To my family, Ruben and my friends

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"FUNCTIONAL ROLE OF ESTROGEN RECEPTORS DURING AGING AND THEIR INVOLVEMENT IN INFLAMMATORY PROCESSES"

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Introduction

Estrogens are female sex hormones, belonging to a group of steroids, and they are responsible for the sexual characteristics of the female (Gruber et al., 2002). They also have effects on bone, cardiovascular system, brain, and skin. The main human estrogens are estrone (E1) **1**, estradiol (E2) **2**, and estriol (E3) **3** (Fig. 1). Biologically the most active and abundant estrogen is estradiol **2**. The estrogen actions occur through the binding of the estrogens to estrogen receptors. The estrogen receptors α and β — the latter found by Gustafsson's group (Kuiper et al., 1996) in 1996 — are present in various tissues. Estrogen fatty acid esters are a lipophilic form of estrogens, thought to function as estrogen storage in adipose tissue. Estrogens are also produced in the male and they play an important role in spermatogenesis, cardiovascular health and bone homeostasis (de Ronde et al., 2003). Estrogens are found in the endocrine systems of all vertebrates (Lange et al., 2002). The horse produces special estrogens, namely equilin **4** and equilenin **5** (Fig. 1). Estrogens also occur in the plant kingdom, in small quantities for example in pomegranate (*Punica granatum*), date palm (*Phoenix dactylifera*), beans (*Phaseolus vulgaris*), and olive tree (*Olea europea*).

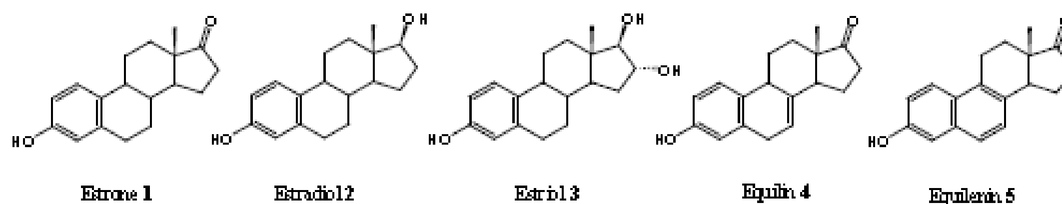


Figure 1. Structures of the main human estrogens estrone 1, estradiol 2, and estriol 3, together with horse estrogens equilin 4 and equilenin 5.

Estrone **1** was first isolated from pregnancy urine and crystallized in 1929 concurrently by two groups, Doisy in the United States and Butenandt in Germany (Butenandt, 1979). Estriol **3** was isolated in 1930 and estradiol **2** in 1936. Estrone **1** (Latin *oestrus* meaning frenzy) was originally named theelin (Greek *thelon* for female), and estriol **3** was called tell (Adam and Rosenheim, 1993), Anner and Miescher (Anner, 1948) announced the first total synthesis of estrone **1** in 1948. Although estrogens have been studied for almost a century, the research continues. Estrogen metabolites and derivatives have been subjects of great interest. The

metabolite 2-methoxyestradiol **6** is an especially promising antitumor agent as well as a potential drug candidate (Lakhani et al., 2003).
Oral and transdermal estrogens are used in hormone replacement therapy (HRT), usually together with progesterone, to relieve menopausal symptoms in women. The benefits and risks of HRT have been extensively evaluated. HRT has beneficial effects on osteoporosis and it is protective against cardiovascular disease, but it increases the risk of breast cancer and may cause thromboembolic disease . Whereas synthetic estrogens are used in HRT in Europe, conjugated equine estrogens extracted from urine of the pregnant horse are common in the United States. These equine products comprise several different estrogens, which are further converted to various metabolites (Turgeon et al., 2004).

ESTROGEN BIOSYNTHESIS

Estrogens are the end products of a long biosynthetic pathway starting from squalene and proceeding through cholesterol **12** to androgens (Ackerman and Carr, 2002). The biosynthetic pathway has been studied by adding, *in vivo* or *in vitro*, ^{14}C-labeled cholesterol, progesterone or androgens and detecting the formed radiolabeled estrogen (Morand and Lyall, 1968). Testosterone **8** is oxidized twice at C-19 by steroid 19-hydroxylase and then aromatized by aromatase (CYP 19) to estradiol (Ackerman and Carr, 2002) 17β-Hydroxysteroid dehydrogenase (17β-HSD) is responsible for the interconversion of estradiol and estrone. The reductive isoform 17β-HSD type 1 converts E_1 **1** to E_2 **2,** and the oxidative isoform 17β-HSD type 2 acts in the opposite way. In women, estrogen biosynthesis occurs mainly in the ovaries in premenopausal women and in adipose tissue in the post menopausal women, but also local biosynthesis occurs in a number of sites including in breast, brain, and bone (Gruber et al., 2002).

ESTROGEN METABOLISM

The oxidative metabolism of estrogens is performed by cytochrome P450 enzyme families mainly in the liver. Oxidation by P450 isoforms can occur at almost every position in the estrogen skeleton and over 40 metabolites have been identified in biological samples from humans or animals or *in vitro* incubations (Zhu and Conney,

1998). In humans, estrogen metabolism consists mainly of the 15 metabolites. Several pathways can be distinguished in estrogen metabolism, the main ones being the 2-hydroxylation and 16α-hydroxylation pathways. A minor pathway is the 4-hydroxylation pathway. The *O*-methylation of catechol estrogens is catalyzed by catechol-*O*methyltransferase (COMT). Eventually the metabolites are converted to inactive, water-soluble glucuronides and sulfates and excreted in the urine or feces.

ESTROGEN RECEPTORS

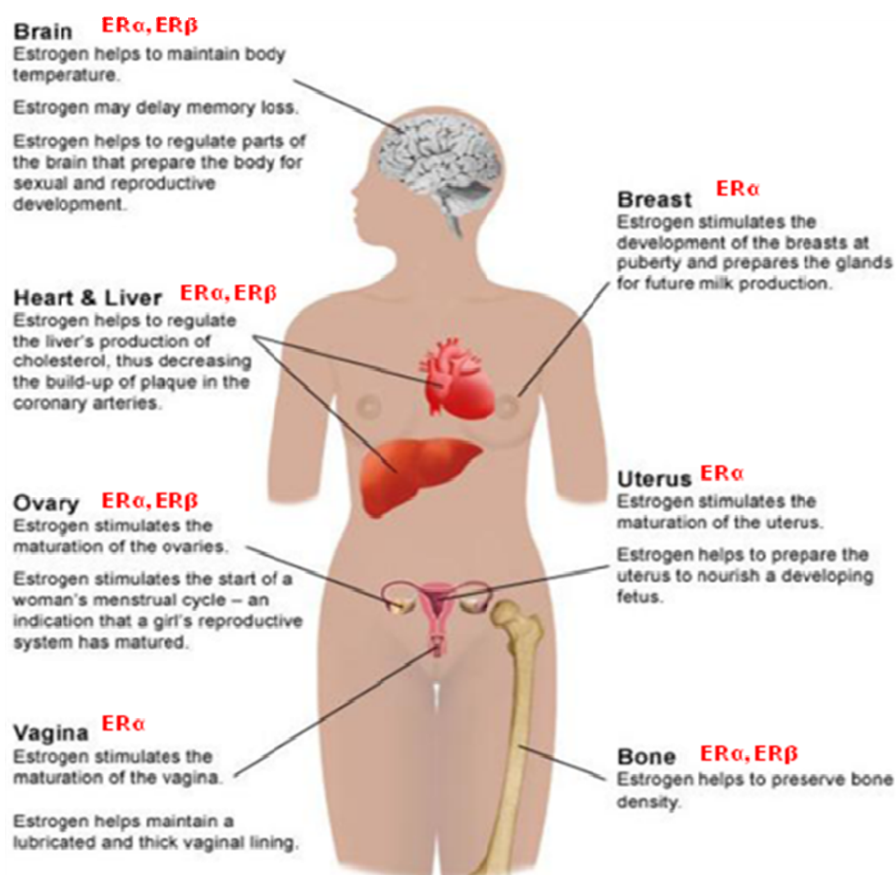


Figure 1. Schematic representation of oestrogen receptor localisation in female human tissues.

Cellular signaling of estrogens is mediated through two ERs, ERα (NR3A1) and ERβ (NR3A2), both belonging to the nuclear receptor (NR) family of transcription factors. Like many other members of the NR family, ERs contain evolutionarily conserved structurally and functionally distinct domains. The central and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas ligand binding occurs in the COOH-terminal multifunctional ligand-binding domain (LBD). The NH2-terminal domain is not

conserved and represents the most variable domain both in sequence and length. Transcriptional activation is facilitated by two distinct activation functions (AF), the constitutively active AF-1 located at the NH2 terminus of the receptor and the ligand-dependent AF-2 that resides in the COOH-terminal LBD. Both AF domains recruit a range of coregulatory protein complexes to the DNA-bound receptor. The two ERs share a high degree of sequence homology except in their NH2- terminal domains, and they have similar affinities for E_2 and bind the same DNA response elements. Ligand-dependent estrogen signaling begins with the binding of estrogen to ER. Thereafter, the cell-specific transcriptional response to estrogen depends on multiple factors, the most immediate being the composition of coregulatory proteins in a given cell and the characteristics of the promoters of estrogen responsive genes. Since hormones are modulators of transcription, the pattern of modulated genes also depends on what other signaling pathways are active in the cell at the time of hormone exposure (Katzenellenbogen et al., 1996; Nilsson et al., 2001; Katzenellenbogen and Katzenellenbogen, 2002).

The identification of the second estrogen receptor ERβ and several receptor isoforms has affirmed the complex nature of estrogen signaling and helped to explain estrogen action in tissues that do not express ERα. ERα and ERβ are products of separate genes located on different chromosomes (Menasce et al., 1993; Enmark et al., 1997). Several splice variants have been described for both receptor subtypes, but whether all the variants are expressed as functional proteins with biological functions is not clear. Most ERα variants differ in their 5'-untranslated region (UTR), not in the coding sequence. In addition, shorter ERα isoforms lacking exon 1 and consequently the NH2-terminal AF-1 (here termed hERα-46 and hERα-36) have been isolated and identified in different cell lines (Flouriot et al., 2000; Wang et al., 2005). These receptor isoforms have not yet been identified or characterized in tissues, and their involvement in regulating estrogen effects *in vivo* remains to be determined. They are, however, interesting research tools since they have the ability to heterodimerize with the full-length ERα and thereby repress AF-1-mediated activity (Flouriot et al., 2000; Wang et al., 2005). Possibly, they may also localize to the plasma membrane and may help to elucidate the mechanisms through which rapid, "nongenomic" estrogen signaling occurs (Li et al., 2003; Wang et al., 2005).

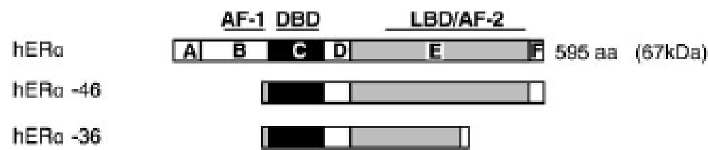
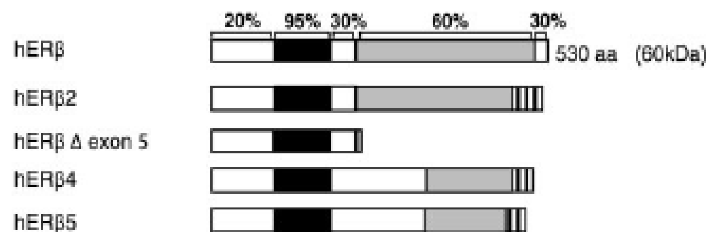


Figure 2. Schematic representation of estrogen receptor alpha isoforms. The domains of the receptors include DBD, LBD, and two transcriptional activation functions, AF-1 and AF-2 as indicated for hERα. Full-length human ERα is 595 amino acids long. Both short ERα isoforms, hERα-46 and hERα-36, lack the NH2-terminal region harboring AF-1.

Unlike ERα, several splice variants of ERβ are expressed in tissues. The 530-amino acid (aa)-long human ERβ isoform is currently regarded as the wild-type ERβ (rat and mouse, 549 aa) (Leygue et al., 1998).

Several alternative ERβ isoforms have been described, and many of these are expressed as proteins in tissues (Fuqua et al., 1999; Saji et al., 2002).

Characterization of the functional isoform pattern in human samples is not complete, but several experiments indicate that ERβ isoforms can differentially modulate estrogen signalling and, as a consequence, impact target gene regulation (Matthews and Gustafsson, 2003; Ramsey et al., 2004; Leung et al., 2006). For example, the human ERβ2 isoform (also named ERβ cx) with 26 unique aa residues replacing the COOH-terminal part of the LBD is unable to bind ligand or coactivators and has no transcriptional activity in reporter assays. ERβ2 dimerizes with preferentially ERα, thereby silencing signaling via this ER isoform (Ogawa et al., 1998). Both species-specific and species-common ERβ isoforms have been identified (Lewandowski et al., 2002).



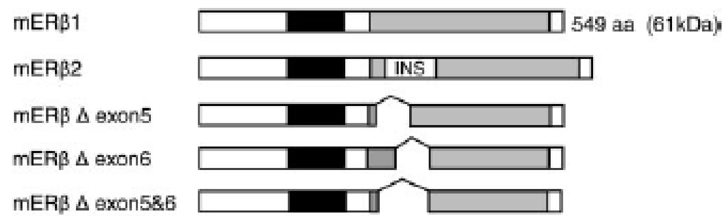


Figure 3. Schematic representation of estrogen receptor beta isoforms. The 530-amino acid-long hERβ isoform is currently regarded as the full-length wild-type hERβ. Note that deletion of the fifth exon giving the hERβ exon 5 isoform results in a frameshift and a termination of the protein before the LBD. The striped fill patterns of the 3' end of hERβ2 (also named hERβcx), hERβ4, and hERβ5 represent the differing COOH terminal regions of these isoforms. The full-length rat and mouse ERβ isoforms are 549 amino acids and have 99% sequence similarity. The mERβ2 and rERβ2 isoforms contain an 18-amino acid insertion in the LBD, causing a significant decrease in ligand binding affinity. Deletion of exon 3 results in rat isoforms unable to bind DNA, and the observed deletion of exon 5 and/or 6 in mice results in isoforms lacking various parts of the LBD.

In the late 1990s, a putative GPCR was cloned by four different groups using highly disparate approaches (Owman et al., 1996; Carmeci et al., 1997; Takada et al., 1997; O'Dowd et al., 1998). The GPCR identified in these studies displayed little homology to other GPCRs. GPR30 mRNA was shown to be expressed in numerous tissues throughout the body (e.g. placenta, lung liver, prostate, ovary, placenta) although substantial contradictions between the tissue expression patterns were reported. Since no ligand was known for this receptor, it was labeled an orphan GPCR. It was not until 2000 that a possible function for this GPCR was identified from experiments demonstrating MAP kinase (Erk1/2) activation by estrogen, as well as ER antagonists, ICI 182,780 and tamoxifen. GPR30 is a G protein-coupled seven-transmembrane receptor, and human GPR30 comprises 375 amino acids with a theoretical molecular mass of approximately 41 kDa. It is thought that the N-terminus is located outside of the cell, and that aspartic acid residues in the terminal region might be modified by glycosylation if GPR30 is localized in the plasma membrane. It is speculated that the ligand associates with the N-terminal domain to activate the receptor. Trimeric G protein is presumed to bind to the 3rd loop of the intracellular domain based on the molecular structure. A PDZ domain appears to be in the C-terminal region of GPR30, but its physiological role is unknown.

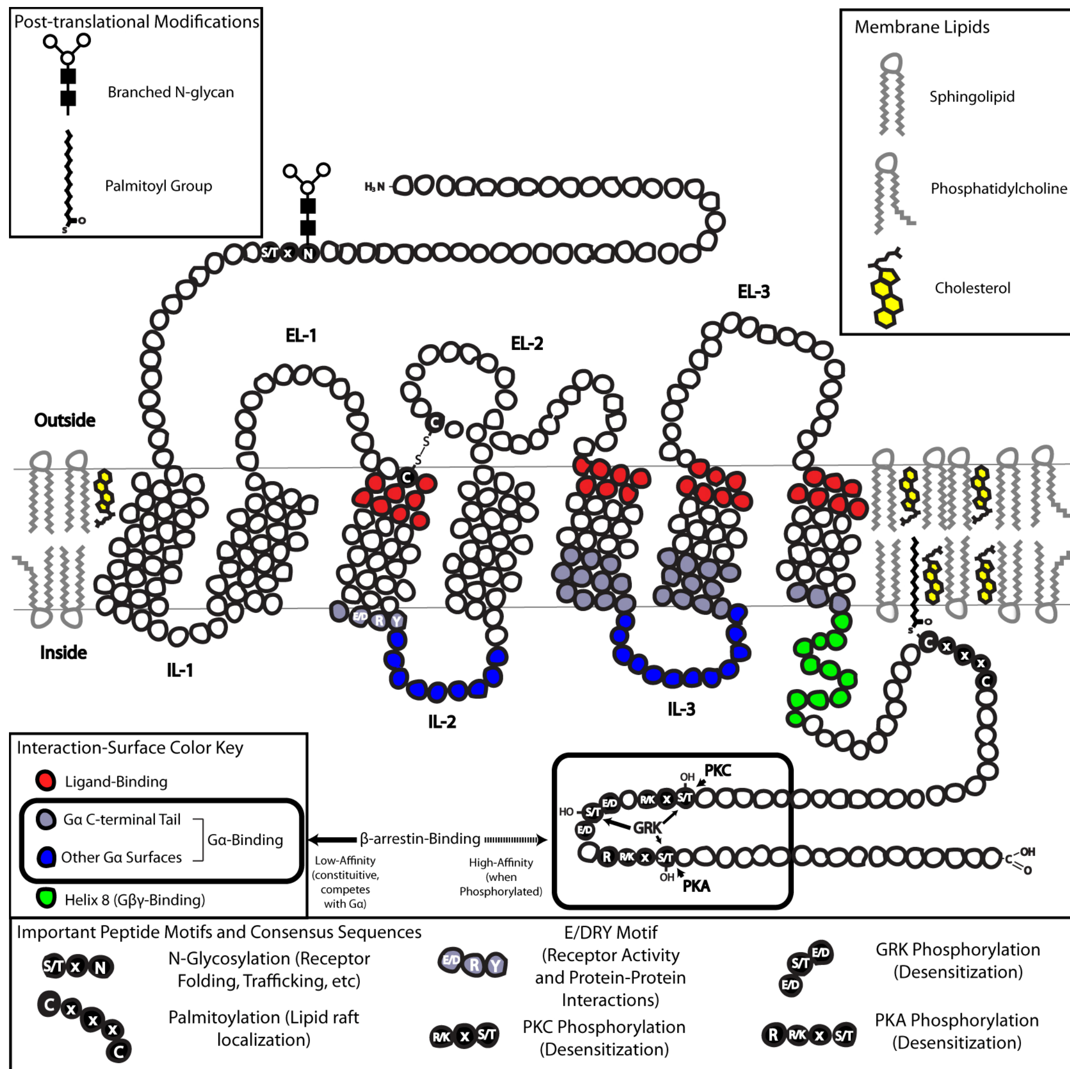


Figure 6. Two-dimensional schematic of a generic GPCR set in a Lipid Raft.

Given that GPR30 and the classical ERs share a common ligand, the question arose as to the relative selectivity of each receptor type for estrogenic substances. Estrogen is a small ligand with no conformational flexibility and limited sites of stereospecific recognition. Thus, it might be predicted that two receptors (with no homology in primary or secondary structure) that both bind estrogen, would bind a similar spectrum of structurally related compounds. As described below, a large number of compounds that bind to classical estrogen receptors have also been demonstrated to bind to or activate GPR30.

Molecular pathways of Estrogen Receptors

There are several distinct pathways by which estrogens and ERs may regulate biological processes (Hall et al., 2001).

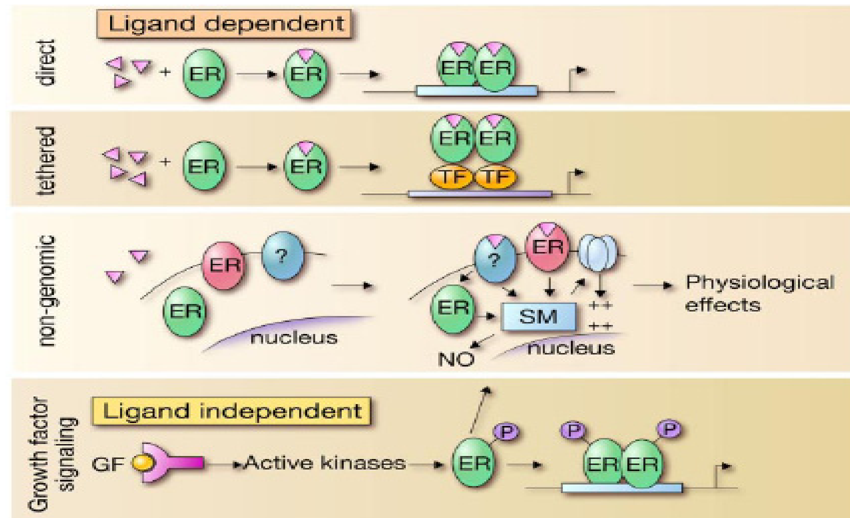


Figure 5. Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs.

Ligand-bound ERs can bind directly to estrogen response elements (ERE), in the promoters of target genes or can interact with other transcription factor complexes like Fos/Jun (AP-1- responsive elements) (Kushner et al., 2000) or SP-1 (GC-rich SP-1 motifs) (Saville et al., 2000) and influence transcription of genes whose promoters do not harbor EREs. Ligand-dependent activation triggers recruitment of a variety of coregulators to the receptor in a complex that alters chromatin structure and facilitates recruitment of the RNA polymerase II (Pol II) transcriptional machinery. The details of ER-transcription factor interactions (i.e., ligand specificity, ER subtype specificity, interaction domains and motifs) remains to be characterized. Interestingly, in certain cell-type and promoter contexts, 4-hydroxytamoxifen and raloxifene, which function as antagonists at EREs, can behave as agonists through these indirect pathways. ERβ, but not ERα, in the presence of E_2 can oppose the actions of 4-hydroxytamoxifen and raloxifene on an AP-1 reporter gene (Paech et al., 1997; Webb et al., 1999). Similar observations have been made for the SP-1 pathway (Zou et al., 1999; Saville et al., 2000).

