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► To cite this version:

Melissa Buscato, Morgane Davezac, Rana Zahreddine, Marine Adlanmerini, Raphaël Métivier, et al.. Estetrol prevents western diet-induced obesity and atheroma independently of hepatic estrogen receptor (ER) α . *AJP - Endocrinology and Metabolism*, 2021, 320 (1), pp.E19-E29. 10.1152/ajpendo.00211.2020 . hal-03006849

HAL Id: hal-03006849

<https://hal.science/hal-03006849>

Submitted on 20 Nov 2020

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1 **Estetrol prevents western diet-induced**
2 **obesity and atheroma independently of hepatic estrogen receptor (ER) α**
3

4 Mélissa Buscato^{1(*)}, Morgane Davezac^{1(*)}, Rana Zahreddine¹, Marine Adlanmerini¹, Raphaël Métivier²,
5 Marianne Fillet³, Gael Cobraiville³, Cedric Moro¹, Jean-Michel Foidart⁴, Françoise Lenfant¹, Pierre
6 Gourdy^{1,5}, Jean-François Arnal^{1(*)#}, Coralie Fontaine^{1(*)#}.

7 1 I2MC, Institut National de la Santé et de la Recherche Médicale (INSERM) U 1048, University of Toulouse 3, Toulouse, France

8 2 CNRS, Univ Rennes, IGDR (Institut de Génétique De Rennes) – UMR 6290, F-35000 Rennes, France

9 3 Laboratory for the Analysis of Medicines, Center for Interdisciplinary Research on Medicines (CIRM), University of Liege, Quartier
10 hopital, Avenue Hippocrate 15, 4000 Liege, Belgium

11 4 Université de Liège, Groupe Interdisciplinaire de Génomprotéomique Appliquée, Liège, Belgique

12 5 Département de Diabétologie, Maladies Métaboliques et Nutrition, CHU de Toulouse, Toulouse, France

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14 (*) the authors equally contributed to this work

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16 **Running head:** Estetrol induces metabolic protection

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22 **Supplemental Material available at:**

23 URL: <https://figshare.com/s/4dc6a3487867f9260a94>

24 DOI: <https://doi.org/10.6084/m9.figshare.12249383>
25
26
27
28

29 **# Corresponding authors:**

30 Jean-François Arnal

31 Tel: +33 5 31 22 40 98

32 E-mail: jean-francois.arnal@inserm.fr

33 Coralie Fontaine

34 Tel: +33 5 31 22 40 89

35 E-mail: coralie.fontaine@inserm.fr

36 INSERM/UPS UMR 1048 - I2MC

37 **Institut des Maladies Métaboliques et Cardiovasculaires**

38 1 avenue Jean Poulhès, BP 84225

39 31432 Toulouse Cedex 4
40

41 **ABSTRACT**

42 Estetrol (E4), a natural estrogen synthesized by the human fetal liver, is currently evaluated in
43 phase III clinical studies as a new menopause hormone therapy. Indeed, E4 significantly
44 improves vasomotor and genito-urinary menopausal symptoms and prevents bone
45 demineralization. Compared to other estrogens, E4 was found to have limited effects on
46 coagulation factors in the liver of women allowing to expect less thrombotic events. To fully
47 delineate its clinical potential, the aim of this study was to assess the effect of E4 on
48 metabolic disorders. Here, we studied the pathophysiological consequences of a western diet
49 (42% kcal fat, 0.2% cholesterol) in ovariectomized female mice under chronic E4 treatment.
50 We showed that E4 reduces body weight gain and improves glucose tolerance in both
51 C57Bl/6 and LDLR^{-/-} mice. To evaluate the role of hepatic ER α in the preventive effect of E4
52 against obesity and associated disorders such as atherosclerosis and steatosis, mice harbouring
53 a hepatocyte-specific ER α deletion (LERKO) were crossed with LDLR^{-/-} mice. Our results
54 demonstrated that, whereas liver ER α is dispensable for the E4 beneficial actions on obesity
55 and atheroma, it is necessary to prevent steatosis in mice. Overall, these findings suggest that
56 E4 could prevent metabolic, hepatic and vascular disorders occurring at menopause,
57 extending the potential medical interest of this natural estrogen as a new hormonal treatment.

58

59 **New & Noteworthy :**

- 60 • Estetrol prevents obesity, steatosis and atherosclerosis in mice fed a western diet
61 • Hepatic ER α is necessary for the prevention of steatosis, but not of obesity and
62 atherosclerosis

63 **Key words:** estrogen receptor, menopause, fatty liver, atherosclerosis, metabolic syndrome

64

65 INTRODUCTION

66 The risk for women to develop metabolic disorders increases significantly after
67 menopause, especially in countries with unbalanced diet habits. Accordingly, postmenopausal
68 women exhibit a higher risk of developing type 2 diabetes, non-alcoholic fatty liver disease
69 (NAFLD) and atherosclerosis (32). Estrogens are well-recognized as key regulators of energy
70 balance and glucose homeostasis. Indeed, cessation of the production of 17- β estradiol (E2)
71 by ovaries at menopause promotes visceral adiposity and insulin resistance resulting in an
72 increased risk of type 2 diabetes (7). In addition, hormonal therapy (HT) was found to reduce
73 the incidence of type 2 diabetes in postmenopausal women (29). In randomized placebo-
74 controlled studies, a significant 21-35% decrease in diabetes occurrence was observed in
75 menopausal women receiving the association of conjugate equine estrogens (CEE) and
76 medroxyprogesterone acetate (MPA) (38). However, HT is associated with two major adverse
77 effects: i) an increased risk of thromboembolic events, probably as a consequence, at least in
78 part, of an increase in hepatic-derived coagulation factors and ii) an increased incidence of
79 breast cancers (39). The identification of safer compounds for HT is thus needed.

80 Estetrol (E4) is a natural estrogen produced only by the human fetal liver from the 9th
81 week gestation. It could be an interesting candidate in oral contraception (14) but also for HT
82 (17). Indeed, E4 treatment did not appear to increase the level of hepatic-derived coagulation
83 factors, and therefore might not increase the risk of thromboembolic events (17, 26). E4 used
84 in clinical studies is synthesized from estrone contained in soy and is identical to the natural
85 hormone with over 99% purity. E4 is currently evaluated in phase III clinical studies for HT
86 and a recent multiple-rising-dose study showed that it had beneficial effects on vasomotor
87 symptoms, bone metabolism and genito-urinary syndrome in menopausal women (10, 11).
88 However, the effect of E4 on metabolic disorders and associated diseases are poorly
89 described.

90 E4 is a natural estrogen but its physiological role remains unknown. As E2, the main
91 natural estrogen in adults, E4 activates the nuclear Estrogen Receptor alpha (ER α) to regulate
92 genomic transcription (1). But in contrast with E2, E4 does not activate membrane ER α and
93 even inhibits these membrane-initiated steroid signals (MISS) effects of E2 (1). Thus, while
94 E2 elicits endothelial NO synthase activation and accelerates endothelial healing, E4 exerts no
95 effect by itself, and even abrogates these E2-induced actions (1). E4 is thus considered as a
96 natural estrogen with selective activation of ER α depending on the tissues, meaning that E4
97 can act as an agonist or an antagonist of ER α in some target cells.

