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<u>HIGHLIGHTS</u>

- Some biological response to E2 displays biphasic dose relationship.
- ERαAF1 plays a key sensitizing role in ERα activity.
- Differences and even contradictory conclusions could be explained by differences of E2 doses.

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<u>ABSTRACT</u>

59 17β-Estradiol (E2) action can be mediated by the full-length estrogen receptor alpha (ERα66),

60 but also by the AF1 domain-deficient ERα (ERα46) isoform, but their respective sensitivity

61 to E2 is essentially unknown. To this aim, we first performed a dose response study using

62 subcutaneous home-made pellets mimicking either metestrus, proestrus or a pharmacological

63 doses of E2, which resulted in plasma concentrations around 3, 30 and 600 pM, respectively.

64 Analysis of the uterus, vagina and bone after chronic exposure to E2 demonstrated dose-

65 dependent effects, with a maximal response reached at the proestrus- dose in wild type mice

66 expressing mainly ERα66. In contrast, in transgenic mice harbouring only an ERα deleted in

67 AF1, these effects of E2 were either strongly shifted rightward (10 to 100-fold) and/or

68 attenuated, depending on the tissue studied. Finally, experiments in different cell lines

69 transfected with ERα66 or 46 also delineated varying profiles of ERα AF1 sensitivity to E2.

70 Altogether, this work emphasizes the importance of dose in the tissue-specific actions of E2

71 and demonstrates the key sensitizing role of AF1 in ERα activity.

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76   **1. Introduction**

77   During her lifetime, a woman is exposed to varying levels of estrogens. At puberty,

78   the onset of the menstrual cycle leads to a cyclical production of estrogens with large

79   fluctuations in estrogen levels. Pregnancy is marked by an extensive increase in estrogens

80   levels whereas menopause, the consequence of the cessation of estrogen production by

81   ovaries in women, results in a sharp decrease in the main estrogen 17-β estradiol (E2) levels.

82   These hormonal variations translate into changes not only in sex targets organs involved in

83   sex secondary characters and reproduction, but also in many other non-reproductive tissues.

84   In particular, estrogens play an important role in the growth and maturation of bone as well as

85   in the regulation of bone turnover in adult. The importance of estrogen for skeletal health is

86   well known and represents an important research area. Indeed, estrogen deficiency is a major

87   risk for development of osteoporosis and leads to increased fracture risk, while estrogen

88   treatment reduces this risk (Almeida *et al.*, 2017). Unfortunately, long-term estrogen therapy

89   is also associated with serious side-effects, mainly increased risk of breast cancer and of deep

90   venous thrombosis (Valera *et al.*, 2018).

91   Mouse models have already contributed to elucidation of the mechanisms of action of

92   estrogens *in vivo* (Guillaume *et al.*, 2017; Hewitt and Korach, 2018; Rooney and van der

93   Meulen, 2017). In general, mice are ovariectomized to eliminate the main source of

94   endogenous estrogens, and to allow studying the effects of doses of exogenous E2 delivered

95   by various devices, such as subcutaneous pellets. A large range of pellets that deliver estrogen

96   from 0.01 mg/pellet (*i.e.,* 0.8 µg/kg/day) to 5 mg/pellet (*i.e.,* 2000 µg/kg/day) are available,

97   and very different doses are used in the literature, often without justification of the dose

98   employed. Furthermore, estrogens have frequently been reported to exert a hormetic or

99   biphasic dose-response relationship, characterized by low-dose stimulation and high-dose

100 inhibition (Strom *et al.*, 2011; Duarte-Guterman *et al.*, 2015). One example of such a

101 dichotomous effect was reported in ischemic stroke; whereas some studies have shown a

102 neuroprotective effect of estrogen, others have reported neurotoxicity, the differences being

103 attributed to the dose (Strom *et al.*, 2009).

104   The different responses to E2 are initiated by their binding to the estrogen receptors

105 (ERs), ERα and ERβ, which belong to the nuclear receptor superfamily and are structurally

106 organized into 6 functional domains (A to F). The E domain supports hormone binding, which

107 induces conformational changes that are required for ER transcriptional activity through the

108 modulation of two activation functions (AFs), AF1 and AF2, located in the A/B and E

109 domains, respectively. In addition to the full-length 66-kDa (ERα66) isoform, a 46-kDa ERα-

110 isoform (ERα46), lacking the N-terminal portion (domains A/B), and thereby AF-1, has been

111 reported to be expressed in various cell types such as human osteoblasts (Denger *et al.*, 2001),

112 macrophages (Murphy *et al.*, 2009), and vascular endothelial cells (Murphy *et al.*, 2009), but

113 also in cancer cells (Chantalat *et al.*, 2016). Mechanisms regulating both the expression of

114 ERα46 and its functions remain essentially unknown. ERα can be generated by either

115 alternative splicing, proteolysis, or an alternative initiation of translation via an internal

116 ribosome entry site (IRES) (Chantalat *et al.*, 2016).

117   ER-mediated transcriptional regulation involves either a direct interaction of ER with

118 specific estrogen-responsive elements (EREs) located in enhancers or near the promoter

119 region of target genes, or an indirect mechanism (tethered) via protein/protein interactions

120 with other transcriptional factors (Hamilton *et al.*, 2017). ERαAF1 and ERαAF2 have been

121 shown to play crucial roles in the transcriptional effects of ERα through the recruitment of

122 coactivators. In cultured cells, the respective roles of ERαAF1 and ERαAF2 appear to depend

123 on the state of cell differentiation (Merot *et al.*, 2004). Using mice selectively deficient in

124 ERαAF1 or ERαAF2, we previously demonstrated that ERαAF2 is absolutely required for all

125 nuclear effects of ERα in response to E2 whereas the role of ERαAF1 is highly tissue

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126   dependent (Abot *et al.*, 2013; Arnal *et al.*, 2013; Billon-Gales *et al.*, 2009; Borjesson *et al.*,

127   2011).

128   However, the potential role of the E2 dose on the different biological effects mediated by the

129   wild-type full length ERα-66 or the AF1/AB domain-deficient ERα has not been evaluated

130   nor taken into account in most *in vivo* studies.

131   In this work, we describe the effect of a 3-week exposure of ovariectomized mice to 3

132   doses of E2 mimicking metestrus (low dose), proestrus (medium dose) or a supra-

133   pharmacological dose on three major tissues: uterus, vagina and bone. This experimental

134   paradigm allowed us to precisely compare tissue responses to these 3 doses. An additional

135   very high dose of E2 was used in transgenic mice harbouring an ERα deleted for AF1

136   (ERαAF1^{0}) and in its littermate wild type mice.

