MOTS-c concentration was  
256 generated and used for calculating their concentration in the samples. The concentration of GSH,  
257 GSH+GSSG, MDA, and MOTS-c in each group was normalized to the total tissue protein  
258 concentration as determined by the Bradford protein assay (Thermo Fisher Scientific).

259 ***Data processing, statistical analysis, and graphical representations***

260 No statistical methods were used to predetermine the sample size. Data are presented as the  
261 means  $\pm$  SEM, and the sample size (n) is listed in the figure legends and indicates the number of  
262 animals in each experiment. Statistical analyses were performed using SPSS version 24.0 for  
263 Windows (SPSS Inc., Chicago, IL). The normal distribution of the data was tested with the Shapiro–  
264 Wilk test. Differences between groups were analyzed by one-way ANOVA followed by Tukey's post-  
265 hoc test for normally distributed data or the Kruskal–Wallis test for skewed data (**Supplemental Table  
266 1**). All p-values less than 0.05 were considered statistically significant.

267 **Results**

268 Because we were most interested in how HAIR induces changes in ferroptosis as opposed to  
269 apoptosis and necroptosis in gravid uterine and placental tissues, we mainly describe the  
270 observations in DHT+INS-exposed pregnant rats vs. control pregnant rats.

271 **Differential regulation of Gpx4 and GSH protein expression in the gravid uterus and placenta**  
272 **exposed to DHT and INS**

273 Gpx4 is present in the cytoplasm, mitochondria and nucleus in mammalian cells (44) and in our  
274 Western blot analysis, the ~20-kDa band represents the cytosolic and mitochondrial Gpx4 protein,  
275 whereas the ~34-kDa band represents the nuclear Gpx4 protein (**Supplemental Fig. 1**). We initially  
276 performed Western blot and immunohistochemical analyses to characterize the tissue and  
277 intracellular localization of Gpx4 protein in rat and human uterine and placental tissues. The Western  
278 blot analysis revealed a predominant band corresponding to cytosolic and mitochondrial Gpx4 (under  
279 denaturing and reducing conditions) in the diestrus uterus of rats and in the non-pregnant secretory  
280 phase and early pregnant decidualized endometria of humans (**Supplemental Fig. 3A**). Our further  
281 immunohistochemical studies showed that while positive immunostaining for cytosolic Gpx4 was  
282 mainly observed in luminal and glandular epithelial cells, Gpx4 immunostaining was additionally  
283 localized to the nucleus of non-decidualized and decidualized stromal cells and myometrial smooth  
284 muscle cells in rats and humans (**Supplemental Fig. 3B-D**). This is consistent with variations in the  
285 cellular compartmentalization of Gpx4 in the cytosol and nucleus between different cell types (44). In  
286 control pregnant rats, immunohistochemical analysis showed that Gpx4 was localized to both the  
287 cytosol and nucleus of different cell types in the gravid uterine decidua, myometrium and placenta  
288 (**Fig. 1C1-C4** and **Supplemental Fig. 2B**). Although the significance of mitochondrial and nuclear  
289 Gpx4 remains to be determined (45), cytosolic Gpx4 has been identified as a central regulator of  
290 ferroptosis (11). We thus evaluated Gpx4 expression and localization in the gravid uterus and  
291 placenta exposed to DHT and INS. The Western blot analysis revealed a significant decrease in  
292 uterine Gpx4 protein abundance in DHT+INS-exposed pregnant rats (**Fig. 1A**). Consistent with this,  
293 we found weaker immunoreactivity of Gpx4 in the cytosolic compartments of decidualized stromal  
294 cells and smooth muscle cells in the gravid uterus of DHT+INS-exposed pregnant rats (**Fig. 1D1-D2**).  
295 Although there was no significant reduction in uterine Gpx4 protein abundance by Western blot  
296 analysis in the pregnant rats treated alone with DHT or INS (**Fig. 1A**), fewer cells of the gravid uterus  
297 showed nuclear immunostaining of Gpx4 when compared to controls (**Fig. 1B**). Although Gpx4  
298 protein abundance by Western blot analysis was not significantly reduced in the placenta of  
299 DHT+INS-exposed pregnant rats (**Fig. 1A**), immunohistochemical analysis revealed that the level of  
300 Gpx4 immunostaining was lower in trophoblast populations of the placenta in DHT+INS-exposed

301 pregnant rats compared to the control pregnant rats (**Fig. 1D3-D4**). In particular, Gpx4  
302 immunostaining was no longer localized to the nuclei of spongiotrophoblasts and glycogen cells of  
303 DHT+INS-exposed pregnant rats (**Fig. 1E3-E4**). There was no alteration of placental Gpx4 protein  
304 abundance in pregnant rats treated with DHT alone or INS alone (**Fig. 1A**). However, similar to what  
305 was seen in DHT+INS-exposed pregnant rats, Gpx4 was no longer localized to the nuclei of  
306 spongiotrophoblasts and glycogen cells in pregnant rats treated with DHT alone (**Fig. 1E3**) but not  
307 INS alone (**Fig. 1F3**).

308 Because Gpx4 uses GSH as a substrate in its peroxidase reaction cycle (46) and because GSH  
309 depletion is one of the key triggers for ferroptosis (10, 11), we measured the levels of GSH and  
310 GSH+GSSG in the gravid uterus and placenta exposed to DHT and INS. As shown in **Figure 2A**, co-  
311 exposure to DHT and INS resulted in decreased GSH levels in the gravid uterus, but not in the  
312 placenta, while increased GSH+GSSG levels were detected in both tissues. Similarly, alterations of  
313 GSH and GSH+GSSG levels were found in the gravid uterus exposed to insulin alone. Levels of GSH  
314 were lower in both the gravid uterus and placenta exposed to DHT alone compared to controls (**Fig.**  
315 **2A**).

316 ***Alterations of ferroptosis-related gene expression, MDA levels, intracellular iron deposition,  
317 and the MAPK signaling pathway***

318 Next, we examined whether maternal exposure to DHT and/or INS alters the expression of pro-  
319 ferroptosis (*Slc1a5*, *Acsl4*, *Gls2*, *Cs*, *Gss*, *Tfrc* and *Ireb2*) or anti-ferroptosis (*Slc7a11*, and *Gclc*)  
320 genes in the gravid uterus and placenta. In pregnant DHT+INS-exposed rats, uterine *Acsl4*, *Slc7a11*  
321 and *Gclc* mRNAs were decreased, while *Tfrx* mRNA was increased (**Fig. 2B**). In comparison with the  
322 control uterus, maternal exposure to DHT alone decreased *Cs*, *Gss*, *Ireb2*, *Slc7a11* and *Gclc* mRNA  
323 expression, whereas exposure to INS alone increased *Gls2* and *Tfrc* mRNAs and decreased *Slc7a11*  
324 mRNA expression (**Fig. 2B**). qPCR analysis also showed that *Gls2* mRNA expression was increased  
325 and *Slc7a11* mRNA expression was decreased in the placenta after co-exposure to DHT and INS. In  
326 comparison with the control placenta, exposure to DHT alone decreased *Cs*, *Gss* and *Slc7a11* mRNA  
327 expression, whereas exposure to INS alone decreased *Gss* and *Gclc* mRNAs in parallel to increased  
328 *Tfrc* and *Slc7a11* mRNA expression (**Fig. 2B**).

329 Given that one of the key consequences of ferroptosis is elevated lipid peroxidation (6, 7), we next  
330 examined the impact of DHT and INS on the levels of MDA, a marker of lipid peroxidation (47), in the

331 gravid uterus and placenta. As shown in **Figure 2C**, co-exposure to DHT and INS resulted in  
332 increased MDA levels in both the gravid uterus and placenta. However, there were no significant  
333 changes in MDA levels between the DHT-exposed rats or the INS-exposed rats and control rats.

334 Whether chronic exposure to DHT and INS can modulate tissue iron deposition was also  
335 examined. Perls' histochemical reaction showed specific cytoplasmic and granular iron storage in rat  
336 uterine epithelial and decidualized stromal cells on GD 6, which is prior to the induction of HAIR  
337 (**Supplemental Fig. 4A1-A2**). These data are consistent with a previous report showing the cellular  
338 expression of ferritin heavy chain, a component of the multi-subunit iron-binding protein ferritin, in the  
339 uterus during early pregnancy (48). In the gravid uterus, iron accumulation was increased in the  
340 external muscle layer, the mesometrial triangle, as well as in the decidua of DHT+INS-exposed  
341 pregnant rats (**Fig. 3B1-B3** and **Supplemental Fig. 4B3-B4**) compared to control pregnant rats (**Fig.**  
342 **3A1-A3** and **Supplemental Fig. 4B1-B2**). Similarly, a significant increase in iron storage in the  
343 mesometrial triangle was also observed in DHT-exposed pregnant rats (**Fig. 3C1-C2**). Further,  
344 granular and cytoplasmic iron-positive staining was barely detectable in the mesometrial decidua in  
345 the DHT-exposed rats and in the INS-exposed rats (**Fig. C2 and D3**). No iron-positive staining was  
346 found in the placental junctional zone in any of the experimental groups (**Fig. 3A4, B4, C4, and D4**),  
347 while intense iron-positive staining was consistently detected in immature erythrocytes within the  
348 placental labyrinth zone in all experimental groups (**Fig. 3A5, B5, C5 and D5**). These results indicate  
349 that the amount of deposited iron was elevated, especially in the gravid uterus, following exposure to  
350 DHT and/or INS.

351 Taking into consideration that the MAPK signaling pathway, including ERK, p38, and c-JUN NH<sub>2</sub>-  
352 terminal kinase (JNK) is involved in the execution of ferroptosis in other cells (10, 49), we evaluated  
353 whether co-exposure to DHT and INS may be linked to activation of the MAPK signaling pathway in  
354 the gravid uterus and placenta. As shown in **Figure 4A**, in the gravid uterus DHT+INS exposure  
355 resulted in an increased abundance of phosphorylated ERK1/2 (p-ERK1/2) and decreased total  
356 ERK1/2, which subsequently resulted in an increased p-ERK1/2:ERK1/2 ratio. Moreover, both p-JNK  
357 and total JNK protein abundance were increased, whereas the p-JNK:JNK ratio remained unchanged  
358 in the DHT+INS-exposed gravid uterus compared to the control uterus (**Fig. 4A**). Additionally, a  
359 similar increase in p-p38 protein abundance and the p-p38:p38 ratio was observed in both the gravid  
360 uterus (**Fig. 4A**) and placenta (**Fig. 4B**) after co-exposure to DHT and INS. These results indicate that

361 both ERK1/2 and JNK signaling are only activated in the gravid uterus, whereas p38 signaling is  
362 activated in both the gravid uterus and placenta after co-exposure to DHT and INS.