In addition to the well-studied transcriptional effects of E_2, there are rapid effects, i.e., occurring within seconds or minutes after addition of E_2 (Wong et al., 2002 Song

et al., 2005; Song and Santen, 2006; Warner and Gustafsson, 2006). These rapid effects include activation of kinases and phosphatases and increases in ion fluxes across membranes. Although these rapid effects have been extensively studied, there is still no consensus as to whether or not the classical ERs are involved (Deecher et al., 2003; Razandi et al., 2004) or whether there is a distinct membrane associated receptor (Doolan and Harvey, 2003). Tools such as pathway-selective ligands (Harrington et al., 2006) or cell lines designed to selectively express ERβ in the nucleus, cytoplasm, or membrane may prove useful in studying these extranuclear ER actions (Santen et al., 2005). Interestingly, evolutionary evidence suggests that early on, estrogen influenced reproduction through ER-independent pathways (Keay et al., 2006) and that the receptor was unresponsive to estrogens and acted as a constitutive transcriptional activator (Keay et al., 2006; Thornton et al., 2003). When the two ER subtypes are coexpressed in cells, ERβ can antagonize ERα-dependent transcription (Matsumoto and Arai, 1979; Matthews et al., 2006). The molecular mechanisms of ERβ-mediated inhibition of ERα signaling are currently under investigation. For example, it was shown that for ERα-mediated regulation of AP-1-dependent transcription, ERβ expression alters the recruitment patterns of c-Fos to AP-1-regulated promoters. Moreover, expression of ERβ and the ERβ variant ERβ2 increases the proteolytic degradation of ERα (Matthews et al., 2006). Collectively, these data suggest that the ERβ-mediated inhibition of ERα activity involves a combination of altered recruitment of key transcription factors and increased ERα degradation. In addition to ligand-induced transcriptional activities of ER, ligand-independent pathways to activate ERs have been described. Growth factor signaling leads to activation of kinases that may phosphorylate and thereby activate ERs or associated coregulators in the absence of ligand (Kato et al., 1995). The growth factor activated pathway is thought to significantly contribute to hormone-independent growth in some tumors (Coutts and Murphy, 1998; Shim et al., 2000).

From these examples, it is clear that estrogen can mediate a multitude of complex rapid cellular activation events. However, not all such responses can be attributed to the classical ERs. Upon agonist binding, GPR30 activates heterotrimeric G proteins, which in turn can activate multiple effectors, including adenylyl cyclase (resulting in cAMP production), Src, and sphingosine kinase (SphK). The latter two pathways appear to be involved in the activation of matrix metalloproteinases (MMPs), which

cleave pro-HB-EGF, releasing free HB-EGF that can then transactivate epidermal growth factor receptors (EGFRs). EGFR activation leads to multiple downstream events, including the activation of phospholipase C (PLC), mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinases (PI3Ks). PLC activation leads to intracellular calcium mobilization through the actions of inositol triphosphate (IP3). The activation of MAPKs and PI3Ks results in the activation of numerous cytosolic pathways as well as the activation of nuclear proteins that are themselves or that regulate transcription factors. Thus, estrogen stimulation can give rise to the transcription of gene targets whose promoters do not contain steroid response elements. The combined effects of these cytosolic signalling and nuclear transcription events often result in cell proliferation.

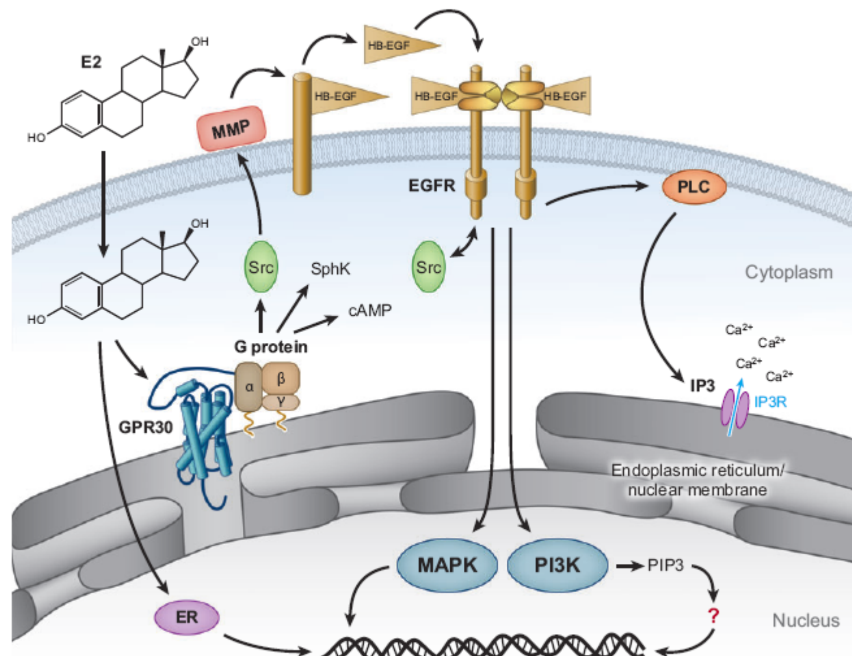


Figure 6. Mechanisms of estrogen-mediated signaling through GPR30.

FUNCTIONAL ROLES OF ERs IN PHYSIOLOGY AND DISEASE

Estrogens acting through ERs exert effects on multiple organs. Epidemiological and retrospective studies have provided important evidence for the diverse roles of estrogens in human physiology and disease. There are gender differences in

occurrence of several diseases. A role of estrogens in these syndromes is evident from the effects of menopause, when estrogen levels decrease, and estrogen replacement therapy. Furthermore, identification of phenotypes of ERα, ERβ, and ERα/ERβ knockout (αERKO, βERKO, αβERKO) mice is in many cases consistent with observations in humans and has provided an added molecular understanding of the function of ERs.

UTERUS

In the uterus, ERα is the predominant isoform, and its presence is necessary for reproductive functions (Couse and Korach, 1999; Matthews and Gustafsson, 2003). Indeed, mice null for ERα are infertile in contrast to ERβ KO mice (Hewitt and Korach, 2003) due in part to the failure of the uteri to develop properly (Couse et al., 1995). Conversely, the unopposed activity of uterine ERα promotes the development and progression of endometrial cancers in humans and animal models (Di Cristofano and Ellenson, 2007). Multiple mechanisms underlie the control of ERα expression and activity; these include regulation at the levels of transcription involving multiple promoters (Kos et al., 2001); alternative splicing (Heldring et al., 2007); post translational modifications via phosphorylation, acetylation, and polyubiquitination (Atsriku et al., 2009; Fu et al., 2004; Masuyama and Hiramatsu, 2004); and post transcriptional silencing by micro-RNA targeting (Castellano et al., 2009). The afore mentioned complex regulation of ERα occurring at many levels predicts that perturbations in any or all of these mechanisms will lead to reproductive dysfunctions and diseases.

Estrogen induces the expression of membrane receptor tyrosine kinase ligands, epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) in the mouse uterus. In addition, estrogen treatment results in activation of the IGF-I receptor signalling pathway (Richards et al., 1996). When IGF-I knockout mice are treated with estradiol, uterine epithelial cells do not undergo mitosis, indicating that activation of the IGF-I pathway is necessary for this uterine response to estrogen (Adesanya et al., 1999). Conversely, EGF or IGF-I administered in an exogenous dose can induce estrogen-like responses, such as epithelial mitogenesis, induction of estrogen target genes and increase in uterine mass (Nelson et al., 1991; Ignar-Trowbridge et al., 1992). The mechanism appears to involve ERα, as studies using

cells *in vitro* show a requirement for ERα for IGF-I or EGF to induce estrogen-responsive reporter constructs (Ignar-Trowbridge et al., 1992; Ignar-Trowbridge et al., 1993). These studies have led to a model of 'cross-talk' in which growth factor receptor activation results in maintenance, amplification or induction of ER transcriptional activity even in the absence of circulating estrogens. αERKO mice were treated with IGF-I and EGF to determine whether ERα is required for uterine responses to these growth factors. In both cases, the necessary membrane growth factor receptors were present and activated by the treatment (Curtis et al., 1996; Klotz et al., 2002). In addition, cfos was induced by EGF in the αERKO, indicating that the EGFR signaling pathway was fully functional. However, no thymidine or bromodeoxyuridine incorporation was induced, indicating that the EGF and IGF-I did not induce a mitogenic response (Curtis et al., 1996; Klotz et al., 2002), and therefore that this response requires ERα. Studies using ovariectomized mice containing an estrogen-responsive luciferase reporter transgene indicated that IGF-I could induce luciferase activity in the uterus in the absence of estrogen (Klotz et al., 2002), demonstrating that the IGF-I treatment results in activation of ER transcriptional activity *in vivo.*

BREAST

ERβ is found in both ductal and lobular epithelial and stromal cells of the rodent. ERα, on the other hand, is only found in the ductal and lobular epithelial cells and not in stroma (Gotteland et al., 1995). It is generally believed that breast tumors depend, at least initially, on the stimulatory effects of estrogens; however, many breast tumors eventually progress to an estrogen-independent growth phenotype.
Various ER transcripts have been found in breast carcinomas (Gotteland et al., 1995). Normal and cancer tissues display a variety of distinct profiles regarding ERα, ERβ, and splice variants at both mRNA and protein levels (Poola and Speirs, 2001). This heterogeneity in ER isoform profiles is suggested to result in variations in estrogen signaling and might affect breast cancer risk, hormone responsiveness, and survival. Some data suggest that the ERβ transcript is down-regulated in breast tumorigenesis, and other studies show regulation of ERβ expression by promoter methylation (Iwao et al., 2000; Zhao et al., 2003). Since promoter methylation is frequently observed in cancer (Garinis et al., 2002), these data suggest that ERβ is a possible tumor

suppressor gene. In vitro studies indicated that ERβ is an important modulator of proliferation and invasion of breast cancer cells, thus supporting the hypothesis that the loss of ERβ expression could be one of the events leading to breast cancer development (Lazennec et al., 2001) However, this hypothesis needs to be confirmed, because it has been shown that ERβ is expressed in the majority of breast tumors, with immunohistochemical staining in approximately 2/3 of breast tumors, similar to the percentage of tumors that express ERα. Currently, only the ERα form is clinically measured for clinical decision making and treatment.

THE CARDIOVASCULAR SYSTEM

As in most tissues studied, estrogen activates multiple molecular signaling pathways in the cells and tissues of the cardiovascular system. Functional ERs are expressed in in the cardiomyocytes of the heart (Mendelsohn and Karas, 2005), in vascular endothelial and smooth muscle cells (SMC). In aortic SMC from diabetic rats the cytokine-mediated iNOS activation and the estrogen response are very different, this effect is associated with an interplay between ERα and ERβ, indeed in diabetic vascular dysfunction there is a significant change in ERα and ERβ relative content.

In vitro transfection experiments show that ER is indispensable to E_2 regulation of iNOS transcription. There is an evidence for a direct interaction of estrogen with the iNOS promoter (Miller et al., 1996), which lacks canonical estrogen responsive elements. As proposed for the effect of estrogens on other molecules of the inflammatory cascade, it is conceivable that iNOS mRNA synthesis is controlled by ERs by interaction with transcription factors such as NF-kB, AP-1, or STATs (Xie et al., 1994; Faulds et al., 2001). Considering a differential effect of the two ER subtypes on iNOS expression, Maggi et al., (Maggi et al., 2003) proposed that the altered ERα/ ERβ ratio reported for diabetic SMC, and in particular the remarkable upregulation of ERβ, underlies the loss of E_2 negative control on iNOS activity. ERα and ERβ expression is increased in diabetic compared with control SMC, with a relative increase in ERα and ERβ protein levels of 1.4- and 2.5-fold, respectively. The increased ERβ expression is associated with the defective response to E_2 and to cytokines observed in diabetes; studies in ERβ-deficient mice describing an important role for this receptor in regulating blood pressure and vascular function (Zhu et al., 2002). The authors showed that ERβ is involved in estrogen-induced

accumulation of iNOS protein in aortic endothelium as well as iNOS gene transcription in normal vessels. This lends support to the view that the altered estrogenic control of iNOS function in diabetic SMC is mediated mainly by isoform beta of estrogen receptor.

The observation that ERα and ERβ protein content is regulated by E_2 in an opposite fashion in aortic SMC provides evidence for the potential divergent effects of the two receptors (Paech et al., 1997; Weatherman and Scanlan, 2001). By upregulating ERα, E_2 may enhance the protective antiinflammatory effects mediated by this receptor subtype in these cells while limiting potential proinflammatory effects with ERα downregulation.

Other intriguing mechanism of action of ligand-activated ERs has been studied for many years: the ability of ERs to signal rapidly, in an apparently non-nuclear manner, resulting in downstream activation of specific kinases and their effector molecules (Mendelsohn, 2000). Rapid, non-nuclear ER signaling in the cardiovascular system has been best studied in ECs, where estrogen and ERs lead to the rapid activation of eNOS, production of nitric oxide, and vasodilation (Mendelsohn, 2000). Over the past 15 years, a number of laboratories have explored the molecular events in this fascinating pathway in vascular ECs, in which a subpopulation of ERs localized to cell membrane–based caveolae (small invaginations of the cell membrane) mediate rapid (15–30 minutes), estrogen induced activation of eNOS via a pathway involving activation of the low-molecular weight G protein, the tyrosine kinase src, and the serine/threonine kinases Akt and MAPK. ERs localized to EC caveolae directly bind to a scaffold protein called striatin, which also binds to caveolin-1, the major structural protein of caveolae; striatin is required for estrogen-ER activation of the rapid, non-nuclear signaling pathway (Qing et al., 2004).

SKELETAL SYSTEM

Among the estrogen target organs, bone has recently drawn increasing attention because postmenopausal osteoporosis induced by estrogen deficiency has emerged as the most widely spread bone/joint disease in developed countries. Osteoporosis in women and men is currently considered a serious disorder of middle-aged and elderly people because of increased risk of bone fracture, often leading to long-term

incapacitation and mortality (Klotz et al., 2002; Ebeling, 2008). Pronounced bone mass decrease due to enhanced or imbalanced bone resorption *vs.* bone formation (high bone turnover) is a typical osteoporotic feature in women with estrogen deficiency or impaired estrogen signaling. The osteoporotic bone phenotype can be experimentally recapitulated in female rodents by ovariectomy (OVX) and consequent estrogen depletion (Harada and Rodan, 2003; Harman, 2006).

Genetically modified models showed that also in the case of bone cells NO signalling is relevant. NO can have both direct effects on cell signaling as well as indirect actions mediated by the reaction products formed when NO interacts with other molecules, such as oxygen or superoxide, thereby playing a central role in the pathophysiology of inflammation and oxidant stress (Nathan, 1992; Payne and Kubes, 1993). iNOS, contrary to what is known for endothelial NOS (eNOS), is not expressed constitutively in the bone of adult mice, but was shown to be induced in osteoblasts and bone marrow by stimulation with proinflammatory cytokines or endotoxin; iNOS is apparently not required for normal trabecular bone development.

The absence of iNOS becomes crucial when bone loss is induced by estrogen depletion, suggesting a predominant role of this enzyme in the osteoporosis consequent to deficient ovarian function. Cuzzocrea et. al., (Cuzzocrea at. al, 2003) suggested a relevant role for iNOS in osteoporosis in agreement with studies carried out on two different models of osteoporosis.

Armour and colleagues (Armour et al., 2001) demonstrated that iNOS is indispensable for the development of osteoporosis consequent to systemic inflammation and proposed that the reduced bone formation mediated by iNOS is due to the dramatic increase in osteoblast apoptosis. Watanuki and colleagues (Watanuki et al., 2002) showed that the decreased bone volume induced by tail suspension is not observed in iNOSKO mice and suggested that the iNOS synthesized in osteoblasts plays a critical role in increasing osteogenic activity in response to the acute increase in mechanical loading after tail suspension.

Cuzzocrea's study pointed to a strong role for iNOS in osteoclast activation after ovariectomy. In smooth muscle cells prolonged exposure to a medium deprived of estrogens by itself is a stimulus sufficient to induce iNOS synthesis (Zancan et al., 1999). In line with this observation, with ovariectomy the prolonged absence of estrogens causes a depression of iNOS synthesis in osteoblasts. Osteoblasts express

ERs (Windahl et al., 2000; Bonnelye et al., 2001), and it is conceivable that the circulating levels of estradiol maintain the receptors in a state of activation sufficient to repress transcription of the iNOS promoter. Sequence analysis of the mouse iNOS promoter does not reveal any estrogen responsive element; however, this promoter contains NF-kB and activating protein1-responsive elements. Estradiol can modulate the activities of promoters controlled by these two transcription factors, it is possible that in the presence of estradiol the ER impairs their positive action on *inos* gene transcription. The absence of the hormone would release the ER inhibitory influence, with consequent pathological increase of iNOS production.

In osteoblasts, an increased iNOS content may lead to local accumulation of nitrites and other products of the inflammatory cascade, which trigger osteoclastogenesis, or alternatively, the NO produced by osteoblasts may also activate p21 *ras* activity in the monocytes and the NF-kB signal transduction pathway necessary for osteoclast differentiation. The study also showed that after estrogen depletion the plasma levels of NO are significantly increased; as it is well known that eNOS is positively regulated by estrogens, this increase could be ascribed to a deficit of the estrogen negative control on the expression of the smooth muscle cell iNOS. Interestingly, the authors demonstrated that iNOS contributes to the increased expression of the cytokines IL-6, IL-1β and TNFα consequent to estrogen deprivation. IL-1, IL-6, and TNFα are known to be potent stimulators of bone resorption. In the absence of an appropriate stimulus such as estrogen depletion iNOS does not have any influence of the systemic synthesis of proinflammatory citokines. These cytokines play an important role in the induction of osteoclastogenesis and bone loss after estrogen depletion. The secretion of both IL-1 and TNFα by peripheral blood monocytes is increased in postmenopausal women (Pacifici et al., 1991), moreover the release of these cytokines can be strongly reduced by iNOS inhibition in shock (Joshi et al., 1996; Kengatharan et al., 1996; Wang et al., 1999). The data suggested that iNOS is in part responsible for bone erosion in ovariectomyzed models, but also that the induction of NO synthesis is a key event in the subsequent activation of inflammatory cascades after estrogen depletion. NO can have both direct effects on cell signaling as well as indirect actions mediated by the reaction products formed when NO interacts with other molecules such as oxygen or superoxide (Kimble et al., 1996). ROS and peroxynitrite produce cellular injury and necrosis via peroxidation

of membrane lipids, protein denaturation, and DNA damage. ROS produce strand breaks in DNA that trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme PARS, resulting in the depletion of its substrate NAD *in vitro* and a reduction in the rate of glycolysis.

NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed the PARS suicide hypothesis (Szabo and Dawson, 1998). There is an evidence that the activation of PARS may also play an important role in inflammation (Szabo et al., 1997; Cuzzocrea et al., 1998). The research demonstrated that absence of the *inos* gene attenuates the increase in PARS activity caused by estrogen deprivation. Thus, they proposed that iNOS induction is also important, at least in part, for the activation of PARS. This enzyme plays a role in the bone erosion after estrogen depletion. Accumulating clinical observations and genetic studies show that male patients defective in either estrogen biosynthesis or function of estrogen receptor α (ERα) display typical pathological conditions of osteoporosis (Smith et al., 1994; Jones et al., 2006). Thus, it is evident that estrogens exert osteoprotective actions and play a significant role in skeletal maintenance in both sexes. The first conventional gene disruption of the mouse ERα locus was achieved in the early 1990s (Couse and Korach, 1999). Paradoxically, however, neither males nor female ERα-deficient mice exhibited typical osteoporotic bone phenotypes (Couse and Korach, 1999; Windahl et al., 2002). Thereafter, the role of ERs in bone health remained obscure. Instead, indirect mechanisms via extraskeletal tissues have been postulated to account for the osteoprotective actions of estrogen (Zaidi, 2007; Pacifici, 2008). It appears that in females, osteoclastic ERα mediates estrogen dependent attenuation of bone resorption through stimulation of apoptosis in osteoclasts. Although primary cultured bone cells from males and females equally respond to estrogen, the osteoclast-selective ablation of ERα in male mice caused neither bone defects nor unclosed epiphyses (Nakamura et al., 2007) that had been consistently observed in male patients with impaired estrogen signaling (Smith et al., 1994; Jones et al., 2006). This discrepancy suggests sex specificity in mechanisms of osteoprotective action of estrogens *in vivo* and raises a hypothesis that in male bones, beneficial effects of estrogens are predominantly mediated by the osteoblastic ERα, rather than through the antiresorptive action of osteoclastic ERα, which is more critical in females. This idea can be tested by cell type-specific

ablation of ERα in males as well as in females to decipher molecular and cellular mechanisms of the anabolic action of estrogens in the skeleton. At the same time, a compensatory action of AR at high concentrations of circulating testosterone may account for different physiological consequences of osteoclast-targeted ERα ablation in male mice.