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2. **Material and Methods**

2.1 Mice

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research and were approved by the local Animal Care and Use Committee. The investigation conforms to the directive 2010/63/EU of the European parliament. Mice were housed in cages in groups of four to six and kept in a specific pathogen-free and temperature-controlled facility on a 12-hour light to dark cycle. Each experimental group included at least six animals. Female C57BL/6J mice were purchased from Charles River Laboratories. ERαAF-1[0] mice were generated as previously described (Billon-Gales *et al.*, 2009). Mice were anesthetized by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg), and ovariectomized or sham operated at 4 weeks of age, before puberty, to avoid any endogenous estrogen exposure. After 2 weeks of recovery, mice were implanted subcutaneously with pellets releasing either vehicle (cholesterol) or various amounts of E2 for 21 days. To deliver increasing doses of estradiol over prolonged periods of time, various amounts of E2 (E2758 Sigma-Aldrich) and cholesterol (C3045 Sigma-Aldrich) were thoroughly mixed in powder form and compacted to obtain pellets (20 mg total) that were then implanted subcutaneously into ovariectomized mice as previously described (Hsieh *et al.*, 1998). Low (LD-E2), medium (MD-E2), high (HD-E2) and very high (VHD-E2) doses correspond respectively to weight/weight ratio of E2/cholesterol: 1/20000, 1/2500, 1/250 and 1/25.

2.2 Hormone assays

Plasma E2 levels were assayed by GC/MS, as described previously (Giton *et al.*, 2015) with minor modifications. Saline water / twice charcoal dextran stripped aged female rabbit serum (1/1, v/v) was used as the matrix for calibrators and quality control (QC) standards. Briefly, each sample (serums, calibration standards, quality controls, and blank matrix) was collected

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163   in an 8 ml borosilicate tube. A spiking solution of deuterated steroid internal standard (IS) (50

164   µl containing 10 pg of E2-d4, except for blank matrix) (CDN Isotopes, Inc., Point-Claire,

165   Canada), and 3 ml of 1-chlorobutane were added to each sample. After fast centrifugation, the

166   upper organic phase was collected on conditioned Hypersep SI 500mg SPE minicolumn

167   (Thermo Scientific, Rockwood, USA). The column and adsorbed material were then washed

168   with ethyl acetate / hexane (6 ml; 1/9, v/v). The second fraction containing E2 was eluted

169   using ethyl acetate / hexane (4 ml; 1/1, v/v), then evaporated at 60°C to dryness. E2 was

170   derivatized with pentafluorobenzoyl chloride (PFBC) (103772-1G, Sigma-Aldrich, Steinheim,

171   Germany). Final extracts were reconstituted in isooctane, then transferred into conical vials

172   for injection into the GC system (GC-2010 Plus, Shimadzu, Japan) using a 50%

173   phenylmethylpolysiloxane VF-17MS capillary column (20m x 0.15mm, internal diameter,

174   0.15µm film thickness) (Agilent Technologies, Les Ulis, France). A TQ8050 (Shimadzu,

175   Japan) triple quadrupole mass spectrometer equipped with a chemical ionization source and

176   operating in Q3 single ion monitoring (SIM) mode was used for detection. The reagent gas for

177   the NCI detection was methane. The GC was performed in pulsed splitless mode with a 1 min

178   pulsed splitless-time. The oven temperature was initially 150°C for 0.50 min, further

179   increased to 305°C at 20°C/min and held at 305°C for 3.60 min, and then to 335°C at

180   30°C/min and held at 335°C for 1.7 min. The injection port and transfer line temperatures

181   were respectively 290 and 280°C. The flow-rate of helium (carrier gas) was maintained

182   constant at 0.96 ml/min. The mass spectrometer CI source temperature was 220°C. The

183   linearity of steroid measurement was confirmed by plotting the ratio of the steroid peak

184   response / internal standard (IS) peak response to the concentration of E2 for each calibration

185   standard. Accuracy, target ions, corresponding deuterated internal control, range of detection,

186   low limit of quantification (LLOQ), and intra & inter assay CVs of the quality control are

187   reported in Supplemental Table 1.

2.3 Determination of total plasma cholesterol

Mice were anesthetized and blood samples were collected from the retro-orbital venous plexus. Total plasma cholesterol was assayed using the CHOD-PAD kit (Horiba ABX, Montpellier, France).

2.4 Histological analysis

Paraffin-embedded transverse sections (4 µm) from formalin-fixed uterine or vaginal specimens were stained as previously described with anti–Ki67 (Abot *et al.*, 2013) (RM-9106; Thermo-scientific). Sections were examined after digitization using a NanoZoomer Digital Pathology®. To examine the proliferative effects of each treatment, the ratio of Ki67–positive epithelial cell/total cell number from two microscopic fields of measurement at x20 magnification for each uterine and vaginal section was evaluated. For uterus, the luminal epithelial height (LEH) of endometrium is the mean of 10 measurements.

2.5 Peripheral quantitative computed tomography (pQCT):

Computerized tomography scans were performed using the pQCT XCT RESEARCH M (version 4.5B, Norland Stratec, Germany) with a voxel size of 70 μm, as previously described (Windahl *et al.*, 1999). To measure trabecular volumetric bone marrow density (BMD), the scan was positioned in the distal metaphysis of the femur at a distance corresponding to 3.4% of the total femur length in the proximal direction from the distal growth plate. This area contains trabecular and cortical bone and the trabecular region was defined as the inner 45% of the total cross-sectional area. The cortical bone parameters were analyzed in the mid-diaphyseal region of femur (Vidal *et al.*, 2000).

2.6 Cell culture and transfection

HeLa, HepG2 and MDA-MB231 cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Biowest) and antibiotics (Invitrogen) at 37 ºC in 5% CO_2. Transfections were carried out using jetPEI reagent according to manufacturer's

213 instructions (Polyplus transfection). One day before transfection, cells were plated in 24-well

214 plates at 50% confluence. One hour prior to transfection, the medium was replaced with phenol

215 red-free DMEM (Invitrogen) containing 2.5% charcoal-stripped FCS (Biowest). Transfection was

216 carried out with 100 ng of the reporter gene C3-LUC, 100 ng of CMV-βGal internal control and

217 50 ng of expression vectors pCR-ERα or pCR-ERαAF-1[0] (Merot *et al.*, 2004). Following an

218 incubation overnight, cells were treated for 24 h with ethanol (vehicle control) or increasing

219 concentration of E2. Cells were then harvested and luciferase and β-galactosidase assays were

220 performed as previously described (Merot *et al.*, 2004).