363 ***Changes in mitochondrial morphology are associated with changes in mitochondria-encoded***  
364 ***gene and protein expression***

365 By TEM (**Supplemental Fig. 5**), we found that compared to controls (**Fig. 5A1**) the mitochondria  
366 showed swelling and collapsed and poorly defined tubular cristae in the gravid rat uterus exposed to  
367 DHT and/or INS (**Fig. 5B1-D1**). Consistent with ferroptosis-related mitochondrial morphology (7, 10,  
368 12), shrunken mitochondria with numerous electron-dense cristae or with the absence of cristae were  
369 only found in the DHT+INS-exposed gravid uterus (**Fig. 5B1 arrows**). TEM also revealed that  
370 treatment with DHT or INS reduced the number of mitochondrial cristae (**Fig. 5C1 and D1**). Further,  
371 ultrastructural analysis showed that mitochondria in the trophoblast within the junctional zone were  
372 significantly affected by exposure to DHT and/or INS (**Fig. 5A2-D2**). For instance, mitochondria  
373 showed blebbing, few or no tubular cristae, and decreased electron density in all treatment groups  
374 (**Fig. 5B2-D2**). However, there was less mitochondrial damage observed in the trophoblast of the  
375 labyrinth zone in all treatment groups compared to controls (**Fig. 5A3-D3**).

376 Based on these morphological observations, the expression of known mitochondria-encoded  
377 genes (*Cisd1*, an anti-ferroptosis gene, and *Dpp4*, a pro-ferroptosis gene (6, 11)) and protein (MOTS-  
378 c, an enhancer of insulin sensitivity (50)) was analyzed by qPCR (**Fig. 5E**) and ELISA (**Fig. 5F**). In the  
379 pregnant rat uterus, DHT+INS-exposure decreased *Cisd1* mRNA expression, increased *Dpp4* mRNA  
380 expression, and decreased the MOTS-c protein level (**Fig. 5E and F upper panel**). In contrast, we  
381 found significantly higher uterine *Cisd1* and *Dpp4* mRNA expression in INS-exposed pregnant rats  
382 (**Fig. 5E upper panel**), but unchanged uterine MOTS-c protein levels in DHT-exposed pregnant rats  
383 compared to controls (**Fig. 5F upper panel**). In the placenta, *Cisd1* mRNA expression was increased  
384 and *Dpp4* mRNA expression was decreased in DHT+INS-exposed pregnant rats compared to  
385 controls (**Fig. 5E lower panel**). A decrease in placental *Dpp4* mRNA expression was also observed in  
386 INS-exposed pregnant rats (**Fig. 5E lower panel**). However, there was no significant difference in  
387 MOTS-c protein levels in the placenta between any of the experimental groups (**Fig. 5F lower panel**).  
388 ***Aberrant regulation of necroptosis-related and anti-/pro-apoptosis-related gene and protein***  
389 ***expression***

390 Different types of cell death are seen in uterine and placental tissue during healthy and  
391 pathological pregnancy (51-54). To extend our observations on the effect of DHT and INS on  
392 ferroptosis and mitochondrial impairment, we analyzed the expression of necroptosis (*Mlk1*, *Ripk1*,  
393 and *Ripk3*), anti-apoptosis (*Bcl2* and *Bcl-xl*) and pro-apoptosis (*Bax*, *Bak*, *Casp3*, and cleaved  
394 caspase-3) mRNAs and proteins (6, 8, 10) in the gravid uterus and placenta. As shown in **Figure 6A**,  
395 DHT+INS-exposure significantly decreased uterine *Ripk1* mRNA expression, while uterine *Mlk1* and  
396 *Ripk3* mRNAs were increased by DHT and/or INS exposure when compared to control pregnant rats  
397 (**Fig. 6A upper panel**). Furthermore, co-exposure to DHT and INS increased *Bcl2*, *Bcl-xl*, and *Bax*  
398 mRNA expression in the gravid uterus, with similar increases in these genes seen in DHT-exposed  
399 and/or INS-exposed pregnant rats compared to controls (**Fig. 6B upper panel**). In DHT+INS-exposed  
400 pregnant rats, *Casp3* mRNA expression and cleaved caspase-3 protein abundance were decreased  
401 in the gravid uterus (**Fig. 6B upper panel and C**). In contrast, in the placenta we found that both  
402 *Ripk1* and *Ripk3* mRNAs were increased in DHT+INS-exposed pregnant rats compared to controls  
403 (**Fig. 6A lower panel**). Furthermore, maternal co-exposure to DHT and INS increased placental *Bcl2*,  
404 *Bcl-xl*, *Bax*, and *Bak* mRNA expression (**Fig. 6B lower panel**). There were, however, no changes in  
405 *Casp3* mRNA expression or cleaved caspase-3 protein abundance in the placenta (**Fig. 6B lower**  
406 **panel and C**). Lastly, similar increases in placental *Bcl2*, *Bcl-xl*, *Bax*, *Bak* and *Casp3* mRNAs were  
407 seen in DHT-exposed and/or INS-exposed pregnant rats compared to controls (**Fig. 6B lower panel**).  
408

## 408 **Discussion**

409 Because PCOS patients frequently suffer from miscarriage and infertility (3, 4), it is important to  
410 understand the molecular mechanisms through which HAIR affects tissues such as the gravid uterus  
411 and placenta. Until now, there have been no reports exploring the relationship between PCOS and  
412 regulated cell death in the uterus and placenta. Our results thus fill an important clinically relevant  
413 knowledge gap by experimentally demonstrating that maternal HAIR can cause the activation of  
414 ferroptosis in the gravid uterus and placenta, although this is mediated through different molecular  
415 and cellular mechanisms. Alterations in the ferroptosis pathway in the uterus and placenta as a result  
416 of maternal HAIR likely contribute to impaired fetal survival seen in experimental animal models, as  
417 well as women with PCOS.

418 Mammalian Gpx4 generally functions as a major antioxidant regulator of systemic and cellular  
419 responses to oxidative stress, and loss of function of Gpx4 protein and depletion of GSH levels are

420 the key mechanisms for triggering ferroptosis (7, 11). *In vivo* knockout studies have shown that mice  
421 lacking the entire *Gpx4* gene are early embryonic lethal (55), and *Gpx4*-deficient male mice are  
422 completely infertile (56). Although the presence of *Gpx4* has been shown in uteri from cows (57, 58)  
423 and pigs (59), the physiological role and localization of *Gpx4* have not been demonstrated in human  
424 and rodent reproductive tissues, including the uterus. Here, we show that *Gpx4* is widely expressed in  
425 the human and rat uterus, including by decidualized stromal cells. Furthermore, *Gpx4* is down-  
426 regulated in the gravid uterus by maternal exposure to DHT and INS. Correspondingly, the levels of  
427 GSH are decreased and GSH+GSSG levels are increased in the uterus by DHT and INS co-  
428 exposure. Taken together, these results suggest that maternal HAIR disrupts the *Gpx4*-GSH  
429 regulatory axis and can result in the induction of ferroptosis in the uterus during pregnancy. The  
430 finding that GSH levels in the uterus were lowest in the INS-only treated rats that showed a non-  
431 significant reduction in *Gpx4* suggests that other pathways and factors such as the antioxidants Nrf1  
432 and Nrf2 (60) might be altered by the treatments and contribute to the resultant changes in GSH  
433 status and should be investigated for causality in the future (also in the placenta of DHT and/or INS-  
434 exposed dams). Indeed, we have previously found altered abundance of antioxidants in pregnant rats  
435 exposed to DHT and/or INS (18, 19). Consistent with previous work on the human placenta (61, 62),  
436 the present study shows that the *Gpx4* protein is highly expressed in the rat placenta during  
437 pregnancy. Although analysis of whole placental homogenates showed no significant decrease in  
438 *Gpx4* levels, immunolocalization revealed loss of *Gpx4* in specific cell types in the placenta (the  
439 glycogen and spongiotrophoblast cells) in response to maternal co-exposure to DHT and INS. The  
440 more minor alterations in *Gpx4* abundance, combined with the high levels of GSH+GSSG and  
441 absence of changes in GSH levels in the placenta, suggest that maternal HAIR induces ferroptosis to  
442 a lesser extent in the placenta compared to the gravid uterus. *Gpx4* is known to protect cells/tissues  
443 against lipid peroxidation by inhibiting lipid-associated hydroperoxides (11). In addition, genetically  
444 ablating or inducing decreased *Gpx4* expression leads to ferroptosis (14, 57). Taken together, our  
445 data therefore suggest that HAIR-induced ferroptosis is mediated by both dysregulation of *Gpx4*  
446 expression and aberrant increases in lipid peroxidation. The induction of uterine and placental  
447 ferroptosis by maternal exposure to DHT and INS may be a novel mechanism contributing to the  
448 malfunction of those tissues and hence impaired fetal development during pregnancy. However, how  
449 maternal HAIR-mediated uterine and placental ferroptosis compromises the growth and development

450 of the fetus is not clear at this time and should be the subject of future investigations. Moreover, future  
451 work should be employed to assess whether the activation of ferroptosis, lipid peroxidation and poor  
452 fetal outcomes by maternal HAIR may be preventable by antioxidant administration.