98 Selective estrogen receptor modulators (SERMs) belong to a family of drugs used in
99 various estrogen-related diseases such as tamoxifen (TAM) for the treatment of breast cancer,
100 raloxifene for the treatment of osteoporosis, or bazedoxifene, in association to estrogens, for
101 the management of postmenopausal symptoms. As E2, SERMs confer protection from
102 metabolic disorders observed in response to high-fat diets (9, 43, 44). However, molecular
103 and cellular targets accounting for their protective action against a metabolic stress appear to
104 differ greatly from that of E2, although mediated by ER α in all cases (9). While E2 improves
105 metabolic homeostasis, at least partially, by increasing hepatic production of fibroblast growth
106 factor 21 (FGF21), bazedoxifene increases hepatic expression of Sirtuin1, PPAR α and AMPK
107 activity (25). In addition, we recently demonstrated that, whereas E2 still prevented obesity
108 and glucose intolerance in liver-specific ER α knock out (LERKO) mice, similar beneficial
109 metabolic actions of tamoxifen were totally abrogated in those mice. Furthermore, and by
110 contrast to E2, the whole-body metabolic protection of tamoxifen is entirely dependent on the
111 hepatokine growth differentiation factor 15 (GDF15) (21). Interestingly, GDF15 is induced in
112 response to chronic exposure to tamoxifen (21) and bazedoxifene (6) but not to E2.
113 Accordingly, ER α binding sites were found in *Gdf15* regulatory region in liver from TAM-
114 treated mice but not from E2-treated mice, due to specific epigenetic modifications (21).

115 The aim of this study was to evaluate the ability of E4 to prevent obesity and
116 associated metabolic and vascular disorders in ovariectomized female mice fed a western diet.
117 We demonstrated that E4 protects against body weight gain and glucose intolerance in both
118 C57Bl/6 and LDLR^{-/-} mice and also prevents steatosis and atherosclerosis. We finally
119 demonstrate that, whereas liver ER α is necessary to the prevention of steatosis in response to
120 E4, it is fully dispensable for E4 protection against obesity and atheroma, highlighting
121 dissociation between global metabolic effect of E4 and its liver impact.

122 **MATERIAL & METHODS**

123 **Mice and surgical procedure**

124 All procedures involving experimental animals were performed in accordance with the
125 principles and guidelines established by the National Institute of Medical Research
126 (INSERM) and were approved by the local Animal Care and Use Committee (CEEA-122 :
127 2014-56). The investigation is consistent with the directive 2010/63/EU of the European
128 parliament. ER α AF2⁰ mouse line was generated by deletion of seven amino acids from helix
129 12 of the AF2 domain as described previously (5). To generate the double-deficient mice,
130 LDLR^{-/-} female mice, purchased from Charles River (L'Arbresle, France), were crossed with
131 AlbCreER $\alpha^{\text{flox/flox}}$ mice (LERKO) (21). WT mice are on a C57Bl/6 background and
132 genetically manipulated mice are systematically compared to their wild type littermates
133 control (n= 7 to 12 per group). Bilateral ovariectomy was performed on 4 weeks of age
134 females, after intraperitoneal injection of ketamine (100 mg.kg⁻¹) and xylazine (10 mg.kg⁻¹).
135 Two weeks after surgery, mice were switched to a western diet (42% kcal fat, 0.2%
136 cholesterol, Envigo) and concomitantly implanted with *s.c.* pellets releasing either vehicle or
137 E4 at 6, 9 and 12 weeks of age to evaluate metabolic and vascular functions.

138

139

140 **Pellet preparation and E4 Dosage**

141 To deliver E4 over prolonged periods of time, E4 (Mithra) and cholesterol (C3045 Sigma-
142 Aldrich) were thoroughly mixed in powder form and compacted to obtain pellets
143 (weight/weight ratio of E4/cholesterol at 1/20). Mice were implanted subcutaneously every 3
144 weeks with E4 pellets (1 mg E4/pellet total) or a vehicle (cholesterol only) pellets as recently
145 described (16). Blood E4 concentration was evaluated as previously described (34).
146 Preliminary experiments determined a 5 ng/ml plasma concentration of E4 after 3 weeks of
147 this treatment, corresponding to the level observed 3h after a single oral administration of E4
148 at 1 mg/kg/day (19) (**Supplementary Figure 1**). This plasma concentration is clinically
149 relevant, as it is comparable to those observed in women receiving chronically a daily
150 administration of 15 mg E4 (2 to 5 fold higher) (Mithra communication study MIT ES 0001).

151 **Metabolic tests**

152 Intra-peritoneal glucose tolerance test (IPGTT) was performed by injecting D-glucose (1 g/kg,
153 i.p) after overnight fasting in 13-week-old mice. Blood glucose concentrations were
154 monitored with a glucose meter (Roche Diagnostic, Meylan, France) at the indicated time
155 points: 30 min prior to (-30), just before (0) and at 30, 60, and 90 min after glucose injection.

156 Basal metabolism was evaluated by indirect calorimetry after 8 weeks of treatment. Mice
157 were individually housed for 48 hours where the first 24 hours corresponded to
158 acclimatization and the subsequent 24 hours to measurement of energy expenditure
159 (Phenomaster; TSE-Systems) at 10-min intervals at constant temperature (22°C). Physical
160 activity was monitored by an infrared photocell beam interruption method.

161 **Liver and perigonadal adipose tissue histology**

162 Liver and perigonadal adipose tissues were quickly excised, fixed in 10% buffered formalin
163 and embedded in paraffin. Sections (3 µm) were stained with haematoxylin/eosin (H&E) and
164 oil red O as previously described (21). Images were obtained with nanozoomer scanner

165 (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with NDP view software
166 (Hamamatsu Photonics, Hamamatsu, Japan).

167 **Liver lipid content analysis**

168 Hepatic levels of triglycerides and cholesterol esters were determined using Bligh & Dyer
169 methodology and lipid extracts were analyzed by gas-liquid chromatography as previously
170 described (21).

171 **Gene expression analysis**

172 Tissues were homogenized using a Precellys tissue homogenizer (Bertin Technol., Cedex,
173 France) and total RNA from tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA).
174 1000 ng for liver and 500 ng for aorta of RNA was reverse transcribed (RT) at 25°C for 10
175 min and then at 37°C for 2 h using the High Capacity cDNA reverse transcriptase kit (Applied
176 Biosystems). For gene expression in aorta and liver, qPCR were performed using SsoFast
177 EvaGreen Supermix (Bio-Rad) with primers efficiency validated using standard curves
178 method (95% < efficiency < 105%) (**Supplemental Table 1**). Gene expression was quantified
179 using the comparative C_t (threshold cycle) method. *Tpt1* and *Ppia* were used as housekeeping
180 gene to normalize the mRNA levels respectively in the aorta and the liver.

181 **Analysis of atherosclerosis lesions**

182 Overnight fasted mice were anesthetized, and blood was collected from the retro-orbital
183 venous plexus before dissecting the heart. Lipid deposition was evaluated at the aortic sinus as
184 previously described (4). Briefly, each heart was frozen on a cryostat mount with OCT
185 compound. One hundred 10 µm thick sections were prepared from the top of the left ventricle,
186 where the aortic valves were first visible, up to a position in the aorta where the valve cusps
187 were just disappearing from the field. After drying for 2 hours, the sections were stained with
188 oil red O and counterstained with Mayer's hematoxylin. Ten sections out of the 100, each
189 separated by 90 µm, were used for specific morphometric evaluation of intimal lesions using

190 a computerized Biocom morphometry system. The first and most proximal section to the heart
191 was taken 90 μm distal to the point where the aorta first becomes rounded. The mean lesion
192 size (expressed in μm^2) of these 10 sections was used for each animal.

193 **Determination of plasma lipids**

194 Plasma triglycerides were assayed using ABX Pentra Triglycerides CP (ABX Pentra, Horiba,
195 Montpellier, France). The high density lipoprotein (HDL) and low density lipoprotein (LDL)
196 fractions were isolated from 10 μl of serum and assayed using the “ABX Pentra HDL Direct
197 CP” method (ABX Pentra, Horiba, Montpellier, France).

198 **Statistics**

199 Results are expressed as means \pm SEM. Statistical analyses were performed using GraphPad
200 Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). T-test was used to
201 analyze the effect of treatment in WT or LDLR^{-/-} mice. *ANOVA-2 factor* were used to analyze
202 the effects of treatment and genotype. Fischer LSD post-test were subsequently performed in
203 case of interaction. $P < 0.05$ was considered statistically significant (* $P < 0.05$, ** $P < 0.01$,
204 *** $P < 0.001$).