221 **2.7 Western Blot**

222 One µg of pCR-ErαWT or pCR-ERαAF1[0] plasmid were transcribed and translated using

223 TNT[®] quick system Promega. Total proteins were separated on a 10% SDS/PAGE gel and

224 transferred to a nitrocellulose membrane. Primary antibody against ERα was purchased from

225 Santa Cruz Biotechnology (SP1 ABCAM, 1/500). Revelation was performed using an HRP-

226 conjugated secondary antibody and visualized by enhanced chemoluminescence (ECL)

227 detection according to the manufacturer's instructions (Amersham Biosciences/GE

228 Healthcare), using ChemiDoc Imaging System (Bio-Rad).

229 **2.8 Statistics**

230 Statistics analysis were performed using GraphPad Prism software (version 6, GraphPad

231 Software, Inc,). To test the different doses of E2, a 1-way ANOVA was performed, followed

232 by a Fisher's LSD's multiple comparison post test. To test the respective roles of E2 treatment

233 and genotype, a 2-way ANOVA was performed. When a significant interaction was observed

234 between the 2 factors, the difference between genotype and treatment was evaluated by a

235 Fisher's LSD's multiple comparison post test. $P<0.05$ was considered statistically significant.

236

237   ## 3. Results

238   **3.1 Dose-response profile to E2 is tissue specific in wild type mice.**

239   We first studied *in vivo* the tissue-specific response to different doses of long-term E2

240   administration (3 weeks) on ovariectomized C57Bl/6 mice (**Figure 1A**). Plasma estradiol

241   measurement allowed us to determine the E2/cholesterol ratio that mimicked either metestrus

242   (low dose, LD-E2 ~1 pg/ml: 3pM) and proestrus (medium dose, MD–E2~10 pg/ml: 30pM) as

243   previously reported (Nilsson *et al.*, 2015), or a pharmacological dose (high dose HD-E2 ~200

244   pg/ml: 600pM) (**Figure 1B**). In mice on a chow diet, E2 treatment had no impact on body

245   mass (**Figure 1C**) or plasma cholesterol content (**Figure 1D**), regardless of the dose

246   administered. The pharmacological dose led to high level of E2 especially in view of the

247   absence of SHBG in mice. Importantly, in mice receiving the high dose of E2, fraction of E2

248   was converted into estrone (E1) and became detectable in the plasma (**Supplementary Table**

249   **2**). By contrast, the level of estrone in mice supplemented with LD-E2 or MD-E2 is below the

250   threshold of 10 pg/ml.

251   Wet weight of the uterus represents the classic endpoint for measuring estrogenicity.

252   As expected, ovariectomy led to uterine atrophy compared to intact mice, and all E2 doses

253   increased uterine weight (**Figure 2A**). The maximal response in terms of weight gain was

254   reached with the MD-E2, with an attenuated response observed using the HD-E2 (**Figure**

255   **2A**). We next evaluated the level of uterine epithelial proliferation using Ki67 staining.

256   Epithelial proliferation in response to E2 was maximal at the medium dose, whereas the

257   pharmacological dose of E2 led to a much lower epithelial proliferation; this represented a

258   much greater decline than that reflected by uterine weight (**Figures 2B and C**). Accordingly,

259   analysis of the height increase of uterine epithelial cells (LEH) as well as stromal proliferation

260   and density confirmed that the medium dose is required to obtain strongest E2 response

261   (**Table 1**).

262   Although the evaluation of morphological changes in the vagina is less used to assess

263   estrogenic exposure in the mouse compared to measurements of uterine parameters, this tissue

264   is another major target for estrogenic action. A maximal response of vagina in terms of weight

265   gain was observed using the medium dose of E2, and it then plateaued with the medium or

266   high dose of E2 (**Figure 2D**). By contrast, a low dose was sufficient to induce maximal

267   epithelial proliferation (**Figures 2E and F**).

268   We next analyzed dose response to E2 treatment in bone, a major non-reproductive

269   target of estrogens. As expected, ovariectomy significantly altered both cortical (**Figure 3A**)

270   and trabecular (**Figure 3B**) bone compartments, but did not impact bone length (**Figure 3C**).

271   Treatment with the lowest dose of E2 was sufficient to restore cortical thickness and cortical

272   bone mineral content (**Figure 3A**) as well as trabecular bone mineral density (**Figure 3B**). As

273   observed in reproductive tissues, the medium dose of E2 induced maximal tissue response

274   thereafter reaching a plateau, with no additional elevation using the pharmacological dose.

275   Altogether, these results demonstrated that using pellets designed to release E2 levels

276   similar to those encountered during metestrus (low dose) and proestrus (medium dose), the

277   low dose of E2 stimulated 3 target tissues (*i.e.,* vagina, uterus and bone), whereas the medium

278   dose was necessary to elicit a maximal effect.

279   **3.2 Involvement of ERαAF1 action in mediating E2 response is dose dependent**

280   Our results highlight that response to E2 is dose-dependent, with differences between tissues.

281   In addition, we previously demonstrated that the role of ERαAF-1 is also tissue-specific, with

282   a crucial role played in uterus and trabecular bone, but not in cortical bone (Abot *et al.*, 2013;

283   Borjesson *et al.*, 2011). We first compared the dose response to E2 on an ERE-driven reporter

284   gene (C3-LUC) in cells expressing either the wild type receptor (ERα66) or the isoform

285   having a deletion of AF1 (ERαAF1^0 = ERα46) (**Figure 4A**) that were introduced into three

286   different cell lines by transient transfection (**Figures 4B-D**). Expression vectors encoding

287   wild type ERα and ERαAF1^0 were expressed at similar level after transfection in these

288   different cell types (**Supplementary Figure 1**). In addition to the previously reported cell-

289   specific differences in the transactivation efficiency between both receptors (Merot *et al.*,

290   2004), we show here the need for higher doses of E2 for ERαAF-1^0 than for ERαWT to

291   induce the activity of the reporter gene. The EC_{50} was about 10 to 100 fold higher for ERαAF-

292   1^0 than for ERαWT, depending of the cell-type (**Figures 4B-D**). In addition, even in higher

293   dose, the maximum response was also much lower using ERαAF-1^0 (**Figure 4B, C**), except

294   for MDA-MB-231 cells (**Figure 4D**).

295   We then decided to systematically assess the role of ERαAF-1 in the estrogenic response. To

296   this end, we used ERαAF1^0 mice expressing a 49-kDa truncated protein lacking the A domain

297   and motifs constituting ERα AF-1 in the B domain (**Figure 5A**) (Billon-Gales *et al.*, 2009).

298   Since some previous studies have used high doses of estrogens *in vivo,* until 5 mg E2/ pellet

299   for 60 day release (Pedram *et al.*, 2014), we have decided to add a very high dose (VHD-E2:

300   ~2000pg/ml *i.e* 2048 pg/ml $\pm$ 242) to evaluate the role of AF-1 function *in vivo*. We

301   previously reported that ERαAF1^0 mice treated with pellets from Innovative Research (0.1

302   mg E2/pellet: 60 day release) presented a moderate uterine hypertrophy that was essentially

303   due to stromal edema, with minimal epithelial proliferation compared to WT mice (Abot *et*

304   *al.*, 2013). Here, we confirm that no uterine hypertrophy was observed in ERαAF1^0 mice in

305   response to LD and MD of E2 (**Figure 5B**). However, HD-E2 or VHD-E2 induced similar

306   increase of uterine weight in ERαAF1^0 and ERαWT mice, demonstrating that the deletion of

307   the ERα AF1 function can be fully compensated by a 10 to 100-fold increase in the E2 dose

308   for some parameters such as uterine weigh (**Figure 5B)**. For several other parameters, such as

309   endometrium epithelial proliferation (**Figure 5C**) and vagina weight gain (**Figure 5D**), E2

310   action was not only shifted rightward but the magnitude of the response was also decreased,

311   remaining below the full effect observed in control mice, even at the VHD-E2

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312   pharmacological dose. In addition, we demonstrate for the first time a role of ERαAF1 in

313   vaginal epithelial proliferation, which as for uterine epithelial proliferation, is highly

314   dependent of E2 dose (**Figure 5E**).