453 Iron can serve as an essential signaling molecule that modulates diverse physiological processes,  
454 and iron homeostasis is required for the normal growth and development of the placenta and fetus  
455 during pregnancy (13, 63). An extensive body of evidence indicates that while iron deficiency is linked  
456 to abnormal pregnancy (13) and increased risk of fetal death (64), iron overload is associated with the  
457 manifestation of PCOS (65). Previous findings by Kim and colleagues indicate that increased  
458 circulating iron levels are associated with metabolic abnormalities, including HAIR, in PCOS patients  
459 (66). Several studies have demonstrated that in addition to its antioxidative property, *Ho1* is a critical  
460 regulator for mobilization of intracellular pools of free iron (67, 68). More recently, we have  
461 demonstrated that maternal co-exposure to DHT and INS suppresses *Ho1* mRNA expression in the  
462 gravid uterus, but not in the placenta (18, 19). While the uptake of transferrin-bound iron, a major  
463 maternal iron source for placental transfer, is mainly mediated through iron import proteins such as  
464 transferrin receptor 1 (Tfr1, Tfrc) (63), our results show that combined exposure to DHT and INS  
465 increases *Tfrc* mRNA expression in association with increased iron deposition in the gravid uterus.  
466 Further, we have provided ultrastructural evidence that shrunken mitochondria with numerous  
467 electron-dense cristae, a key feature of ferroptosis-related mitochondrial morphology are present in  
468 the gravid uterus. However, the placentas of the same animals exhibited increased mRNA expression  
469 of *Cisd1*, a mitochondrial iron export factor, but no change in *Tfrc* mRNA or iron accumulation.  
470 Ferroptosis can be induced by excessive accumulation of free iron in tissues and cells (69) and our  
471 findings support the notion that, in response to exposure to DHT and INS, aberrant iron accumulation  
472 and activation of ferroptosis occurs in the gravid uterus but not in the placenta. The synthesis of heme  
473 and iron-sulfur clusters is controlled by different intracellular compartments, including the cytosol and  
474 mitochondria (69). Further investigations are needed to determine which cellular compartments  
475 contribute to the defective utilization of iron and increased ferroptosis observed in the gravid uterus  
476 under conditions of HAIR.

477 Given that aberrant accumulation of intracellular iron induces oxidative stress (69) and  
478 subsequently results in multiple modes of cell death (70), it is not surprising that, in addition to  
479 ferroptosis, apoptosis (a non-inflammatory form of cell death) and necroptosis (a pro-inflammatory

480 form of cell death) may also be involved in HAIR-induced fetal loss in pregnant rats. Indeed, pregnant  
481 rats co-exposed to DHT and INS exhibited decreased *Casp3* mRNA expression and cleaved  
482 caspase-3 protein abundance in the uterus, but not in the placenta, even though selectively increased  
483 expression of anti-apoptotic genes (*Bcl2* and *Bcl-xl*) and pro-apoptotic genes (*Bax*) was observed in  
484 both tissues. We suspect that suppression of apoptosis might serve as a compensatory mechanism to  
485 protect against increased ferroptosis in order to maintain homeostasis of the gravid uterus after  
486 exposure to DHT and INS. This is supported by studies assessing the interaction and interplay of  
487 different cell death pathways in cancer research (71). Ferroptosis and necroptosis are two different  
488 forms of regulated necrosis (8, 10). Necroptosis requires mitochondrial ROS generation and is  
489 primarily regulated by the *Ripk1*, *Ripk3*, and *Mlk1* proteins (9, 46). We found that in DHT+INS-exposed  
490 pregnant rats the level of ROS (19) and expression of *Ripk1* and *Ripk3* mRNAs was increased in the  
491 placenta, but not in the gravid uterus ((18) and this study). Therefore, it is tempting to speculate that  
492 the activation of necroptosis in response to PCOS-related HAIR might serve to counteract the  
493 ferroptosis pathway in the placenta. Additionally, both ferroptosis and necroptosis might intersect and  
494 crosstalk with HAIR-induced oxidative damage and subsequently result in increased fetal loss. It  
495 remains to be determined whether HAIR-induced pregnancy loss is due to increased iron-mediated  
496 uterine ferroptosis or to necroptosis-related defects in the placenta, or both.

497 In this study, we found that some pro-ferroptosis genes such as *Acsl4*, *Tfrc* and *Dpp4* were  
498 oppositely regulated in the uterus by co-exposure to DHT and INS. However, several anti-ferroptosis  
499 genes, including *Slc7a11*, *Gcls*, and *Cisd1* were downregulated in the gravid uterus after co-exposure  
500 to DHT and INS. These results suggest that the suppression of anti-ferroptosis gene transcription  
501 might play a dominant role in promoting ferroptosis in this tissue under conditions of HAIR. Compared  
502 to the gravid uterus, the placenta showed a distinct profile of ferroptosis-related gene changes in  
503 response to the combined DHT and INS exposure. Furthermore, we often observed contrasting  
504 expression patterns of pro- and anti-ferroptosis genes in the gravid uterus and placenta with exposure  
505 to DHT or INS alone compared to the combined exposure. We do not know the exact reason for these  
506 inconsistencies; however, we do know that the expression of ferroptosis-related genes and proteins  
507 are only assessed at one gestational age in pregnant rats when they display HAIR (18, 19). In  
508 addition, perhaps components of HAIR may act synergistically or through separate pathways to bring  
509 about divergent effects on gene expression and signaling pathways to regulate the ferroptosis

510 process. Overall, our findings demonstrate the complexity and challenges in establishing direct roles  
511 and patterns linking individual pro-/anti-ferroptosis genes to the ferroptosis pathway in the gravid  
512 uterus and placenta in response to DHT and/or INS *in vivo*. Future work should therefore investigate  
513 the tissue-specific and time-dependent changes in ferroptosis-related gene expression in the gravid  
514 uterus and placenta during the hormonal manipulation. In comparison to the single treatment groups  
515 (DHT or INS), specific changes within the maternal uterus and placenta appeared to be driven by  
516 hyperandrogenism, insulin resistance, or both (co-treatment with DHT and INS) and reflected the  
517 complexity in working with tissues from animals in which many physiological parameters may be  
518 altered. Indeed, experiments utilizing gene and pathway inhibitors in uterine decidual cells and  
519 placental trophoblasts would be beneficial in future studies that aim to explore the causality of  
520 changes observed regarding ferroptosis and iron metabolism. Work is also required to assess  
521 whether elevated ferroptosis in the uterus contributes to the placental dysfunction in the rat dams with  
522 HAIR due to DHT and INS, which would be aided by a time-course analysis.

523 Recently, Zhang and colleagues reported that oxidative stress-induced ferroptosis contributes to  
524 the pathogenesis of preeclampsia (72). Because decreased Gpx4, GSH, and SLC7A11 protein levels  
525 and increased MDA content are seen in the preeclamptic placenta in humans and rats (72), our  
526 findings together with this report support the notion that defective ferroptosis is involved in the  
527 pathophysiological processes of female reproductive disorders.

528 In summary, our findings suggest maternal exposure to DHT and INS alters the ferroptosis pathway  
529 in the gravid uterus and placenta; however, this occurs via different regulatory mechanisms and  
530 signaling pathways. For instance, in contrast to the placenta, increased ferroptosis in the gravid  
531 uterus in response to DHT and INS was related to decreased Gpx4 and GSH abundance, altered  
532 expression of ferroptosis-associated genes (*Acs14*, *Tfrc*, *Slc7a11*, and *Gclc*), increased MDA and iron  
533 deposition, upregulation of the ERK/p38/JNK pathway and mitochondrial *Dpp4* expression, and the  
534 appearance of typical ferroptosis-related mitochondrial morphology. In addition, DHT and INS were  
535 associated with reduced activation of apoptosis in the uterus and increased necroptosis in the  
536 placenta. The concomitant presence of different forms of regulated cell death would be expected to  
537 disrupt uterine and placental function and play a role in the fetal loss observed in DHT+INS-exposed  
538 pregnant rats. Both the maternal uterine decidua and placenta play essential roles in embryo  
539 implantation and successful pregnancy (73, 74). Therefore, while the present study improves our

540 understanding of the impact of HAIR on regulated cell death in specific tissues during pregnancy,  
541 more preclinical and clinical studies are needed to further investigate the molecular and functional  
542 connectivity between the maternal decidua and the placenta and between the placenta and fetus  
543 under conditions of PCOS.

544 **Abbreviations**

545 PCOS, polycystic ovary syndrome; HAIR, hyperandrogenism and insulin resistance; DHT, 5α-  
546 dihydrotestosterone; INS, human recombinant insulin; Gpx4, glutathione peroxidase 4; GSH,  
547 glutathione (reduced state); GSSG, glutathione disulfide (oxidized state); JNK, c-JUN NH2-terminal  
548 kinase; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; GD, gestational day; TEM,  
549 transmission electron microscopy.

550 **Author contributions**

551 Study design and supervision: LRS. Study conduct: YZ, MH, WJ, GL, JZ, BW, PC, XL, YH, LS,  
552 XW, and LRS. Data collection: YZ, MH, WJ, GL, JZ, BW, JL, XL, and LRS. Data analysis: YZ, MH, JL,  
553 and LRS. Data interpretation: SL, ANS, LS, MB, LRS, and HB. Drafting the manuscript: YZ, MH, and  
554 LRS. Revising the manuscript: SL, ANS, MB, LRS, and HB. YZ, MH, LRS, and HB take responsibility  
555 for the integrity of the data analysis. All authors have read and approved the final version of the  
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570 **Conflicts of Interest**

