

Estetrol is a weak estrogen antagonizing estradiol-dependent mammary gland proliferation

C Gérard, S Blacher, L Communal¹, A Courtin¹, E Tskitishvili, M Mestdagt, C Munaut, A Noel, A Gompel^{1,2}, C Péqueux* and J M Foidart*

Laboratory of Tumor and Development Biology, GIGA-Cancer, Institute of Pathology, University of Liège, CHU-B23, B-4000 Liège, Belgium

¹INSERM-UMRS 938, Université Pierre et Marie Curie (UPMC), F-75005 Paris, France

²Gynaecological Endocrinology Unit, Paris Descartes University, Hôpitaux Universitaires, F-75006 Paris, France

*C Péqueux and J M Foidart contributed equally to this work

Correspondence should be addressed to C Péqueux
Email
C.Pequeux@ulg.ac.be

Abstract

Estetrol (*E*₄) is a natural estrogen produced exclusively by the human fetal liver during pregnancy. Its physiological activity remains unknown. In contrast to ethinyl estradiol and estradiol (*E*₂), *E*₄ has a minimal impact on liver cell activity and could provide a better safety profile in contraception or hormone therapy. The aim of this study was to delineate if *E*₄ exhibits an activity profile distinct from that of *E*₂ on mammary gland. Compared with *E*₂, *E*₄ acted as a low-affinity estrogen in both human *in vitro* and murine *in vivo* models. *E*₄ was 100 times less potent than *E*₂ to stimulate the proliferation of human breast epithelial (HBE) cells and murine mammary gland *in vitro* and *in vivo* respectively. This effect was prevented by fulvestrant and tamoxifen, supporting the notion that ER α (ESR1) is the main mediator of the estrogenic effect of *E*₄ on the breast. Interestingly, when *E*₄ was administered along with *E*₂, it significantly antagonized the strong stimulatory effect of *E*₂ on HBE cell proliferation and on the growth of mammary ducts. This study characterizes for the first time the impact of *E*₄ on mammary gland. Our results highlight that *E*₄ is less potent than *E*₂ and exhibits antagonistic properties toward the proliferative effect of *E*₂ on breast epithelial cells. These data support *E*₄ as a potential new estrogen for clinical use with a reduced impact on breast proliferation.

Key Words

- ▶ estetrol
- ▶ mammary gland
- ▶ proliferation
- ▶ estrogen receptor alpha
- ▶ agonist/antagonist
- ▶ menopause
- ▶ contraception

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Introduction

17 β -estradiol (*E*₂) stimulates postnatal mammary gland development by driving and coordinating proliferation and differentiation through paracrine interactions (Anderson *et al.* 1998, Russo & Russo 1998, Brisken & O'Malley 2010). Prolonged exposure to endogenous estrogens in case of early age at menarche or late menopause increases the risk of breast cancer development, while an early menopause has a protective effect

(Fenton 2006, Rudel *et al.* 2011). Interestingly, early pregnancy, which is associated with extremely high levels of estrogen exposure to women, exhibits a dual effect on the risk of the breast cancer development (Lambe *et al.* 1994).

A major harmful effect of oral estrogen for menopausal hormone therapy (MHT) or combined oral contraceptive (COC) users is an imbalance in hemostasis leading

to a global enhancement of thrombin generation that increases the risk of venous thromboembolism (Canonica 2014, O'Brien 2014, Scarabin 2014). In addition, several studies have linked the use of exogenous estrogen preparations to the risk of initiating breast cancer (Stahlberg *et al.* 2004, Lai *et al.* 2011, Turkoz *et al.* 2013). The combined administration of equine estrogen plus progestin, and, to a lesser extent, estrogen alone to postmenopausal women increases the risk of breast cancer (Rossouw *et al.* 2002, Anderson *et al.* 2006, Foidart *et al.* 2007, Sturdee *et al.* 2011). A new estrogen with a safer profile is therefore required to improve women's health during and after their fertile lifetime.

Estetrol (*E*₄) is a human-specific natural estrogen produced only during pregnancy by the fetal liver (Coelingh Bennink *et al.* 2008a, Holinka *et al.* 2008a). It was initially identified by Hagen *et al.* (1965), but its physiological role during pregnancy remains to be defined. *E*₄ maternal plasma levels reach 1 ng/ml (3 nM), and 0.5–3.8 mg/day of *E*₄ have been measured in urine at the end of pregnancy, suggesting that exposure to a high level of *E*₄ is not toxic. *E*₄ binds to both estrogen receptors, with a higher affinity for estrogen receptor alpha (ER α – ESR1) (Visser *et al.* 2008). In experimental animal models and in clinical studies in women, *E*₄ acts as an estrogen to prevent vaginal dryness, osteoporosis, and hot flushes (Coelingh Bennink *et al.* 2008b, Holinka *et al.* 2008b, Visser *et al.* 2008). In contrast to *E*₂, it exhibits a high oral bioavailability with an elimination half-life of 28 h in humans. It does not stimulate the production of nor bind to the sex hormone-binding globulin (Hammond *et al.* 2008) and it has no or only a minimal impact on liver function. When used at 5–20 mg in a phase 2 clinical trial (Foidart, JM. Congress of Eur. Soc. Gynecol. 2013, personal communication), *E*₄ induces minimal changes compared with ethinyl estradiol (EE) in binding cortisol globulin, angiotensinogen, triglycerides, or estrogen-sensitive coagulation proteins. Due to these unique pharmacological properties, *E*₄ appears to be suitable as a potential drug for contraception or MHT. However, nothing is known about the potential impact of *E*₄ on the normal breast.