205 **RESULTS**

206 **ER α AF2 is necessary to mediate E4 beneficial effect against western-diet induced** 207 **obesity and steatosis.**

208 To evaluate the metabolic impact of E4, we first determined whether E4 was able to confer
209 protection against western diet-induced obesity as previously described in response to E2 and
210 SERMs. To this aim, ovariectomized WT female mice were fed a western diet (42% kcal fat,
211 0.2% cholesterol) and concomitantly implanted with subcutaneous E4 pellets or a vehicle for

212 9 weeks (**Figure 1A**). Weekly body weight measurements revealed that E4-treated mice are
213 protected against western diet-induced obesity from the first weeks of treatment (**Figure 1B**).

214 After 7 weeks of western diet feeding, E4 treatment improved glucose tolerance in overnight-
215 fasted mice, as illustrated by lower plasma glucose levels at 30, 60 and 90 min following
216 glucose challenge, resulting in a 20% decrease in the Area Under the Curve (AUC) for
217 glucose (**Figure 1C**). At the end of the protocol, E4-treated mice were remarkably leaner
218 (**Supplementary Figures 2A and 2B**). Fasting glucose levels were significantly lower in E4-
219 treated mice (**Supplementary Figure 2C**), while insulin levels tended to be lower, although
220 this difference did not reach statistical significance (**Supplementary Figure 2D**).
221 Nevertheless, insulin sensitivity was preserved in E4-treated mice compared to the vehicle
222 group, as attested by a lower HOMA-IR mean value (**Figure 1D**). Uterine impregnation was
223 chosen as a positive control of estrogenic action and, as expected, ovariectomy led to uterine
224 atrophy, whereas chronic E4 treatment increased uterine weight (**Figure 1E**). E4 treatment led
225 to reduced accumulation of subcutaneous, perigonadal and mesenteric adipose tissues (**Figure**
226 **1F**). The mean area of adipocytes from perigonadal adipose tissue was also significantly
227 lower in E4-treated mice compared to control mice (**Figure 1G**). Moreover, protection
228 conferred by E4 included a lower lipid accumulation in the liver (**Figure 1H**). Accordingly,
229 mRNA levels of genes involved in lipid metabolism, including cholesterol metabolism and
230 lipoprotein assembly (i.e. *Cd36*, *Pltp* and *Apoa4*) were decreased in the liver from E4-treated
231 mice compared to vehicle control mice (**Supplementary Figure 3**). E4 treatment also
232 regulated expression of some hepatokines with an increase in Growth differentiation factor 15
233 (*Gdf15*) but a decrease in Fibroblast growth factor 21 (*Fgf21*) and Angiotensin-like 6
234 (*Angptl6*) mRNA levels. (**Supplementary Figure 3**).

235 To further explore the beneficial effect of E4 on metabolism, we then analyzed basal
236 metabolism by indirect calorimetry after 8 weeks of E4 treatment. As expected, both control

237 and E4-treated mice elicited a marked increase in physical activity during the dark (active)
238 phase (**Figure 2A**). More importantly, during this period the physical activity was
239 significantly higher in E4-treated mice than in the control group. Accordingly, E4 treatment
240 induced higher energy expenditure during the dark phase (**Figure 2B**). Altogether, these
241 results suggest that E4 protects ovariectomized WT female mice from western diet-induced
242 obesity at least in part by increasing locomotor activity and energy expenditure.

243 To evaluate the role of ER α and more particularly of its transcriptional activity in this E4
244 metabolic protection, we then used ER α AF2⁰ mice. In this mouse model, nuclear action of
245 ER α is disrupted due to the deletion of seven amino acids in the helix 12 while membrane
246 initiated signalling are preserved, as previously reported (2) (**Figure 3A**). As expected, E4 led
247 to an increase of uterine weight in ER α AF2^{+/+} mice but not in ER α AF2⁰ mice, reinforcing the
248 demonstration of the crucial role of the nuclear function of ER α in uterine response to E4 (1)
249 (**Figure 3B**). Likewise, although E4 prevented body weight gain (**Figure 3C and 3D**) and
250 accumulation of subcutaneous, perigonadal and mesenteric adipose tissues (**Table 1**) in the
251 control ER α AF2^{+/+} mice, this beneficial action of E4 was no longer observed in ER α AF2⁰
252 mice. The protective effect of E4 against lipid accumulation in the liver was also abrogated in
253 ER α AF2⁰ mice compared to their ER α AF2^{+/+} littermates (**Figure 3E and Table 1**),
254 highlighting the role of ER α and its nuclear/AF2-dependent action in E4 metabolic protection.

255 **Estetrol prevents obesity as well as associated vascular and hepatic metabolic disorders**
256 **induced by western diet in ovariectomized LDLR^{-/-} mice.**

257 We then evaluated the effect of E4 on lipid lesion deposits at the aortic sinus using LDLR^{-/-}
258 mice known to develop both atherosclerosis and steatosis in response to western diet (3, 24,
259 40, 42) (**Figure 4A**). As previously observed in WT C57Bl/6 mice, E4 also prevented diet-
260 induced obesity (**Figure 4B**), improved glucose tolerance (**Figure 4C**) and protected the liver
261 against triglycerides and cholesterol ester accumulation (**Figures 4D and 4E**) in this LDLR^{-/-}

262 mouse model. Importantly, this metabolic protection was associated with less fatty streak at
263 the aortic sinus (**Figures 4F and 4G**) and a decreased expression of endothelial activation
264 markers, such as Vascular Cell Adhesion Molecule 1 (*Vcam1*) and Intercellular Adhesion
265 Molecule 1 (*Icam1*) (**Figure 4H**) in the aorta from E4-treated mice. Moreover, E4 treatment
266 also decreased mRNA expression of pro-inflammatory cytokines such as interleukin (*Il1 β*),
267 macrophage inflammatory protein 1-alpha (*Mip1 α*), and of the macrophage marker (*Cd68*)
268 (**Figure 4H**). Altogether, these results demonstrate that, in addition to the protection against
269 diet-induced obesity and steatosis, E4 also conferred atheroprotection.

270 **Hepatic ER α is necessary for E4-mediated prevention of steatosis but dispensable for its**
271 **protective action against obesity and atherosclerosis.**

272 Hepatic ER α has been proposed to contribute to the protective effect of E2 on both
273 atherosclerosis (49) and steatosis (13, 21, 23, 31), and is also necessary for the prevention of
274 diet-induced obesity in response to some SERMs such as TAM (21). Chronic E4 treatment
275 did not regulate ER α protein level in liver from chow diet-fed mice (**Supplementary Figure**
276 **4A**). However, ER α protein level was decreased in liver from mice on western diet compared
277 to mice on chow diet but this effect was prevented by E4 treatment (**Supplementary Figure**
278 **4B**). We then evaluated the transcriptional response induced by E4 on a set of ER α target-
279 genes previously identified in liver (35). To avoid the bias associated to the protective action
280 of E4 against steatosis under western diet feeding, the transcriptional response induced by E4
281 was studied in liver from chow diet-fed mice. Most of the known ER α target genes tested
282 were found to be regulated by E4 (**Supplemental Table 2**). The comparison of the
283 transcriptional responses induced by E4, the full agonist E2 or the well-known SERM, TAM,
284 identified subsets of genes specifically regulated by E4 or regulated by both E4 and TAM but
285 not by E2 (**Supplemental Table 2**). Importantly, ChIP-qPCR analyses revealed the
286 recruitment of ER α induced by E4 in the regulatory region of several of these genes, further

287 highlighting the direct role of ER α in this transcriptional response to E4 (**Supplementary**
288 **Figure 5**).