571 The authors indicate no potential conflicts of interest

572

573 **References**

- 574 1. Azziz, R., Carmina, E., Chen, Z., Dunaif, A., Laven, J. S., Legro, R. S., Lizneva, D., Natterson-  
575 Horowitz, B., Teede, H. J., and Yildiz, B. O. (2016) Polycystic ovary syndrome. *Nat Rev Dis  
576 Primer* **2**, 16057
- 577 2. Lizneva, D., Suturina, L., Walker, W., Brakta, S., Gavrilova-Jordan, L., and Azziz, R. (2016)  
578 Criteria, prevalence, and phenotypes of polycystic ovary syndrome. *Fertil Steril* **106**, 6 - 15
- 579 3. Palomba, S., de Wilde, M. A., Falbo, A., Koster, M. P., La Sala, G. B., and Fauser, B. C. (2015)  
580 Pregnancy complications in women with polycystic ovary syndrome. *Hum Reprod Update* **21**,  
581 575-592
- 582 4. Chakraborty, P., Goswami, S. K., Rajani, S., Sharma, S., Kabir, S. N., Chakravarty, B., and Jana,  
583 K. (2013) Recurrent pregnancy loss in polycystic ovary syndrome: role of  
584 hyperhomocysteinemia and insulin resistance. *PLoS One* **8**, e64446
- 585 5. Rosenfield, R. L., and Ehrmann, D. A. (2016) The Pathogenesis of Polycystic Ovary Syndrome  
586 (PCOS): The Hypothesis of PCOS as Functional Ovarian Hyperandrogenism Revisited. *Endocr  
587 Rev* **37**, 467-520
- 588 6. Tang, D., Kang, R., Berghe, T. V., Vandenabeele, P., and Kroemer, G. (2019) The molecular  
589 machinery of regulated cell death. *Cell Res* **29**, 347-364
- 590 7. Dixon, S. J., Lemberg, K. M., Lamprecht, M. R., Skouta, R., Zaitsev, E. M., Gleason, C. E., Patel,  
591 D. N., Bauer, A. J., Cantley, A. M., Yang, W. S., Morrison, B., 3rd, and Stockwell, B. R. (2012)  
592 Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* **149**, 1060-1072
- 593 8. Choi, M. E., Price, D. R., Ryter, S. W., and Choi, A. M. K. (2019) Necroptosis: a crucial  
594 pathogenic mediator of human disease. *JCI insight* **4**, e128834
- 595 9. Tonnus, W., Meyer, C., Paliege, A., Belavgeni, A., von Massenhausen, A., Bornstein, S. R.,  
596 Hugo, C., Becker, J. U., and Linkermann, A. (2019) The pathological features of regulated  
597 necrosis. *J Pathol* **247**, 697-707
- 598 10. Xie, Y., Hou, W., Song, X., Yu, Y., Huang, J., Sun, X., Kang, R., and Tang, D. (2016) Ferroptosis:  
599 process and function. *Cell Death Differ* **23**, 369-379
- 600 11. Stockwell, B. R., Friedmann Angeli, J. P., Bayir, H., Bush, A. I., Conrad, M., Dixon, S. J., Fulda,  
601 S., Gascon, S., Hatzios, S. K., Kagan, V. E., Noel, K., Jiang, X., Linkermann, A., Murphy, M. E.,  
602 Overholtzer, M., Oyagi, A., Pagnussat, G. C., Park, J., Ran, Q., Rosenfeld, C. S., Salnikow, K.,  
603 Tang, D., Torti, F. M., Torti, S. V., Toyokuni, S., Woerpel, K. A., and Zhang, D. D. (2017)  
604 Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease.  
605 *Cell* **171**, 273-285
- 606 12. Li, J., Cao, F., Yin, H. L., Huang, Z. J., Lin, Z. T., Mao, N., Sun, B., and Wang, G. (2020)  
607 Ferroptosis: past, present and future. *Cell Death Dis* **11**, 88
- 608 13. Ng, S. W., Norwitz, S. G., and Norwitz, E. R. (2019) The Impact of Iron Overload and  
609 Ferroptosis on Reproductive Disorders in Humans: Implications for Preeclampsia. *Int J Mol  
610 Sci* **20**
- 611 14. Burton, G. J., and Jauniaux, E. (2018) Pathophysiology of placental-derived fetal growth  
612 restriction. *Am J Obstet Gynecol* **218**, S745-S761
- 613 15. Spencer, S. J., Cataldo, N. A., and Jaffe, R. B. (1996) Apoptosis in the human female  
614 reproductive tract. *Obstet Gynecol Surv* **51**, 314-323
- 615 16. Luo, L., Gu, F., Jie, H., Ding, C., Zhao, Q., Wang, Q., and Zhou, C. (2017) Early miscarriage rate  
616 in lean polycystic ovary syndrome women after euploid embryo transfer - a matched-pair  
617 study. *Reprod Biomed Online* **35**, 576-582
- 618 17. Rees, D. A., Jenkins-Jones, S., and Morgan, C. L. (2016) Contemporary Reproductive  
619 Outcomes for Patients With Polycystic Ovary Syndrome: A Retrospective Observational  
620 Study. *J Clin Endocrinol Metab* **101**, 1664-1672
- 621 18. Hu, M., Zhang, Y., Guo, X., Jia, W., Liu, G., Zhang, J., Li, J., Cui, P., Sferruzzi-Perri, A. N., Han,  
622 Y., Wu, X., Ma, H., Brannstrom, M., Shao, L. R., and Billig, H. (2019) Hyperandrogenism and

623 insulin resistance induce gravid uterine defects in association with mitochondrial  
624 dysfunction and aberrant ROS production. *Am J Physiol Endocrinol Metab* **316**, E794-E809

625 19. Zhang, Y., Zhao, W., Xu, H., Hu, M., Guo, X., Jia, W., Liu, G., Li, J., Cui, P., Lager, S., Sferruzzi-  
626 Perri, A. N., Li, W., Wu, X. K., Han, Y., Brannstrom, M., Shao, L. R., and Billig, H. (2019)  
627 Hyperandrogenism and insulin resistance-induced fetal loss: evidence for placental  
628 mitochondrial abnormalities and elevated reactive oxygen species production in pregnant  
629 rats that mimic the clinical features of polycystic ovary syndrome. *J Physiol* **597**, 3927-3950

630 20. Sun, X., Niu, X., Chen, R., He, W., Chen, D., Kang, R., and Tang, D. (2016) Metallothionein-1G  
631 facilitates sorafenib resistance through inhibition of ferroptosis. *Hepatology* **64**, 488-500

632 21. Lai, Q., Xiang, W., Li, Q., Zhang, H., Li, Y., Zhu, G., Xiong, C., and Jin, L. (2017) Oxidative stress  
633 in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with  
634 polycystic ovary syndrome. *Front Med* **12**, 518-524

635 22. Banuls, C., Rovira-Llopis, S., Martinez de Maranon, A., Veses, S., Jover, A., Gomez, M., Rocha,  
636 M., Hernandez-Mijares, A., and Victor, V. M. (2017) Metabolic syndrome enhances  
637 endoplasmic reticulum, oxidative stress and leukocyte-endothelium interactions in PCOS.  
638 *Metabolism* **71**, 153-162

639 23. Victor, V. M., Rocha, M., Banuls, C., Alvarez, A., de Pablo, C., Sanchez-Serrano, M., Gomez,  
640 M., and Hernandez-Mijares, A. (2011) Induction of oxidative stress and human  
641 leukocyte/endothelial cell interactions in polycystic ovary syndrome patients with insulin  
642 resistance. *J Clin Endocrinol Metab* **96**, 3115-3122

643 24. Agarwal, A., Aponte-Mellado, A., Premkumar, B. J., Shaman, A., and Gupta, S. (2012) The  
644 effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol* **10**, 49

645 25. Schoots, M. H., Gordijn, S. J., Scherjon, S. A., van Goor, H., and Hillebrands, J. L. (2018)  
646 Oxidative stress in placental pathology. *Placenta* **69**, 153-161

647 26. Gao, M., Yi, J., Zhu, J., Minikes, A. M., Monian, P., Thompson, C. B., and Jiang, X. (2019) Role  
648 of Mitochondria in Ferroptosis. *Mol Cell* **73**, 354-363 e353

649 27. Jelinek, A., Heyder, L., Daude, M., Plessner, M., Krippner, S., Grosse, R., Diederich, W. E., and  
650 Culmsee, C. (2018) Mitochondrial rescue prevents glutathione peroxidase-dependent  
651 ferroptosis. *Free Radi Biol Med* **117**, 45-57

652 28. Zhang, J., Bao, Y., Zhou, X., and Zheng, L. (2019) Polycystic ovary syndrome and  
653 mitochondrial dysfunction. *Reprod Biol Endocrinol* **17**, 67

654 29. Ding, Y., Jiang, Z., Xia, B., Zhang, L., Zhang, C., and Leng, J. (2018) Mitochondria-targeted  
655 antioxidant therapy for an animal model of PCOS-IR. *Int J Mol Med* **43**, 316-324

656 30. Hu, M., Zhang, Y., Guo, X., Jia, W., Liu, G., Zhang, J., Cui, P., Li, J., Li, W., Wu, X., Ma, H.,  
657 Brannstrom, M., Shao, L. R., and Billig, H. (2019) Perturbed ovarian and uterine  
658 glucocorticoid receptor signaling accompanies the balanced regulation of mitochondrial  
659 function and NFkappaB-mediated inflammation under conditions of hyperandrogenism and  
660 insulin resistance. *Life Sci* **232**, 116681

661 31. Feng, Y., Weijdegard, B., Wang, T., Egecioglu, E., Fernandez-Rodriguez, J., Huhtaniemi, I.,  
662 Stener-Victorin, E., Billig, H., and Shao, R. (2010) Spatiotemporal expression of androgen  
663 receptors in the female rat brain during the oestrous cycle and the impact of exogenous  
664 androgen administration: a comparison with gonadally intact males. *Mol Cell Endocrinol* **321**,  
665 161-174

666 32. Zhang, Y., Sun, X., Sun, X., Meng, F., Hu, M., Li, X., Li, W., Wu, X. K., Brännström, M., Shao, R.,  
667 and Billig, H. (2016) Molecular characterization of insulin resistance and glycolytic  
668 metabolism in the rat uterus. *Sci Rep* **6**, 30679

669 33. Silfen, M. E., Denburg, M. R., Manibo, A. M., Lobo, R. A., Jaffe, R., Ferin, M., Levine, L. S., and  
670 Oberfield, S. E. (2003) Early endocrine, metabolic, and sonographic characteristics of  
671 polycystic ovary syndrome (PCOS): comparison between nonobese and obese adolescents. *J  
672 Clin Endocrinol Metab* **88**, 4682-4688