The aim of this work was to investigate the proliferative activity of *E*₄ on the mammary cells in an *in vitro* model of isolated human breast epithelial (HBE) cells and an *in vivo* model of mouse mammary gland growth, coupled with a robust quantification performed by computer-assisted image analysis. To our knowledge, our study is the first one to describe the impact of *E*₄ on normal breast.

Materials and methods

Chemicals, reagents, and steroids

HAM's F10 medium, FCS, NaHCO₃ solution, Hank's balanced salt solution, and sterile trypsin solution were obtained from Life Technologies. EDTA, penicillin–streptomycin, cholera toxin, transferrin, insulin, triiodothyronine, cortisol, estradiol, tamoxifen, ICI 182 780 (fulvestrant), hyaluronidase, and carmin were obtained from Sigma–Aldrich. Epidermal growth factor and collagenase were obtained from Boehringer Mannheim Chemical Corp. Human serum was obtained from the 'Etablissement Français du Sang' of the Saint Antoine Hospital (Paris, France). [Methyl-³H]thymidine was obtained from GE Healthcare (Buckinghamshire, UK). *E*₄ was supplied by Uteron Pharma (Liège, Belgium).

Ethical approval

Human mammary epithelial cells were obtained from women undergoing reduction mammoplasty for aesthetic purposes. All patients gave their informed consent for the studies according to the French law on clinical experimentation ('Comité de protection des Personnes', biomedical project 11826). All of the animal procedures of this study were approved by the animal ethical committee of University of Liège (Belgium).

Human mammary epithelial cell culture

Specimens of normal breast epithelial cells were obtained from women (aged 15–42 years). The patients had no history of breast disease and pathological examination of the tissue showed only normal breast tissue. Briefly, the tissue was digested with collagenase (0.15%) and hyaluronidase (0.05%) in HAM's F10 medium, and then filtered consecutively through 300 and 150 μ m sieves to retain undigested tissue. The cells were pelleted, distributed into T25 plastic flasks, and maintained at 37 °C in a humidified atmosphere with 5% CO₂. The HBE cells were maintained in HAM's medium containing NaHCO₃ (0.24%), penicillin–streptomycin (1%), cortisol (5 ng/ml), T3 (6.5 ng/ml), cholera toxin (10 ng/ml), transferrin (5 mg/ml), insulin (0.016 U/ml), EGF (10 ng/ml) and 5% human serum. Proliferation tests were assessed with these fresh cells.

Proliferation assay

Forty-eight hours after exposure to hormones, cells were incubated with 2 μ Ci of [methyl-³H]thymidine for 48 h

at 37 °C. After incubation, the cells were washed twice with PBS and twice with 5% trichloroacetic acid. The cells were incubated in 5% TCA for 15 min at 4 °C and lysed in NaOH 0.1 M for 15 min at 37 °C. The total cell lysate was added to 25 µl of scintillation liquid and radioactivity was counted with a Beckman (Fullerton, CA, USA) LS-500-CE β counter.

Prepubertal mouse study experimental design

C57BL/6 female prepubertal mice were purchased from Janvier Laboratory (Saint-Berthevin, France). Five mice per cage were housed under a standard 12-h photoperiod. Food and water were provided *ad libitum*. Mice were ovariectomized (OVX) at 28 days of age under isoflurane anesthesia to remove endogenous hormone production. Oral treatment was initiated at 35 days of age for a period of 6 or 14 days. Mice were fed by gavage with peanut oil containing 5% ethanol (vehicle, negative control) or *E*₄ (0.3, 1, 3, or 10 mg/kg per day) alone or in combination with *E*₂ (1 mg/kg per day). *E*₂ and *E*₄ were dissolved in absolute ethanol and brought to the final concentration with peanut oil (5% EtOH in peanut oil). At the end of treatment, mice were killed; mammary tissues were dissected for whole-mount preparation and epithelial RNA isolation or paraffin-embedded for histological analyses. Plasma was isolated from blood (cardiac puncture) and circulating hormone concentration was determined by mass spectrometry (ATC Pharma, Liège, Belgium). The animals were weighed before and after treatment. The experimentation was performed with five animals in each group.

Mammary gland whole mount

Murine mammary gland growth is a particularly sensitive method largely used to study the impact of various compounds directly on the mammary epithelium (Ayyanan *et al.* 2011) and to define the molecular mechanisms driving mammary gland development (Mallepell *et al.* 2006, Beleut *et al.* 2010). One of the fourth inguinal mammary glands was removed in one piece and spread onto a glass slide. The removed gland was subjected to whole-mount fixation, defatting, and staining. The mammary glands were fixed in Carnoy's fixative (ethanol, chloroform, glacial acetic acid; 6:3:1) for at least 4 h and defatted in acetone overnight. They were then rehydrated through a series of graded alcohols and stained with carmine overnight. The whole-mount glands were then dehydrated sequentially through 70, 90, and 100% ethanol for 20 min each and cleared in xylene for at

least 2 h. Digital pictures of mammary glands immersed in xylene were taken using a Leica M80 microscope with a Leica IC80 HD digital camera attached.