289 Then, to directly evaluate the role of hepatic ER α , we analyzed the metabolic effects of
290 chronic E4 treatment in mice harbouring a hepatocyte-specific ER α deletion (LERKO) bred
291 with atheroprone LDLR^{-/-} mice. Efficiency of ER α deletion in hepatocytes was confirmed by
292 the 80 % decrease of ER α mRNA level measured by qPCR in LERKO-LDLR^{-/-} mice
293 compared to the control littermate mice (**Supplementary Figure 6**). Ovariectomized
294 LERKO-LDLR^{-/-} mice treated or not with E4 were fed a western diet for 9 weeks. The
295 protection conferred by E4 against western diet-induced obesity was fully preserved in
296 LERKO-LDLR^{-/-} mice (**Figure 5A and Supplementary Figure 7**), demonstrating that
297 hepatic ER α is not required to induce this beneficial action of E4. Hence, E4 reduced fat mass
298 accumulation to a similar extent in WT-LDLR^{-/-} and LERKO-LDLR^{-/-} mice, at both
299 subcutaneous and visceral sites (**Figure 5C**). In addition, E4 exposure protected similarly
300 WT-LDLR^{-/-} and LERKO-LDLR^{-/-} female mice from glucose intolerance (**Figure 5B**) and
301 atherosclerosis (**Figure 5D**) induced by western diet. Plasma HDL- and LDL-cholesterol
302 profiles were also similar in WT-LDLR^{-/-} and LERKO-LDLR^{-/-} mice whereas the decrease in
303 plasma triglycerides induced by E4 treatment in WT-LDLR^{-/-} mice was totally abrogated in
304 LERKO-LDLR^{-/-} mice (**Figure 5E**). In addition, whereas E4 treatment prevented intrahepatic
305 accumulation of triglycerides and cholesterol ester in WT-LDLR^{-/-} control mice, this effect
306 was abolished in LERKO-LDLR^{-/-} mice (**Figures 6A and 6B**). Accordingly, analysis of lipid
307 droplets revealed that the liver protection conferred by E4 treatment was no longer observed
308 in LERKO-LDLR^{-/-} mice (**Figure 6C**), demonstrating the crucial role of hepatic ER α to the
309 protection against steatosis in response to E4.

310 **DISCUSSION**

311 E4 is an estrogen currently considered for its highly promising clinical potential, in particular
312 as a new HT for menopause. In this therapeutic indication, phase III development is ongoing
313 but phase II clinical investigations already provided positive results (18). Indeed, in a
314 multiple-rising-dose study involving postmenopausal women, E4 improved vasomotor
315 symptoms and vaginal cytology, and a dose-dependent estrogenic effect was observed on
316 endocrine parameters, bone turnover markers, lipids and lipoproteins, along with only a small
317 effect on haemostatic parameters (10, 11). However, although this is an important issue to
318 consider for HT, the ability of E4 to prevent postmenopausal metabolic disorders was still
319 unknown. Indeed, the decline in endogenous estrogens at menopause leads to a redistribution
320 of body fat, characterized by accumulation of visceral adiposity (7, 29, 41), which contributes
321 to an increased risk of cardiovascular diseases, type 2 diabetes and steatosis (15). The major
322 finding of this study is that a chronic E4 exposure prevents body weight gain, glucose
323 intolerance and steatosis in ovariectomized C57Bl/6 mice submitted to a western diet. These
324 findings were confirmed in the atheroprone LDLR^{-/-} mouse model in which E4 treatment also
325 prevented lipid accumulation at the aortic sinus highlighting the atheroprotective effect of E4
326 in such an obesity context.

327 We previously reported that E4 was able to activate the nuclear actions of ER α but not its
328 membrane initiated signalling (1). Here, we showed that ER α AF2 is necessary for the
329 prevention of weight gain and associated disorders in response to E4, further demonstrating
330 the importance of nuclear activation of ER α in E4-mediated action. However, to which extent
331 this action is mediated through ERE-dependent pathway or involves other signalling pathways
332 such as protein-protein interaction with other transcription factors as AP-1 or SP1 remains to
333 be determined. Indeed, several studies highlighted the importance of the non-classical
334 genomic ER α signalling pathways in the beneficial action of E2 against metabolic
335 disturbances (36, 47).

336 Our observations in LERKO mice demonstrated that hepatic ER α is not necessary to the
337 beneficial effects of E4 on body weight gain, glucose tolerance and atheroma. The increase in
338 physical activity and energy expenditure conferred by E4 is reminiscent of the effects of a
339 chronic E2 treatment that we and others previously reported (20, 28) and rather suggests a
340 central effect of E4. Indeed, numerous studies have reported the crucial role of ER α in
341 specific area of the central nervous system in mediating the regulation of energy balance in
342 response to E2 (12, 33, 45, 46). ER α could also affects metabolism by controlling
343 mitochondrial dynamics and function in both white and brown adipose tissues (48) and the
344 role of ER α in adipocytes could also contribute to the preventive effect of E4 against obesity
345 and metabolic dysfunction.

346 Hepatic ER α is necessary for the prevention of steatosis conferred by E4. Nevertheless, we
347 cannot exclude the contribution of other nuclear receptors since crosstalk of ER α with other
348 nuclear receptor such as LXR and GR have been reported to mediate part of the protective
349 effect of E2 against steatosis (22, 37). Interestingly, we found that E4 chronic treatment
350 induces *Gdf15* mRNA level in the liver, correlated with a direct binding of ER α on its
351 promoter. We recently reported such a regulation in response to tamoxifen but not to E2 due
352 to epigenetic modification (21). *Gdf15* induction by tamoxifen is sufficient to prevent diet-
353 induced obesity, type 2 diabetes, and NAFLD (21). However, by contrast to tamoxifen,
354 selective deletion of hepatic ER α does not impact here the beneficial actions of E4 on body
355 weight gain and glucose tolerance suggesting that GDF15 is not the main effector of E4
356 action. More generally, we reported that E4 displays a specific transcriptional program in the
357 liver which differs from those of E2 and TAM. This underlines that each molecule displays a
358 highly specific profile of gene regulation in the liver, consistently with their differential action
359 on hepatic coagulation factors in humans (26, 30). In addition to the nuclear activity of ER α ,
360 selective activation of membrane ER α using “pathway preferential estrogens” (PaPEs) was

361 also reported to prevent steatosis (8, 27) and to regulate a specific pattern of genes that differs
362 from E2, through activation of mTOR and MAPK signalling pathways independently of ER α
363 DNA binding (27). Altogether, these results demonstrate that different ER α subfunctions can
364 be involved in a similar biological effect depending on the nature of the ligand through
365 specific transcriptional program.

366 To conclude, this study demonstrates that E4 confers metabolic and vascular protection in
367 mice. Together with the protection against osteoporosis and the limited impact on coagulation
368 factors associated with E4 administration, our results complete the really promising clinical
369 potential of this estrogen as a new hormonal treatment for menopause.

370 **ACKNOWLEDGMENTS**

371 The staff of the animal facilities and of the “Plateforme d’expérimentation fonctionnelle” is
372 acknowledged for their skillful technical assistance. We acknowledge the skillful technical
373 assistance of Anexplo-Genotoul, GeT-TQ Genopole and MetaToul-lipidomique.

374 **GRANTS**

375 The work at INSERM U1048 was supported by INSERM, CHU and Université de Toulouse
376 III, Faculté de Médecine Toulouse-Rangueil, ANR (NOSE4), Fondation de France (FDF,
377 N°00086486), Fondation pour la Recherche Médicale (FRM ECO201806006691), Institut
378 Universitaire de France and Mithra Pharmaceuticals. Work at the CNRS UMR6290 was
379 supported by the CNRS and University of Rennes1.

380 **DISCLOSURES**

381 JM. Foidart is a consultant at Mithra, the company that develops estetrol-based women's
382 healthcare products. JF Arnal at INSERM U 1048 / University of Toulouse 3 has received a
383 research grant from Mithra. The remaining authors have no disclosures to report.