673 34. Fassnacht, M., Schlenz, N., Schneider, S. B., Wudy, S. A., Allolio, B., and Arlt, W. (2003)  
674 Beyond adrenal and ovarian androgen generation: Increased peripheral 5 alpha-reductase  
675 activity in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **88**, 2760-2766  
676 35. Zhang, Y., Meng, F., Sun, X., Sun, X., Hu, M., Cui, P., Vestin, E., Li, X., Li, W., Wu, X. K., Jansson,  
677 J. O., Shao, L. R., and Billig, H. (2018) Hyperandrogenism and insulin resistance contribute to  
678 hepatic steatosis and inflammation in female rat liver. *Oncotarget* **9**, 18180-18197  
679 36. Maliqueo, M., Lara, H. E., Sanchez, F., Echiburu, B., Crisosto, N., and Sir-Petermann, T. (2013)  
680 Placental steroidogenesis in pregnant women with polycystic ovary syndrome. *Eur J Obstet  
681 Gynecol Reprod Biol* **166**, 151-155  
682 37. Sir-Petermann, T., Maliqueo, M., Angel, B., Lara, H. E., Perez-Bravo, F., and Recabarren, S. E.  
683 (2002) Maternal serum androgens in pregnant women with polycystic ovarian syndrome:  
684 possible implications in prenatal androgenization. *Hum Reprod* **17**, 2573-2579  
685 38. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R.,  
686 Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. (2009) The MIQE  
687 guidelines: minimum information for publication of quantitative real-time PCR experiments.  
688 *Clin Chem* **55**, 611-622  
689 39. Zhang, Y., Hu, M., Meng, F., Sun, X., Xu, H., Zhang, J., Cui, P., Morina, N., Li, X., Li, W., Wu, X.  
690 K., Brannstrom, M., Shao, R., and Billig, H. (2017) Metformin Ameliorates Uterine Defects in  
691 a Rat Model of Polycystic Ovary Syndrome. *EBioMedicine* **18**, 157-170  
692 40. Maiorino, M., Scapin, M., Ursini, F., Biasolo, M., Bosello, V., and Flohe, L. (2003) Distinct  
693 promoters determine alternative transcription of gpx-4 into phospholipid-hydroperoxide  
694 glutathione peroxidase variants. *J Biol Chem* **278**, 34286-34290  
695 41. Tramer, F., Micali, F., Sandri, G., Bertoni, A., Lenzi, A., Gandini, L., and Panfili, E. (2002)  
696 Enzymatic and immunochemical evaluation of phospholipid hydroperoxide glutathione  
697 peroxidase (PHGPx) in testes and epididymal spermatozoa of rats of different ages. *Int J  
698 Androl* **25**, 72-83  
699 42. Hu, M., Zhang, Y., Feng, J., Xu, X., Zhang, J., Zhao, W., Guo, X., Li, J., Vestin, E., Cui, P., Li, X.,  
700 Wu, X. K., Brannstrom, M., Shao, L. R., and Billig, H. (2018) Uterine progesterone signaling is  
701 a target for metformin therapy in PCOS-like rats. *J Endocrinol* **237**, 123-137  
702 43. Meguro, R., Asano, Y., Odagiri, S., Li, C., Iwatsuki, H., and Shoumura, K. (2007) Nonheme-iron  
703 histochemistry for light and electron microscopy: a historical, theoretical and technical  
704 review. *Arch Histo Cyto* **70**, 1-19  
705 44. Conrad, M., Schneider, M., Seiler, A., and Bornkamm, G. W. (2007) Physiological role of  
706 phospholipid hydroperoxide glutathione peroxidase in mammals. *Biol Chem* **388**, 1019-1025  
707 45. Forcina, G. C., and Dixon, S. J. (2019) GPX4 at the Crossroads of Lipid Homeostasis and  
708 Ferroptosis. *Proteomics* **19**, e1800311  
709 46. Brigelius-Flohe, R., and Maiorino, M. (2013) Glutathione peroxidases. *Biochim Biophys Acta*  
710 **1830**, 3289-3303  
711 47. Gawel, S., Wardas, M., Niedworok, E., and Wardas, P. (2004) [Malondialdehyde (MDA) as a  
712 lipid peroxidation marker]. *Wiad Lek* **57**, 453-455  
713 48. Zhu, L. J., Bagchi, M. K., and Bagchi, I. C. (1995) Ferritin heavy chain is a progesterone-  
714 inducible marker in the uterus during pregnancy. *Endocrinology* **136**, 4106-4115  
715 49. Hattori, K., Ishikawa, H., Sakauchi, C., Takayanagi, S., Naguro, I., and Ichijo, H. (2017) Cold  
716 stress-induced ferroptosis involves the ASK1-p38 pathway. *EMBO Rep* **18**, 2067-2078  
717 50. Kim, S. J., Xiao, J., Wan, J., Cohen, P., and Yen, K. (2017) Mitochondrially derived peptides as  
718 novel regulators of metabolism. *J Physiol* **595**, 6613-6621  
719 51. Welsh, A. O. (1993) Uterine cell death during implantation and early placentation. *Microsc  
720 Res Tech* **25**, 223-245  
721 52. Haider, S., and Knofler, M. (2009) Human tumour necrosis factor: physiological and  
722 pathological roles in placenta and endometrium. *Placenta* **30**, 111-123

723 53. Tassell, W., Slater, M., Barden, J. A., and Murphy, C. R. (2000) Endometrial cell death during  
724 early pregnancy in the rat. *Histochem J* **32**, 373-379

725 54. Sharp, A. N., Heazell, A. E., Crocker, I. P., and Mor, G. (2010) Placental apoptosis in health  
726 and disease. *Am J Reprod Immunol* **64**, 159-169

727 55. Imai, H., Hirao, F., Sakamoto, T., Sekine, K., Mizukura, Y., Saito, M., Kitamoto, T., Hayasaka,  
728 M., Hanaoka, K., and Nakagawa, Y. (2003) Early embryonic lethality caused by targeted  
729 disruption of the mouse PHGPx gene. *Biochem Biophys Res Commun* **305**, 278-286

730 56. Schneider, M., Forster, H., Boersma, A., Seiler, A., Wehnes, H., Sinowitz, F., Neumuller, C.,  
731 Deutsch, M. J., Walch, A., Hrabe de Angelis, M., Wurst, W., Ursini, F., Roveri, A.,  
732 Maleszewski, M., Maiorino, M., and Conrad, M. (2009) Mitochondrial glutathione peroxidase  
733 4 disruption causes male infertility. *FASEB J* **23**, 3233-3242

734 57. Ramos, R. S., Oliveira, M. L., Izaguirry, A. P., Vargas, L. M., Soares, M. B., Mesquita, F. S.,  
735 Santos, F. W., and Binelli, M. (2015) The periovulatory endocrine milieu affects the uterine  
736 redox environment in beef cows. *Reprod Biol Endocrinol* **13**, 39

737 58. Baithalu, R. K., Singh, S. K., Kumaresan, A., Mohanty, A. K., Mohanty, T. K., Kumar, S.,  
738 Kerketta, S., Maharana, B. R., Patbandha, T. K., Attupuram, N., and Agarwal, S. K. (2017)  
739 Transcriptional abundance of antioxidant enzymes in endometrium and their circulating  
740 levels in Zebu cows with and without uterine infection. *Anim Reprod Sci* **177**, 79-87

741 59. Dalto, D. B., Roy, M., Audet, I., Palin, M. F., Guay, F., Lapointe, J., and Matte, J. J. (2015)  
742 Interaction between vitamin B6 and source of selenium on the response of the selenium-  
743 dependent glutathione peroxidase system to oxidative stress induced by oestrus in pubertal  
744 pig. *J Trace Elem Med Biol* **32**, 21-29

745 60. Lu, S. C. (2009) Regulation of glutathione synthesis. *Molecular aspects of medicine* **30**, 42-59

746 61. Mistry, H. D., Kurlak, L. O., Williams, P. J., Ramsay, M. M., Symonds, M. E., and Broughton  
747 Pipkin, F. (2010) Differential expression and distribution of placental glutathione peroxidases  
748 1, 3 and 4 in normal and preeclamptic pregnancy. *Placenta* **31**, 401-408

749 62. Mistry, H. D., Wilson, V., Ramsay, M. M., Symonds, M. E., and Broughton Pipkin, F. (2008)  
750 Reduced selenium concentrations and glutathione peroxidase activity in preeclamptic  
751 pregnancies. *Hypertension* **52**, 881-888

752 63. Cao, C., and Fleming, M. D. (2016) The placenta: the forgotten essential organ of iron  
753 transport. *Nut Rev* **74**, 421-431

754 64. Guo, Y., Zhang, N., Zhang, D., Ren, Q., Ganz, T., Liu, S., and Nemeth, E. (2019) Iron  
755 homeostasis in pregnancy and spontaneous abortion. *Am J Hematol* **94**, 184-188

756 65. Escobar-Morreale, H. F. (2012) Iron metabolism and the polycystic ovary syndrome. *Trends  
757 Endocrinol Metab* **23**, 509-515

758 66. Kim, J. W., Kang, K. M., Yoon, T. K., Shim, S. H., and Lee, W. S. (2014) Study of circulating  
759 hepcidin in association with iron excess, metabolic syndrome, and BMP-6 expression in  
760 granulosa cells in women with polycystic ovary syndrome. *Fertil Steril* **102**, 548-554 e542

761 67. Poss, K. D., and Tonegawa, S. (1997) Heme oxygenase 1 is required for mammalian iron  
762 reutilization. *Proc Natl Acad Sci U S A* **94**, 10919-10924

763 68. Kovtunovych, G., Eckhaus, M. A., Ghosh, M. C., Ollivierre-Wilson, H., and Rouault, T. A.  
764 (2010) Dysfunction of the heme recycling system in heme oxygenase 1-deficient mice:  
765 effects on macrophage viability and tissue iron distribution. *Blood* **116**, 6054-6062

766 69. Galaris, D., Barbouti, A., and Pantopoulos, K. (2019) Iron homeostasis and oxidative stress:  
767 An intimate relationship. *Biochimica et biophysica acta. Mol Cell Res* **1866**, 118535

768 70. Lei, P., Bai, T., and Sun, Y. (2019) Mechanisms of Ferroptosis and Relations With Regulated  
769 Cell Death: A Review. *Front Physiol* **10**, 139