Image processing and morphological measurements

To quantify the morphological changes occurring under the various treatments in mouse mammary gland, we developed an original computer-assisted method of quantification. Digital pictures of mammary gland whole mounts allowed an assessment of morphological features by image analysis. Image processing and measurements were implemented with the image analysis toolbox of Matlab7.9, Mathworks (Natick, MA, USA). Mammary gland structure was extracted using the Frangi multiscale filter (Frangi *et al.* 1998) that allows the identification of elongated objects having a characteristic thickness, in images presenting nonhomogeneous intensities. The resulting images were then binarized, i.e., pixel values belonging to mammalian glands take the value equal to 1 and 0 background pixels. Finally, morphological filters (Soille 1999) were used to eliminate noise remaining in the images. The resulting binary images were then systematically compared with the original ones and corrected manually if required. Total area of the epithelial network, also named ductal tree (mm²), was calculated from binary images that were further skeletonized (Soille 1999) in order to measure the total length (mm). Total area and total length of the ductal tree are two parameters reflecting the growth rate of the mammary gland. Furthermore, to evaluate gland differentiation, proliferating units of the mammary gland named terminal end buds (TEBs) were automatically detected on mammary glands collected after 6 and 14 days. As they appear as the darkest features located at the periphery of the mammary gland, binary images of TEBs were obtained using an appropriate threshold and a size filter. Only buds with diameter >100 µm were considered as TEB (LaRocca *et al.* 2011). From those images, the total number of TEBs were measured.

Ki67, ER α , and progesterone receptor immunohistochemistry

To carry out histological analysis, samples were fixed in 4% formalin for 2 h and stored in 70% ethanol before paraffin embedding. The sections were cut at 6 µm. For detection of Ki67, ER α , and progesterone receptor (PGR) in the mammary gland, the slides were deparaffinized in xylene and rehydrated through graded alcohols. For antigen retrieval, sections were heated at 126 °C under

1.4 Bar in 10 mM citrate buffer for 10 min. The sections were then treated with 3% H₂O₂ for 20 min to block endogenous peroxidase activity, washed with PBS, and incubated with 10% BSA for 1 h. After blocking, the sections were incubated with the Ki67 antibody (TEC-3 rat monoclonal IgG) from Dako (Glostrup, Denmark), the ER α antibody (MC-20 rabbit polyclonal IgG) from Santa Cruz Biotechnology, or the PGR antibody (SP2 rabbit monoclonal IgG) from Thermo Scientific (Waltham, MA, USA) at 1:50, 1:25, and 1:400 dilutions respectively. The slides were then incubated with biotinylated secondary antibodies for 30-min, followed by 30 min incubation with streptavidin–peroxidase conjugate. Antigen–antibody complex was visualized by incubation with 3,3'-diaminobenzidine 5. The slides were counterstained with hematoxylin, dehydrated, and mounted using a mounting medium from Labonord (Templemars, France). Positively stained cells appeared brown while negative cells were blue.

Epithelial cell isolation from mouse mammary gland

Mice were killed by cervical dislocation and both fourth mammary glands were dissected. The proximal and distal parts of the mammary glands were collected in DMEM/F12 supplemented with collagenase (0.10%) and hyaluronidase (0.05%), but the central part containing the lymph node was discarded. Tissue was minced and incubated under constant agitation for 1 h at 37 °C. After centrifugation at 450 *g* for 5 min, organoids were suspended in cold HBSS, and then five pulse centrifugations reaching 450 *g* were performed. The final pellet should contain mainly epithelial structures. Single cells, fibroblasts, and blood cells were discarded with the supernatant.

Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen) from the epithelial cells isolated from mouse mammary gland, following the manufacturer's protocol. RT and real-time quantitative PCR were performed using specific primers and Brilliant SYBR GREEN QPCR master mix on a Roche apparatus. Sequence primers for target genes were (5' to 3'): glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) forward GTTCACCCACTAATAGGG and reverse GGAT-TCTGACTTAGAGGCGTT; *Pgr* forward GCCACAG-TATGGCTTTGATTCTT and reverse GCAACACCGTC-AAGGGTTCTCATA; *Mki67* (Ki67) forward GATAGCT-TCTGTGCTGACCCTGAT and reverse CCCTGATGA-GTCTTGCTAACACT; *Ccnd1* (cyclin D1) forward

GAGCTGCTGCAAATGGAAGCTGCTT and reverse GTTC-TCATCCGCCTCTGGCATTCTT. Gene expression values were normalized to housekeeping gene *Gapdh*.

Statistical analyses

All quantitative experimental data are expressed as mean \pm S.D. or mean \pm S.E.M. Statistical analyses were conducted with GraphPad Prism Software (La Jolla, CA, USA) using one-way ANOVA followed by Student–Newman–Keuls's test or using Kruskal–Wallis followed by Dunn's test, with regard to heteroscedasticity. A *P* value of ≤ 0.05 was considered as statistically significant.

Results

E₄ is an estrogen with low affinity, which stimulates HBE cell proliferation at high doses

Exposure of HBE cells for 96 h to 10 nM *E₂* elicited a maximal cell proliferation increase by 60% (Fig. 1A). A tenfold higher *E₂* concentration failed to further stimulate proliferation. 10 nM *E₄* did not increase HBE cell proliferation (Fig. 1B). A 100-fold higher concentration was necessary to stimulate the proliferation rate to the same extent, indicating that *E₄* is a low affinity estrogen in HBE cells.