ENDOCRINE SYSTEM

The most powerful but indirect indicator that estrogens display antidiabetic action comes from the observation that the overall prevalence of diabetes is lower in premenopausal women, a trend that is reversed after menopause (Wild et al., 2004). The hypothesis that this gender dimorphism is partially related to β-cell function stems from the observation that it is more pronounced in syndromes with severe insulin deficiency. Indeed, common autoimmune diseases usually show a female predominance except for type 1 diabetes mellitus (T1DM) (Beeson, 1994). T1DM is characterized by a male predominance in populations of European origin (ratio 1.7) (Gale and Gillespie, 2001). More interestingly, the male predominance develops after puberty, whereas puberty is associated with a decreased incidence in girls (Blohme et al., 1992). In adults, the best example of sex dimorphism is that of ketosis-prone diabetes (also called idiopathic diabetes, characterized by a propensity to acute insulin deficiency) (Mauvais-Jarvis et al., 2004). In ketosis-prone diabetes, male predominance is high (75%) and women are protected unless they are in a hypoestrogenic state (Mauvais-Jarvis et al., 2004; Louet et al., 2008). Together, the observations described above in diabetic syndromes with insulin deficiency suggest that the ovarian hormones, and especially estrogens, are protective against β-cell apoptosis and insulin deficiency. The antidiabetic actions of estrogen were confirmed in two large, randomized, double-blind, and placebo-controlled trials. The Women's Health Initiative study, which included more than 15,000 women, showed a 20% decrease in the incidence of diabetes in the estrogen replacement therapy (ERT) group at 5 yr (Margolis et al., 2004). More impressively, the Heart and Estrogen/Progestin Replacement Study, which focused on 3000 women with a predisposition to oxidative stress and coronary heart disease, and thus at high risk of developing type 2 diabetes mellitus T2DM, resulted in a 35% reduction in the incidence of diabetes in the ERT group at 4 yr (Kanaya et al., 2003). Because

hyperglycemia cannot develop without β-cell failure, the observation that ERT prevents diabetes suggests that estrogens improve β-cell function or survival via direct or indirect mechanisms. In fact, 14 studies assessed the effect of ERT on β-cell function in postmenopausal women. More than half of them have reported an improvement in glucose-stimulated insulin secretion. Early studies suggested that E_2 administration to cultured rat islets had limited effects on insulin release (Costrini and Kalkhoff, 1971; Sorenson et al., 1993). More recent data from cultured mouse islets indicate that E_2 increases islet insulin content and secretion (Alonso-Magdalena et al., 2008). However, the most promising and direct effect of E_2 on islets is to enhance survival. E_2 protects against oxidative stress and proinflammatory cytokines induced apoptosis (Contreras et al., 2002; Eckhoff et al., 2003; Eckhoff et al., 2004; Le May et al., 2006). ERα and ERβ are expressed in rodent and human β-cells. Although they can be found in the nucleus, they exhibit a predominant extranuclearlocalization (Nadal et al., 2000; Le May et al., 2006; Alonso-Magdalena et al., 2008; Liu et al., 2009). Mouse and human islets express both the long 66-kDa isoform and a shorter 58-kDa isoform, whereas mouse clonal β-cells express only the long 66-kDa isoform (Le May et al., 2006). E_2-activated ERα prevents islet apoptosis via an estrogen response element- independent pathway in mouse and human islets. This is mediated via activation of extranuclear and perhaps membrane forms of ERα. ERα and ERβ both favour survival with a predominant ERα effect (Liu et al., 2009). Although the precise signalling pathways are still under investigation, ERs prevent apoptosis independently of gene transcription or *de novo* protein synthesis, suggesting that this cytoprotection happens independently of nuclear events (Liu and Mauvais-Jarvis, 2009). In cultured human islets, Contreras and co-workers (Contreras et al., 2002; Eckhoff et al., 2004) reported that E_2 inhibits the nuclear factor- kB and the stress activated kinase, c-Jun N-terminal kinase. ERα is also important to insulin transcription. Exposure to physiological concentrations of E_2 increased β-cell insulin gene expression, insulin content, and insulin release via an ERα-dependent mechanism involving Src and ERK kinases (Alonso-Magdalena et al., 2008). Thus, the elevation in serum E_2 concentration during pregnancy may participate to the islet adaptation to the increased metabolic demand. The effect of E_2 on β-cell proliferation *in vivo* is poorly studied. In one study, E_2 promoted β-cell hypertrophy but no expansion of the β-cell population, which improved diabetes in

partially pancreatectomized rats (Zhu et al., 1998). In another study, E_2 treatment increased β-cell proliferation in ovariectomized rats (Choi et al., 2005). In STZ-challenged, hyperglycemic mice, E_2 treatment did not induce β-cell proliferation (Le May et al., 2006), and there was no effect of E_2 on β-cell proliferation in cultured islets (Alonso-Magdalena et al., 2008).

Ten years ago, Nadal et al. (Nadal et al., 1998; Nadal et al., 2000) reported the existence of a membrane G protein-coupled receptor in β-cells unrelated to ERα and ERβ, which was probably GPR30. A more recent report has revealed that GPER-deficient mice display altered E_2-stimulated insulin release from isolated islets associated with impaired glucose stimulated insulin secretion *in vivo* (Martensson et al., 2009). Liu et al. found that elimination of GPR30 predisposes to STZ-induced islet apoptosis in female mice and that pharmacological activation of GPR30 by a selective agonist, G1, prevents oxidative stress-induced apoptosis in cultured mouse and human islets (Liu et al., 2009). Thus, GPR30 is important to E_2 effects on islet function and survival.

IMMUNE SYSTEM

In postmenopausal women changes in the immune system have been attributed to estrogen deprivation. There is an increase in pro-inflammatory serum markers (IL1, IL6, TNF-alpha), an increasing response of the body's cells to these cytokines, a decrease in CD4 T and B lymphocytes and in the cytotoxic activity of NK cells. Thus attenuated immune response and higher susceptibility to microbial invasion and infection are characteristic in postmenopausal women. In addition, IL-6 has been related to bone resorption by osteoclast activation and also seems to be associated with other diseases that occur more often in menopause such as diabetes, atherosclerosis and cardiovascular disease. IL7 is another cytokine that might be associated with IL-6 and bone turnover but more studies are needed previously to drawn definitive conclusions. Epidemiological and clinical studies indicate a positive action on cellular immune response of hormonal replacement at menopause, thus being a potential influence on the development and course of autoimmune disorders.

Lymphocyte

Estradiol can modulate lymphocyte cytokine production, cytokine receptor expression, and activation of effector cells (Salem, 2004; Pernis, 2007; Karpuzoglu and Zouali, 2009). ERα is expressed in most immune cells both at baseline and at increased levels after estrogen administration (Rider et al., 2006; Stygar et al., 2006; Inui et al., 2007). It can be detected in thymocytes, bone marrow non hematopoietic cells, T cells, B cell precursors, and circulating B cells (Haruki et al., 1983; Stimson, 1988; Kawashima et al., 1992; Smithson et al., 1995; Igarashi et al., 2001; Shim et al., 2006). One recent study examined ERα expression in resting and activated PBMC subsets and found that ERα was expressed at higher levels in CD4+ T cells than B cells. Instead, B cells expressed high levels of estrogen receptor beta (ERβ). CD8+ T cells expressed both ERα and ERβ at low, but equivalent levels (Phiel et al., 2005). Since multiple studies have demonstrated estradiol responsivity of immune cells, one could infer that T cells would mediate this response via ERα, whereas B cells would respond via ERβ. The functional differences between ERα and ERβ are as yet unclear, but these data suggest that immune cells have the ability to respond to estradiol or alternative ligands with potentially disparate effects on T cells versus B cells due to variance in expression of ERs.

Thurmond et al. provided evidence that, while ERα is required for regulating the number of B cells reaching maturity, it is not essential for maintaining the normal proportion of hematopoietic progenitors (Thurmond et al., 2000).

Less is known regarding effects of ERα on T cells, however, a number of studies have established a role for estrogen and ERα in the thymus and early T cell development. For example, estrogen can cause thymic atrophy (reduction in size and cellularity) (Rijhsinghani et al., 1996; Okasha et al., 2001), and this effect is ERα-dependent (Staples et al., 1999). Estrogen also influences the production of CD4+CD8+ double positive cells, which requires both ERα and ERβ (Erlandsson et al., 2001).

Unlike B cells, estrogen effects on the T cell repertoire selection and eradication of autoreactive T cells has not been studied in detail. More is known about the effect of estrogen on mature T cells and, as with B cells, it is quite complex. Multiple studies have shown a role for estrogen in influencing T helper responses (Th1 vs. Th2) and the effect appears to be dose-dependent, i.e., low doses of estrogen stimulate a Th1

response and higher doses promote a Th2 response (Bebo et al., 2001; Maret et al., 2003; Delpy et al., 2005). *In vivo*, estrogen administered to ovariectomized female mice resulted in increased antigen-specific CD4+ T cell responses and in the selective development of interferon-gamma (IFNγ)-producing cells. Using ERα−/− mice, the researchers went on to show that ERα in the hematopoietic compartment was necessary for this enhanced Th1 cell responsiveness. Estrogen also stimulates the production of regulatory T cells. Tai et al. (Tai et al., 2008) observed that ERα is expressed in CD4+CD25− T cells and that physiologic doses of estrogen increased expression of Foxp3 and IL-10 genes in vitro, as well as converted T cells from CD4+CD25−into CD4+CD25+ T cells indicating a role for ER. In the experimental autoimmune encephalitis (EAE) animal model for MS, ERα is required for the protective effect of estrogen (Polanczyk et al., 2004). The ability of estrogen to protect against EAE correlated with its ability to up-regulate FoxP3, and was dependent on Erα. Estrogen induced the expression of FoxP3 and potentiated the regulatory activity of CD4+CD25+ T cells. The ability of estrogen to protect against EAE correlated with its ability to up-regulate FoxP3, and is dependent on ERα.

Dendritic cells

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that capture antigens and stimulate immune responses by naïve T cells. DCs not only initiate T cell responses, but also influence the nature of those responses (Th1 vs. Th2), as well as maintenance of self-tolerance. Data from multiple studies suggest that DCs and other APCs are modulated, both in number and function, by sex steroids, most notably estradiol (Hoek et al., 1997; Saito et al., 1998; Salem et al., 2000; Medina et al., 2001; Wira et al., 2002; Nalbandian and Kovats, 2005). *In vivo*, APC number and function are also variable with different estrogen levels in the female rat reproductive tract (Prabhala and Wira, 1995). Consistent with this, both ERα and ERβ are expressed in most APCs including macrophages, monocytes, and DCs, as well as DC progenitors (Komi and Lassila, 2000; Sapi et al., 2002; Mor et al., 2003; Paharkova-Vatchkova et al., 2004; Mao et al., 2005; Harkonen and Vaananen, 2006; Carreras et al., 2008), however, as with most cell types, there appears to be differential regulation of ERα vs. ERβ. For example, in murine *ex vivo* cultures of DCs, ERα is expressed in both major subsets of CD11c+ cells whereas ERβ is expressed in only one of the two (Mao et al., 2005). This may provide a basis for the differential

regulation of these two DC types by estrogen; they exhibit distinct phenotypes with respect to capacity for co-stimulatory molecule and MHC expression, and antigen internalization. In contrast, Komi et al. used blood from healthy human subjects to isolate PBMCs for culture, and found that immature and mature DCs expressed both ERα and ERβ mRNA regardless of their developmental stage (Komi and Lassila, 2000). Estradiol promotes the differentiation of DCs from bone marrow. In a recent study utilizing murine bone marrow precursor cells, Paharkova et al. demonstrated that differentiation of DCs was inhibited in the absence of steroid hormone supplemented media and restored with the addition of estradiol. Additionally, DC differentiation was inhibited in the presence of an ER antagonist or in ERα−/− bone marrow cells, demonstrating a role for ERα in this process (Paharkova-Vatchkova et al., 2004).

Monocytes and macrophages

Both monocytes and macrophages are key phagocytes and provide early recognition of pathogens as part of the innate immune system. Their function is profoundly affected by the environment to which they are exposed, including not only cytokines and chemokines but also steroid hormones. Evidence suggests that estrogen has effects on monocyte and macrophage immune function. It has been known for many years that ERs are expressed in monocytes (Weusten et al., 1986; Suenaga et al., 1996; Suenaga et al., 1998). However, their response to estrogen and whether ERα or ERβ expression dominates appears to be dependent on their stage of differentiation.

Regulation of apoptosis is considered an important mechanism for controlling the number of monocytes available and therefore the intensity of the physiological response to infection or wound healing. Failure in the control of programmed cell death may become particularly relevant in tumor growth, autoimmune, and chronic inflammatory disorders, where apoptosis has been identified as a key factor in disease progression or remission (Said et al., 1997; Musgrove et al., 1998).

Some years ago Vegeto et. al, used the premonocytic U937 cell line, which can be differentiated *in vitro* toward a macrophage-like phenotype, as a model system to investigate whether sex steroid hormones played a role in the manifestation of apoptosis. Their results suggested that both estrogen and progesterone, by interacting with their cognate receptor, partially prevent the onset of TNFα-induced apoptosis. ER and PR are expressed in U937 and can be regulated by steroid hormones, under

both physiological and pathological conditions (Pacifici et al., 1991; Olsen and Kovacs, 1996). With this study Vegeto et al., have shown that TNFα exerts an antiproliferative and apoptotic activity, when physiological concentrations of estradiol or progesterone were added together with TNFα, 30% cells were induced to survive the apoptotic signal and to proliferate.

This cellular system suggested that this effect is mediated by intracellular hormone receptors: *1*) nanomolar concentrations of hormones are effective in inducing cell survival; *2*) the ER antagonist ICI 182,780 is able to block estrogen activity; and *3*) incubation of U937 cells with estrogen resulted in a decrease in Nip2 mRNA. The results on expression and function of the sex steroid receptors in U937 cells provide important informations on this cellular system and represent an interesting background for understanding the genetic mechanism underlying the antiapoptotic mechanism (Legdeur et al., 1996). The authors demonstrated that Nip-2, is regulated by estradiol and that encodes for a protein involved in the onset of apoptosis (Boyd et al., 1994), Nip-2 can be modulated by estrogen in U937 cells; this result suggests that the genetic pathway of response to steroid hormones is conserved among different cell types. Characterization of TNFα activity has shown two distinct mechanisms of action in induction of apoptosis by this cytokine: an initial, transcription-independent activity, which operates through the activation of proteases, and a subsequent transcription dependent antiapoptotic activity by activation of the NF-kB factors (Wallach, 1997). Vegeto et al., reported that interference between estradiol and TNFα signaling pathways occurs only when E_2 is added along with or 2 h before the cytokine, this suggest that induction of apoptosis and/or cell survival might be regulated by overlapping genetic targets of TNFα and ER transcriptional activity. Negative interference between ER and TNFα signaling pathways has been reported for the interleukin-6 promoter, where transcriptional interference has been proposed to occur through direct physical interaction and reciprocal transcriptional silencing between specific members of the NF-kB family and ER. Interaction between PR and the NF-kB family has also been demonstrated; in addition, PR and STAT5, a member of the JAK/STAT family of latent transcription factors that are activated by numerous extracellular signals, have been shown to interact *in vitro* and to reciprocally interfere when artificially coexpressed in cells, providing further evidence for the hypothesis that the steroid receptors and membrane receptor-

associated second messengers can communicate and reciprocally modulate transcriptional efficacy of the respective activating signals (Richer et al., 1998). The results of this study have provided the first evidence for receptor-mediated transcriptional and cellular responses of myeloid cells to estrogen and progesterone. After this study in 2005 Ghisletti et al. (Ghisletti et al., 2005) demonstrated that 17β-estradiol inhibits inflammatory gene expression by controlling NF-kB intracellular localization, this factor has a central role in several biological pathways that govern immunity. The Ghisletti's study showed that E_2 prevents inflammatory gene transcription induced by inflammatory agents by inhibiting NF-kB intracellular transport, an immediate-early event in the inflammatory signaling cascade. This is a novel mechanism in the control of NF-kB activation that is not shared by conventional anti-inflammatory drugs. In addition, they showed that the effect of E_2 on NF-kB is mediated by the intracellular receptor ERα through a non genomic signaling pathway that involves the activation of PI3K. This discovery was the first report showing an inhibitory effect of E_2 on the intracellular transport of proteins toward the nucleus. Ghisletti et. al., showed that the decreased activity of p65 induced by E_2 is due to the cytoplasmic sequestration of this transcription factor. NF-kB activation requires the degradation of its inhibitory protein, Ik-B; NSAIDs that are inhibitors of NF-kB activation mediate this effect by blocking IKK activity and thus suppressing Ik-Bα phosphorylation and degradation. In this way, NF-kB remains inactive in the cytoplasm in association with Ik-B proteins. In contrast to this mechanism, the Maggi's laboratory observed that inhibition of NF-kB nuclear accumulation by E_2 occurs through the blocking NF-kB intracellular transport without affecting Ik-Bα degradation. It has been shown that NF-kB may utilize microtubules as cytoskeletal tracks to massively migrate from the cytoplasm to the nucleus in response to inflammatory signals. The study, also, showed that the effect of E_2 is specific for macrophages. The action of E_2 in inflammation requires the activation of a specific intracellular receptor. By showing that ERα mediates the inhibition of NF-kB transport, Ghisletti provided the mechanism to explain this receptor-specific anti-inflammatory activity. The effect of E_2-activated ERα on NF-kB involves a non genomic signaling pathway. Moreover, the authors demonstrated that the action of E_2 fails when the hormone and LPS are added at the same time, demonstrating that the target of hormone action is the early-phase activation of LPS

signaling. Phosphoinositides in the plasma membrane, including PIP3, play critical roles in regulating the interplay between cytoskeleton dynamics and cell reactivity and shape in response to extracellular signals. The study showed that E_2 rapidly stimulates the production of PIP3 in macrophages and that this induction uncouples the inflammatory signaling to the transport system of NF-kB. In light of the widespread biological responses regulated by PIP3, their results suggested that E_2 may be a master regulator of intracellular pathways activated at the plasma membrane of inflammatory cells by lipid secondary messengers. This notion also leads the authors to speculate on whether the action of the hormone on PIP3 signaling pathways may be compromised in inflammatory-based pathologies that do not benefit from E_2 action. The relevant and unique role played by E_2 in inflammation may reflect the endocrine activity of this hormone, which could act as a protective agent against inflammatory insults in order to favor pregnancy and grant offspring sustenance during a woman's fertile life. At menopause, the development of chronic degenerative diseases, such as those affecting bone, vessel walls, or the brain, is strictly associated with an increased proinflammatory reaction, indicating that E_2 deprivation may indeed facilitate the onset of chronic inflammatory reactions. In summary, the Ghisletti's finding of a novel mechanism of E_2 action in inflammation supports the unique role of E_2 in the control of innate immunity .

ERs IN THE BRAIN

For a long time the presence of ERs was ascribed in hypothalamic and preoptic area, to regulate endocrine functions and sex related behavior.

In the late 1980's several studies showed that estrogens and progesterone were involved also in the modulation of functions not strictly related to endocrine control, indeed, it was demonstrated that these hormones partecipate in the control of motor areas and limbic areas. Maggi and Perez, in 1984, on this postulate found that receptors for progesterone and estrogens are present in numerous extrahypothalamic brain regions, this finding had given a new impulse to studies estrogens regulation of brain functions (Maggi and Perez, 1984). A recent study of Maggi's laboratory (Stell et al., 2007), demonstrated that ERs localized in both reproductive and non reproductive areas of the brain are transcriptionally active and susceptible to

regulation by estrogens. The study also showed, for the first time, that the extent of ER activity in the brain of unstimulated male and female mice is comparable and quite elevated (Foidart et al., 1995). This observation suggested that in brain, the sex hormone receptor has functions that are beyond the control of reproduction. Supporting this view, the activity of ER is quite sustained in males and females and is observed independently from the association of each given brain region to reproductive functions. The finding of high ER activity in male brain is not surprising in view of the widespread expression of aromatase in this tissue; furthermore, the authors believe that ligand-independent transcriptional activation of ER may occur, triggered by a crosstalk with growth factors and other molecules inducing ER phosphorylation (Ciana et al., 2003; McCaffrey et al., 2003; Maggi et al., 2004; Mussi et al., 2006 ;Ciana et al., 2007).
The study clearly shows the major influence of the estrous cycle on ER activity in several brain areas: the effect observed in limbic areas such as the hippocampus is of particular interest with regard to the effects of estrogens on memory and affective disorders. Interestingly, ER activity in the amygdala is high in both sexes: this is consistent with the report that ERs are most expressed in amygdaloid nuclei. We can study the dynamics of ER activity with a functional method to precisely localize and quantify ER transcriptional activity in mouse brain, enabling the quantitative measurement of luciferase activity in selected brain regions created in our laboratory. The method can be easily expanded to the study of brain activity of transcription factors, protein–protein interaction, or neural cell differentiation or migration, on availability of the suitable reporter animals.
ERα is generally expressed in the hippocampus, preoptic area, and most of the hypothalamus, the bed nucleus of stria terminalis, the ventromedila nucleus and the posterodorsal part of the medial amygdala, whereas it is sparse or absent from the cerebral cortex and cerebellum. ERβ is widely distributed in cell nuclei within selected regions of the brain including the olfactory bulb, cerebral cortex, septum, preoptic area, bed nucleus of the stria terminalis, amygdala, paraventricular hypothalamic nucleus, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, and cerebellum. Extranuclear immunoreactivity is detected in several areas including fibers of the olfactory bulb, CA3 stratum lucidum, and CA1 stratum radiatum of the hippocampus, the presence of hormonally regulated ERs has

also been found in glial cells, in microglia, and in neural stem cells (Couse et al., 1997; Shughrue and Merchenthaler, 2001) Expression of ERs has also been observed in other cell types within the CNS that participate in the inflammatory reaction, namely endothelial cells and circulating leukocytes (Vegeto et al., 2003). Cerebellum, hippocampus and the amygdala, are regions responsible for anxiety, depression, and learning and memory, ERβ has key role in these regions but also in the serotonergic neurons of the dorsal raphe nucleus (DRN) in the midbrain. The DRN is the primary part of the midbrain housing serotonergic cell bodies that project to other parts of the brain. In postmenopausal women serotonergic activity is low and can be increased by administration of E_2 (Halbreich et al., 1995). E_2 influences several aspects of serotonin function: release, metabolism, reuptake, synthesis and receptor modification (Osterlund, 2009). With this profound influence of E_2 on the serotonergic system it is not surprising that ovariectomy, menopause, antiestrogen treatment, GnRH analog treatment, and postpartum and premenstrual periods are associated with depression (N-Wihlback, 2006). It is also easy to understand why selective serotonin reuptake inhibitors (SSRIs) are effective antidepressants. Interestingly, coadministration of E_2 (Schneider et al., 2001) or raloxifene (Grigoriadis et al., 2005; Sugiyama et al., 2007; Yokoyama et al., 2008) with SSRIs augments their antidepressant action. There is now a consensus that the DRN is ERα-poor or ERα-negative, whereas it is strongly ERβ-positive, both at the mRNA (Lu et al., 1999; Gundlah et al., 2000; Gundlah et al., 2001) and protein levels (Mitra et al., 2003; Nomura et al., 2005; Vanderhorst et al., 2005). Although ERα and ERβ are coexpressed in many other regions of the brain, there is a clear segregation of the ERs at the level of the midbrain. ERβ was found to increase Tph2 mRNA expression in rat brain, and administration of a selective ERβ ligand, diarylproprionitrile (DPN), decreased depression-like behavior of rats (forced swim test) (Donner and Handa, 2009). Therefore, not only is ERβ present in the DRN but it has clear functions in the serotonergic neurons in the DRN. In contrast to the DRN, which is ERα-negative and ERβ positive, many other parts of the brain express both ERs.

Maggi and Zucchi analyzed the distribution of estrogen receptor (ER) via immunoenzymatic assay in the brain of ovariectomized rats and found the presence of large amounts of ER-like immunoreactive material in the cytosol of the hippocampus: a brain area described to contain little estrogen-binding activity. The

protein detected in the hippocampus by the specific antibody is indistinguishable from the rat ER in its response to hormonal treatments and in its electrophoretic mobility. The presence of elevated amounts of ER in such an important part of the limbic system had creates new possibilities for interpreting the role played by this sex hormone in the central nervous system of rat. In areas such as the hippocampus, the amygdala and the hypothalamus, the role of E_2 signaling is more complex. Maggi et. al. (Maggi et al., 2004), think that existence of two subtypes of ER in the same cell permits fine-tuning of E_2 signaling; therefore, by investigating the interaction between the two ERs (e.g. their direct interaction and/or interactions of molecules downstream in their signaling pathways) they might understand some of the ambiguities in the physiological effects of E_2. If they presume that ERα and ERβ have opposite effects in some situations then they can begin to explain several puzzling actions of E_2.