384 **AUTHORS CONTRIBUTION**

385 MB, MD, RZ, RM performed and analyzed experiments. MF and GC performed estetrol
386 dosage. CM and JMF provided intellectual guidance in all phases of the study. FL, PG, MA,
387 JFA and CF designed the studies, analyzed experiments, wrote the manuscript and oversaw
388 the project.

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581 **FIGURE LEGENDS**

582 **Figure 1: Estetrol (E4) prevents western diet (WD)-induced obesity and steatosis in wild-**
583 **type (WT) female mice.**

584 Four-week-old ovariectomized female C57Bl/6 mice were implanted subcutaneously with E4
585 or Vehicle (Veh) pellets and concomitantly fed a western diet from the age of 6 to 15 weeks
586 (A). Body weight was measured every week (B). Intraperitoneal glucose tolerance test
587 (IPGTT) was performed at 13 weeks. AUC (area under the curve) is expressed as means \pm
588 SEM (C). HOMA-IR score (D), uterine weight (E) and adipose tissue distribution (F) (SC:
589 subcutaneous, PG: perigonadal and Mes: mesenteric) were evaluated at the end of the
590 protocol. Representative photomicrographs of perigonadal adipose tissue sections
591 (haematoxylin-eosin staining, scale bars: 400 μ m) and quantification of adipocyte mean area
592 (G) and representative images of haematoxylin and eosin (H/E) staining on liver sections
593 (scale bars: 330 μ m) and intrahepatic triglycerides measurement (H). *t*-test were used to
594 analyze the effect of treatment, except for body weight gain and glucose tolerance test for
595 which two-way ANOVA of variance test with repeated measures and a *Fisher's LSD post test*
596 were performed. (E4 exposure *versus* vehicle **P < 0.01, ***P < 0.001, n=5/6 mice/group).

597 **Figure 2: Estetrol (E4) enhances energy expenditure in wild-type (WT) female mice fed**
598 **a western diet.**

599 Daily profile of physical activity (A), and energy expenditure (B) measured in metabolic
600 chambers. Results are expressed as means \pm SEM measured each hour. Two-way ANOVA
601 test revealed a significant interaction between the 2 factors (treatment and periods), a *Fisher's*

602 *LSD* multiple comparison test was performed (Veh versus E4: *P < 0.05, ***P < 0.001, n=5/6
603 mice/group).

604 **Figure 3: E4 prevents western diet-induced obesity and steatosis through ER α AF2**
605 **activation.**

606 Schematic representation of the ER α AF2⁰ mice model (A). Four-week-old ovariectomized
607 ER α AF2⁰ female mice and their littermates were implanted subcutaneously with E4 or
608 Vehicle pellets and concomitantly fed a western diet from the age of 6 to 15 weeks.
609 Representative images of uteri (scale bars: 1cm) (B) and mice (C) at the end of the protocol
610 and cumulative weekly body weight gain (D) are presented. Representative images of
611 haematoxylin and eosin (H/E) staining of liver sections (scale bars: 310 μ m) (E). Results are
612 expressed as means \pm SEM measured each week. Two-way ANOVA of variance test with
613 repeated measures and a *Fisher's LSD post test* was performed (***P < 0.001, n=6/8
614 mice/group).

615 **Figure 4: Estetrol (E4) prevents atherosclerosis and hepatic steatosis in LDLR^{-/-} female**
616 **mice fed a western diet.**

617 Four-week-old ovariectomized female LDLR^{-/-} mice were implanted subcutaneously with E4
618 or Vehicle (Veh) pellets and concomitantly fed a western diet from the age of 6 to 15 weeks
619 (A). Cumulative weekly body weight gain is presented (B). Intraperitoneal glucose tolerance
620 test was performed at 14 weeks. AUC (area under the curve) is expressed as means \pm SEM
621 (C). Representative images of haematoxylin and eosin (H/E) staining on liver sections (scale
622 bars: 100 μ m) (D) and intrahepatic triglycerides and cholesterol ester measurement (E).
623 Representative image (F) and quantification of lipid deposition at the aortic sinus after Oil
624 Red O staining (scale bars: 1mm) (G). mRNA level of atherosclerotic markers in the aorta
625 were quantified by qPCR and normalized to *Tpt1* mRNA levels (H). Results are expressed as

626 means \pm SEM. *t*-test were used to analyze the effect of treatment, except for body weight gain
627 evolution and glucose tolerance test for which two-way ANOVA of variance test with
628 repeated measures and a *Fisher's LSD post test* was performed. (E4 exposure *versus* vehicle
629 *P<0.05, **P < 0.01, ***P < 0.001, n=5/6 mice/group).

630 **Figure 5: Hepatic ER α is not necessary for the beneficial effect of E4 on metabolic**
631 **disorders and atherosclerosis in LDLR^{-/-} female mice fed a western diet.**

632 Four-week-old ovariectomized female LERKO-LDLR^{-/-} mice and their littermates were
633 implanted subcutaneously with E4 or Vehicle (Veh) pellets and concomitantly fed a western
634 diet from the age of 6 to 15 weeks. Cumulative weekly body weight gain evolution (A),
635 intraperitoneal glucose tolerance (B) and adipose tissue distribution (C) are presented.
636 Quantification of lipid deposition at the aortic sinus (scale bars: 1mm) (D) and plasmatic
637 HDL-cholesterol, LDL-cholesterol and triglycerides content (E) were quantified. Two-way
638 ANOVA were performed to evaluate interaction between Genotype (Gen) and treatment (E4).
639 When a significant interaction between the 2 factors were revealed, a *Fisher's LSD* post-test
640 was performed (Veh *versus* E4: ***P < 0.001, n=6 mice/group).

641 **Figure 6: Hepatic ER α is necessary for the beneficial effect of E4 on hepatic steatosis in**
642 **LDLR^{-/-} female mice fed a western diet**

643 Four-week-old ovariectomized female LERKO-LDLR^{-/-} mice and their littermates were
644 implanted subcutaneously with E4 or Vehicle (Veh) pellets and concomitantly fed a western
645 diet for 9 weeks from the age of 6 to 15 weeks. Quantification of intrahepatic triglycerides (A)
646 and cholesterol ester (B) content. Representative images of haematoxylin and eosin (H/E)
647 (scale bars: 160 μ m) and Oil Red O staining (scale bars: 510 μ m) on liver sections (C). Two-
648 way ANOVA were performed to evaluate interaction between Genotype (Gen) and treatment
649 (E4). When a significant interaction between the 2 factors were revealed, a *Fisher's LSD* post-
650 test was performed (Veh *versus* E4: ***P < 0.001 n=6 mice/group).

Table 1: Metabolic parameters of ER α AF2^{+/+} vs ER α AF2⁰ mice treated with E4 or vehicle and fed a western diet

	ER α AF2 ^{+/+}		ER α AF2 ⁰		Two-factor ANOVA P, Interaction
	Veh	E4	Veh	E4	
Body weight (g)	38.3 \pm 2	24.5 \pm 0.9 ***	37.7 \pm 0.7	37.1 \pm 1.2	*** p<0.0001
Uterine weight (mg)	8.5 \pm 1.2	109.2 \pm 21 ***	5.5 \pm 0.7	5.1 \pm 0.5	*** p<0.0001
Subcutaneous Tissue (% body weight)	7.3 \pm 0.3	2.5 \pm 0.2 ***	8 \pm 0.3	7.1 \pm 0.2 *	*** p<0.0001
Perigonadal Tissue (% body weight)	5 \pm 0.1	1.2 \pm 0.05 ***	4.8 \pm 0.2	4.6 \pm 0.4	*** p<0.0001
Mesenteric Tissue (% body weight)	2.4 \pm 0.3	1 \pm 0.1 ***	2.9 \pm 0.2	2.6 \pm 0.1	** p=0.0015
Intrahepatic Triglycerides (μg/mg of liver)	60.6 \pm 3.6	25.1 \pm 5.7 *	50.7 \pm 18.2	74.3 \pm 11.8	* p=0.015

Four-week-old ovariectomized ER α AF2⁰ female mice and their littermates were implanted subcutaneously with E4 or vehicle (Veh) pellets and concomitantly fed a western diet from the age of 6 to 15 weeks. Body weight, uterine weight, subcutaneous, perigonadal and mesenteric tissues weight and intrahepatic triglycerides were evaluated. Results are expressed as means \pm SEM. Two-way ANOVA were performed to evaluate interaction between Genotype and treatment (E4). Fisher's LSD post-test were performed (Veh versus E4: *P < 0.05, **P<0.01, ***P < 0.001 n=6/8 mice/group).