E₄ promotes mammary gland growth and proliferation in OVX mice

The *in vivo* impact of a daily oral treatment with *E₄* was analyzed on mammary gland of OVX mice after 6 or 14 days and quantified using an original computer-assisted

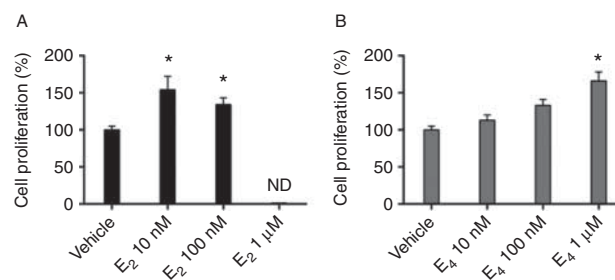
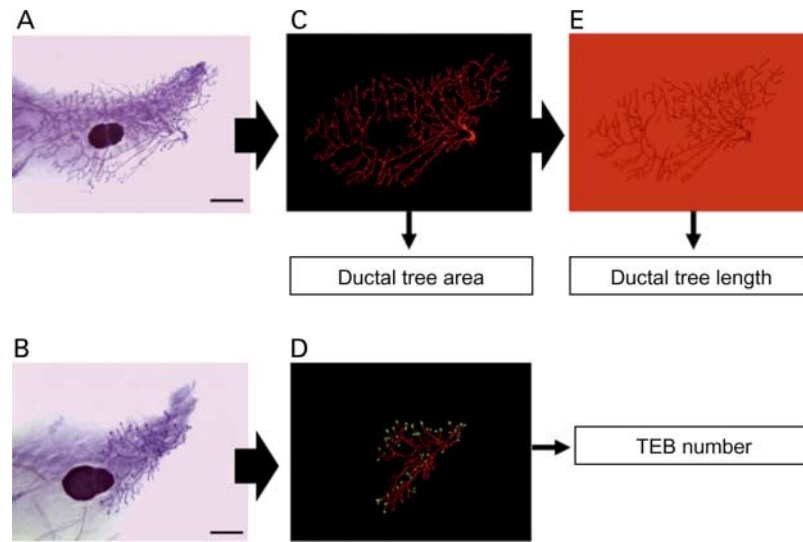


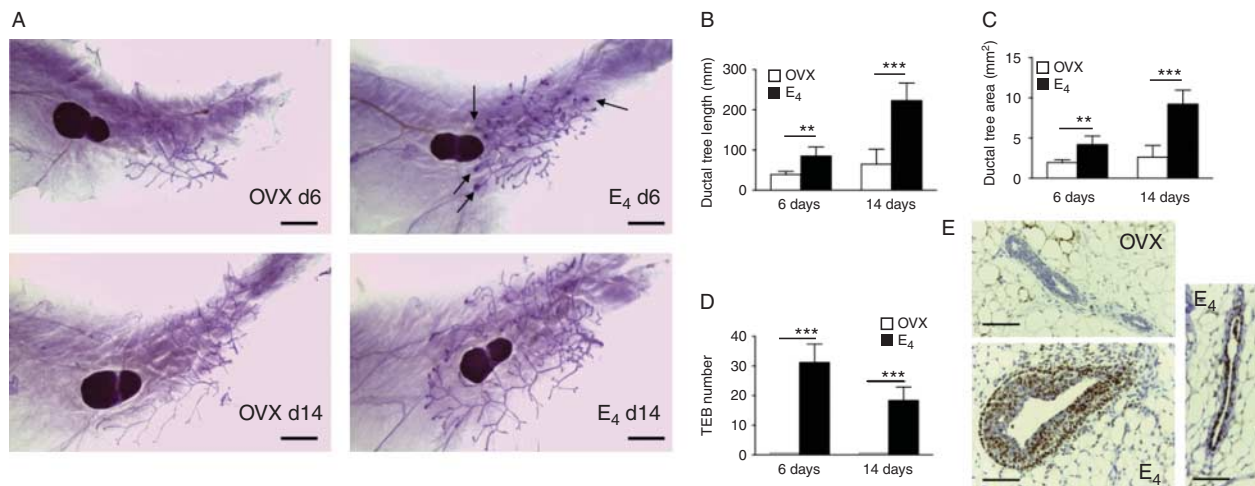
Figure 1 Effect of *E₄* and *E₂* on HBE cell proliferation. Histograms of HBE cell proliferation determined by [³H]thymidine incorporation after 96 h of treatment. (A) Treatment with *E₂* (10 and 100 nM). (B) Treatment with *E₄* (10 nM, 100 nM, and 1 μ M). Data are expressed as % of control normalized at 100%, mean \pm S.E.M., *n* = 3. **P* < 0.05 vs vehicle. HBE, human breast epithelial, ND, not determined.

**Figure 2**

Computer-assisted method of mammary gland quantification. Morphological parameters of the mammary glands were quantified with a computer-assisted method. Whole-mount mammary glands after 14 days (A) and 6 days (B) of estrogenic treatment. (C) Binary image of the matched whole-mount mammary gland used to measure the total area occupied by