Brain functions can be broadly divided into three categories: affection (mood or emotion), behavior, and cognition (learning and memory). Estrogen has a strong influence over all three. The effects of E_2 on emotion (anxiety and/or depression-like behavior) are inconsistent and paradoxical. Past studies report that E_2 can reduce, increase, or have no effect on performance in several behavioral tests of anxiety and/or depression-like behavior (Imwalle et al., 2005; Toufexis et al., 2007). There is increasing evidence that ERα activation is anxiogenic (Morgan and Pfaff, 2001; Lund et al., 2005; Toufexis et al., 2007) whereas ERβ activation is anxiolytic (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Walf and Frye, 2005; Toufexis et al., 2007; Frye et al., 2008; Walf et al., 2008; Donner and Handa, 2009; Osborne et al., 2009) and/or antidepressive (Walf et al., 2004; Rocha et al., 2005; Walf and Frye, 2007; Hughes et al., 2008; Walf et al., 2008; Donner and Handa, 2009). Thus E_2 (that can act at both receptors) should, and does, have unpredictable effects on emotion. In studies of aggressive behavior, for example, ERα and ERβ show dramatically opposing effects. Based on the behavior of $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice it has been suggested that ERα enhances aggressive behavior (Ogawa et al., 1997; Ogawa et al., 1998) whereas ERβ reduces aggression (Ogawa et al., 1999; Nomura et al., 2002). Further, ERβ, but not ERα, is believed to enhance cognitive performance in several learning tasks (Frye et al., 2008; Walf et al., 2008; Osborne et al., 2009). Rissman et al. (Rissman et al., 2002) reported that wild-type female mice with or without E2

showed the same performance of spatial learning tested in the Morris water maze (MWM) as ERβ$^{-/-}$ females. However, when ERβ$^{-/-}$ females were treated with E_2 there was impaired spatial learning. These results could be interpreted to mean that, in the absence of ERβ, E_2-activated ERα interferes with spatial learning.

One should not try to oversimplify the role of ERα and ERβ in the brain, notably because anxiety and depression-like behavior are tightly connected with spatial learning ability and aggressive behavior, and all behaviors are also affected by sexual influences related to reproductive function. Furthermore, it is important to note that timing of the activation of ERs is crucial. For example, when neonatal mice are treated with the ERβ-specific agonist DPN there is increased anxiety and aggression during adulthood (Patisaul and Bateman, 2008). To date, despite its importance, information about the temporal expression of ERβ in the brain throughout life is very limited. If author's hypothesis is right, all mouse experiments addressing the role of the two estrogen receptors should include ERαβ$^{-/-}$ and aromatase knockout (ArKO; Cyp19a1$^{-/-}$) mice because, when one observes a functional impairment in an ERa$^{-/-}$ or ERβ$^{-/-}$ mouse, it is difficult to tell whether the phenotype is due to loss of one receptor or to the overactivity of the receptor which remains. For example, a phenotype observed in ERβ$^{-/-}$ mice, but not observed in ERαβ$^{-/-}$ or ArKO mice, is a strong indication that this phenotype is caused by overactivity of ERα rather than by the absence of ERβ itself. Several studies suggested that estrogen increases the risk of gynecological tumors (Morch et al., 2009) there has been a lively debate on the safety of hormone replacement therapy in postmenopausal women. After the discovery of ERβ, and confirmation of its presence in the brain, many researchers raised the question of whether ERβ-specific ligands can be used as novel therapeutic agents with the desirable effects of estrogen but without risk of tumor incidence. The involvement of estrogen in pain has been demonstrated (Chakrabarty et al., 2008; Fan et al., 2007), and pain disorders might be treated using ERβ agonists. One study has shown that E_2-activated ERβ dampens endogenous pain caused by increased nociceptive neuronal activity (Spooner et al., 2007), and another has demonstrated that ERβ agonists alleviate chronic but not acute inflammatory pain states (Gardell et al., 2008).

ERβ is also a promising novel drug target for treatment of hypertension (HT) and panic disorder. Because ERβ$^{-/-}$ mice are hypertensive it is possible that ERβ regulates

vasopressin and/or oxytocin in the magnocellular neurosecretory cells of the hypothalamus that are ERβ-positive but ERα-negative (Somponpun and Sladek, 2002). Several different types of drugs with multiple targets are available for control of HT. However, for HT and cardiac palpitations caused by psychiatric disorders, such as panic disorder, no effective treatment is yet available. Depression, anxiety, hypertension and epilepsy, and alterations in cognition, appetite control, and pain threshold, are not isolated phenomena but frequently coexist. Development of effective ERβ-targeted drugs would probably provide significant benefits to many patients suffering from psychiatric disorders.

INTERACTIONS OF ESTRADIOL AND INSULIN-LIKE GROWTH FACTOR-I SIGNALING IN THE NERVOUS SYSTEM

Insulin-like growth factor-I (IGF-I) is a hormone of the somatotrophic axis and a local factor produced in many tissues, including the nervous system (LeRoith, 2008).
IGF-I has pleiotropic actions in the brain and influences neuronal development, synaptic plasticity, neuroendocrine regulation, adult neurogenesis and cognition (Aberg et al., 2006; Fernandez et al., 2007; Aleman and Torres-Aleman, 2009). IGF-I is also a potent neuroprotective molecule (Carro et al., 2001; Aberg et al., 2006). IGF-I is actively transported across the blood–brain barrier (Carro et al., 2006). Therefore, brain function is factor (BDNF) (Carro et al., 2000; Ding et al., 2006) and vascular endothelial growth factor (VEGF) (Lopez-Lopez et al., 2004; Lopez-Lopez et al., 2007). In addition, IGF-I interacts with the ovarian hormone estradiol in the regulation of multiple events in the nervous system. IGF-I interacts with estradiol in the regulation of developmental events in the nervous system. An interaction between these two factors may contribute to the generation of structural sex differences in the brain by the regulation of the survival and differentiation of developing neurons in brain areas involved in the regulation of neuroendocrine events and reproduction (Carrer and Cambiasso, 2002).
Several studies have demonstrated an interdependence of ERs and IGF-I receptor in the promotion of the survival and differentiation in primary cultures of developing hypothalamic neurons (Duenas et al., 1996). Both estradiol and IGF-I promote

neuronal survival and differentiation in primary neuronal cultures grown in a defined medium deprived of serum and hormones.

Different neurodegenerative conditions are associated with modifications in serum IGF-I levels (Busiguina et al., 2000; Carro et al., 2000). Furthermore, mice with low levels of serum IGF-I, as a consequence of specific targeted disruption of the IGF-I gene in the liver, had reduced neurogenesis in the hippocampus together with impaired spatial learning (Trejo et al., 2008). Moreover, the disruption of IGF-I input to the brain promotes amyloidosis, cognitive disturbance, hyperphosphorylated Tau deposits, gliosis and synaptic protein loss. This finding supports the hypothesis that disrupted IGF-I signalling may be involved in the pathology of Alzheimer’s disease. Indeed, systemic IGF-I promotes brain b-amyloid clearance, stimulating the neuronal release of the mmolecule and the transport into the brain of bamyloid carrier proteins that will take the molecule out of the brain (Carro et al., 2002; Carro and Torres-Aleman, 2004). Therefore, decreased systemic IGF-I levels may result in an impaired b-amyloid clearance. In addition to the neuroprotective effects of peripheral IGF-I, local IGF-I produced in the nervous system may also play a role in neuroprotection. Brain injury induces the synthesis of IGF-I and estradiol by reactive astrocytes (Hwang et al., 2004; Garcia-Segura, 2008; Saldanha et al., 2009) and up-regulates ERs, IGF-I receptors, and IGF-binding proteins in reactive glia (Garcia-Ovejero et al., 2002; Chung et al., 2003). Therefore, estradiol and IGF-I released by reactive glia may act directly on these cells or on neighbouring neurons, regulating reactive gliosis, neuronal survival and the reorganization of neural tissue after injury. Indeed, IGF-I and estradiol interact to regulate the plastic response of the brain after injury and during neurodegenerative conditions.

IGF-I promotes neuronal survival and inhibits neuronal apoptosis in vitro and in vivo in a variety of experimental models of neurodegeneration (Carro et al., 2003; Trejo et al., 2004; Aberg et al., 2006) *In vivo*, IGF-I has also been shown to be a neuroprotective factor against a variety of neurodegenerative conditions, including hypoxic-ischaemic brain injury (Guan et al., 1993; Guan et al., 2003), excitotoxicity (Carro et al., 2001) and cerebellar ataxia (Fernandez et al., 1998; Fernandez et al., 1999; Fernandez et al., 2005). IGF-I neuroprotective effects are exerted by the activation of the main intracellular signalling pathways associated with IGF-I receptors, the MAPK and the PI3K/Akt pathways (Guan et al., 2003). In particular,

the inhibition of GSK3b activity, which is downstream of the PI3K/Akt pathway, seems to be an essential step in the neuroprotective mechanism (Brywe et al., 2005).
The interaction of IGF-I and estradiol in neuroprotection has been assessed in ovariectomized rats in vivo, using systemic administration of kainic acid to induce degeneration of hippocampal hilar neurons, an experimental model of excitotoxic cell death. Both the systemic administration of estradiol and the intracerebroventricular infusion of IGF-I prevent hilar neuronal loss induced by kainic acid. The neuroprotective effect of estradiol is blocked by the intracerebroventricular infusion of an IGF-I receptor antagonist, while the neuroprotective effect of IGF-I is blocked by the intracerebroventricular infusion of the ER antagonist ICI 182780. Similar results have been obtained in ovariectomized rats after the unilateral infusion of 6-hydroxdopamine into the medial forebrain bundle to lesion the nigrostriatal dopaminergic pathway. Pre-treatment with estradiol or IGF-I prevents the loss of substantia nigra compacta neurons, the decrease in dopaminergic innervations of the striatum and the related motor disturbances. Blockage of IGF-I receptor by the intracerebroventricular administration of the IGF-I receptor antagonist JB1 attenuates the neuroprotective effects of both estrogen and IGF-I (Quesada and Micevych, 2004). In addition, the neuroprotective action of estradiol against 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) toxicity in the nigro-striatal system of male mice is associated to the regulation of IGF-I receptor signalling (D'Astous et al., 2006). These findings suggest that the neuroprotective actions of estradiol and IGF-I after brain injury depend on the co-activation of both ERs and IGF-I receptor in neural cells. Furthermore, in a model of global cerebral ischaemia, in which both estradiol and IGF-I prevent neuronal loss in hippocampal CA1, simultaneous treatment with both factors do not have an additive effect (Traub et al., 2009). This suggests that both factors may use the same signalling mechanisms to exert neuroprotection.

E_2 AND NEUROPROTECTION

In the last few years a clear and relevant protective role of estrogens against neural cell death has been delineated, as extensively illustrated by animal and cellular models of neurodegeneration (Behl et al., 1997; Meda et al., 2000; Bebo et al., 2001).

This beneficial effect of estrogens can be explained by their neurotrophic and antiapoptotic functions and anti-inflammatory potential.

NEUROTROPHIC ACTIVITY

Some studies propose that the trophic activities of estrogens during the maturation of the CNS may continue to exist in the adult brain and ensure that neurons maintain the synaptic connections indispensable for neural signaling and survival. Toran-Allerand demonstrated that estradiol treatment of explants cultures of cerebral cortex and hypothalamus stimulates extensive neurite outgrowth (Toran-Allerand et al., 1980). Since then, several studies in dissociated neurons in culture or in neuroblastoma cells showed that estradiol increases cell viability, differentiation, neurite outgrowth, and spine density and controls the ability of neurons to extend neurites and to form synaptic connections with other cells via dendritic spines (Maggi et al., 2004). Estrogens were shown to modulate the synthesis of growth factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), transforming growth factor-beta (TGF-β), and related receptors, TrkA and TrkB, in neurons and astroglia (Cardona-Gomez et al., 2000) and this *de novo* synthesis of growth factors is required for neurite formation.

ANTI-APOPTOTIC ACTION

Estradiol protects neurons against cell apoptosis by regulating the expression of anti- and proapoptotic proteins, as observed in primary neuronal cell cultures, tumor-derived neuronal cell lines, mixed neuron/astrocyte cell culture, and organotypic explants. Several of the known antiapoptotic genes, such as Bcl-2 and BclXL, are transcriptionally activated by the hormone through the classic mechanism of transcriptional regulation, as EREs are present in the promoter sequence of these genes (Garcia-Segura et al., 1998; Dong et al., 1999; Gollapudi and Oblinger, 1999; Pike, 1999). Accordingly, proapoptotic genes (bax, bad, bcl-Xs) are down-modulated by estrogens, thus indicating that the antiapoptotic activity of estradiol controls the balance between apoptotic and antiapoptotic genes (Patrone et al., 1999; Pike, 1999). In addition, estrogen acts on antiapoptotic protein activity by an indirect mechanism, as shown in the case of BNIP2, a protein that inactivates bcl-2 through protein-

protein interaction (Boyd et al., 1994), which is negatively modulated by estrogens in different cellular systems (Garnier et al., 1997; Vegeto et al., 1999; Meda et al., 2000).

ANTI-INFLAMMATORY POTENTIAL

Recent data provided by *in vivo* and *in vitro* studies suggested that estrogens exert a protective effect against brain disorders by influencing the inflammatory response. This anti-inflammatory hypothesis also stemmed from the evidence that menopause, which is characterized by the drastic drop in estrogen levels, results in an increased incidence of inflammatory pathologies of brain and other tissues.

The anti-inflammatory properties of female steroid hormones is demonstrated *in vivo* in animal models of CNS inflammation, that is, experimental autoimmune encephalomyelitis (EAE, the animal model of MS), brain ischemia, globoid cell leukodistrophy, and experimental brain inflammation. Treatment with physiological doses of estrogen before the onset of disease downregulates the expression of inflammatory factors, including cytokines, chemokines, and their receptors, (Matejuk et al., 2001; Matsuda et al., 2001) apolipoprotein E (Horsburgh et al., 2002), and other modulators of leukocyte migration, such as matrix metalloproteinase-9, complement receptor-3, and scavenger receptor-A50,51; moreover, estradiol strongly opposes the influx of leukocytes into the CNS, which is a distinctive sign of ongoing inflammation in these pathologic conditions (Weissman et al., 1993; Jansson and Holmdahl, 1998; Ito et al., 2001; Horsburgh et al., 2002).

Both chronic or acute–traumatic brain diseases are known to be under estrogen control. These hormones may influence brain development dysfunctions (autism), neurotransporter impairments (depression, anorexia and bulimia), neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease, Amyotrophic lateral sclerosis), traumatic episodes and injuries (epilepsy and skull trauma), immune system dysfunctions (multiple sclerosis), and ischemic damage (ictus).

The prominent role of estrogens in these CNS disorders has been hypothesized, and in some cases for a long time, based on the evidence that the incidence, course, and gravity of these disorders were strongly dependent on the plasma level fluctuations of these hormones, as it occurs during the menstrual phases, after parturition, or at menopause; additional indications arise from the comparison between age-matched

males and females in the manifestation of disease. Several examples of these observations are present in the literature, relating to mood disorders, (Gregoire and Drahmoune, 2000) psychotic episodes (Huber et al., 2001), Alzheimer's disease (AD) (Henderson, 1997), Parkinson's tremors (Tsang et al., 2000), amyotrophic lateral sclerosis (ALS) (Veldink et al., 2003), or ischemic insults.

It is now well established that estrogens are involved in the control of the inflammatory response. The following description summarizes some examples of brain diseases characterized by an inflammatory reaction state, in which estrogens were shown to be involved by molecular, cellular, and pathophysiological evidence.

ALZHEIMER'S DISEASE

The inflammatory component plays a relevant role in this disease, in which there is a clear activation of the resident macrophage cell population. In human biopsies immunohistochemical analyses revealed a strong activation of microglia around senile plaques, the main pathologic feature of this disorder (Kalaria and Perry, 1993). Activation of microglia, increased levels of inflammatory mediators, and cells associated with amyloid deposition have also been observed in animal models of AD (Bomemann, 2001); yet, the precise role of inflammation in AD progression is still debated. Clinical trials involving the use of nonsteroidal anti-inflammatory drugs reduced the incidence of AD (Stewart et al., 1997; Anthony et al., 2000; Yip et al., 2005). On the other hand, many studies support the idea that microglia are beneficial to the diseased brain (Turrin and Rivest, 2006), through the release of neurotrophic factors (Nguyen et al., 2002) and phagocytosis of amyloid deposits (Rogers and Lue, 2001). The incidence of AD is higher in women than in men and the progression of this disease has different features in the two sexes (Barnes et al., 2005; Liu et al., 2005). Epidemiological evidences suggest protective effects of the hormone replacement therapy (HRT) on the onset of this pathology and some experimental analyses confirmed this evidence demonstrating the prevention of cerebral structure degeneration by estrogens (Henderson et al., 1994; Henderson, 1997). On the contrary, recent clinical trials on women taking progesterone–estrogen combination therapy suggested an increased risk of dementia (Yaffe, 2003). Recently, a model of brain estrogen-deficient AD mice was generated by crossing the aromatase knockout mice with an AD transgenic mouse line of AD (Yue et al., 2005). Absence of the

enzyme for the synthesis of estrogens specifically in brain areas resulted in the early onset of pathology and in increased β-amyloid peptide deposition.

PARKINSON'S DISEASE

Parkinson's disease (PD), a degenerative pathology of dopaminergic neurons localized in the substantia nigra pars compacta (SNc), is also characterized by the presence of activated microglia surrounding Lewy's bodies, α-synuclein accumulation elements. Some clinical studies suggested that this neuroinflammatory reaction can be a critical factor for the development of this disease (Casals et al., 1998; Ling et al., 2002). Immunological analyses of brain biopsies from PD patients showed the presence of activated microglia cells, with increased HLA-DR and CR3 receptor expression, without reactive astroglyosis (Banati et al., 1998; Mirza et al., 2000); in PD tissues, the levels of ROS, IL-1β, IL-6, and TNF-α are increased.(Jenner and Olanow, 1998). Frequency of PD is high in men having a ratio of 1:5 or 3:7 (concerning the ethnic provenience) compared to that in women, who show low symptom gravity and need lower doses of levodopa (Lyons et al., 1998). In women, pathological symptoms get worse with reduced estrogen levels during the menstrual cycle (Quinn and Marsden, 1986). Furthermore, symptoms seem to increase in women, which interrupts their HRT. Several retrospective clinical and epidemiological studies tried to connect estrogen treatment with onset and severity of the pathology. These results are discordant: some show indications of a late onset and a decrease in disease risk with estrogen, (Benedetti et al., 2001) whereas other observations show no difference in these parameters, but an amelioration in cognitive faculty (Marder et al., 1998; Thulin et al., 1998). Also, the prospective studies do not reach a definitive conclusion, as one shows that estrogens do not provide significant symptomatic variations (Strijks et al., 1999), while other studies indicate that hormone treatment reduces the levodopa dose after only 10 days of therapy (Blanchet et al., 1999) and that prolongs the follow-up period (Tsang et al., 2000). One recent clinical study demonstrated that estrogen therapy has a beneficial effect, establishing that women treated with estrogens have a low pathology risk than that of the not-treated ones (Currie et al., 2004).

AMYOTROPHIC LATERAL SCLEROSIS

ALS is a neurodegenerative disease that involves primary cerebellar and spinal cord motorneurons. Recent studies provided evidence for the involvement of neuroinflammatory processes in this disease. Tissues from ALS patients show a widespread activation of microglia and astrocytes. In tissues, blood, or cerebrospinal fluid from ALS subjects there is an abundant expression of proinflammatory markers like TNF-α, IL-1β IL-6, IL-2, IFN-γ RANTES, and the COX enzyme. These data are confirmed also in animal models of mice and rats (Hensley et al., 2002; Chen et al., 2004; Malaspina and de Belleroche, 2004; Xie et al., 2004).

AGING AND MENOPAUSE

In the developed world, mean life expectancy for women since 1900 has increased from 50 to 81.7 years. Particularly striking is the remarkable increase in the proportion of women over fifty in the population, which has tripled since the turn of the 19th century. Popolation projections estimated approximately 1,200 million in the world to be aged 50 years and older in 2030. The numbers of postmenopausal women in the developing world are anticipated to increase much more rapidly than those in the industrialized world. From 1990 to 2030 the proportion of postmenopausal women in more developed countries is expected to decline from 40 to 24 percent, whereas it will increase from 60 to 76 percent in less-developed countries. The most profound and universal alteration in the mature aging endocrine system occurs in women and is due to menopause.

Accelerated population aging has led to a major epidemiological transition in the leading causes of death from infections and acute diseases to the chronic and degenerative diseases of old age (such as malignant neoplasms cardiovascular and cerebrovascular diseases, osteoporosis and dementia). Now aging is appreciated as the eterogeneous product of a genetic disposition being revealed under varariable enviromental, behavioural, psychosocial, and economics conditions, many of which are amenable to profound change with existing as well as emerging strategies.

Aging leads to a significant deterioration of the physiological systems, including the immune, nervous and endocrine systems, as well as of the immune-neuroendocrine network (De la Fuente, 2008). The age-related decline in immunity, known as

immunosenescence, involves innate and adaptive immune responses, but concerns especially T cell functions (Gruver et al., 2007; Kumar and Burns, 2008; De la Fuente and Miquel, 2009). As regards the endocrine system, aging involves a progressive decrease in the secretion of several hormones such as estrogens (Arlt and Hewison, 2004). These hormones, due to the widespread distribution of their receptors throughout the brain (Aloysi et al., 2006; Morrison et al., 2006), also play a key role in the neurobiology of aging (Morrison et al., 2006) as they exert a great influence on a broad array of brain regions, mostly areas associated with cognition, memory and emotional processing (as mood and affect). All these areas constitute important sites of agree-lated neurodegenerative changes, such as neuronal loss and compensatory gliosis (Ferrari and Magri, 2008). Other physiological age-related changes commonly described among the elderly include balance dysfunctions, reduced speed, shorter step length and muscular weakness due to skeletal mass reduction (El Haber et al., 2008). According to all the above, one essential concept that must be taken into consideration when studying the aging process is the concept of "biological age," which arises as a consequence of the different rates of physiological changes in the members of a population of the same chronological age and suggests that chronological and biological age do not necessarily coincide (De la Fuente and Miquel, 2009). Therefore, the assessment of biological age requires the use of biomarkers to determine the level of senescence and life expectancy. The immune system has been proposed as a good marker of health, biological age and predictor of life span, since a good maintenance of several immune functions is related to a longer life span (Wayne et al., 1990; Guayerbas et al., 2002).