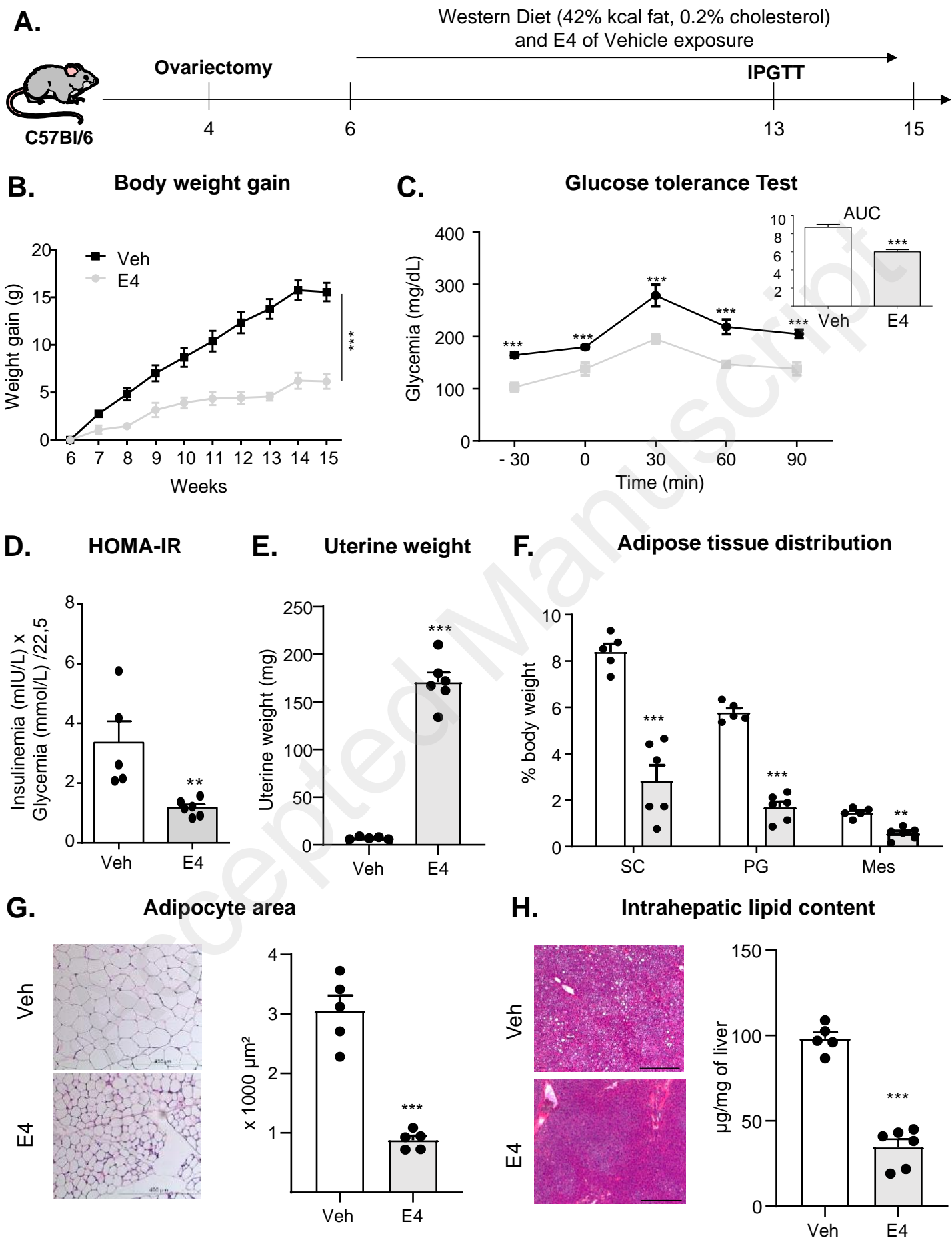
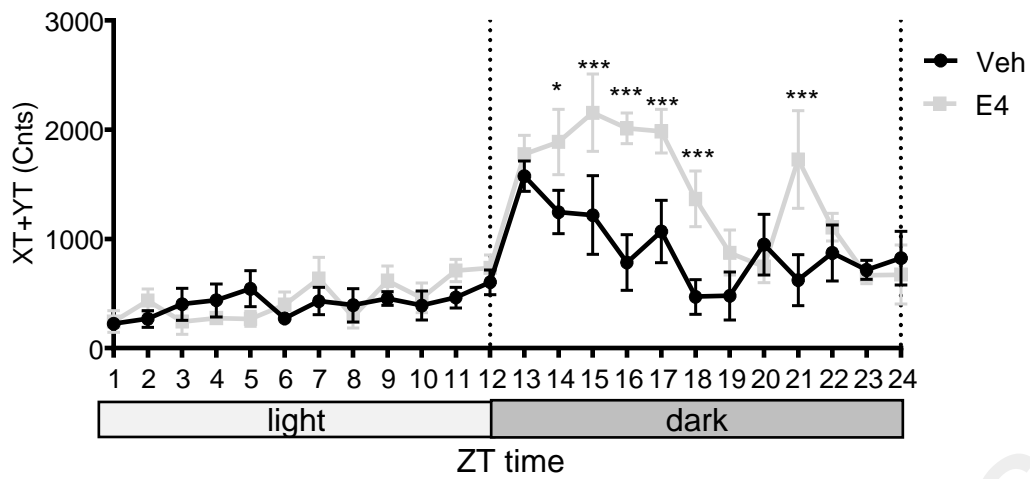
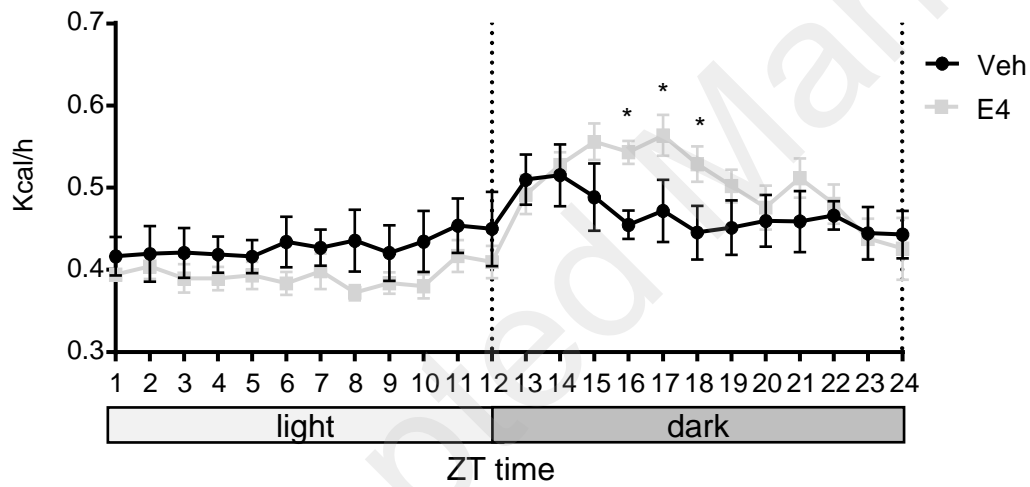