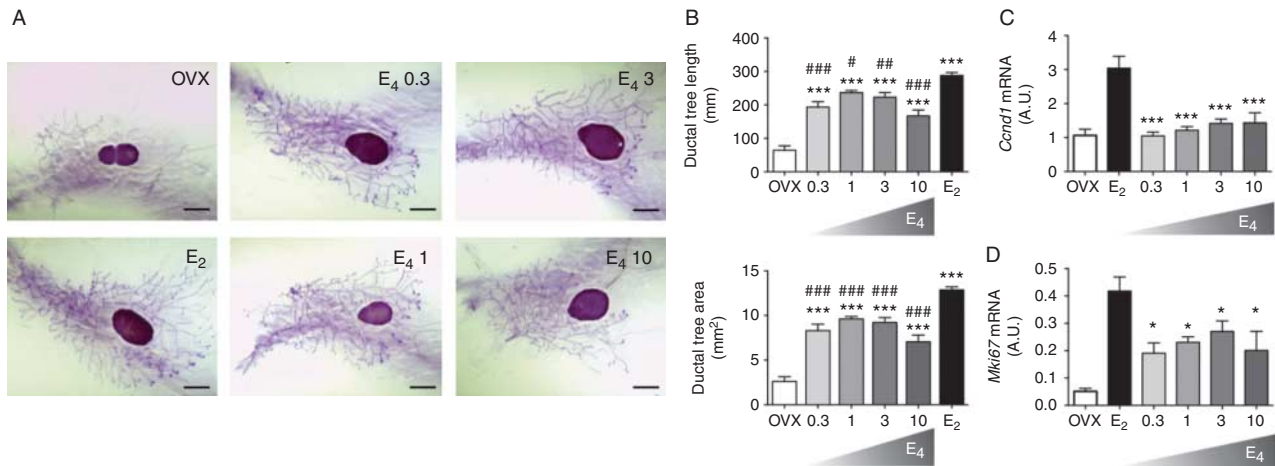
image analysis method (Fig. 2). *E*₄ (3 mg/kg per day) induced the growth of the prepubertal epithelial ducts and the appearance of TEBs (Fig. 3A). The mammary gland growth rate was quantified by measuring the total length

and total area of the epithelial ductal tree (Fig. 3B and C). After 6 days of treatment, the average total length and area of the epithelial network were increased by 2.18- and 2.13-fold in *E*₄-treated mice respectively. TEBs were

**Figure 3**

Effect of *E*₄ on mouse mammary gland growth and proliferation. (A) Whole-mount mammary gland from ovariectomized mice orally treated with vehicle (OVX) or from ovariectomized mice orally treated with *E*₄ at 3 mg/kg per day (*E*₄) for a period of 6 (d6) or 14 (d14) days. TEBs are pointed by arrows. Scale bars=2 mm. (B, C, and D) Computer-assisted quantification of the morphological parameters of whole-mount mammary glands from ovariectomized mice orally treated with vehicle (OVX) or from ovariectomized mice orally treated with *E*₄ 3 mg/kg per day (*E*₄) for a period of 6 (d6) or 14 (d14) days. Mammary gland morphology was assessed with

three parameters: total length (B) and area (C) of the ductal tree, and number of TEBs (D). Note the total lack of TEBs in control groups (OVX). Data are presented as mean \pm s.e.m., $n=5$. ** $P<0.01$, *** $P<0.001$ vs OVX. (E) Histological sections of mammary gland, stained with an anti-Ki67 antibody, from OVX or *E*₄ (3 mg/kg per day)-treated mice after 6 days. Lower left panel, TEB section; right panel, epithelial duct section. Scale bars=100 μ m. TEB, terminal end bud. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JOE-14-0549>.

**Figure 4**

Comparison between the effect of *E*₄ and *E*₂ on mammary gland growth. Mice were ovariectomized and orally treated with vehicle (OVX), with *E*₂ 1 mg/kg per day (*E*₂) or with *E*₄ ranging from 0.3 to 10 mg/kg per day (*E*₄) for a period of 14 days. (A) Whole-mount mammary gland, scale bars = 2 mm. (B) Computer-assisted quantification of morphological parameters of whole mount mammary glands. Ductal tree length and area data are

expressed as mean \pm s.e.m., $n=5$. *** $P<0.001$ vs OVX; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs *E*₂. (C) *Ccnd1* (cyclin D1) mRNA and (D) *Mki67* (*Ki67*) mRNA expression level of epithelial cells isolated from mammary gland. Results are expressed in arbitrary units (A.U.), mean \pm s.e.m., $n=5$, * $P<0.05$, *** $P<0.001$ vs *E*₂. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JOE-14-0549>.

completely absent in the vehicle-treated group (OVX), although an average of 29.4 ± 2.2 TEBs/gland ($n=10$ glands from five mice) was identified in the *E*₄-treated group (Fig. 3D). After 14 days of treatment, the mean total length and the area of the epithelial network were increased by 4.9- and 3.5-fold in *E*₄-treated mice respectively. As classically observed in this model (Sternlicht *et al.* 2006), the number of TEBs remained higher in the estrogen-treated mice compared with the untreated ones, but to a lower extent than after 6 days. Indeed, after 14 days of treatment, most of TEBs had regressed and became histologically simpler. The proliferative status of the *E*₄-treated mammary glands was confirmed by Ki67 immunolabeling (Fig. 3E). In contrast to what was observed for mammary gland from OVX mice that did not express Ki67, the labeling revealed the presence of proliferating cells in TEBs and in ducts of *E*₄-treated mice.