315   Finally, we reconsidered the question of the role of ERαAF1 in bone, and, as previously

316   described, we demonstrated that ERαAF1 is dispensable for cortical response to E2 regardless

317   of the dose used, as revealed by the absence of interaction between genotype and E2 treatment

318   (**Figures 6A and B**). It should be however noted a significant effect of genotype due to a

319   smaller cortical thickness and a smaller cortical BMC in ERαAF1[0] mice, independently on

320   E2. By contrast, ERαAF1 is required for the increase of bone mineral density (BMD) in

321   response to E2 in the trabecular compartment regardless of the dose (**Figure 6B**). We

322   confirmed that E2 has no impact on bone length in both WT and ERαAF1[0] mice but

323   interestingly in ovariectomized mice, bone lengths from ERαAF1[0] were smaller than in WT

324   mice, as previously reported in older ERαAF1[0] mice (Borjesson *et al.*, 2012) (**Figure 6C**).

325   Altogether, these data definitely demonstrate that ERαAF1 is necessary for the full E2

326   response in uterus, vagina and the trabecular bone compartment. However, depending on the

327   phenotypic feature, ERαAF1 deficiency can be partially or fully overcome by using high E2

328   levels.

329   **4. Discussion**

330   To understand the physiology of estrogens, as well as to optimize the use of female

331   sex hormones in medicine, it is crucial to precisely delineate the sensitivity of the tissues to

332   estrogen levels. It is a common practice to ovariectomize mice to eliminate the main source of

333   endogenous estrogens, and subsequently to administer E2 exogenously. Subcutaneous

334   implantation of slow-release pellets is the most convenient and efficient way to deliver

335   estrogen to mice; it avoids the stress associated with daily subcutaneous injections. In our

336   study, the low dose of E2 (metestrus level) stimulated vagina, uterus and bone tissues in

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337   ovariectomized mice, whereas the medium dose (proestrus level) was necessary to elicit a

338   maximal effect. However, use of a supraphysiological dose (HD-E2) did not confer any

339   further stimulation compared to the medium dose in these tissues. The HD of E2 even induced

340   a lesser response than the MD of E2 in the uterus, but not in bone nor in the vagina. There are

341   numerous examples of estrogen hormesis, *i.e.* lowering activity levels while increasing doses,

342   concerning a wide variety of endpoints, including cerebral (Strom *et al.*, 2011; Strom *et al.*,

343   2009; Strom *et al.*, 2010), mammary gland differentiation (Vandenberg *et al.*, 2006) and

344   insulin sensitivity (Gonzalez *et al.*, 2002). High concentrations of E2 can also lead to

345   increased levels of metabolites, such as estrone (**Supplementary Table 2**), which can also

346   influence the biological responses through either dependent or independent ER mechanisms

347   (Thomas and Potter, 2013).

348   Remarkably, despite a significant effect on cortical thickness and BMC as well as on

349   trabecular BMD, E2 even at a very high dose had no impact on bone length in our

350   experimental conditions. This result differs from previous studies that reported a reduced bone

351   length and growth plate width using high dose of E2 in older mice (Borjesson *et al.*, 2012;

352   Borjesson *et al.*, 2010). These apparent discrepancies underline the importance of the age of

353   ovariectomy, age of mouse studied, and timing of E2 supplementation.

354   E2 effects are mediated by hormone binding to ERα, ERβ or GPR30 (Miller *et al.*,

355   2017). ERα also exists as two isoforms, designated ERα66 and ERα46, based on their

356   respective molecular weight, and both isoforms were reported to regulate E2-mediated

357   proliferation in the mouse uterus (Abot *et al.*, 2013). The expression of ERα46 in the rat

358   uterus was demonstrated even before the use of antibodies against ERα (Faye *et al.*, 1986).

359   Importantly, we recently demonstrated that ERα46 is frequently and sometimes abundantly

360   expressed in ERα-positive breast tumors, but the mechanisms of its generation as well as

361   pathophysiological significance of its expression are essentially unknown (Chantalat *et al.*,

362 2016). Furthermore, the specific signalling events associated with this AF1-deficient isoform

363 as well as its interactions with the full length ERα66 remain unclear. Given the complexity of

364 estrogen-mediated actions, which goes far beyond the single-receptor situation on which the

365 linear dose-response model is based, it is not surprising that E2 can elicit hormetic responses.

366 In particular, varied affinities for distinct receptors having different intrinsic activity levels

367 would essentially lead to a "mixture" effect, where the summation of their individual dose-

368 response curves would lead to composite behaviour that could be non-monotonic (Miller *et*

369 *al.*, 2017). In particular, we previously proposed that ERα46 counteracts ERα66 on E2-

370 induced cell proliferation (Abot *et al.*, 2013; Penot *et al.*, 2005).

371   The structural difference between ERα66 and ERα46, according to our current

372 knowledge, resides mainly in the absence of AF1 in ERα46 (Chantalat *et al.*, 2016).

373 Accordingly, both receptors exhibit different transactivation efficiencies in a cell-specific

374 manner (Merot *et al.*, 2004). It has been proposed that ERα46 could have a repressive role in

375 AF1-permissive contexts through interference with ERα66 binding to DNA (Penot *et al.*,

376 2005). The present study deepens the comparison between both ERα forms by revealing a

377 major difference in their transcriptional sensitivity to the dose of E2. To reach the maximal

378 level of ligand-dependent transcriptional activity, ERα46 requires an E2 dose 10 to 100 times

379 higher than does ERα66. Several studies have shown that N-terminal truncated forms of ERα,

380 including ERα46, have an affinity for E2 which is similar to that found for the wild-type

381 ERα66, indicating that the hormone binding domain is able to function independently of the

382 N-terminal half of the ER (Kumar *et al.*, 1986; Carlson *et al.*, 1997; Lin *et al.*, 2013).

383 However, differences in ERα binding to DNA and in coactivator and/or corepressor

384 recruitment could explain the distinctively lower sensitivity of ERα46 *versus* ERα66 to the

385 dose of E2. We previously reported that although the qualitative overall recruitment of

386 cofactors is quite similar between ERα46 and ERα66, there is a quantitative differential

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387   modulation with some preference for cofactor binding to ERα66 over ERα46 (Chantalat *et al.*,

388   2016). ERα46 is also able to recruit transcriptional repressors in a ligand-independent manner

389   (Metivier *et al.*, 2004). In accordance with the specific binding of coregulators, we reported

390   that some genes were specifically regulated by either the ERα46 or ERα66 isoforms,

391   demonstrating that the absence of ERαAF1 affects specific transcriptional programs rather

392   than leading to an overall decreased activity (Chantalat *et al.*, 2016) .