Figure 1

A.**Physical activity****B.****Energy expenditure****Figure 2**

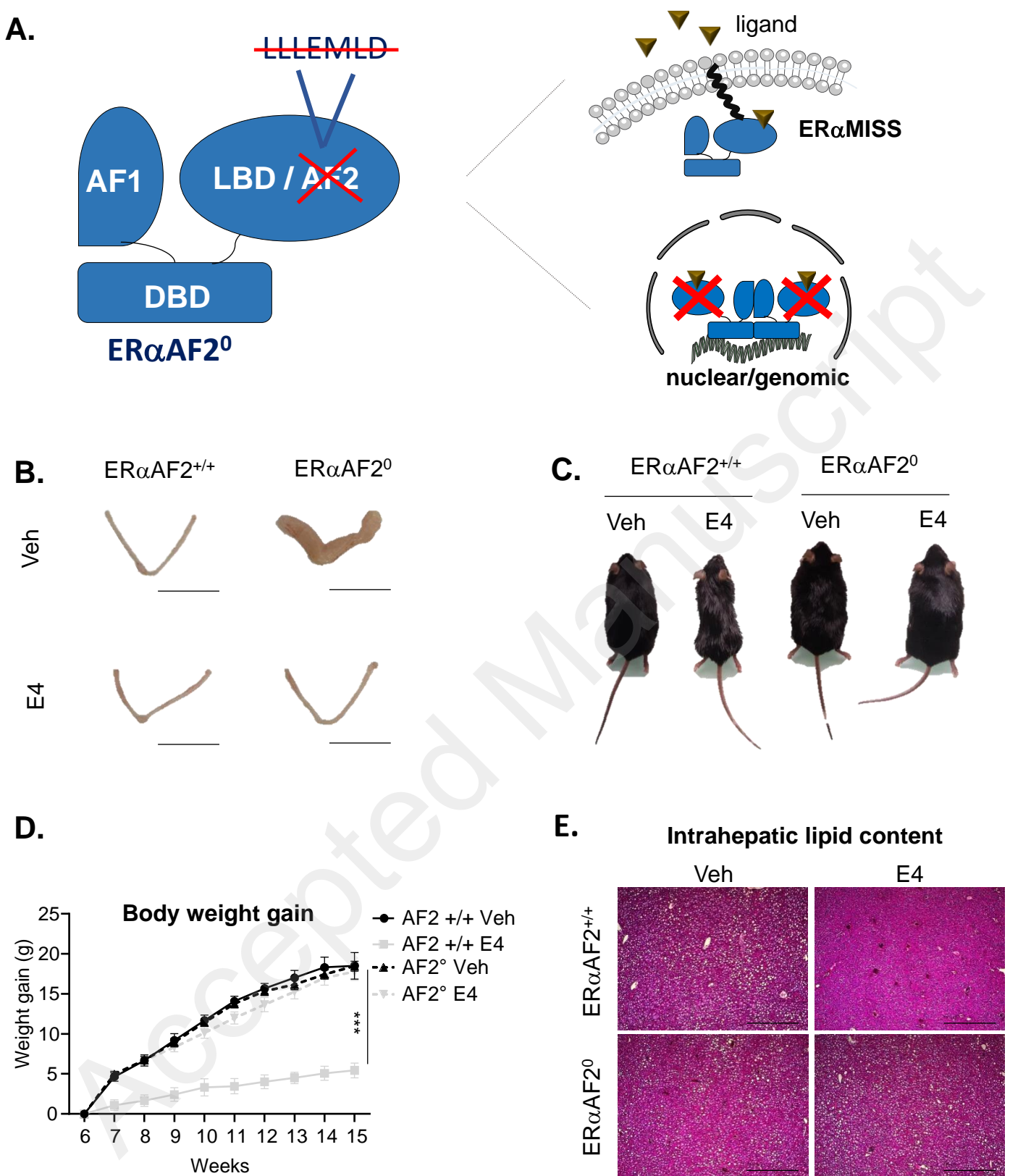


Figure 3

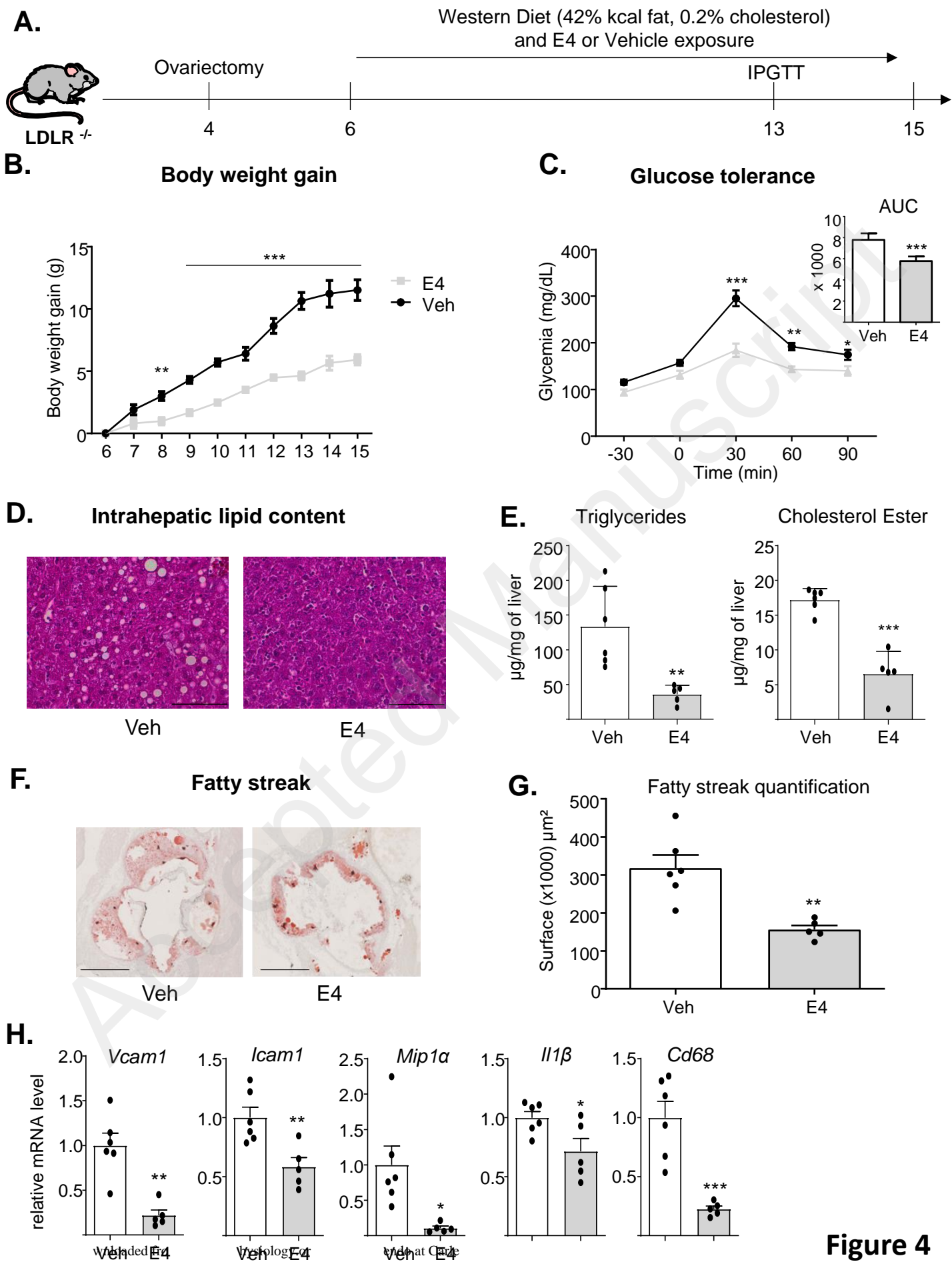