*E*₄ has a weaker stimulatory effect on mammary gland than *E*₂

We then compared the effect of *E*₄ to a physiological concentration of *E*₂. OVX mice were orally treated with *E*₄ (0.3, 1, 3, or 10 mg/kg per day) or with *E*₂ (1 mg/kg per day) during 14 days (Fig. 4A). The serum levels of *E*₂ and *E*₄ measured 3 h after oral administration are given in Table 1. The concentration range used for *E*₄ was established to start within the physiological and

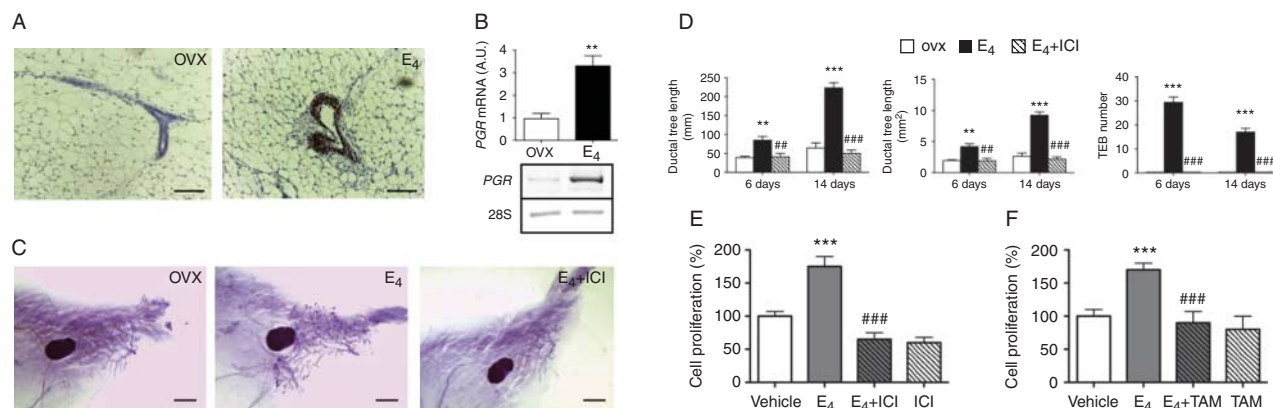
therapeutic ranges and to exceed them (Coelingh Bennink *et al.* 2008a). Despite doses of *E*₄ resulting in a plasma concentration up to 300-fold over that of *E*₂, *E*₄ promoted epithelial growth only by 55–75% of the total length of the gland area achieved with *E*₂ (Fig. 4B). The expression of cyclin D1 mRNA, which is induced by *E*₂ and correlates with proliferation (Liu *et al.* 2002), was significantly lower in mammary epithelial cells of mice treated with *E*₄ than that with *E*₂, and is even comparable with untreated mice (Fig. 4C). *Ki67* mRNA that is overexpressed during cell proliferation was also significantly lower (Fig. 4D).

ER α drives *E*₄-mediated stimulation of mammary gland growth

While ER β is dispensable for mammary gland elongation (Forster *et al.* 2002), *E*₂ contributes to mammary epithelium proliferation and morphogenesis by paracrine

Table 1 Circulating levels of *E*₂ and *E*₄ measured 3 h after oral administration

	Oral administration (mg/kg)	Serum concentration (mean \pm s.d., pg/ml)	Serum concentration (mean \pm s.d., nM)	n	<i>E</i> ₄ / <i>E</i> ₂ ratio
<i>E</i> ₂	1	171 \pm 15	0.63 \pm 0.06	3	–
<i>E</i> ₄	0.3	2253 \pm 775	7.5 \pm 2.5	8	12
<i>E</i> ₄	1	6533 \pm 2409	22.5 \pm 7.9	8	36
<i>E</i> ₄	3	18 484 \pm 5318	61 \pm 17	8	97
<i>E</i> ₄	10	71 832 \pm 23 944	236 \pm 79	4	374

**Figure 5**

ER α drives E₄ activity on mammary gland growth and HBE cell proliferation. (A) Histological sections of mammary gland, stained with an anti-PGR antibody, from OVX or E₄ (3 mg/kg per day)-treated mice after 6 days, magnification 20 \times . Scale bars = 100 μ m. (B) *Pgr* mRNA expression level of epithelial cells isolated from mammary gland from OVX or E₄ (3 mg/kg per day)-treated mice after 6 days. Data are expressed as mean \pm s.e.m., n = 5. ***P* < 0.01 vs OVX. (C) Whole-mount mammary gland from ovariectomized mice orally treated with vehicle (OVX) or with E₄ 3 mg/kg per day alone (E₄) or associated with s.c. injection of ICI 182 780 used at 30 mg/kg (E₄+ICI) for a period of 6 days. Scale bars = 2 mm. (D) Computer-assisted quantification

of morphological parameters of whole-mount mammary glands obtained as in (C). Ductal tree length and area data and TEB number are expressed as mean \pm s.e.m., n = 5. ***P* < 0.01, ****P* < 0.001 vs OVX; ###*P* < 0.01, ####*P* < 0.001 vs E₄. (E and F) Proliferation of HBE cells treated with vehicle, E₄ (1 μ M), and/or with ICI 182 780 (ICI, 1 μ M) or tamoxifen (TAM, 1 μ M) measured by [³H]thymidine incorporation. Results are expressed as % of vehicle normalized at 100%, mean \pm s.e.m., n = 3. ****P* < 0.001 vs vehicle, ####*P* < 0.001 vs E₄. TEB, terminal end bud. A full colour version of this figure is available via <http://dx.doi.org/10.1530/JOE-14-0549>.

signaling through ER α (Mallepell *et al.* 2006). When activated by E₂, ER α promotes the expression of PGR in the mammary epithelium (Beleut *et al.* 2010). In order to define whether E₄ activates mammary epithelium ER α , the expression of PGR was evaluated *in vivo*. PGR proteins were expressed in the mammary epithelium of E₄-treated mice, but not of OVX untreated mice (Fig. 5A). *Pgr* mRNA was overexpressed under E₄ treatment (Fig. 5B). In addition, the pure ER α antagonist ICI 182 780 completely blocked the elongation of the ductal tree induced by E₄ (Fig. 5C). Quantification of total ductal tree and length and TEB number confirmed these observations (Fig. 5D). *In vitro* both ICI 182 780 and tamoxifen (an ER α antagonist in the breast) also abolished the stimulating effect of E₄ on HBE cell proliferation (Fig. 5E and F). Altogether, these *in vitro* and *in vivo* results support that E₄ exerts genomic effects through ER α and that this receptor is involved in the proliferative effect of E₄ on human mammary epithelial cells and mouse mammary gland.