393   Using mice harbouring an ERα deleted for AF1 (ERαAF1[0]), we previously

394   demonstrated that ERαAF1 is necessary to induce uterine hypertrophy, suggesting that

395   ERα46 is not able to mediate E2 responses in uterus (Abot *et al.*, 2013). Here, we

396   demonstrate that with a HD of E2, the increase in uterine weight is similar between ERαAF1[0]

397   and ERαWT, although an attenuated response is observed in terms of epithelial proliferation.

398   By contrast, physiological LD or MD doses of E2 leading to levels encountered during the

399   estrous cycle were unable to induce a uterine response in ERαAF1[0] mice in contrast to

400   ERαWT mice.   These results demonstrate that the involvement of ERαAF1 in the E2

401   response is highly dependent on ligand dose, and that different signalling pathways regulate

402   epithelial proliferation and uterine weight gain. Similarly, the response to E2 in vagina and

403   trabecular bone are dependent of ERαAF-1, since this response is not only shifted rightward

404   but also decreased in intensity, suggesting that these tissues can be used as an index of

405   ERαAF-1-dependent estrogenicity.

406   There is also evidence that amino acid phosphorylation located in the ERαAF-1

407   domain, regulates numerous aspects of ERα function, including protein stability, hormone

408   binding, receptor dimerization, DNA binding, protein-protein interactions, and transcription

409   activity (Orti *et al.*, 1992; Lannigan, 2003; Weigel and Moore, 2007). In particular, Ser118

410   has been implicated in ERα mediated transcriptional regulation since a partial or complete

411   attenuation of E2-induced transcription has been observed in ERαS118A transfected cells

412   (Ali *et al.*, 1993; Duplessis *et al.*, 2011). Appropriate transgenic mouse studies should be

413   developed to determine the importance of ERα Ser118 phosphorylation *in vivo*.

414

5. Conclusion

416   We describe here the dose-effect of E2 on sexual targets and bone thanks to a simple, reliable

417   and unexpansive slow release device. Our results demonstrate that the estrogenic effect on

418   tissues is highly dependent on the dose used. Although the precise role of ERαAF1 both in

419   physiology and in pathology remains to be clarified, the present work reveals that ERαAF1

420   provides an important layer of regulation through the sensitization of the response to

421   estrogens. The role of ERαAF1 can be even more spectacular in the action of some selective

422   ER modulators (SERMs) (Arao and Korach, 2019). For instance, tamoxifen selectively

423   activates ERαAF1, and this activation is required for the beneficial effects of this SERM on

424   arteries (Fontaine *et al.*, 2013) and metabolism (Guillaume *et al.*, 2017), while at the same

425   time it prevents the recurrence of breast cancer. Whereas antagonist activity of tamoxifen is

426   essentially through the ERαAF2 blockade, molecular mechanism of its partial agonist activity

427   that involved ERαAF1 activation is not fully understood (Arao and Korach, 2019). Here, we

428   report that importance of ERαAF1 domain in estrogenic response depend on both considered

429   cell type/ tissues and ligand dose, two parameters which could be determinant in the agonist

430   activity of E2 or SERM that control ERαAF-1-mediated physiological response.

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433 **Acknowledgments**

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588 **FIGURE LEGEND**

589 **Figure 1: Plasma estradiol (E2) and cholesterol concentrations in mice implanted with**

590 **E2/cholesterol pellets**.

591 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted

592 subcutaneously into ovariectomized mice for a period of 3 weeks (**A**). Plasma E2 (**B**), body

593 weight (**C**) and cholesterol content (**D**) of the mice were evaluated.

594 **Figure 2: E2 dose response analysis of uterine and vaginal parameters**

595 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted

596 subcutaneously into ovariectomized mice for a period of 3 weeks and uterus (**A, B, C**) and

597 vagina (**D, E, F**) were analyzed. Uterine (**A**) and vagina (**D**) weight. Percentage of uterine (**B**)

598 and vagina (**E**) Ki67-positive epithelial cells. Results are expressed as means ± SEM. To test

599 effect of E2 exposure, one-way ANOVA and a Fisher's LSD's post test were performed (E2

600 exposure *versus* placebo *P < 0.05, ***P < 0.001, difference in E2 concentration [†††]P<0.001

601 n=10/12 mice/group). Representative Ki-67 detection in transverse uterus (**C**) and vagina (**F**)

602 sections.

603 **Figure 3: E2 dose response analysis of bone parameters**

604 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted

605 subcutaneously into ovariectomized mice for a period of 3 weeks and femurs were analyzed.

606 Cortical thickness and cortical bone mineral content (BMC) (**A**) in the mid-diaphyseal part of

607 femur. Trabecular bone mineral density (BMD) in the distal metaphyseal part of femur (**B**)

608 and Bone length (**C**). Results are expressed as means ± SEM. To test effect of E2 exposure,

609 one-way ANOVA and a Fisher's LSD's post test were performed (E2 exposure *versus* placebo

610 **P < 0.01, ***P < 0.001, difference in E2 concentration [†††]P<0.001, n=6/7 mice/group).

611 **Figure 4: Dose response of E2 transcriptional activity in cultured cells expressing ERα66**

612 **or ERα46.**

613 Schematic representation of the wild-type ERα66 (ERαAF1$^{+/+}$) and ERα (ERαAF1^{0}) isoforms

614 and expression of these isoforms obtained from in vitro translation pCR-ERαWT or pCR-

615 ERαAF1^{0} vectors performed with the TNT Coupled Reticulocyte Lysate System (**A**). HepG2

616 (**B**), HeLa (**C**) and MDA-MB231 (**D**) cells were transiently transfected with the C3-Luc

617 reporter constructs in the presence of pCR-ERαWT, pCR-ERαAF1^{0}, or empty pCR vectors.

618 Cells were treated with the indicated dose of E$_2$ or vehicle (Ctrl) for 24 h. Normalized

619 luciferase activities were expressed as fold increase above values measured with empty pCR

620 and vehicle. Data correspond to the mean values ± SEM of at least three separate transfection

621 experiments. Two-way ANOVA followed by Fisher's LSD's multiple comparison test were

622 performed (ERαAF1$^{+/+}$ *versus* ERαAF1^{0}: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

623 **Figure 5: The role of ERαAF1 in uterine and vagina is dependent of E2 dose**

624 Schematic representation of the wild-type ERαAF1$^{+/+}$ and ERαAF1^{0} mice constructs (**A**).

625 Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose

626 (VHD) of E2 were implanted subcutaneously into ERαAF1^{0} ovariectomized mice during 3

627 weeks. Uterus (**B, C**) and vagina (**D, E**) were analyzed. Uterine (**B**) and vagina (**D**) weight.

628 Percentage of uterine (**C**) and vagina (**E**) Ki67-positive epithelial cells. Representative Ki-67

629 detection in transverse uterus sections. Results are expressed as means ± SEM. Two-way

630 ANOVA followed by Fisher's LSD's multiple comparison test were performed (ERαAF1$^{+/+}$

631 *versus* ERαAF1^{0}: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n=6/8 mice/group).