770 71. Riegerman, M., Bradbury, M. S., and Overholtzer, M. (2019) Population Dynamics in Cell  
771 Death: Mechanisms of Propagation. *Trend Cancer* **5**, 558-568

772 72. Zhang, H., He, Y., Wang, J. X., Chen, M. H., Xu, J. J., Jiang, M. H., Feng, Y. L., and Gu, Y. F.  
773 (2020) miR-30-5p-mediated ferroptosis of trophoblasts is implicated in the pathogenesis of  
774 preeclampsia. *Redox Biol* **29**, 101402

775 73. Schatz, F., Guzeloglu-Kayisli, O., Arlier, S., Kayisli, U. A., and Lockwood, C. J. (2016) The role  
776 of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy  
777 outcomes and abnormal uterine bleeding. *Hum Reprod Update* **22**, 497-515

778 74. Sharma, S., Godbole, G., and Modi, D. (2016) Decidual Control of Trophoblast Invasion. *Am J  
779 Reprod Immunol* **75**, 341-350

780 **Figure legend**

781 **Figure 1. Regulation and localization of Gpx4 protein in pregnant rats exposed to DHT and/or**  
782 **insulin at GD 14.5.** Western blot analysis of Gpx4 protein expression in the uterus and placenta (A, n  
783 = 9/group). In all plots, values are expressed as means  $\pm$  SEM. Significant differences ( $p < 0.05$ )  
784 within each group are denoted by different letters, and the same letter between groups indicates lack  
785 of statistical significance. N.S., not significant. Tissue sections were stained with hematoxylin and  
786 eosin (H&E, B1-4). Histological analysis by Gpx4 immunostaining in the gravid uterus (Mt and Md)  
787 and placenta (Jz and Lz) (C1-F4). Images are representative of 8–10 tissue replicates per group. Mt,  
788 mesometrial triangle; Md, mesometrial decidua; Jz, junctional zone (maternal side); Gc, glycogen  
789 cells; Sp, spongiotrophoblast cells; Lz, labyrinth zone (fetal side); Mv, maternal blood vessel; Fv, fetal  
790 blood vessel. Scale bars (100  $\mu$ m) are indicated in the photomicrographs. DHT, 5 $\alpha$ -  
791 dihydrotestosterone; INS, insulin.

792 **Figure 2. Alteration of GSH, GSH+GSSG, ferroptosis-related gene expression, and MDA in**  
793 **pregnant rats exposed to DHT and/or insulin at GD 14.5.** ELISA analysis of GSH, GSH+GSSG,  
794 and MDA in the uterus and placenta (A, n = 8/group). qPCR analysis of uterine and placental genes  
795 involved in modulating ferroptosis (B, n = 7–8/group). In all plots, values are expressed as means  $\pm$   
796 SEM. Significant differences ( $p < 0.05$ ) within each group are denoted by different letters, and the  
797 same letter between groups indicates lack of statistical significance. DHT, 5 $\alpha$ -dihydrotestosterone;  
798 INS, insulin.

799 **Figure 3. Iron deposition in the uterus and placenta of pregnant rats exposed to DHT and/or**  
800 **insulin at GD 14.5.** Gravid uterine and placental tissues from pregnant rats treated with vehicle (A1-  
801 5), DHT+INS (B1-5), DHT (C1-5), or INS (D1-5) are shown. The sections were stained by DAB-  
802 enhanced Perls' staining for iron accumulation. Yellow arrowheads indicate iron-positive staining.  
803 Images are representative of eight tissue replicates per group. Mt, mesometrial triangle; Md,  
804 mesometrial decidua; Jz, junctional zone (maternal side); Lz, labyrinth zone (fetal side). Scale bars  
805 (100  $\mu$ m) are indicated in the photomicrographs. DHT, 5 $\alpha$ -dihydrotestosterone; INS, insulin.

806 **Figure 4. Changes in the expression of proteins involved in the ferroptosis-related MAPK**  
807 **signaling pathway in pregnant rats exposed to DHT and/or insulin at GD 14.5.** Western blot  
808 analysis of ERK, p38, and JNK protein expression and their phosphorylated forms in the uterus and  
809 placenta (n = 9/group). In all plots, values are expressed as means  $\pm$  SEM. Significant differences ( $p$

810 < 0.05) within each group are denoted by different letters, and the same letter between groups  
811 indicates lack of statistical significance. N.S., not significant. DHT, 5 $\alpha$ -dihydrotestosterone; INS,  
812 insulin.

813 **Figure 5. Electron microscopy and mitochondria-mediated ferroptosis-related gene and**  
814 **protein expression in pregnant rats exposed to DHT and/or insulin at GD 14.5.** Mitochondrial  
815 ultrastructural defects in the uterus (A1, B1, C1, and D1, mesometrial decidua) and placenta  
816 (junctional (A2, B2, C2, and D2) and labyrinth zones (A3, B3, C3, and D3)). Images are  
817 representative of two tissue replicates. Md, mesometrial decidua; Jz, junctional zone (maternal side);  
818 Lz, labyrinth zone (fetal side). Red asterisks indicate mitochondria, and white arrows indicate  
819 shrunken mitochondria with electron-dense cristae. Scale bars (500 nm) are indicated in the  
820 photomicrographs. qPCR analysis of mitochondrial genes involved in modulating ferroptosis (E, n =  
821 8/group). ELISA analysis of MOTS-c content (F, n = 8/group). In all plots, values are expressed as  
822 means  $\pm$  SEM. Significant differences ( $p < 0.05$ ) within each group are denoted by different letters,  
823 and the same letter between groups indicates lack of statistical significance. N.S., not significant.  
824 DHT, 5 $\alpha$ -dihydrotestosterone; INS, insulin.

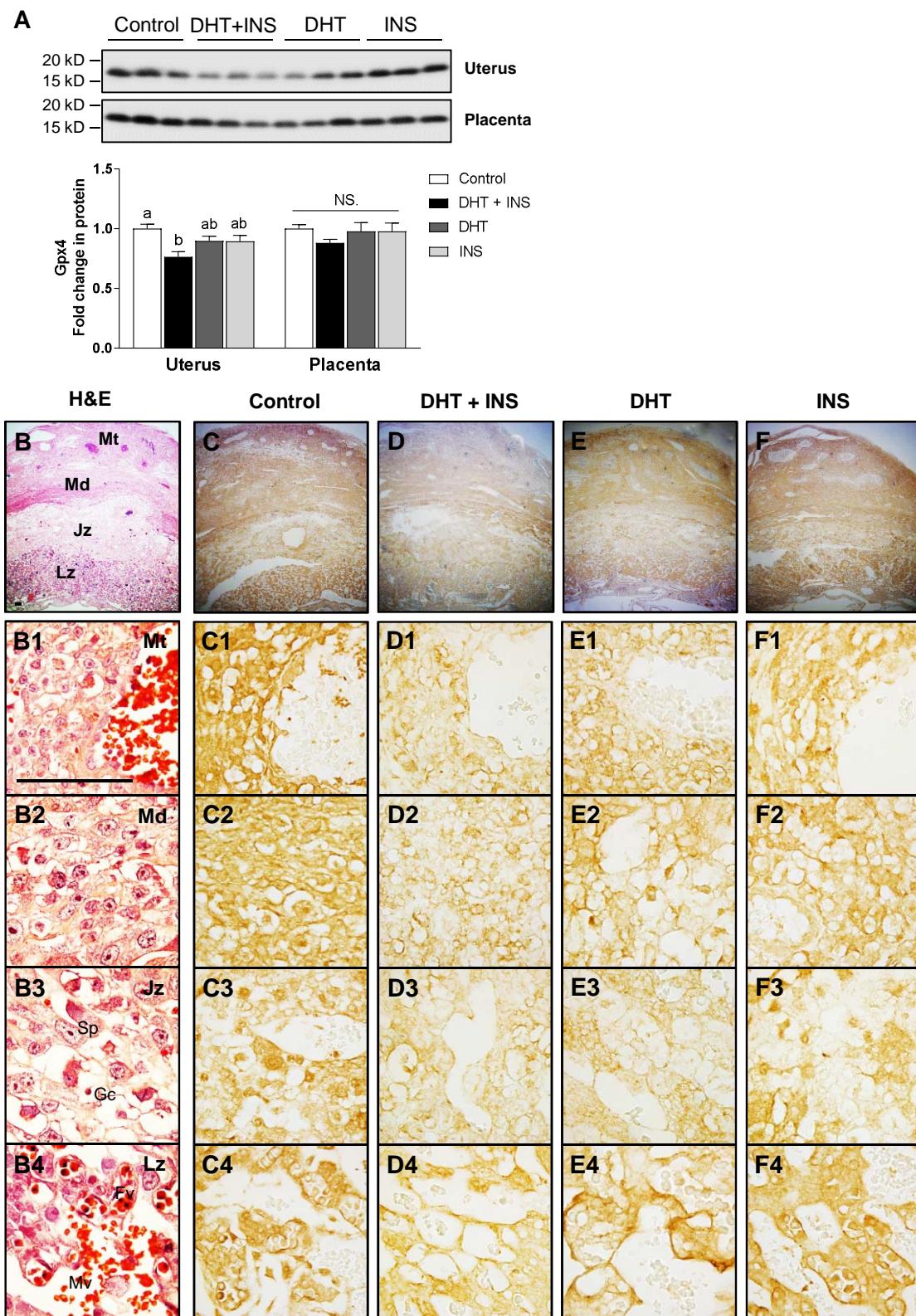
825 **Figure 6. The regulatory pattern of necroptosis-related and pro-/anti-apoptosis-related gene**  
826 **and protein expression in pregnant rats exposed to DHT and/or insulin at GD 14.5.** qPCR  
827 analysis of *Milkl*, *Ripk1*, *Ripk3*, *Bcl2*, *Bcl-xl*, *Bax*, *Bak*, and *Casp3* mRNA in the uterus and placenta (A  
828 and B, n = 8/group). Western blot analysis of cleaved caspase-3 protein expression in the uterus and  
829 placenta (C, n = 9/group). In all plots, values are expressed as means  $\pm$  SEM. Significant differences  
830 ( $p < 0.05$ ) within each group are denoted by different letters, and the same letter between groups  
831 indicates lack of statistical significance. N.S., not significant. DHT, 5 $\alpha$ -dihydrotestosterone; INS,  
832 insulin.