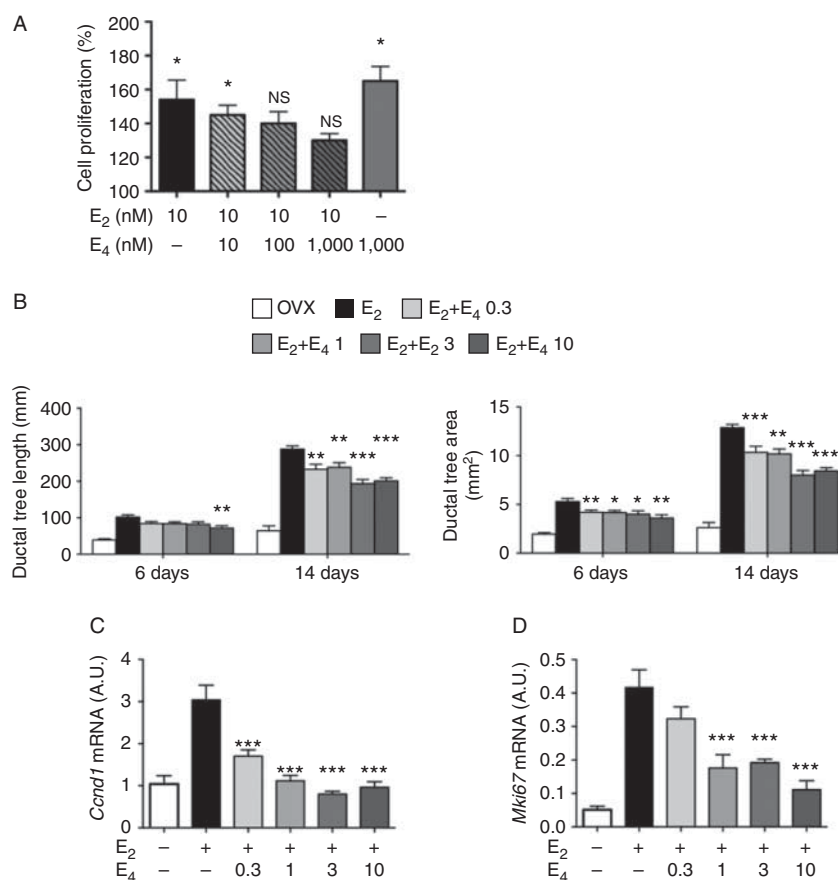
E₄ partially antagonizes the stimulatory effect of E₂ on HBE cell proliferation and mammary gland growth

We then evaluated the impact of a combined administration of E₄ and E₂ on HBE cell proliferation and on mammary gland growth. HBE cells were exposed

in vitro to a maximal stimulatory concentration of E₂ (10 nM) combined with increasing concentrations of E₄ (10–1000 nM). E₄ antagonized the effect of E₂ in a dose-dependent pattern (Fig. 6A). *In vivo* mice received a daily oral physiological dose of E₂ (1 mg/kg per day) either alone or in combination with E₄ at a concentration of 0.3, 1, 3, or 10 mg/kg per day. After 14 days of treatment, exposure to the combination of E₂+E₄ decreased the total length and the area of the epithelial network induced by treatment with E₂ alone by 17–38% (Fig. 6B). In addition, the combination of E₂+E₄ decreased cyclin D1 and *Ki67* mRNA expression by 23–100% in mammary epithelium (Fig. 6C and D) on E₂ treatment. Altogether, these results support that E₄ is able to partially antagonize the effect of E₂ on breast epithelium proliferation *in vitro* and *in vivo*.

Discussion

Despite benefits in contraception and MHT, debates over harmful effects of estrogen have been intensified. Even if they are required for breast physiology, particularly during pregnancy in preparation for lactation, their mammary impact is usually considered to be deleterious because of their mitogenic activity. Thus, there is considerable interest in developing COC or MHT with new estrogen having minimal venous thromboembolism impact and

**Figure 6**

Effect of combination of *E*₂ and *E*₄ on HBE cell proliferation and mammary gland growth. (A) Effect of *E*₂, *E*₄, and their combination (*E*₂ + *E*₄) on HBE cell proliferation measured by [³H]thymidine incorporation and expressed as % of vehicle normalized at 100%, mean ± s.e.m. (*n* = 3). **P* < 0.05, NS, not significant vs vehicle. (B) Computer-assisted quantification of morphological parameters of whole-mount mammary glands from ovariectomized mice orally treated with vehicle (OVX), *E*₂ 1 mg/kg per day alone (*E*₂), or associated with *E*₄ used at 0.3, 1, 3, or 10 mg/kg per day (*E*₂ + *E*₄) for a period

of 6 or 14 days. Ductal tree length and area data are expressed as mean ± s.e.m., *n* = 10 glands from five mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs *E*₂ of the corresponding period of 6 or 14 days. (C) *Ccnd1* (cyclin D1) mRNA and (D) *Mki67* (*Ki67*) mRNA expression level of epithelial cells isolated from mammary gland of ovariectomized mice orally treated for 14 days with vehicle or with *E*₂ 1 mg/kg per day alone or associated with *E*₄ doses ranging from 0.3 to 10 mg/kg per day. Data are expressed as mean ± s.e.m., *n* = 5. ****P* < 0.001 vs *E*₂.

lower breast proliferative capacity. In this study, we report that *E*₄ acts on breast proliferation through ERα less efficiently than *E*₂, *in vitro* and *in vivo*. More interestingly, when coadministered, *E*₄ is able to partially antagonize the *E*₂-induced proliferation of HBE cells and the mouse mammary gland growth.