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634 **Figure 6: E2 dose response analysis of trabecular and cortical bone parameters in wild-**

635 **type ERαAF1$^{+/+}$ and ERαAF1^{0} mice.**

636 Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose

637 (VHD) of E2 were implanted subcutaneously to ERαAF1^{0} ovariectomized mice for a period

638 of 3 weeks and femurs were analyzed. Cortical thickness and cortical bone mineral content

639 (BMC) in the mid-diaphyseal part of femur. 2-way ANOVA test revealed no significant

640 interaction, the overall effect of the treatment and genotype was analyzed (**A**). Trabecular

641 bone mineral density (BMD) in the distal metaphyseal part of femur (**B**) and Bone length (**C**).

642 Results are expressed as means ± SEM. Since two-way ANOVA test revealed a significant

643 interaction between the 2 factors (E2 exposure and genotype), a Fisher's LSD's multiple

644 comparison test were performed (ERαAF1$^{+/+}$ *versus* ERαAF1^{0}: **P < 0.01, ***P < 0.001,

645 n=6/8 mice/group) (**B, C**).

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A.

B. Plasma E2
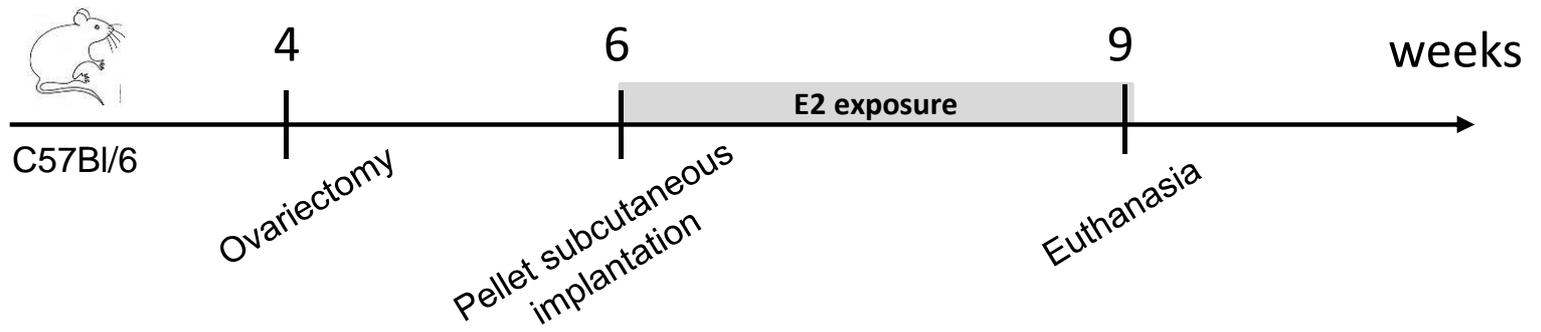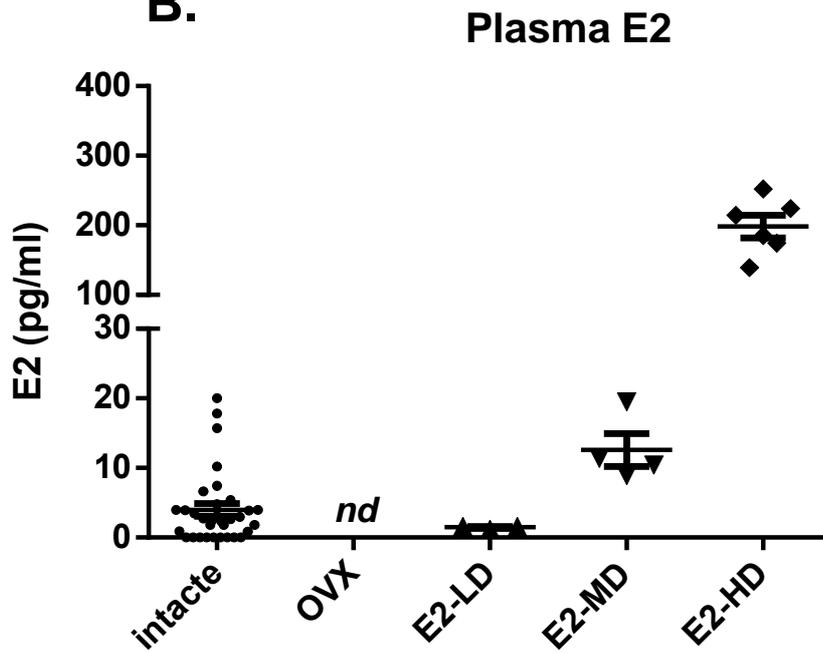
C. Body weight
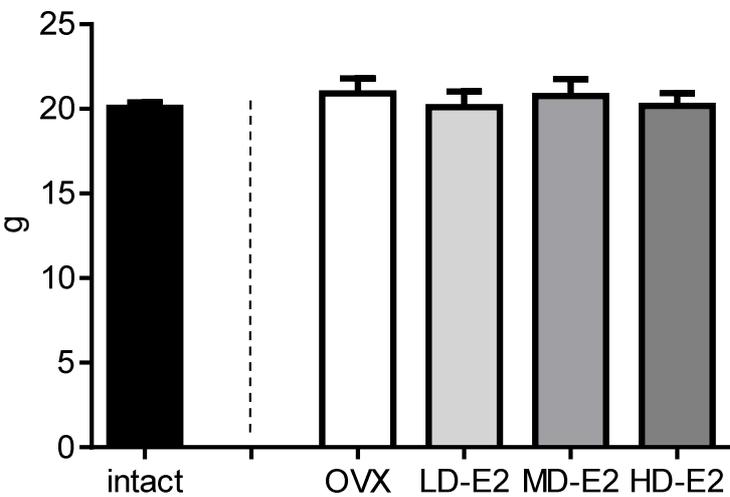
D. Plasma Cholesterol
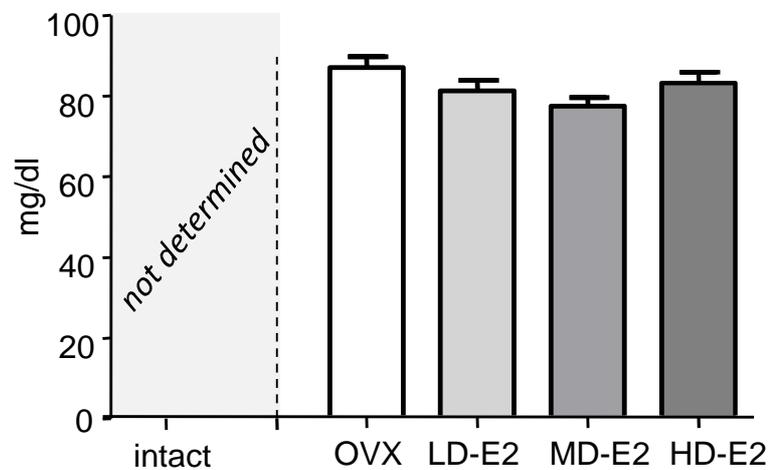
Figure 1
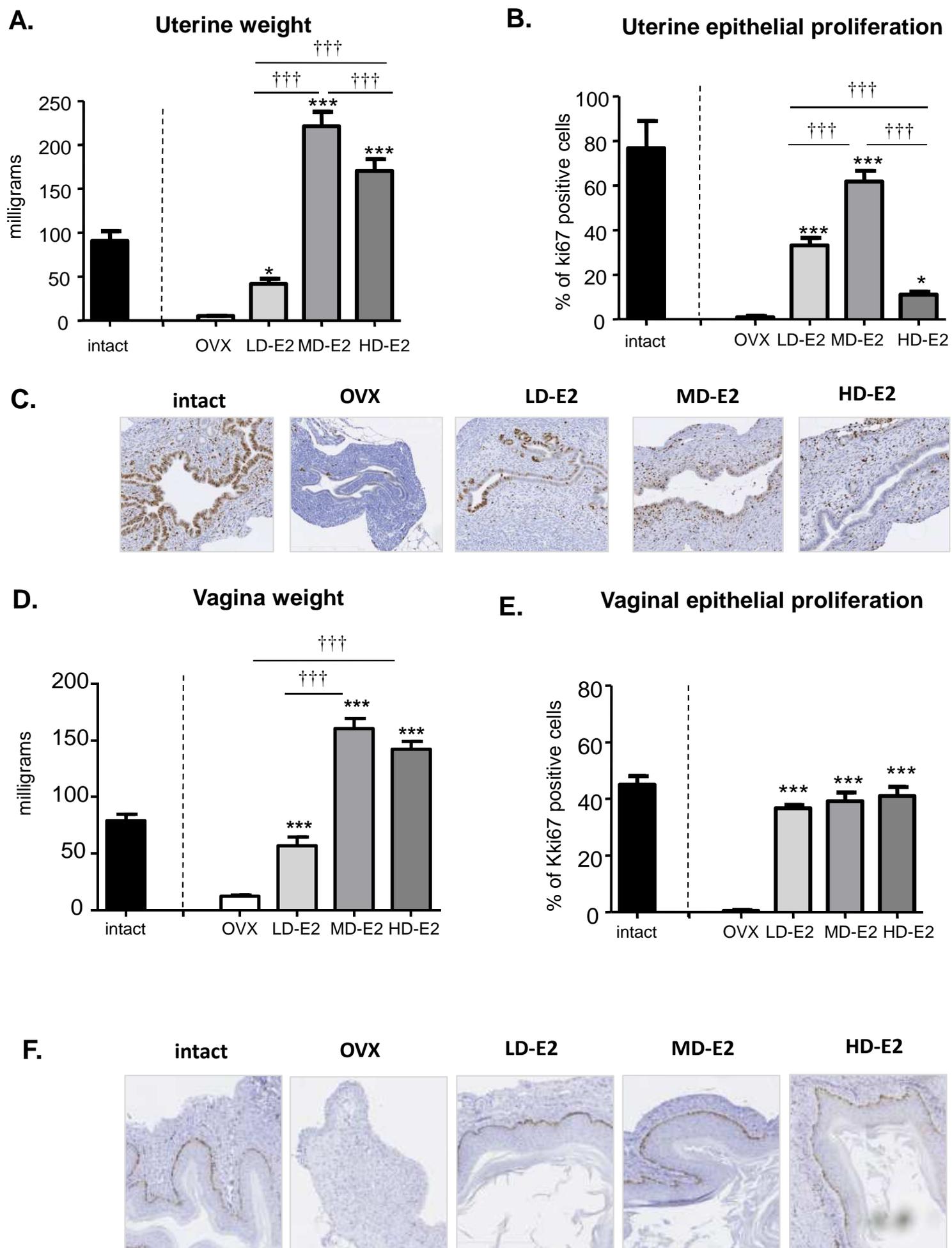
Figure 2