Figure 4

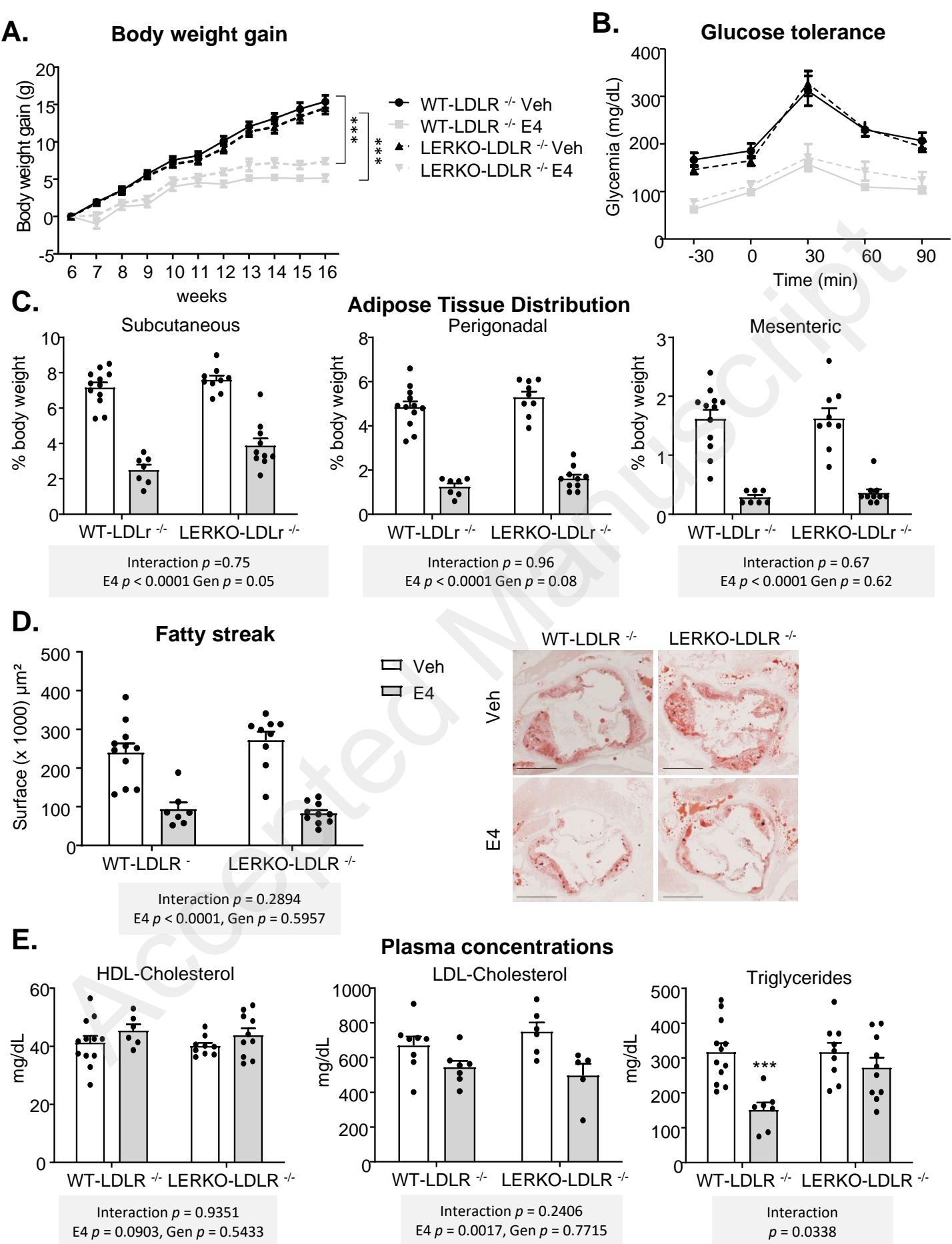


Figure 5

Intrahepatic lipid content

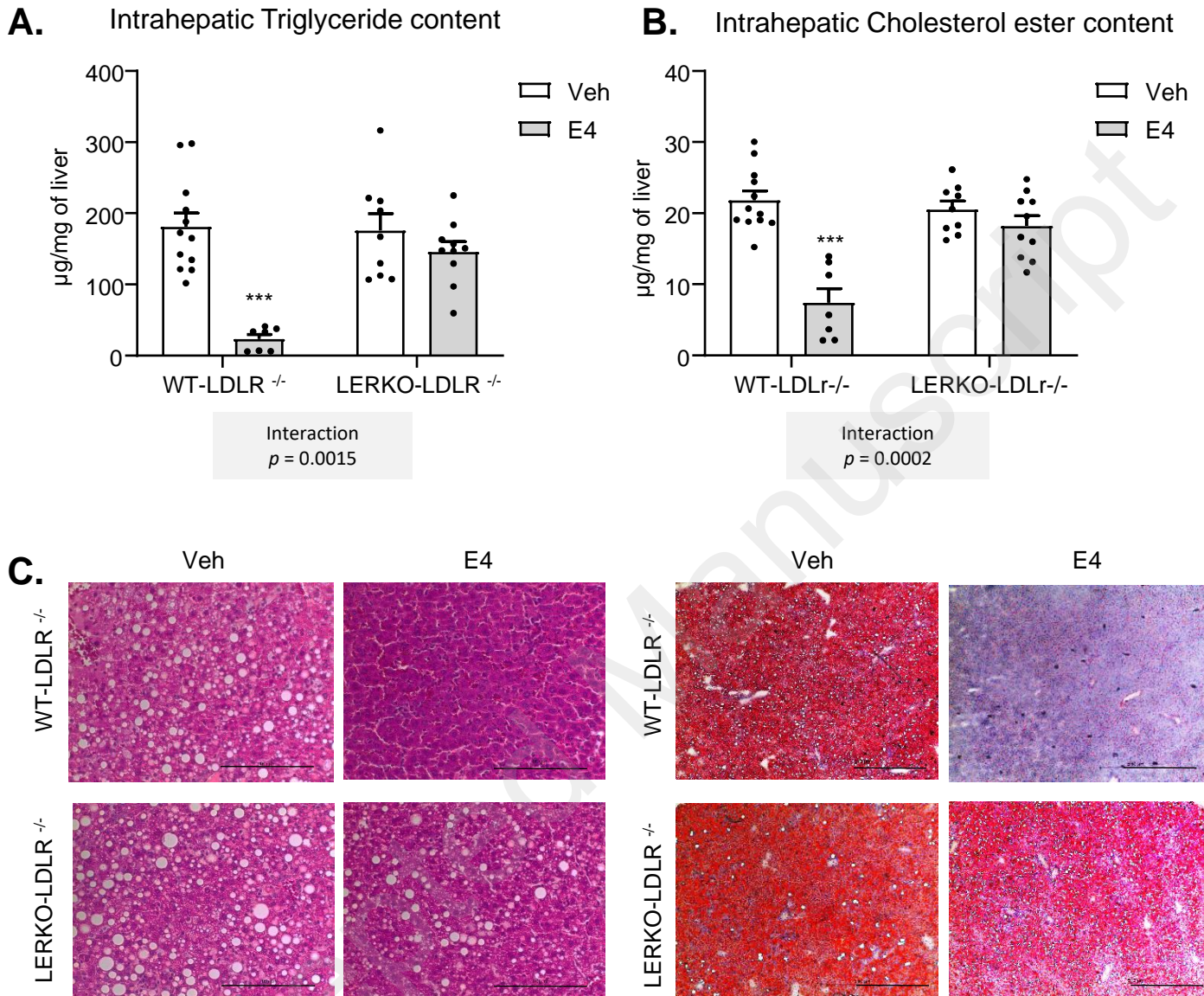


Figure 6