**Table 1.** Primer sequences used for qPCR measurement.

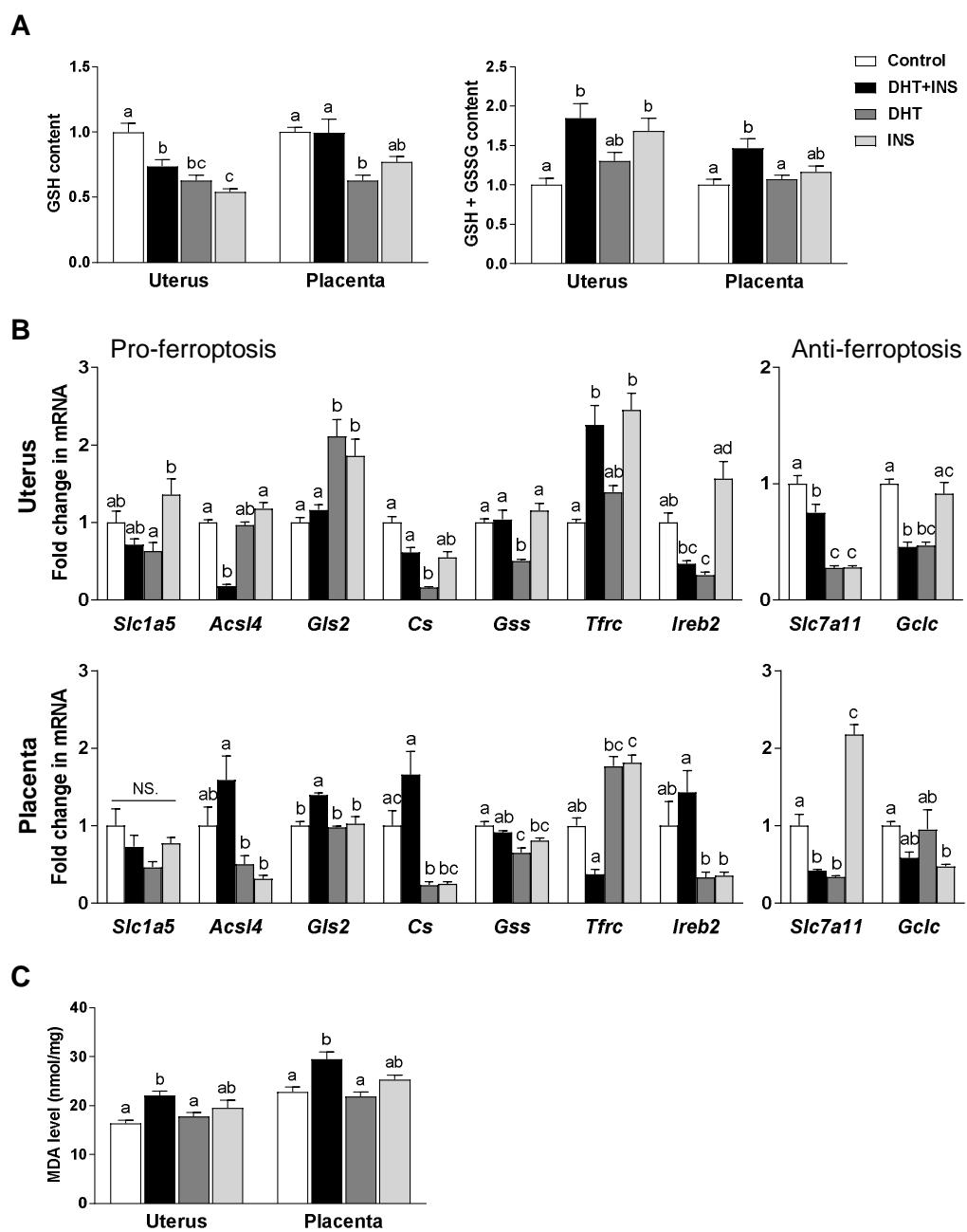
Gene	Primer Sequence (5'-3')		Reference Sequence	Product Size (bp)
<i>Slc1a5</i>	Forward	TCGGGACCTCTTAGCTCT	NM_175758.3	90
	Reverse	TGAACCGGCTGATGTGTTG		
<i>Acsl4</i>	Forward	CTCCTGCTTACCTACGGCT	NM_053623.1	97
	Reverse	ACAATCACCCCTTGCTTCCCT		
<i>Gls2</i>	Forward	GGCCAAGTCAAACCCAGATC	NM_001270786.1	153
	Reverse	TAGTCGGTGCCTAAGGTGC		
<i>Cs</i>	Forward	AGTGCCAGAAACTGCTACCT	NM_130755.1	117
	Reverse	GTGAGAGCCAAGAGACCTGT		
<i>Gclc</i>	Forward	AAGCCATAAACAAAGCACCCC	NM_012815.2	116
	Reverse	CGGAGATGGTGTGTTCTTGTG		
<i>Gss</i>	Forward	ATGCCGTGGTGCTACTGATT	NM_012962.1	107
	Reverse	TCTTCGGCGGATTACATGGA		
<i>Tfrc</i>	Forward	AGGCTCCTGAGGGTTATGTG	NM_022712.1	204
	Reverse	AGATGAGGACACCAATTGCA		
<i>Ireb2</i>	Forward	TGTTTGAAGAAGCCGACCTG	NM_022863.2	97
	Reverse	ACTCCCCACCCAAAGAATTCC		
<i>Slc7a11</i>	Forward	GTGCCCGGATCCAGATTTTC	NM_001107673.2	270
	Reverse	TGATGGCCATAGAGATGCAGA		
<i>Cisd1</i>	Forward	GCTAAAGAGAGTCGCACCAAAG	NM_001106385.2	113
	Reverse	CGGCAATACACGGCCTTATC		
<i>Dpp4</i>	Forward	GGCTGGTGCAGAAGATTAA	NM_012789.1	135
	Reverse	GACCTGTTGGGTTCTATC		
<i>Bcl2</i>	Forward	TTGCAGAGATGTCCAGTCAG	NM_016993.1	125
	Reverse	GAACCTCAAAGAAGGCCACAATC		
<i>Bcl-xl</i>	Forward	GGTGGTTGACTTCTCTCCTAC	NM_031535.2	116
	Reverse	TCTCCCTTCTGGTTCACTTTC		
<i>Bax</i>	Forward	GATGGCCTCCTTCCTACTTC	NM_017059.2	96
	Reverse	CTTCTTCCAGATGGTGAGTGAG		
<i>Bak</i>	Forward	GATGCCCTCAGCCTATTAAAG	NM_053812.1	115
	Reverse	CAGGAAGCCAGTCAAACCA		
<i>Casp3</i>	Forward	GACTGGAAAGCCGAAACTCT	NM_012922.2	97
	Reverse	TGCCATATCATCGTCAGTCC		
<i>Mlkl</i>	Forward	GGAACTGCTGGATAGAGACAAG	XM_008772570.2	117
	Reverse	CTGATTTCCGTGGAGTGT		
<i>Ripk1</i>	Forward	CAGGTACAGGAGTTGGTATGG	NM_001107350.1	108
	Reverse	TGTATGGCATGGGGTATG		
<i>Ripk3</i>	Forward	ACTGAGAGGAGAGGAAAGGAAG	NM_139342.1	107
	Reverse	CTGGAGGGTAGAGTATGTGGAA		
<i>Gapdh</i>	Forward	TCTCTGCTCCTCCCTGTTCTA	NM_017008.4	121
	Reverse	GGTAACCAGCGTCCGATAC		

*Slc1a5*, solute carrier family 1 member 5; *Acsl4*, acyl-CoA synthetase long-chain family member 4; *Gls2*, glutaminase 2; *Cs*, citrate synthase; *Gclc*, glutamate-cysteine ligase catalytic subunit; *Gss*, glutathione synthetase; *Tfrc*, transferrin receptor; *Ireb2*, iron responsive element binding protein 2; *Slc7a11*, solute carrier family 7 member 11; *Cisd1*, CDGSH iron sulfur domain 1; *Dpp4*, dipeptidylpeptidase 4; *Bcl2*, b-cell lymphoma 2; *Bcl-xl*, b-cell lymphoma-extra large; *Bax*, bcl-2-like protein 4; *Bak*, bcl-2 homologous antagonist killer; *Casp3*, caspase 3; *Mlkl*, mixed lineage kinase domain like pseudokinase; *Ripk1*, receptor interacting serine/threonine kinase 1; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase.