A.

Cortical compartment
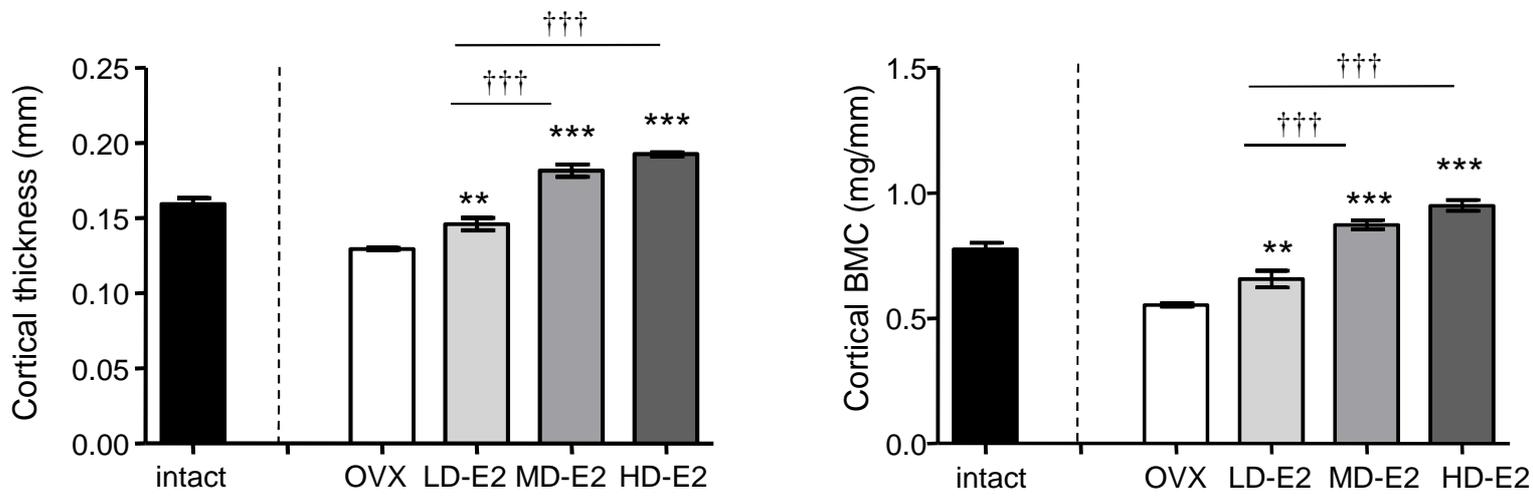
B. **Trabecular compartment**
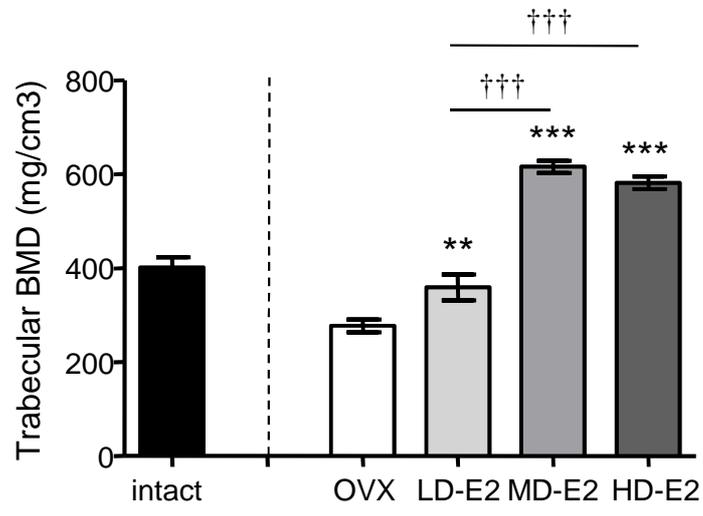
C. **Bone length**

Figure 3

Figure 4

Figure 5

A.