Although during the last decades of the 20th century human life expectancy in developed countries has increased from approximately 75 to 83 years, the age at which women encounter their major age-related hormonal change, that is, menopause, has remained essentially constant at around 50 years (Miquel et al., 2006). Therefore, many women will spend over one-third of their lives in the postmenopausal state. Since estrogens have a regulatory role on many organs, the rapid decline in their circulating levels associated to menopause triggers many physiopathological reactions, making women more prone to experience disease and disability (Amin et al., 2003). Thus, chronic deficiency of sex hormones has many implications in a wide variety of non-reproductive functions, and among the most

studied symptoms we can cite hot flashes, skin aging and high risk of osteoporosis and cardiovascular disease (Miquel et al., 2006). There have also been described different psycho-emotional symptoms that somehow overlap with depressive symptoms and include disturbed sleep, lack of concentration, anxiety, irritability, frustration, mood lability, depression and fatigue (Rasgon et al., 2005; Sarkaki et al., 2008). Moreover, estrogens have been shown to influence the development, regulation and normal functions of the immune system (Islander et al., 2003; Rehman and Masson, 2005). These hormones modulate lymphoid cell growth, differentiation and proliferation, antigen presentation, cytokine and antibody production, NK activity and cell survival (McMurray, 2001). Thus, the repercussions of menopause on female health have become a subject of increasing interest.

To tackle this question, a great deal of research has been done during the last few years in murine experimental models as a first approach to clarify the repercussions of menopause. Since rodents become anovulatory at a mature age (10–12 months old) but maintain a basal gonadal steroid secretion, in contrast to what happens in women (Nelson, 2008), ovariectomy in those animals became the best tool to mimic human ovarian hormone loss. Not surprisingly, during the last years there has been a great increase in the number of published work focusing on the consequences of ovariectomy, mainly in rats, on different physiological functions or systems, such as the central nervous system, vascular function, hepatocytes, bone, skin (Castillo et al., 2005; Perez-Martin et al., 2005; Castillo et al., 2006; Tresguerres et al., 2008) and immune function (De la Fuente et al., 2004; Baeza et al., 2007; Baeza et al., 2009).

DEFINITION OF MENOPAUSE

Female reproductive senescence is a lifelong process that begins before birth and *culminates with ovarian follicular depletion and the menopause. The word* "menopause" ("ménopausie") was used for the first time in 1816 by Guardanne. Initially, the phenomenon of menopuase was explained as a deficiency of ganglionic regulatory functions. In 1910, Marshall recognized that the ovary should be classified as an endocrine organ.The menopause results from reduced secretion of the ovarian hormones oestrogen and progesterone, which takes place as the fi nite store of ovarian follicles is depleted. Natural menopause is diagnosed after 12 months of amenorrhoea not associated with a pathological cause.

Menopause can also be induced by surgery, chemotherapy, or radiation. Initially, the menstrual cycle lengths become irregular, and follicle-stimulating hormone (FSH) concentrations rise in response to decreased concentrations of ovarian hormones. As the menopausal transition progresses, menstrual cycles are missed and ultimately stop, as does ovulation. For some women, 3 consecutive months of amenorrhoea, or mean cycle lengths longer than 42 days, are predictors of impending menopause (Dudley et al., 1998; Taffe and Dennerstein, 2002). Several terms have been used to describe the events that take place during the menopausal transition. A model developed at the Stages of Reproductive Aging Workshop (STRAW) (Soules et al., 2001) described seven stages of reproductive ageing, which were subdivided into reproductive stages, characterised by regular menstrual cycles; menopausal transition stages, with variable menstrual cycles and high FSH values; and postmenopause stages, beginning with the final menstrual period, and lasting until the end of life. The menopausal transition usually begins when women are in their mid-to-late 40s, and can last several years, most commonly 4–5 years. The final menstrual period generallyn happens when women are between 40 and 58 years old, and a final menstrual period before 40 years of age is regarded as premature. The age at which women have their final menstrual period varies across large surveys done in different countries.

For many years, the menopausal transition was viewed to be simply the end product of accelerated oocyte depletion. Moreover, hypothalamic-pituitary axis (HPA) dysfunction was thought to reflect a compensatory response to the gradual decline in the number and quality of remaining oocytes. However, recent studies challenge the conventional belief that ovarian aging is the sole determinant of when females begin the transition into reproductive senescence and raise questions about the sequence of pathophysiological events that initiate reproductive quiescence (Hall, 2007). There is now a convincing body of literature in primates and non-primates that support a role for HPA dysfunction independent of ovarian aging in the transition into reproductive senescence (Brann and Mahesh, 2005; Hall, 2007). Moreover, aberrant responsiveness of the HPA to estrogen feedback and the subsequent generation of abnormal patterns of gonadotropin release may in itself accelerate ovarian follicular exhaustion (Klein et al., 1996). Defining the physiological and cellular mechanisms that initiate female reproductive senescence is an area of intense scientific interest.

Understanding the mechanisms that propel women into the menopause may offer opportunities for interventions that delay menopause-related increases in disease morbidity and thus improve the overall quality of life for aging women.

NON HUMAN MODELS OF FEMALE REPRODUCTIVE AGING

Rodents are useful models for studying female reproductive physiology because they exhibit a high degree of genetic and physiologic similarity to humans, have a relatively short lifespan, and homogeneous strains are widely available at low cost (Wu et al., 2005). The rodent estrous cycle exhibits similar patterns of cyclic changes in serum LH, FSH, estradiol and progesterone levels as the human menstrual cycle. The primary differences are the very short luteal phase and the absence of menses when pregnancy does not occur in rodents. Although reproductive aging manifests somewhat differently in rodents andhumans (e.g., oocyte depletion does not occur before rodents become reproductively senescent) (Wise et al., 2002), several fundamental similarities are seen in perimenopausal women and middle-aged rodents: (1) one of the first physiological signs of impending reproductive senescence is elevated FSH levels (Cooper et al., 1980; Burger et al., 1996; Guthrie et al., 1996; Santoro et al., 1998; Anzalone et al., 2001); (2) elevated FSH is associated with attenuated granulosa cell production of inhibin B; (3) middle-aged rats (Cooper et al., 1980) and women (Weiss et al., 2004) exhibit altered hypothalamic-pituitary axis responsiveness to estrogen positive feedback; (4) altered patterns of gonadotropin secretion occur long before overt ovarian failure; and (5) both humans and rodents develop highly variable cycle lengths with ovarian steroid production preceding reproductive quiescence (Wise et al., 2002). Because so man ychanges in reproductively aging rodents parallel those in aging women (Hall, 2007; Veldhuis, 2008) investigators have used female rodents, especially rats, to explore the role of the HPA in the onset of female reproductive senescence. Consequently, much of what we know about the HPA and female reproductive aging is derived from studies in rats and mice (Downs and Wise, 2009).

MODEL SYSTEMS TO STUDY ER PHYSIOLOGICAL ACTIVITIES

In the last twenty years the use of cell systems and engineered cells has tremendously aided our studies on the intracellular mechanisms of estrogen and ER action in both reproductive and non-reproductive tissues. However these systems do not allow to get an insight on the exact physiological functions of these receptors. The availability of mice in which ERα (ERKO), ERβ (BERKO) or both receptors have been inactivated (DERKO) provided the opportunity, for the first time, to gain an insight of the potential functions of these receptors in reproductive and non-reproductive organs (Couse and Korach, 1999; Dupont et al., 2000).These studies proved that malfunctioning of ERs is associated to major deficits of the skeletal, cardiovascular, nervous and immune systems. However, in these systems the understanding of ER functions is revealed by mutant phenotypes which often are the resultant of several adaptive changes. Furthermore, these systems do not allow to examine the physiological relevance of ER activity at selected period of life, like for instance at menopause. Reporter animals provide us the opportunity to overcome these shortcomings because enable to visualize in real time the state of ER activity in specific organs (Maggi and Ciana, 2005). Knowing when and where the ER is in the transcriptionally activated state directs the study toward a subsequent evaluation of the mechanism and of the direct consequences of ER activation (e.g. characterization of regulated genes) which will then allow to gain the desired insight on ER function. We have recently generated a reporter mouse, ERE-Luc, by integrating into the genome of C57BL/6 mice a transgene in which a luciferase gene is driven by a promoter containing a multimerized ERE and a minimal TK promoter (Ciana et al., 2001). The ubiquitous expression of the transgene is ensured by the presence of MAR insulator sequences bordering the transgene (Sturchler-Pierrat et al., 1997). This mouse model was generated to expressly report on the state of ER transcriptional activity on ERE genes and therefore does not provide any information of the activities of ER on other intracellular signalling molecule. This models system has revealed of great utility to visualize ER state of activation particularly in cycling female mice where it was possible for the first time to determine that the mechanism of ER activation differs in reproductive and non-reproductive organs (Ciana et al.,

2003). These findings open several questions on the actual state of activity of ERs after cessation of ovarian functions in non-reproductive organs.

AIM OF THE THESIS

The aim of my studies was to evaluate the effect of aging and blockade of ovarian functions on estrogen receptor transcriptional activity and ER anti-inflammatory action .

In **specific aim #1** we proposed to study genes driven by ERE-containing promoters: endogenous as well as surrogate reporters; within **specific aim #2** we proposed to provide support to the theory that lack of estrogen anti-inflammatory activity is a major component for the onset of pathologies associated with menopause (osteoporosis, atherosclerosis, metabolic and neurological dysfunctions).

Results

ER ACTIVITY DURING THE MENOPAUSE TRANSITION AND IN AGING.

We still do not know the extent to which ER activity is affected by menopause. It is conceivable that the lack of circulating estradiol induces up-regulation of the ERs, and, as a consequence, the minimal quantities of estrogens produced by organs other than the ovaries (kidney or fat) might be sufficient to maintain the activity of estrogen receptors. On the other hand, previous studies in young, cycling female mice showed that in non-reproductive organs the transcriptional activity of ERs is regulated by factors other than 17β-estradiol (E_2) (Ciana et al., 2003). The knowledge of the exact state of ER transcriptional activity during the menopause transition and in aging is of primary relevance to the design of future therapies aimed at re-establishing the exact mechanisms responsible for ER activity in non-reproductive organs.

a) Experimental groups:

to study the effect of aging and the relevance of ovarian function in thus process we carried out studies in intact (sham operated) and ovx (at 5 month) mice. ER activity was evaluated at month 6, 12, 18 and 22.

b) Experimental protocol:

to overcome any dietary influence in our studies on ER state of activity, we generated a completely synthetic diet which did not activate ERs in the ERE-Luc mice. These results led us to establish a protocol to be followed to study ER activity in ERE-Luc mice. Mice are maintained with regular chow and then shifted to the synthetic food 48 hours before the experiment. This time is sufficient to eliminate all estrogenic compounds present in the food. To evaluated ER transcriptional activity in aging and after ovx we first studied the expression of the reporter luciferase (by measuring luciferase mRNA and luciferase enzymatic activity *ex vivo* and *in vivo*. Next we evaluated the expression of ER endogenous genes such as Prothymosin alpha (PTMA) (Martini et al., 2000) and Progesteron Receptor (PgR) known to be a direct target of ER. To verify that ER synthesis was not modulated by aging, we investigated ER mRNA content in the tissues selected for our studies: i.e. aorta, liver and in a brain region.

1. AGING EFFECT ON ERα EXPRESSION IN SPECIFIC TISSUES.

The study of the changes of ERα expression during female aging demonstrated that aging does not cause a generalized decreased of receptor synthesis, indeed Fig.1 shows a significant increase of ERα content in aorta and uterus at 22 months of age, in liver ER is decreased while no significant change was observed in hippocampus. These data suggest that ER expression with age changes in function of the tissue taken in consideration but not in relation to the specific role plaied by the organ itself.

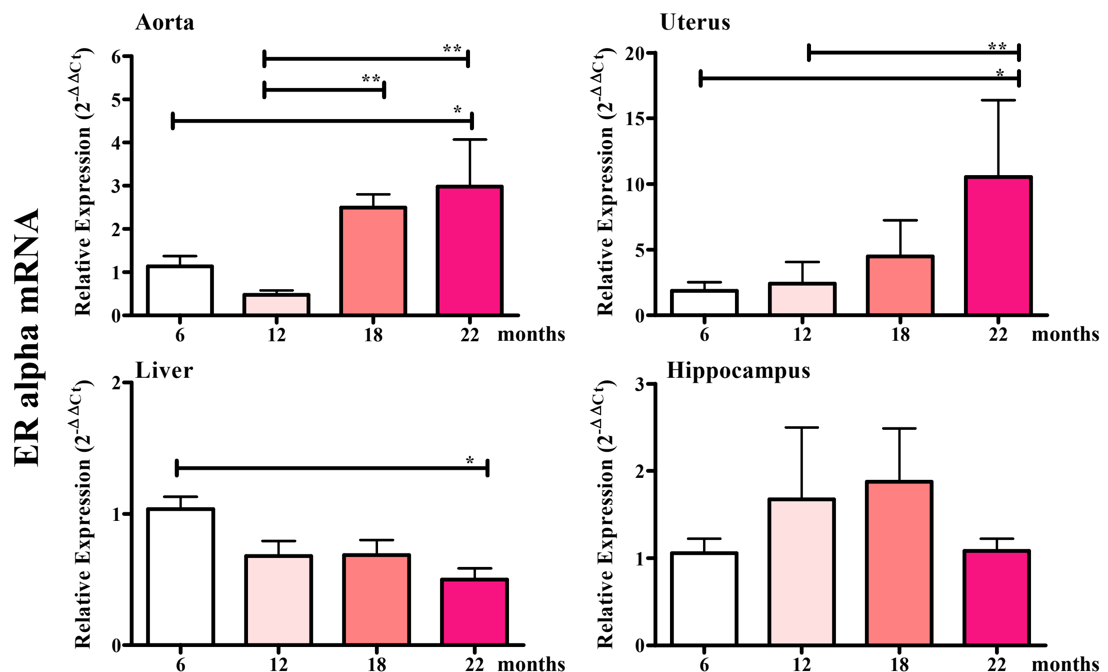


Figure 1 Aging does not affect negatively ER synthesis .
RT-PCR detection of ER alpha RNA levels in aorta, uterus, liver and hippocampus. Groups of six female animals were operated at 5 months of age (sham operated mice) and at due time (6, 12, 18 and 22 months) were euthanized for RT-PCR analysis on extract tissues. The data are expressed as relative expression and calculated by $\mathbf{2}^{-\Delta\Delta Ct}$ method (Livak et al., 2001). *$p<0.05$ versus group at 22 months of age as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

2. ER ACTIVITY CHANGES WITH AGING

a) Study of ER activity on the surrogate target ERE-Luc.

First, we evaluated ER activity by measuring luciferase mRNA and activity in mice of different ages. As shown in Fig.2 (A, B), Luciferase mRNA content in aorta, liver

and uterus did not change significantly in relation to age, while the activity of the protein decreased in strict association with age.

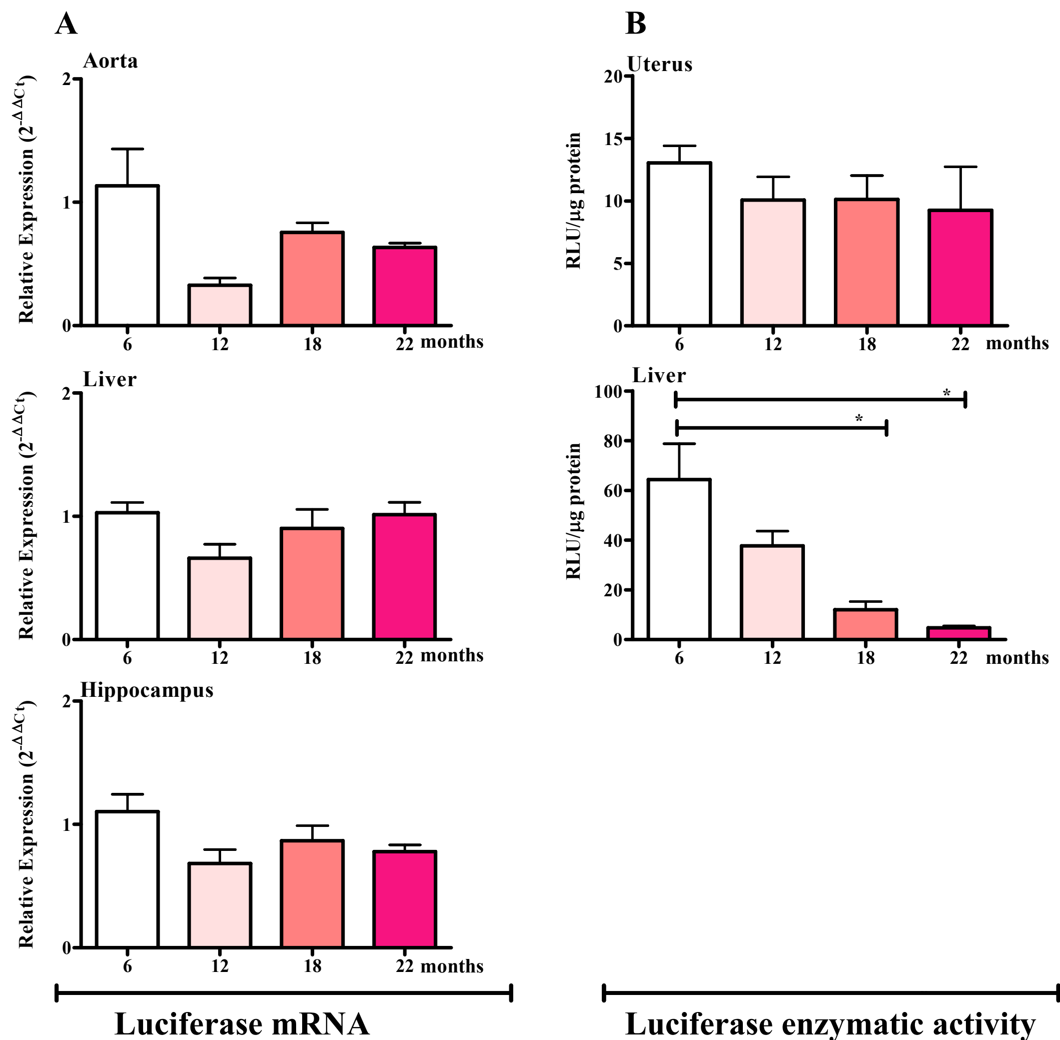


Figure 2 Evaluation of ER activity by study the expression and the activity of Luciferase. At month 6, 12, 18 and 22, six sham mice were sacrificed to collect different tissues. (A) RT-PCR analysis of Luciferase content, the data are expressed as relative expression, the aging does not influence Luciferase mRNA. (B) Luciferase enzymatic assay, the luminescence data, normalized over protein content of each sample are expressed as relative light units for µg of protein. The activity of protein decreases with aging in liver, *$p<0.05$ versus groups at 18 and 22 months of age as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

b) Study of ER activity on endogenous target genes.

Next, we measured the mRNA of the well known targets of E_2-ER the proliferation marker PTMA and PgR (Fig.3) unfortunately this receptor is not expressed in the liver and therefore the analysis was carried out only in three of the tissues selected.

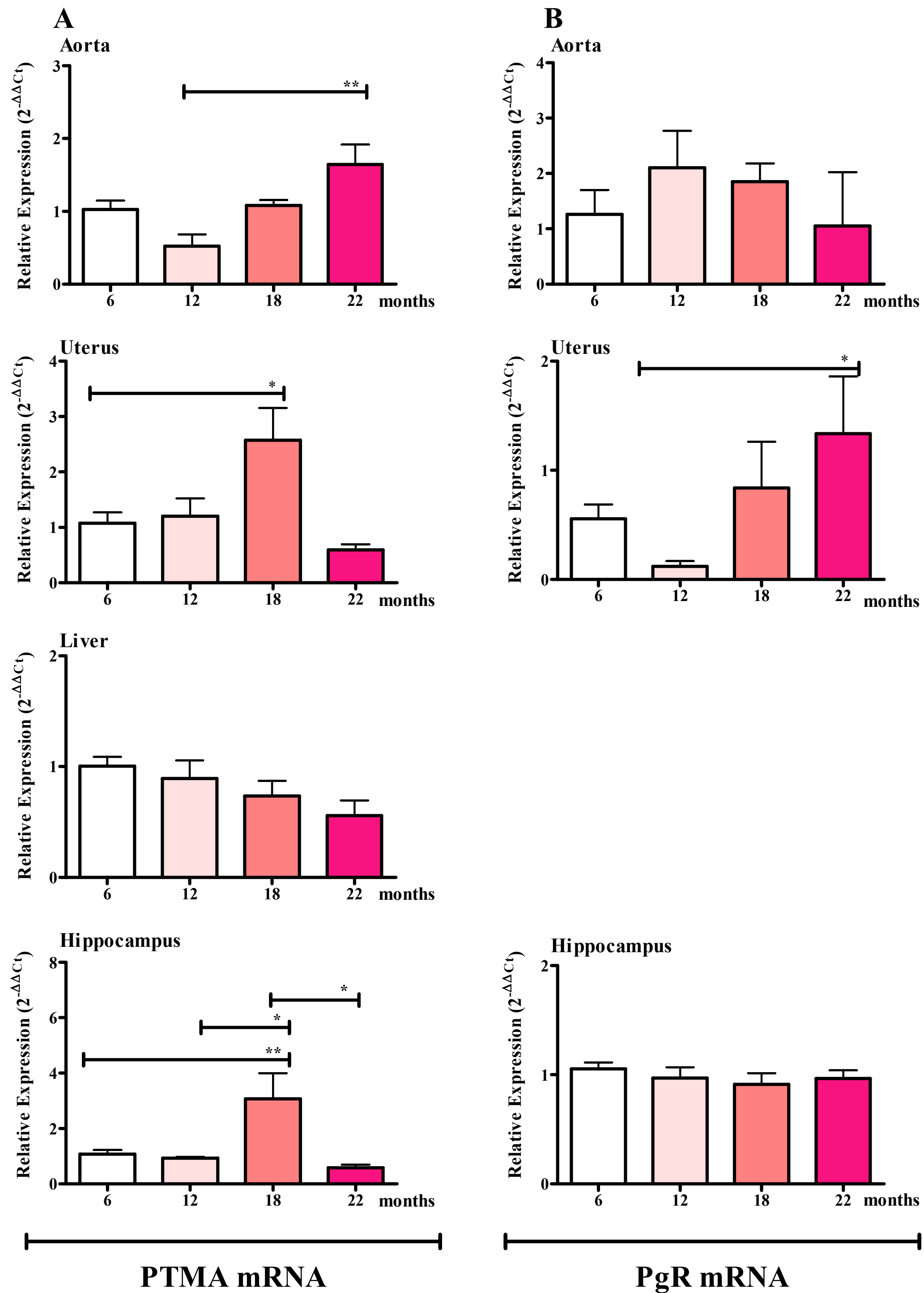


Figure3. Evaluation of ER activity by study the expression of endogenous target genes. (A) Real time PCR of PTMA on aorta, uterus, liver and hippocampus, (B) Real time PCR of PgR on aorta, uterus and hippocampus. Data are expressed as relative expression, *P<0.05, **P<0.01 versus mice at 18 and 22 months of age.