Similar to *E*₂, *E*₄ stimulated the proliferation of human mammary epithelial cells *in vitro* and murine breast growth *in vivo* through ERα, because ICI 182 780 and tamoxifen blocked this effect. This ERα-dependent activation induced PGR expression, a well-described process regulated by *E*₂ (Horwitz *et al.* 1978, Vienonen *et al.* 2002). However, *E*₄ stimulated breast proliferation with a 100-fold weaker efficacy than *E*₂ *in vitro* as well as *in vivo*.

This is consistent with competitive binding studies showing that *E*₄ exhibits considerably lower affinity than *E*₂ toward ERα (Martucci & Fishman 1976, Tseng & Gurbide 1976, 1978, Visser *et al.* 2008), and with the observation that *E*₄ was less potent than *E*₂ to increase breast cancer cell line proliferation and migration (Jozan *et al.* 1981, Giretti *et al.* 2014, Liu *et al.* 2014).

The dose range of *E*₄ administered orally in this study was defined to cover and exceed the physiological levels of *E*₄ detected in the plasma of pregnant women (3 nM), in fetal blood (45 nM) (Coelingh Bennink *et al.* 2008a), as well as in serum from a clinical trial where 5–20 mg/day *E*₄ was administered to women of reproductive age (Foidart, JM. Congress of Eur. Soc. Gynecol. 2013, personal

communication). This range of *E*₄ doses induced circulating levels of approximately ten- to 400-fold higher than those of *E*₂ tested in this study. Despite these large differences, the stimulation of mammary gland growth by *E*₄ remained at most 55% of that by *E*₂. *E*₄ was unable to increase cyclin D1 expression, an ER α -inducible gene that governs *E*₂-dependent epithelial cell growth in mammary gland (Liu *et al.* 2002, Casimiro *et al.* 2013). At these concentrations, *E*₄ has been reported to significantly prevent osteoporosis and hot flushes in preclinical rat models (Visser & Coelingh Bennink 2009). Altogether, these results highlight that *E*₄ could be suitable to control reproduction, osteoporosis, and vasomotor symptoms with limited impact on breast proliferation.

The concomitant exposure to *E*₂ and *E*₄ *in vitro* and *in vivo* revealed that *E*₄ partially antagonized the proliferation induced by *E*₂ on HBE cell proliferation and on mammary gland growth. This antagonistic effect was also evidenced for cyclin D1 and *Ki67* mRNA expression. It was maximal *in vitro* and *in vivo* when the dose of *E*₄ was 100 times higher than that of *E*₂. Giretti *et al.* (2014) recently showed that *E*₄ administration to malignant human breast adenocarcinoma T47-D cells weakly stimulated their cytoskeleton remodeling and migration. However, when *E*₂ was present, *E*₄ counteracted the stimulatory actions of *E*₂. Estriol (*E*₃), another estrogen associated with pregnancy, has also been described as a weak estrogen with mixed agonist/antagonist activity (Clark & Markaverich 1984, Melamed *et al.* 1997).

The precise molecular mechanism by which *E*₄ antagonizes *E*₂ on epithelial breast cell proliferation has not been evaluated in this study. The molecular and kinetic basis for the mixed agonist/antagonist activity of *E*₃ has been suggested to be the consequence of a complex equilibrium among several ER α species: unliganded hER α monomers and dimers; *E*₂-hER α monomers and dimers; *E*₃-hER α monomers and dimers; and *E*₃-*E*₂-hER heterodimers. It is plausible that similar plurality of ER α complexes occurs in the presence of *E*₄+*E*₂ and causes variable interactions with estrogen-responsive elements (ERE) comparable to those described with *E*₂+*E*₃ (Melamed *et al.* 1997). A differential activation by *E*₂ and *E*₄ of nuclear (genomic) and extra-nuclear (nongenomic) effects of ER α could also be involved as reported in uterus, vasculature, and breast cancer cells in the study reported by Abot *et al.* (2014).

Whatever the complex mechanisms of ER α activation by *E*₄+*E*₂ may be, our quantitative analyses demonstrate that *E*₄ acts on ER α in the breast as a weak estrogen agonist and as an *E*₂ antagonist. Our data contribute to the

emerging hypothesis that *E*₄ may be a naturally occurring ER modulator in the breast that may be considered as a potential new estrogen for clinical use with a reduced impact on breast proliferation.

Declaration of interest

J M F is Professor at the University of Liege and Scientific Consultant for Uteron Pharma. M M is Scientific Consultant for Uteron Pharma.

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Author contribution statement

C G designed and performed experiments, analyzed data, and prepared the figures and wrote the manuscript. S B developed and performed all computer assisted quantification analysis. L C and A C contributed to *in vitro* experiments. A G supervised *in vitro* experiments and reviewed the manuscript. E T, M M, C M, and A N gave scientific advices along the study. C P supervised experiment design, analyzed data, and wrote the manuscript. J M F initiated and supervised the project and critically reviewed the manuscript.

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