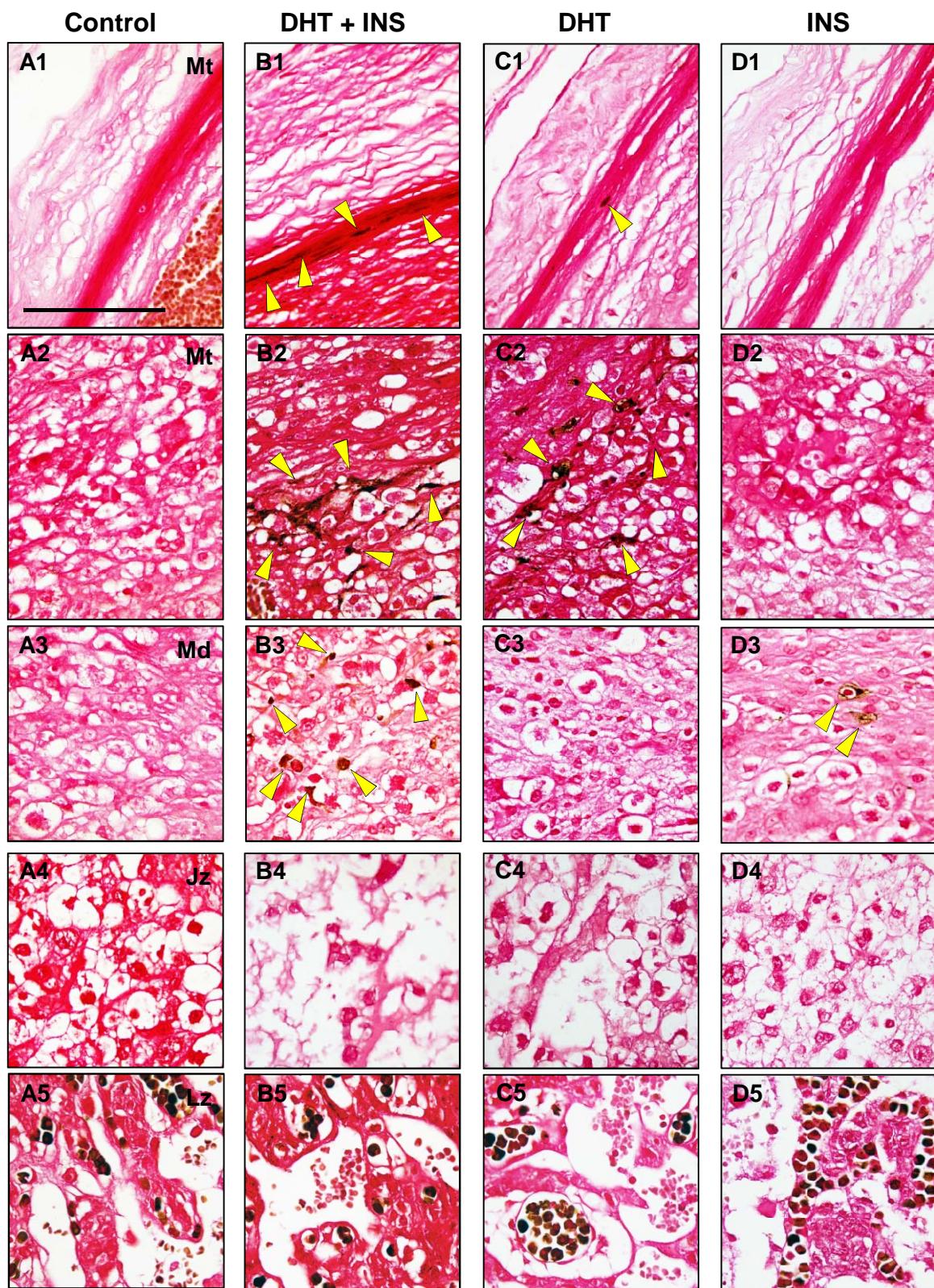
**Figure 1**



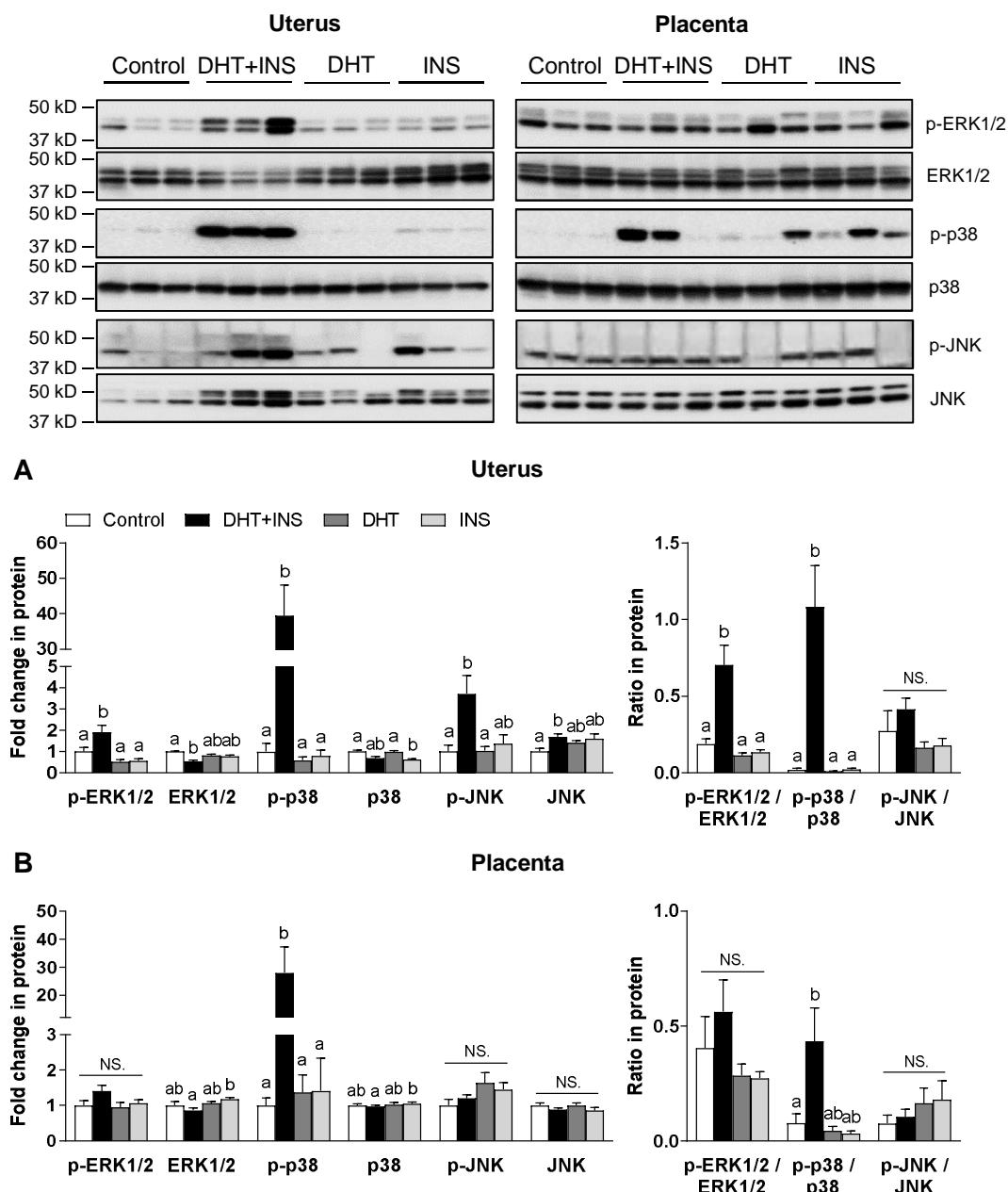
**Figure 2**



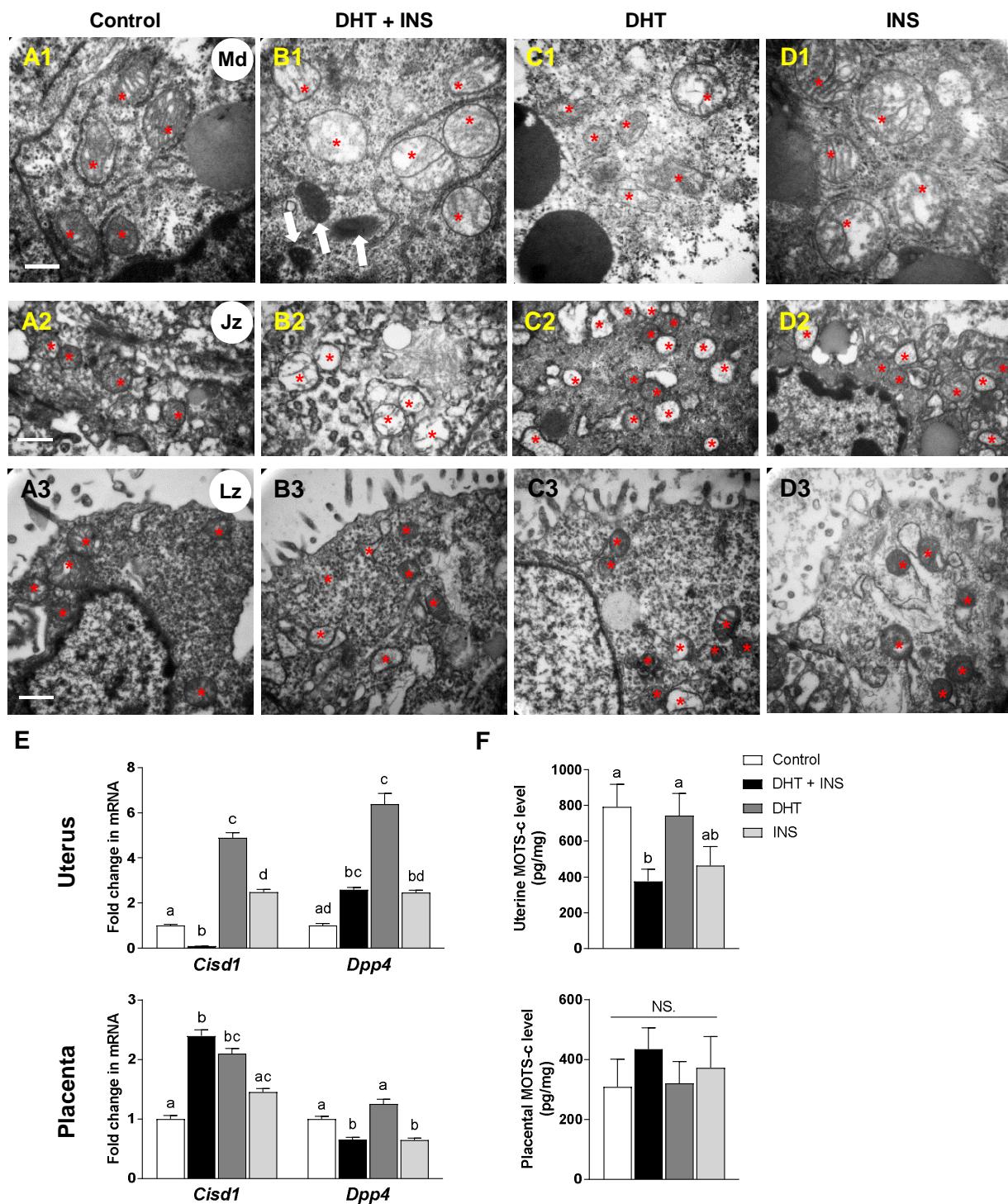
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