PTMA has been shown to serve biological function, this gene may have a dual role both intracellulary and extracellulary. Intracellulary PTMA acts both in cytoplasm and in nucleus, in this site PTMA affects the activity of several gene transcription; it

plays an important role in transcription regulation and promotes transcriptional activity of the estrogen receptor by sequestering a repressor of ER from the ER complex. It has been proposed that expression of PgR determination indicates a responsive estrogen receptor (ER) pathway.

The analysis of the profile of PTMA and PgR mRNA accumulation in aging tissues was unexpectedly quite diverse and with the exception of uterus where we observed an increased expression of both PTMA and PgR with aging. However this change had not been observed when luciferase activity was measured. In the aorta PTMA expression increased with age, but no increase in PgR and luciferase was observed. Similarly PgR and Luciferase was observed were not affected by aging, while PTMA not increased at 18 month of age. In liver PTMA showed a tendency to decrease similarly to what observed with luciferase.

The increased ER activity in the uterus of 18 month old female mice was further substained by the study of uterus weight which was clearly influenced by age and at 18 month which was increased by 95% with respect to 6 months old mice (Fig.4).

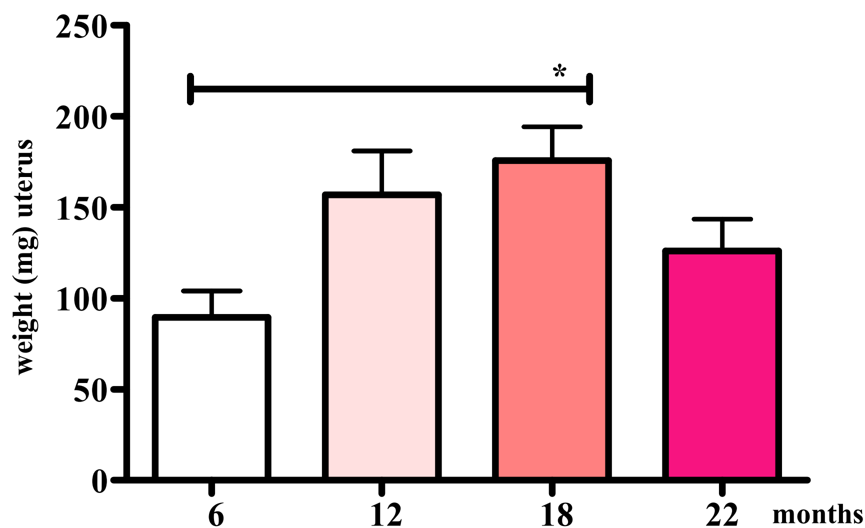


Figure 4. Uterus weight increases during aging. At due time uterus weight of sham mice was evaluated with an analytical balance. Data are expressed as mg of uterus, *P<0.01 versus animals at 6 months.

EFFECT OF LONG TERM OVARIECTOMY ON ER ACTIVITY

In several of the tissues analyzed, ovx had a significant effect on ERα expression, particularly in mice 12 and 18 month old which had been ovx at 5 month of age (Fig.5). A significant increase of ER expression was found in particular in aorta, uterus and hippocampus. In liver there was a trend to increase at 12 months which did not result significant.

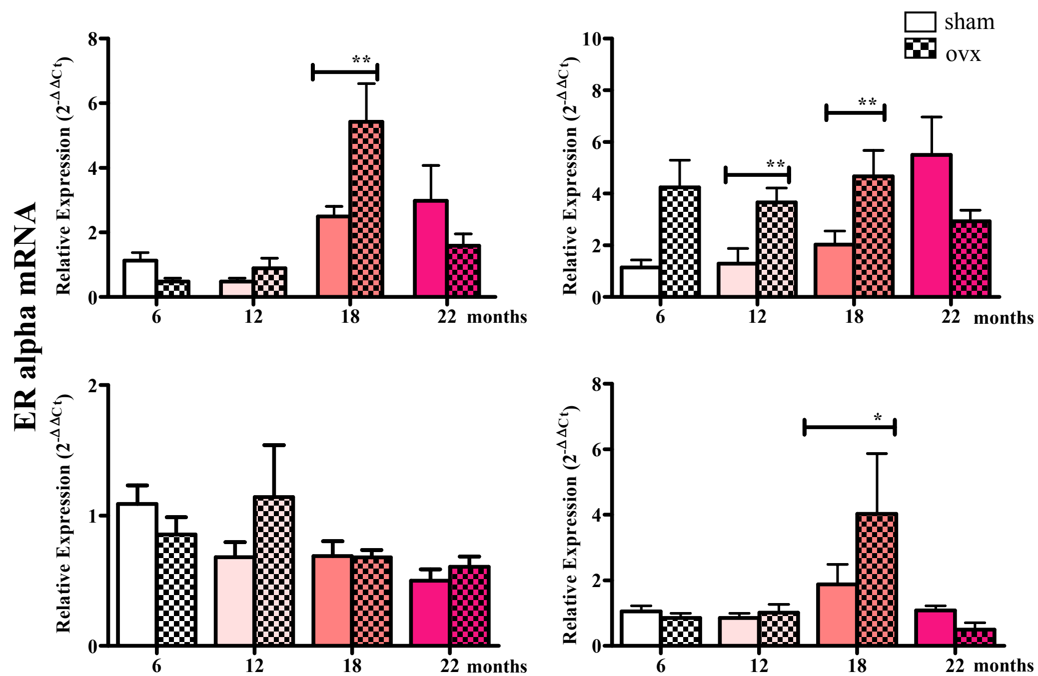


Figure 5. Ovariectomy influences expression of ERalpha in aging ERE-Luc female mice. The 5 month old mice were ovariectomyzed and then at 6, 12, 18 and 22 months were euthanized to collect the tissues. The mRNA levels of ERα change with ovx and the trend is different in reproductive and non reproductive tissues in mice at various ages. *P <0.05, **P<0.01, ***P<0.001 sham versus ovx as calculated by two-way ANOVA followed by Bonferroni post-hoc test.

a) Study of ER activity on the surrogate target ERE-Luc after ovariectomy.

The increased synthesis of ER alpha did not appear to influence significantly ER activity on the surrogate reporter luciferase considering both mRNA (Fig. 6A) and enzymatic activity (Fig. 6B) . Even if a trend to increase was observed in aorta and uterus at 18 month of age.

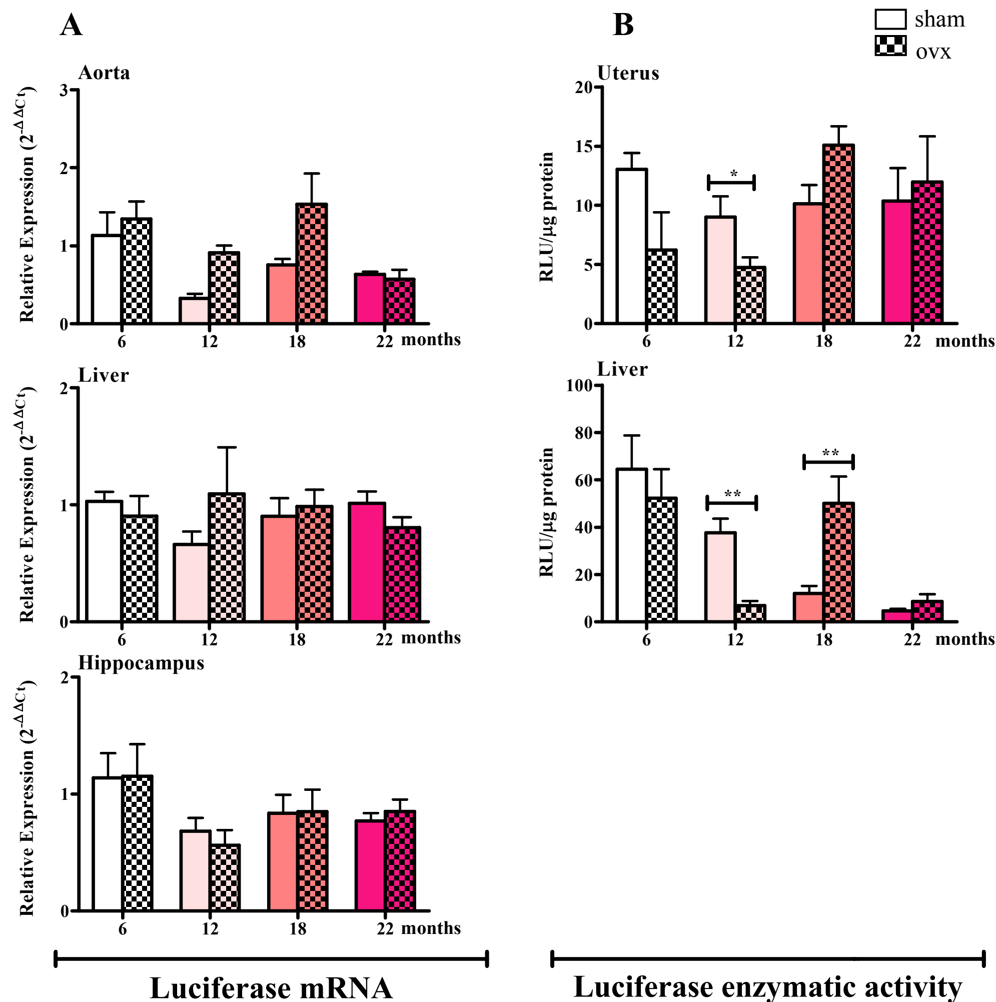


Figure 6. Evaluation of ER activity by study the expression and the activity of Luciferase. in aging ovx mice. (A) RT-PCR analysis of Luciferase content, the data are expressed as relative expression, the ovariectomy does not influence negatively Luciferase mRNA. (B) Luciferase enzymatic assay, the luminescence data, normalized over protein content of each sample are expressed as relative light units for µg of protein. The activity of protein increases with aging in uterus and in liver after ovaries failure.

b) Study of ER activity on endogenous target genes in aging ovx mice .

Conversely, when we studied the expression of endogenous target genes we observed the effect of the increased synthesis of ER alpha on the target tissues taken in consideration. PTMA mRNA content changed with ovariectomy in aorta, uterus and

hippocampus; in liver we observed a similar trend between sham and ovx of PTMA mRNA. PgR content increased significantly only in uterus. In aorta at 12 months and the expression of receptor in ovx group was higher than sham group; in hippocampus ovx and sham PgR levels did not change(Fig. 7).

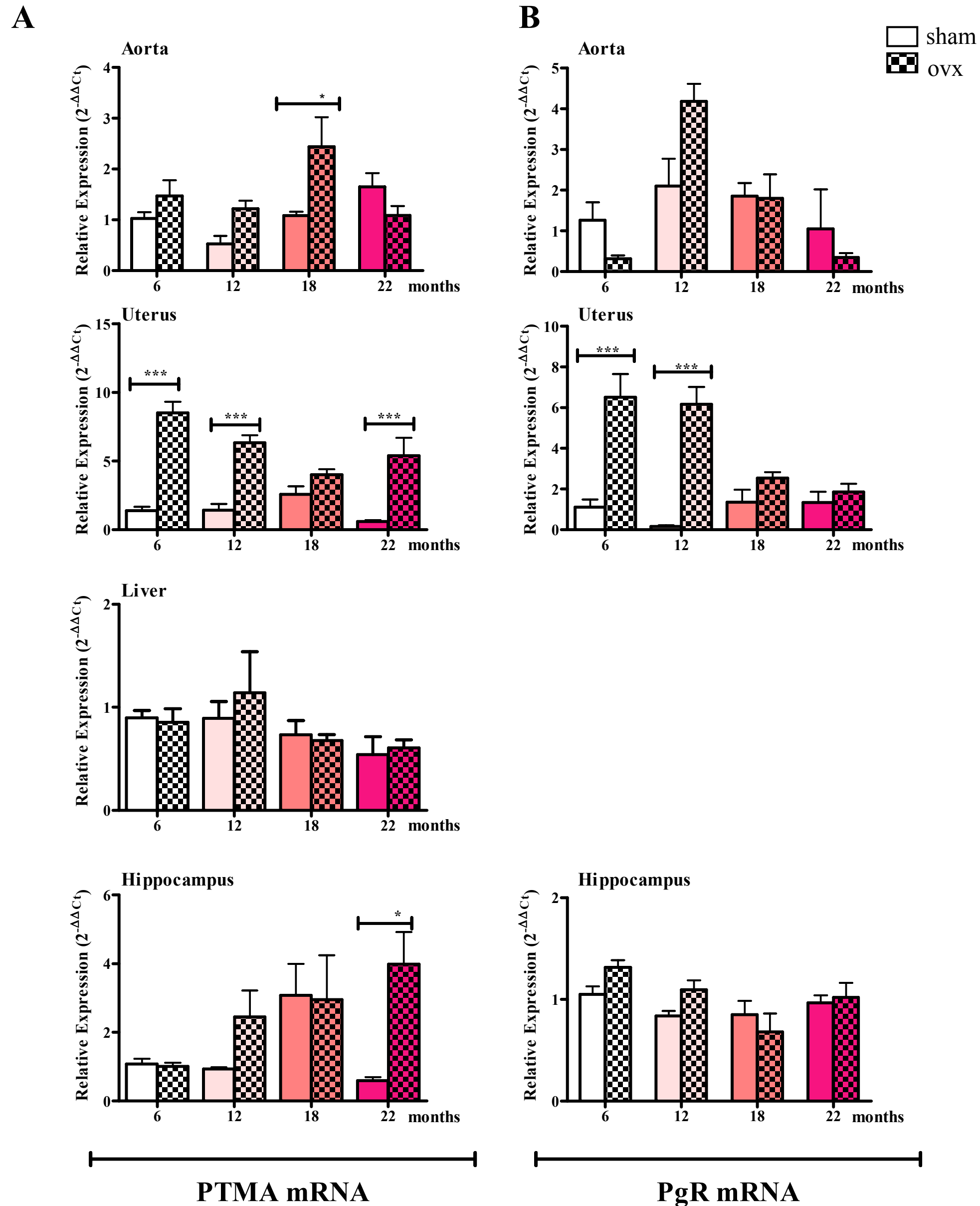


Figure 7. Evaluation of ER activity by study the expression of endogenous target genes in aging ovx mice. (A) Real time PCR of PTMA on aorta, uterus, liver and hippocampus in mice after ovariectomy (at 5 month), (B) Real time PCR of PgR on aorta, uterus and hippocampus during menopause. Data are expressed as relative expression, *$P<0.05$, ***$P<0.01$ sham versus ovx as calculated by two-way ANOVA followed by Bonferroni post-hoc test.

COMPARATIVE ANALYSIS OF ER ACTIVITY IN AGING FEMALE AND MALE MICE.

Several disorders are characterized by the gender difference . The different phases in the reproductive life or female hormonal instability seem to play a role in the gender difference. From these observations, we decided to compare the state of ER transcriptional activity after gonadectomy in aging female and male mice.

a) Experimental groups:

to study the effect of aging and the relevance of gonadal function in gender difference we carried out studies in intact (sham operated) and ovx/orx (at 5 month) mice. The pattern ER activity was evaluated at month 6 and 20.

b) Experimental protocol:

To evaluated ER transcriptional activity in aging and after gonadectomy we treated mice with luciferine and after 20 minutes the whole body photon emission was analysed and luciferase activity was quantificated as counts per unit of time and area [cts/(cm^{2}s)]. Then we compared data obtained in different animals or in the same animal at different time points with a template mask enabling to evaluate, reproducibly, photon emission from selected body areas: head, limbs, tail, chest, abdomen and thymus.

In young female mice after ovariectomy, ER activity decreased significantly in bone (head, limb and tali), in genital and in chest; in young male gonadectomised this activity decreased only in chest and abdomen (Fig,8; Fig.9). At 20 months of age we observed a sexual dimorphism:in the ER activity; interestingly in ovariectomized females the activity was increased significantly in breast, chest and abdomen while in gonadectomized males the activity of receptor was decreased in all areas. (Fig.10, Fig.11). In aged gonadectomized male mice may decreases the concentration of other factors that activate the receptor, while in aged female mice the ovariectomy may activate a pathway of ER activation regulated by factors other than 17β-estradiol.

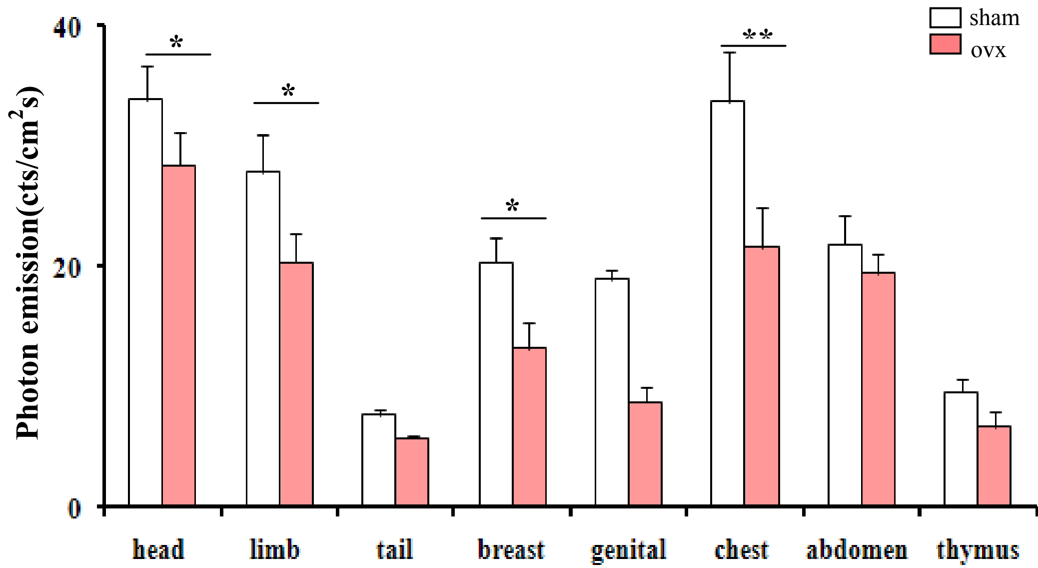


Figure 8. Ovariectomy decreases luciferase expression in selected organs of female mice at 6 months of age. ERE-Luc female mice were bilaterally ovariectomized (n=6) and luciferase pattern was obtained by bioluminescence in sham operated and in ovx. Data are expressed as photon emission *P<0.05, ***P<0.01 sham versus ovx as calculated by t Test.

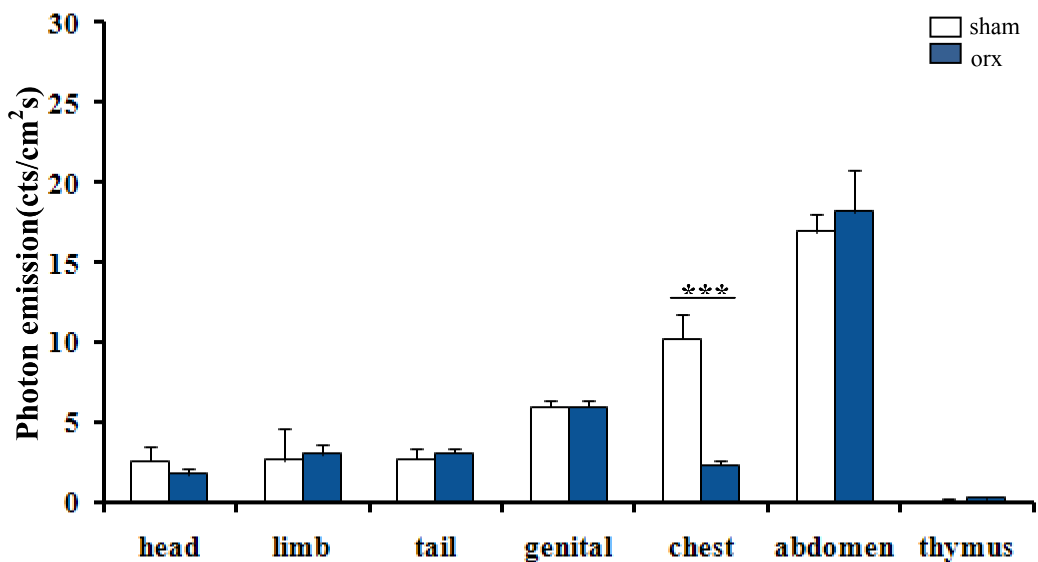


Figure 9. Orchidectomy reduces ER activity in male ERE-Luc mice at 6 months of age. Male mice were bilaterally orchiedectomized (n=6) and luciferase pattern was obtained by bioluminescence in sham and orx. Data are expressed as photon emission *P<0.001 sham versus orx as calculated by t Test.

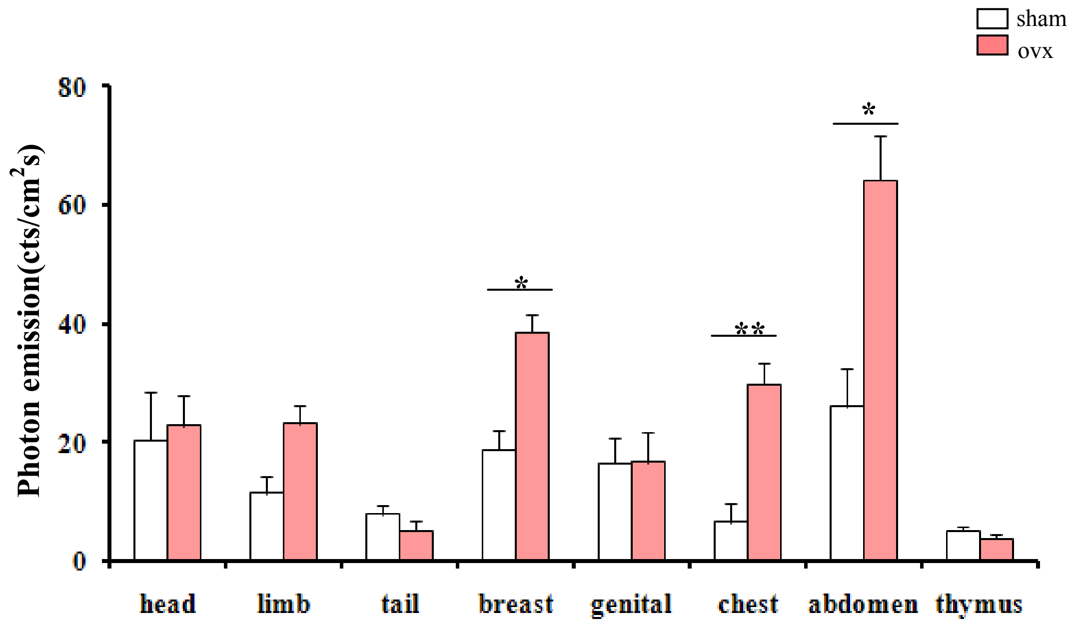


Figure 10. Ovariectomy increases ER activity in female mice at 20 months of age. ERE-Luc female mice were bilaterally ovariectomized (n=6) from 14 months and luciferase pattern was obtained by bioluminescence in both groups. Data are expressed as photon emission *P<0.05, ***P<0.01 sham versus ovx as calculated by t Test.

