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

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Running head: Estetrol induces metabolic protection

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ABSTRACT

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

New & Noteworthy :

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

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

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).

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

MATERIAL & METHODS

Mice and surgical procedure

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

Pellet preparation and E4 Dosage

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

Metabolic tests

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

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

Liver and perigonadal adipose tissue histology

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

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

Liver lipid content analysis

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

Gene expression analysis

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

Analysis of atherosclerosis lesions

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

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

Determination of plasma lipids

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

Statistics

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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DISCLOSURES

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

AUTHORS CONTRIBUTION

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

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FIGURE LEGENDS

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

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

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

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

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

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

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

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

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

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

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

Four-week-old ovariectomized female LERKO-LDLR^{-/-} 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. Cumulative weekly body weight gain evolution (A), intraperitoneal glucose tolerance (B) and adipose tissue distribution (C) are presented. Quantification of lipid deposition at the aortic sinus (scale bars: 1mm) (D) and plasmatic HDL-cholesterol, LDL-cholesterol and triglycerides content (E) were quantified. Two-way ANOVA were performed to evaluate interaction between Genotype (Gen) and treatment (E4). When a significant interaction between the 2 factors were revealed, a *Fisher's LSD* post-test was performed (Veh *versus* E4: ****P* < 0.001, n=6 mice/group).

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

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

Diagram illustrating the structure of ERαAF2. The structure consists of three main domains: AF1 (Activation Function 1), DBD (DNA-Binding Domain), and LBD / AF2 (Ligand-Binding Domain / Activation Function 2). The AF1 domain is connected to the DBD domain, which is connected to the LBD / AF2 domain. A red 'X' is drawn over the LBD / AF2 domain, indicating a mutation or deletion. Above the LBD / AF2 domain, the sequence LLEMLD is shown with a red line through it, and a blue 'V' shape is positioned below it.

B.

	ER α AF2 ^{+/+}	ER α AF2 ⁰
Veh
E4

C.

ER α AF2 ^{+/+}		ER α AF2 ⁰	
Veh	E4	Veh	E4

D.

Body weight gain

Y-axis: Weight gain (g)

X-axis: Weeks

Legend:

- AF2 +/+ Veh
- AF2 +/+ E4
- ▲ AF2° Veh
- ▼ AF2° E4

*** p < 0.001

Weeks	AF2 +/+ Veh (g)	AF2 +/+ E4 (g)	AF2° Veh (g)	AF2° E4 (g)
6	0	0	0	0
7	4.5	1.5	4.5	1.5
8	6.5	2.0	6.5	2.0
9	9.0	2.5	9.0	2.5
10	11.5	3.5	11.5	3.5
11	14.0	3.5	14.0	3.5
12	15.5	4.0	15.5	4.0
13	17.0	4.5	17.0	4.5
14	17.5	5.0	17.5	5.0
15	18.0	5.0	18.0	12.0

E. Intrahepatic lipid content

Figure 3

Figure 4

Figure 5

Intrahepatic lipid content

Figure 6