Cortical compartment

Interaction $p = 0.365$
Treatment $p < 0.0001$
Genotype $p = 0.0063$

Interaction $p = 0.054$
Treatment $p < 0.0001$
Genotype $p < 0.0001$

Cortical thickness (mm)

0.25
0.20
0.15
0.10
0.05
0.00

OVX   LD-E2   MD-E2   HD-E2   VHD-E2

Cortical BMC (mg/mm)

1.0
0.8
0.6
0.4
0.2
0.0

OVX   LD-E2   MD-E2   HD-E2   VHD-E2

B.

Trabecular compartment

Interaction $p < 0.0001$

Trabecular BMD (mg/cm3)

800
600
400
200
0

OVX   LD-E2   MD-E2   HD-E2   VHD-E2

**   ***   ***

C.

Bone length

Interaction $p < 0.0001$

Femoral length (mm)

20
15
10
5
0

OVX   LD-E2   MD-E2   HD-E2   VHD-E2

***

☐ ERα-AF1$^{+/+}$ ■ ERα-AF1^{0}

Figure 6

	OVX	LD-E2	MD-E2	HD-E2
Uterine LEH (μm)	12.1 +/- 0.6	20.2 +/- 1.2 **	37.6 +/- 1.9 ***	40.5 +/- 2.1 ***
Uterine Stromal Density	257 +/- 1.14	116.7 +/- 44.6 ***	87 +/- 35.5 ***	72.7 +/- 34.5 ***
Uterine Stromal proliferation (%Ki67positive cells)	0.3 +/- 0.5	8 +/- 5.6	42.1 +/- 11 ***	21.6 +/- 8 ***

Results are expressed as means ± SEM. To test the respective roles of each treatment, one-way ANOVA and a Fisher's LSD's *post test* were performed (treatment versus placebo **, $P < 0.01$, ***, $P < 0.001$)

Table 1: Analysis of uterine parameters in C57Bl/6 mice

| Accucary (%)	Analyte	Target ion analyte /IS (amu)	Range (pg/ml) R^2	Mean (pg/ml) 17 runs *Intra- & Inter assay CVs (%)*			
				LLOQ	Low QC	Middle QC	High QC
94-107	E2	660/664	0.2- 56.0 0.9994	0.22 17.3 -19.6	2.87 2.9-4.2	6.07 2.6-3.7	12.88 2.3-3.33

LLOQ : low limit of quantification; QC : quality control

Supplementary Table 1 : GC/MS plasma analytical control validation

	OVX	LD-E2	MD-E2	HD-E2
Estrone (E1)	< 10pg/ml	< 10pg/ml	< 10pg/ml	22,5 +/- 5,4

Results are expressed as means ± SEM.

Supplementary Table 2: GC/MS plasma Estrone (E1) dosage
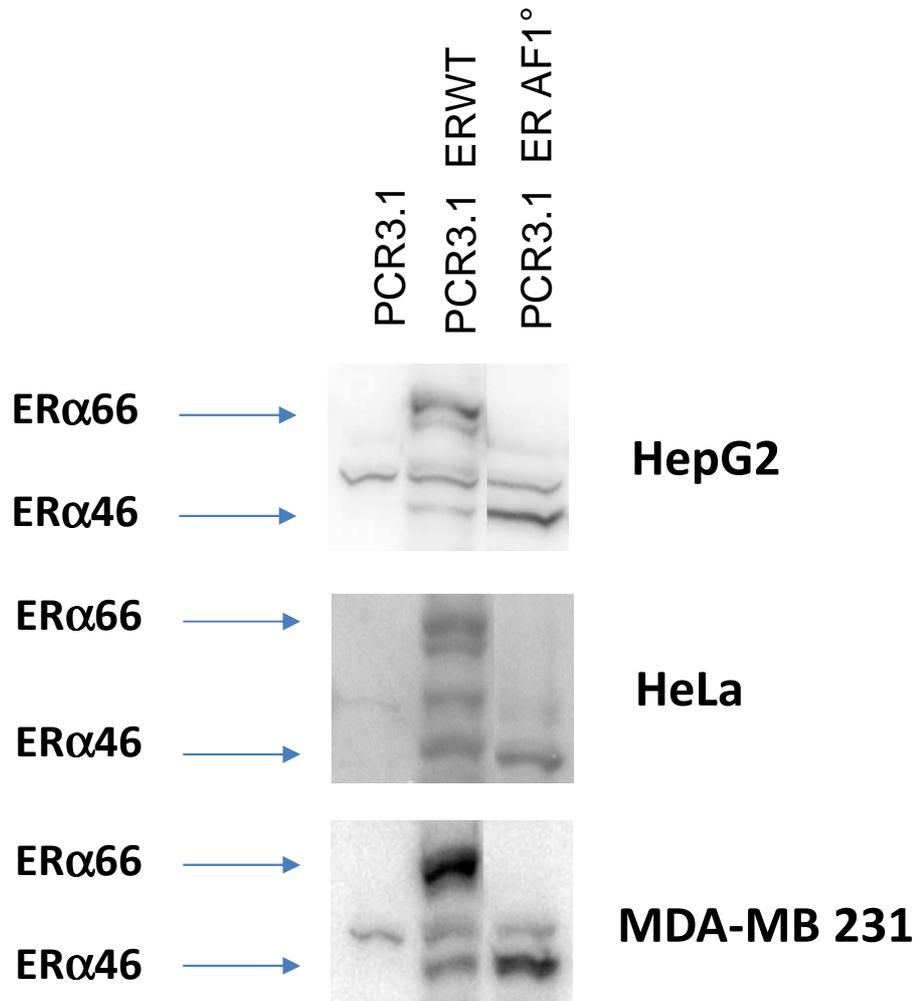
Supplementary Figure 1: Western blot analysis was performed on an equal amount of cellular extracts prepared from cells transfected with empty vectors (lane 1) expression vectors encoding wild type (lane 2), or ERαAF1^0 mutant (lane 3).

HIGHLIGHTS

- Some biological response to E2 displays biphasic dose relationship.

- ERαAF1 plays a key sensitizing role in ERα activity.

- Differences and even contradictory conclusions could be explained by differences of E2 doses.