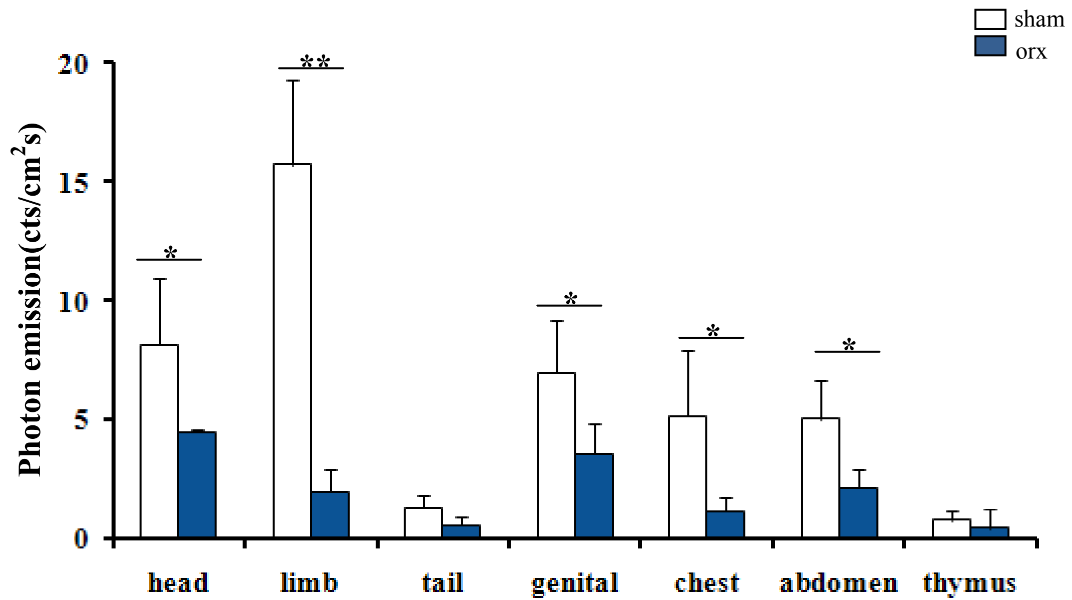


Figure 11. Orchidectomy affectes negatively ER activity in in aging male ERE-Luc mice. Male mice were bilaterally orchiedectomized (n=6) at 5 months of age and luciferase pattern was obtained by bioluminescence in sham and orx. Data are expressed as photon emission *p<0.05 sham versus orx as calculated by t Test.

INFLAMMATION AND AGING

Inflammation is a common component of all pathologies associated with menopause (osteoporosis, arteriosclerosis, diabetes, dementias). The inflammatory state triggers biochemical alterations and leads to tissue degeneration. Quenching the chronic production of inflammatory mediators and limiting the damage induced by free radicals and toxic agents is expected to slow down the progression of most aging diseases. Estrogens were shown to play a strong anti-inflammatory activity in *in vitro* and *in vivo* model systems: Several years ago we observed that ER is expressed in macrophages, in smooth muscle cells in culture estrogens may inhibit the synthesis of iNOS induced by selected inflammatory stimuli. This led us to further investigate the potential anti-inflammatory action of estrogens using a model of carageenan induced pleurisy, estrogen deprivation-dependent osteoporosis and microglia inflammation in response to lipopolysaccharide (LPS) treatment (Vegeto et al., 2003; Vegeto et al., 2006; Vegeto et al., 2002). In all of these studies estradiol appeared to exert an anti-inflammatory activity via ERα, but not ERβ, and the anti-inflammatory effect was observed only when E_2 was administered before the inflammatory stimuli. Next we investigated on the molecular mechanism of estrogen-ERα anti-inflammatory action and showed that E_2 prevents NF-kB transcriptional activity by inhibiting its transport to the nuclear compartment in inflammatory cells. This activity was mediated by ERα through a non-genomic, phosphatidylinositol-3-OH kinase-dependent pathway that does not modify Ik-Bα degradation, thus indicating a novel mechanism for estrogen action that is not shared by other anti-inflammatory drugs. The peculiarity of this mechanism may suggest that estrogens exert an unique function in inflammation.

Yet, it is still unknown to which extent a prolonged reduction of the levels of circulating estradiol influences the state of inflammation in the different organs.

Due to the considerable length of time required before the manifestation of the symptoms of an inflammatory disorders associated to menopause, it is still unclear when the inflammatory process starts to be established. We therefore proposed a study aimed at understanding the extent to which menopausal transition is associated with an increased basal inflammatory status of the different tissues.

a) Experimental groups:

to have a direct assessment of the effects induced by short and long term lack of sex hormones in basal inflammatory process, groups of animals were been ovariectomized at 5 months of age and inflammatory signs evaluated at 6, 12,18 and 22 months of age in specific tissues.

b) Experimental protocol:

to evaluate a basal inflammatory status associated with aging and menopause we first studied the expression of four inflammatory markers: TNFα and IL1β which are known to be involved in the acute inflammatory response and MCP1 and MIP2 which are involved respectively in the recruitment of macrophages and neutrophils in the chronic inflammatory response.

It s well known that the brain is one of the organs where inflammation, driven by brain microglia and by astrocytes, plays a relevant role after menopause, thus we evaluated the expression of microglia and by astrocytes in different brain areas. Using a cryostat, we collected brain sections from different levels of the brain which we analysed by immunohistochemistry using antibodies directed against activated microglia (i.e., Mac-1 for complement 3 receptor), and GFAP, a marker for astrocytes.

1. AGING EFFECT ON INFLAMMATORY MARKERS EXPRESSION IN SPECIFIC TISSUES

The expression of all markers of inflammation increased significantly at 22 months of age (Fig.12, 13, 14).

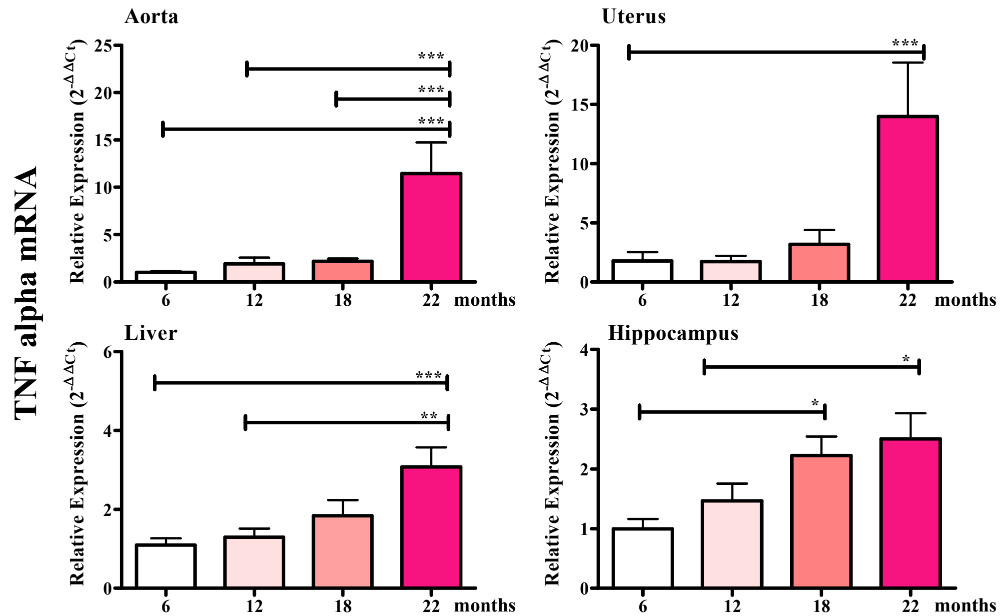


Figure 12. Aging increases TNFα synthesis.
RT-PCR detection of TNF alpha RNA levels in aorta, uterus, liver and hippocampus. Groups of six female animals were operated at 5 months of age (sham operated mice) and at due time (6, 12, 18 and 22 months) were euthanized for RT-PCR analysis on extract tissues. The data are expressed as relative expression and calculated by $\mathbf{2}^{-\Delta\Delta Ct}$ method (Livak et al., 2001). *P<0.05 versus group at 22 months of age as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

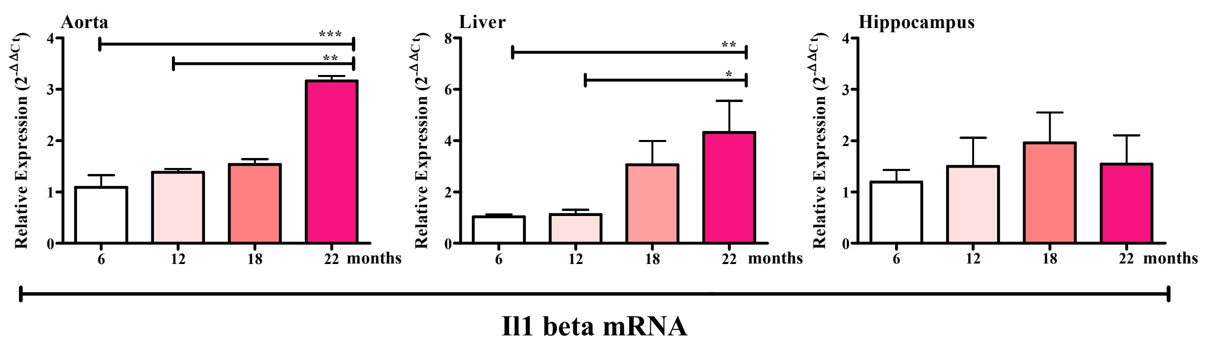


Figure 13. Aging affects Il1 beta synthesis.
The expression of Il1 beta was measured by semiquantitative real-time PCR assay on total mRNA extracted from aorta, liver and hippocampus. Bars represent the mean ± SEM of at least six mice. *P<0.05; **P<0.01,***P<0.001 versus 22m sham. P values were calculated with ANOVA followed by Bonferroni's test.

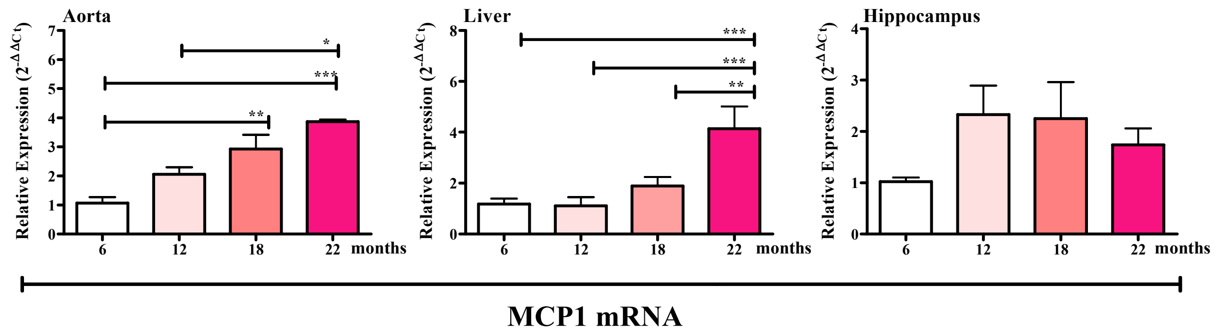


Figure 14. Aging increases MCP1 mRNA content.
The expression of chemokine was quantified by real-time RT-PCR and was significantly increased by age in aorta and liver. Graphs represent mean ± SEM. *P < 0.05; **, **P < 0.01; ***, P < 0.001 young versus aged mice.

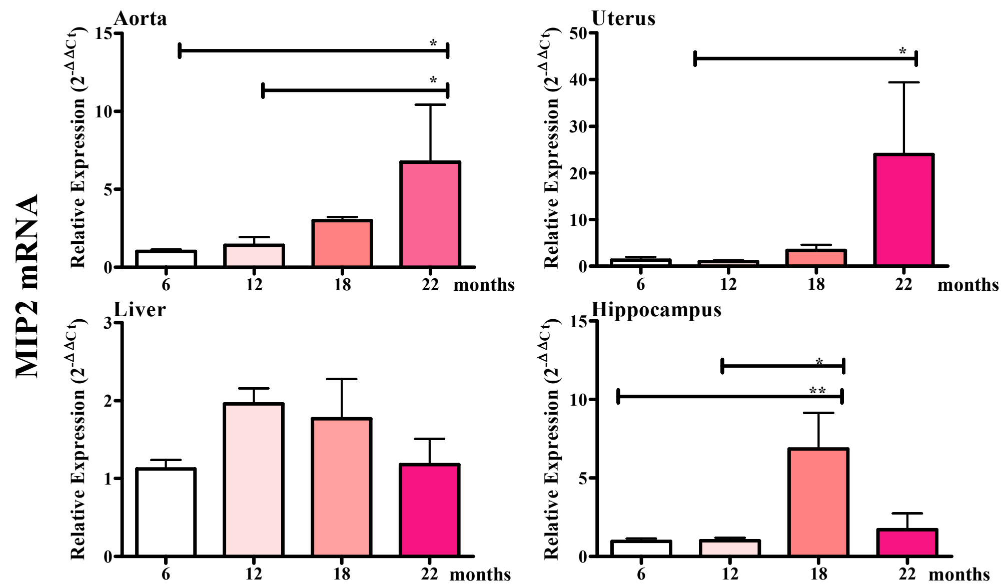


Figure 15. Aging increases MIP2 synthesis.
mRNA accumulation of the *mip2* gene was measured by real-time PCR in aorta, uterus, liver and hippocampus of aged mice. Bars represent the mean ± SEM of 6 mice. *P<0.05 versus group at 22 months of age as calculated by one-way ANOVA followed by Bonferroni post-hoc test.

2. EFFECT OF ESTROGEN DEPRIVATION ON INFLAMMATION

In ovariectomized female mice there was not a clear factor of modulation, at 22 months the trend was that ovariectomy reduced inflammation while in uterus but mainly in hippocampus the expression of citokines and chemokines increased (Fig.16).

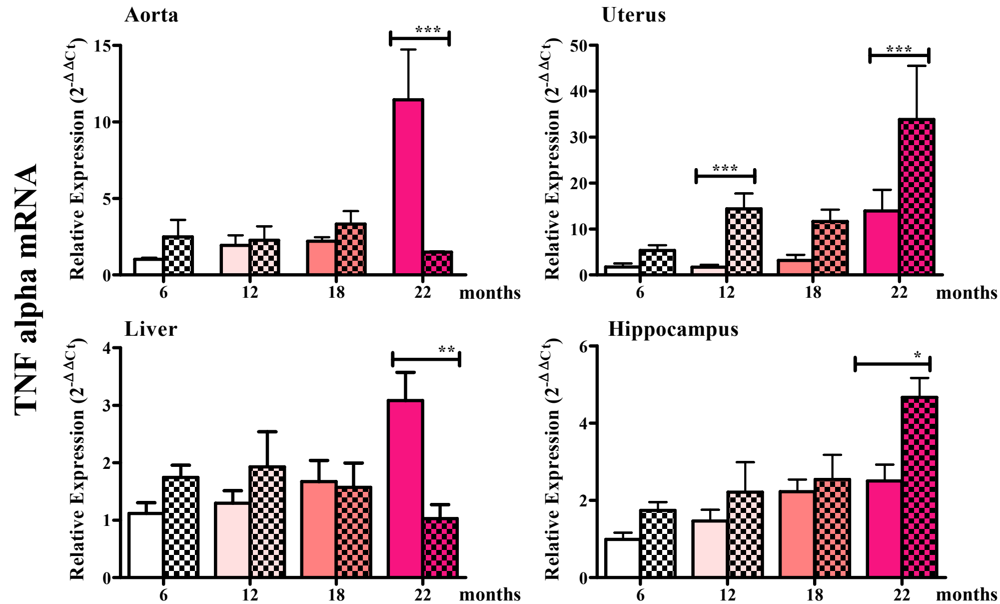


Figure 16. Ovariectomy affects TNFα synthesis.
RT-PCR detection of TNF alpha RNA levels in aorta, uterus, liver and hippocampus. Groups of six female animals were ovariectomized at 5 months of age and at due time (6, 12, 18 and 22 months) were euthanized for RT-PCR analysis on extract tissues. In aorta and liver ovx reduced the gene expression at month 22 while in uterus and hippocampus the trend is opposite. The dotted bars represented a ovx grup and*P < 0.05; **, **P < 0.01; ***, P < 0.001 sham versus ovx. P values were calculated with two way ANOVA followed by Bonferroni post-hoc test.

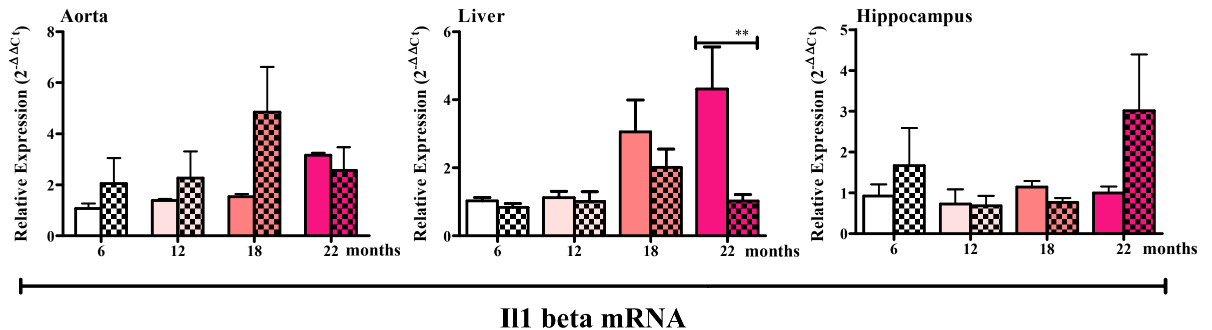


Figure 17. Estrogen deprivation acts on IL1 beta mRNA content.
The expression of Il1 beta was measured by semiquantitative real-time PCR assay on total mRNA extracted from aorta, liver and hyppocampus in ovx mice. Bars represent the mean ±

SEM of at least six mice. **P<0.01 sham versus ovx. P values were calculated with two wayANOVA followed by Bonferroni's test.

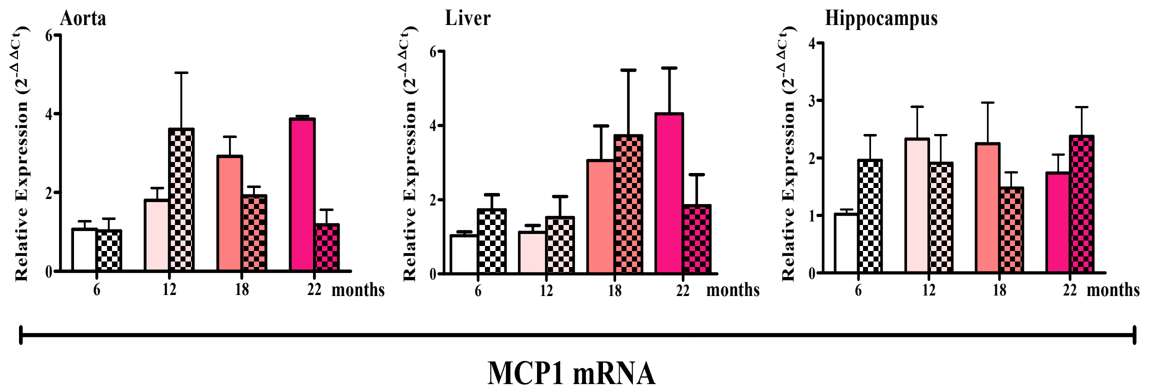


Figure 18. Ovariectomy does not affected the synthesis of MCP1 mRNA content.
mRNA accumulation of the *mcp1* gene was measured by real-time PCR in aorta, liver and hippocampus of sham and ovx mice.

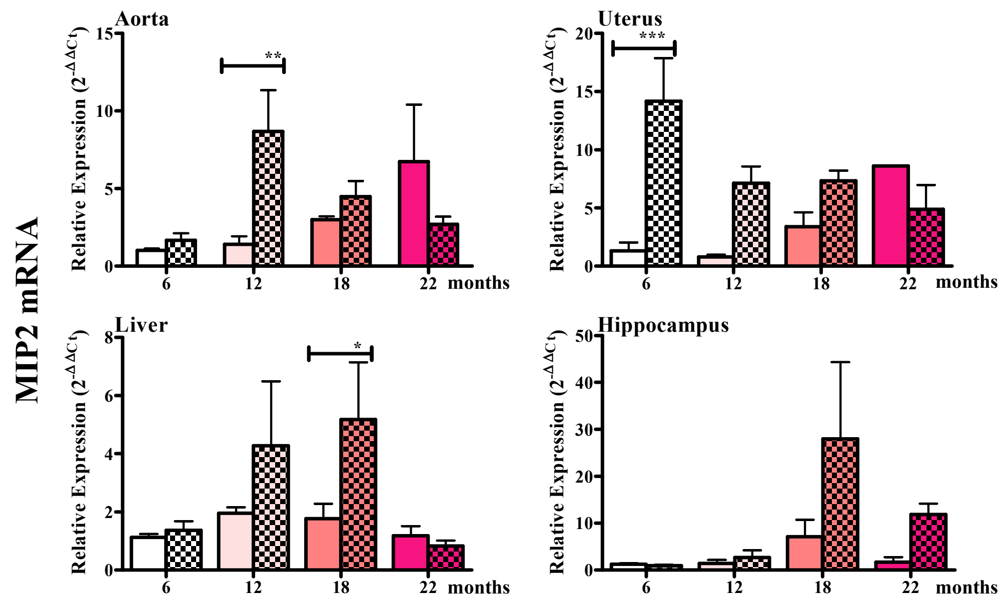


Figure 18. Estrogen deprivation has a different effect on MIP2 expression.
mRNA content was evaluated by real-time RT-PCR and was significantly increased by ovariectomy at 6, 12, 18 months respectively in aorta, uterus and liver. *P < 0.05; **, **P < 0.01; ***, P < 0.001sham versus ovx. P values were calculated with two way ANOVA followed by Bonferroni post-hoc test.

3. EFFECT OF AGING AND ESTROGEN DEPRIVATION ON MICROGLIAL CELLS AND ASTROCYTES.

We performed an immunoistochemical study of different brain areas: frontal cortex, straitum, hypothalamus and hyppocampus, in sham and ovariectomized ERE-Luc mice from 6 to 22 months of age. In hippocampus there were morphological

differences among astocytes between ovariectomized and sham operated mice, indeed in ovx animals astrocytes seem with thick filaments, typical filaments of reactive astrocytes associated a state of inflammation. Also microglial cells were more reactive in ovariectomy group, but either reactive cells growth in number in group of sham while in ovx animals the number was similar in all time points (Fig.20).

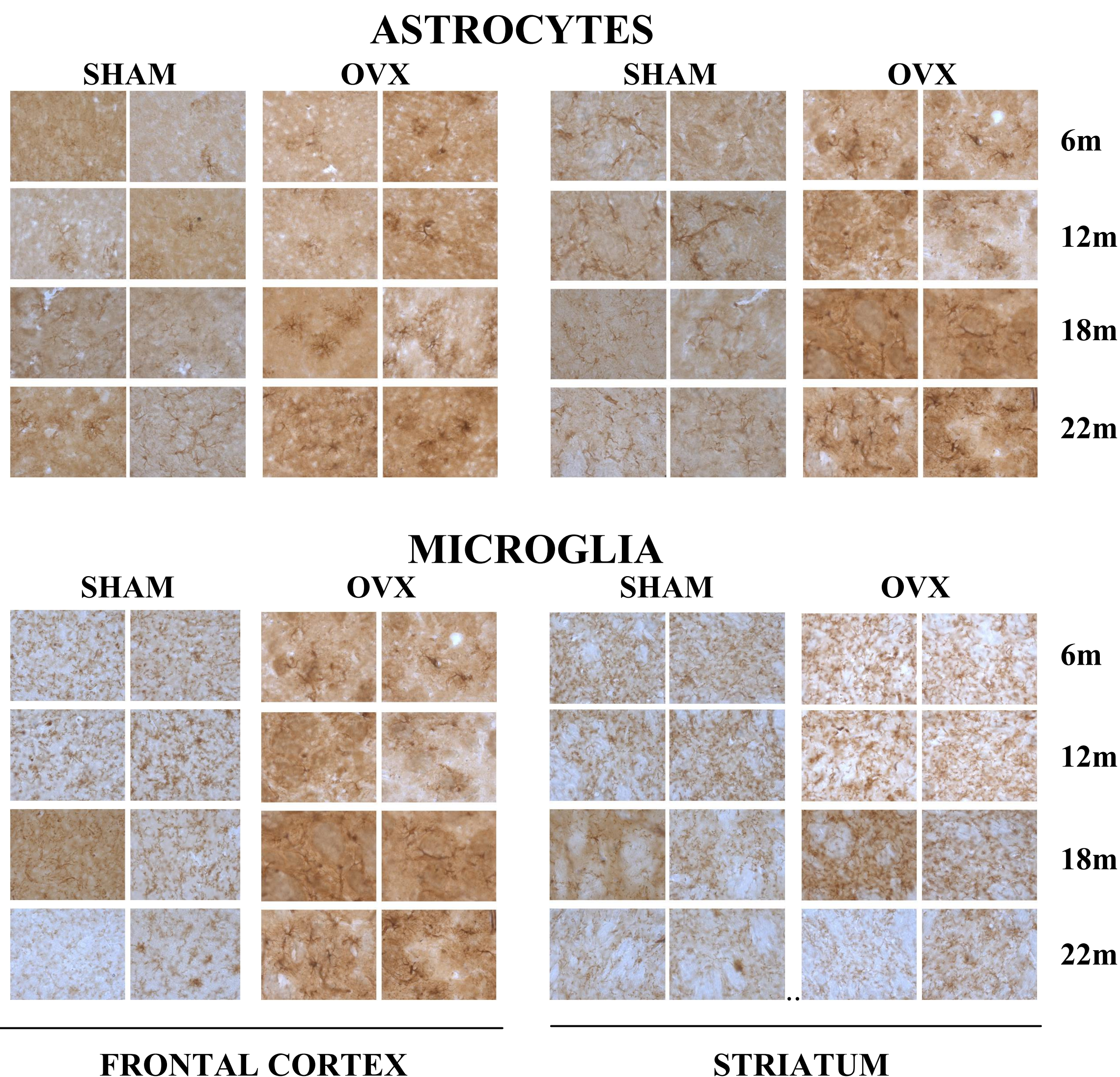
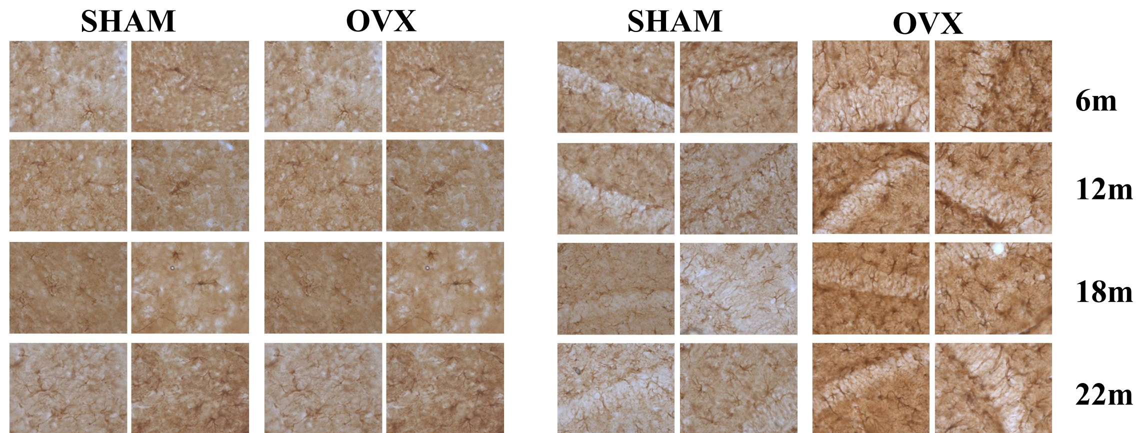
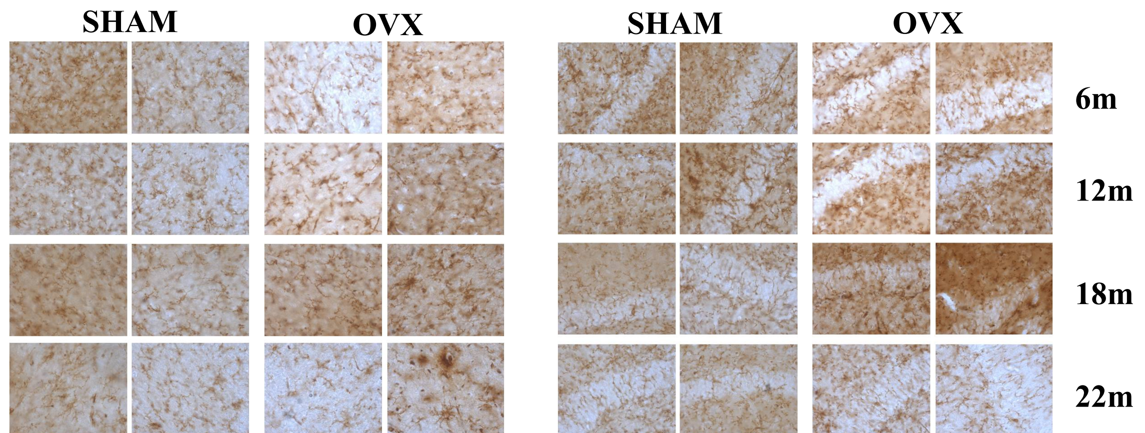


Figure 19. Aging and estrogen deprivation affects number and morphological aspect of astrocytes and microglial cells in frontal cortex and striatum. Immunohistochemical assay was performed in brain areas of aging female ERE-Luc mice sham and ovx with GFAP and MAC-1 markers. Astrocytes were morphological different between two group at all time points (top panel). The number of microglia cells was higher in ovx than sham at 6,12,18 and 22 months (bottom panel).

ASTROCYTES



MICROGLIA



HYPOTALAMUS **HIPPOCAMPUS**

Figure 20. Aging and ovariectomy affects number and morphological aspect of astrocytes and microglial cells in hypothalamus and hippocampus. Immunohistochemical assay was performed in brain areas of aging female ERE-Luc mice sham and ovx with GFAP and MAC-1 markers. Number of astrocytes were increased in sham from 6 to 22 months of age; in ovx these cells were morphological different with aging (top panel). Microglia cells were reactive both in sham and ovx group (bottom panel).

ROLE OF BRAIN INFLAMMATORY STIMULI ON INFLAMMATORY GENE EXPRESSION DURING AGING AND IN MENOPAUSE

Several studies revealed a higher number of reactive microglia cells in the brain of female as compared with male mice and that ovariectomy increases microglia activation. In addition, we observed that the brain of ERα-knock out mice show an increased expression of complement-3 receptor by microglia cells as compared to age-matched wild-type littermates, suggesting that the lack of the endogenous mediator of estrogen anti-inflammatory activity results in a partial reactivity of brain macrophages. Because of our expertise, we selected the brain as a paradigmatic organ in which to study the decreased defence to inflammatory stimuli due decreased circulating estradiol.

The acute response of the brain following inflammatory stimuli is supposed to eliminate the toxic insult together with restraining cell damage and restoring tissue integrity. However, it is not known whether this response is similarly maintained or is somehow impaired in the aging brain. Thus we decided to evaluate the acute response of the brain to inflammatory agents in mice of different ages and hormonal statuses**.**

a) Experimental groups:

To evaluate the inflammatory response following an inflammatory stimulus in the brain in mice during aging, we induced brain inflammation by intracerebroventricular (icv) injection of lypopolisaccharide (LPS: a potent inflammatory agent) or saline in female intact and ovariectomized (at month 2 or 5) ERE-Luc mice at different ages.

b) Experimental protocol:

After three hours to icv, hippocampus were collected for real time PCR experiments on inflammatory cytokines and chemokines: TNFα, Il1b, MCP1 and MIP2

Our results in the hippocampus showed that the levels of the four cytokines are:

TNFα production did not increase with ovariectomy at 18 months of age,

IL1β production didnot increase with estrogen deprivation,

MCP1 production increased with ovariectomy at 12 and 18 months of age,

MIP2 production increased only at 12 months of age with estrogen deprivation.

Furthermore, all inflammatory analyzed markers with a short term ovariectomy (performed at 11 months of age), decreased with aging (Fig. 21).

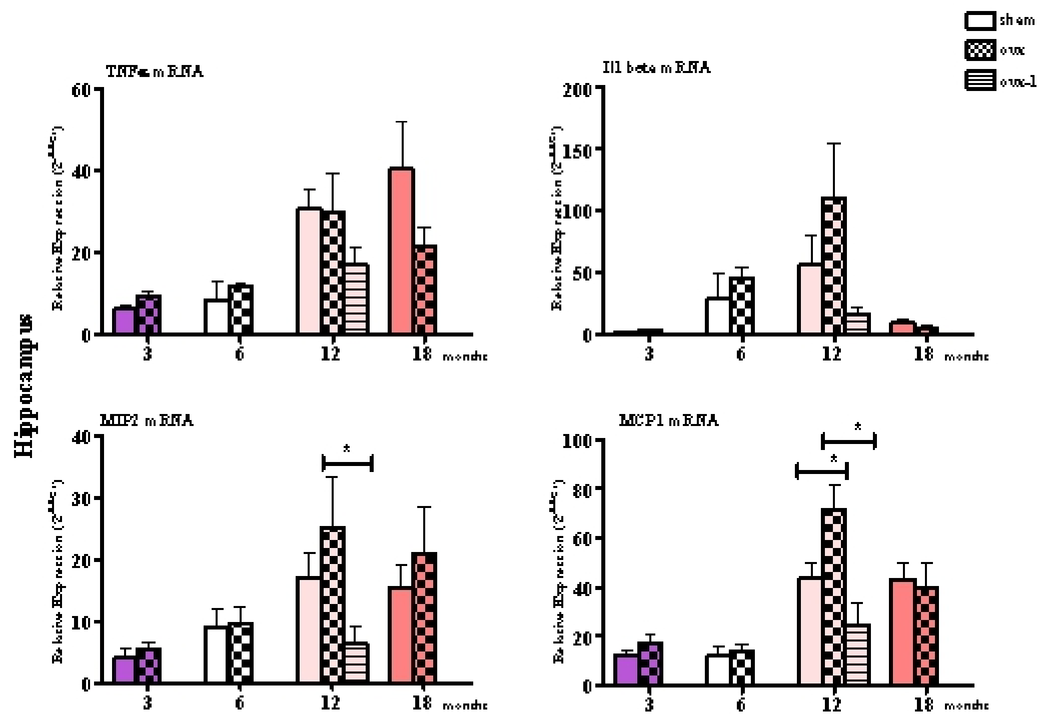


Fig. 21 Effect of long and short term ovariectomy on Expression of TNFα, Il1beta, MCP1 and MIP2 in aging ERE-Luc female mice in hippocampus after inflammatory stimuli. The mice are treated with LPS after three hours to injection, hippocampus was removed and expression of chemokines and cytokines was valuated by RT-PCR.
*$P < 0.05$ sham versus ovx and ovx-1; *$P < 0.05$ ovx versus ovx-1; P values were calculated with two way ANOVA followed by Bonferroni post-hoc test.

Discussion

In our study we evaluated ER activity during aging in ERE-Luc mice. The study shows that in aged mice ERα is still synthesized at the rate observed in young and adult mice (hippocampus) or at higher rate (uterus and aorta). In the liver we measured a decrease of ERα content with time: at 22 months of age the amount of hepatic estrogen receptor is about 50% lower than at month 6. With age, ovariectomy further increases ERα content in uterus, aorta and hippocampus, but not in the brain.

The study of luciferase, as well as the two ER gene targets: prothymosine (PTMA) and Progesterone Receptor (PgR), showed that the ER present in aged tissues is fully functional from the transcriptional point of view, indeed in hippocampus, aorta and uterus of mice at 18 and 22 month of age PTMA mRNA content is higher than in young mice, PgR mRNA increases with age (22m) only in the uterus. The fact that, in some tissues, luciferase accumulates at the different rate than endogenous genes target for the ER has to ascribe to the different complexity of the promoters regulating the Pol II activity: in the ERE-Luc mice the construct carrying the luciferase reporter was conceived to be a very precise indicator of the state of ER transcriptional activity, thus to highlight ER action in the absence of other factors (thus the construct is composed of a very simple promoter with a multimerized ERE and a minimal TK driving the luciferase). On the other hand, each natural, endogenous gene is under the control of very complex promoters, where the final transcriptional activity is determined by a number of regulators which activity may change significantly spatiotemporally.

The increased content of PTMA mRNA in the uterus of mice at 18 months of age is in line with the uterus weight previously reporter and attributed to a hyperactivation of the production of estrogen by the ovaries (Sherman et al., 2007). However PTMA mRNA content is also increased significantly by ovariectomy, which leads to a decrease of uterus weight. We hypothesize that the increase PTMA mRNA after ovx may be due to inflammatory processes occurring in this organ and leading to proliferation of inflammatory cells.

We were intrigued by the observation that, in aged female mice, a reduction of circulating levels of estrogens induced by ovariectomy was associated with an increased ER activity in several organs. To further study this phenomenon, we gonadectomised male and female mice at the age of 5 months and we measured luciferase activity by *in vivo* imaging at 6 or 20 months of age. As expected

luciferase activity was higher in females than in males in both groups of age, however a few observations were unexpected:

1. aging was associated with a decrease of photon emission in females, but not in males;
2. in young female animals ovariectomy reduced significantly luciferase content in several organs (bone, genital area and liver); however this was not the case in aged mice, where ovariectomy was mainly associated with an increase of luciferase activity;
3. gonadectomy did not affect luciferase activity in young males (with the exception of the chest), but clearly decreased photon emission in aged mice.

Clearly, in young females, the ovaries are the main source of endogenous ligands for the estrogen receptor and it was expected that gonadectomy would have induced a general decrease of luciferase synthesis; in males, the enzyme converting testosterone into estrogens is present in several organs and therefore was not surprising to observe that the surgical removal of the gonads did not affect luciferase activity. More puzzling were the results in aged animals, where ER activity was slightly decreased in the females. However ovariectomy was not associated with decreased luciferase activity, indicating that, with age, sources other than the gonads are generating signals able to activate the estrogen receptor transcriptionally. The opposite was true for the aged males where gonadectomy decreased ER activity in most of the organs studied, suggesting that in males the aging process leads the gonads to become the only source of estrogens.

Taken togheter, these data indicate a different regulation of estrogen receptor in both sexes and particularly the existence of different factors that are active on ER in aged females.

On these bases our data support the possibility of an activation of the estrogen receptor independent from plasmatic estrogens, that during aging are partially replaced by other factors active on ERs (i.e., IGF1 and other growth factors).

In our study we also tested the hypothesis that with aging the loss of the anti-inflammatory activity of estrogens may explain the increased susceptibility to inflammatory disorders (i.e., osteoporosis, atherosclerosis, diabetes, certain neurodegenerative disorders), reported by epidemiological studies in women.

Our results, on the expression of inflammatory genes in selected tissues, agree previous preclinical and epidemiological studies that indicate that the aging process is connected with a significant increase in pro-inflammatory factors. Our study focused primarily on TNFα, IL1 beta, MCP1 and MIP2. The mRNA of all these inflammatory mediators was shown to increase progressively with aging.

To evaluate the influence of estrogens on the expression of inflammatory genes, we measured the content of mRNA encoding for inflammatory mediators in different tissues of ovariectomized females. Most interestingly, we observed that in ovariectomized mice the activity of the inflammatory genes, increased with aging in uterus and hippocampus, while decreased in aorta and liver. Due to the relevance of inflammatory processes in the CNS, we next focused on the effect of ovariectomy in the hippocampus by IHC studies the state of reactivity of microglia and astrocytes, cells known to play a relevant role in neuroinflammation. Our data show morphological differences between astrocytes in the hippocampus and striatum in ovariectomized compared to sham operated mice already at the age of 6 months: astrocytes in ovx group are reactive with tick processes. At 12 months of age astrocytes are activated and proliferating in the hypothalamus of ovariectomized animals; at 18 and 22 months the differences in morphology and number of astrocytes affect all the brain areas. Also microglia presents a morphological activation in all the brain areas, as observed in astrocytes.

Finally we investigated the extent to which the susceptibility to an inflammatory stimulus changed during aging and if the ovariectomy was playing a role in this phenomenon. In the hippocampus TNFα production increases with aging, MIP2 and MCP1 espression changes at 12 months and is similar at 18 months, whereas mRNA levels of IL1 beta are not affected by aging.

Ovariectomy does not seem to influence the inflammatory process indeed:

1. TNFalpha production does not increase with OVX at 18 months of age;
2. IL1 beta production does not increase with OVX;
3. MCP1 production increases with OVX at 12 and 18 months of age;
4. MIP2 production increases with OVX only at 12 months of age.

The present study, for the first time, provides a systematic analysis on the activity of estrogen receptor in females during aging in reproductive and non-reproductive organs. The main merit of the study is to have demonstrated that ER is still expressed

in aged tissues and its activity may be stimulated to an extent similar to what observed in young tissues: the finding that ER is still synthesized and active in aged mammals could provide an explanation for the negative effects observed in the women of the NIH Women Health Study (Women's Health Initiative Memory Study). Infact, several cardiovascular effects were observed in the very first year of the study, particularly in women who had undergone menopause several years before the beginning of the investigation. Indeed, it could be hypothesized that the administration of a full dose of therapeutic hormone might have triggered an abnormal response, leading to the pathological outcome reported.

The data so far obtained on the effect of ovariectomy on inflammation need further analysis and study: apparently ovariectomy in aged mice does not clearly increase the susceptibility to inflammatory stimuli. However, the study also shows that in aged mice signals other than estrogens have the ability to modulate ER activity: it would be most relevant to identify the molecular nature of these stimuli in order to achieve a better understanding on their exact physiological significance and relevance to ER activity in both reproductive and not reproductive tissues.

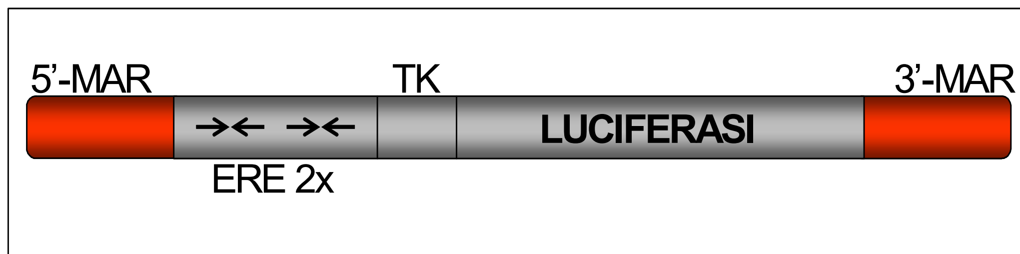
Materials and methods

Chemical used

Ketamine (Imalgene 500) from Merial (Tolouse France), and xilazine (Rompun) from Bayer (Shawnee Mission, Kansas, USA), D-luciferin (Beetle luciferin potassium salt) from Promega (Milan, Italy), Escherichia coli LPS (serotype 0.111:B4 from Sigma (Milan, Italy).

ERE-Luc reporter mouse system

The ERE-Luc mice were generated to obtain the ubitous expression of an estrogen-regulated reporter gene. In the following image (Fig.1) we describe the structure of the trangene used for the generation of the reporter mouse:



a) the trangene is flanked by two insulators sequences (MAR sequence) that create open chromatin domains permissive to gene expression. They constitute a barrier against acetylation and methylation events, thus preventing the position effects and ensuring an ubiquitous expression of the trangene;
b) the estrogen-inducible promoter is generated using deleted mutants of the minimal promoter from the thymidine kinase (*tk*) gene from *Herpes simplex* virus linked to a combination of two palinfromic receptor-responsive elements (EREs). The two EREs are spaced 8bp and located 55bp from the *tk* promoter; this structure provides the desired low basal trascription and an high estrogen-induced reporter expression;
c) the reporter gene sequence

The activation of estrogen-receptors through specific binding ligands leads to the translocation of the receptor complex into the nucleus where it interacts with several co-factors and binds the estrogen responsive elements of the promoter. In this case luciferase is produced and can be detected by different assays such as

immunohistochemistry, that allows tissue and cellular localization of the ERE activation, quantitative enzymatic assays on tissue extract or optical imaging technology. This last methodology is the most used in our lab because, by the use of a charge-coupled device (CCD), it allows an easy and real-time visualization of the bioluminescence produced by luciferase expression in living animals.

After an injection of luciferine the whole body photon emission can be analysed and luciferase activity can be quantificated as counts per unit of time and area [cts/(cm^2s)].To be able to compare data obtained in different animals or in the same animal at different time points we generated a template mask (Fig.2) enabling to evaluate, reproducibly, photon emission from selected body areas.

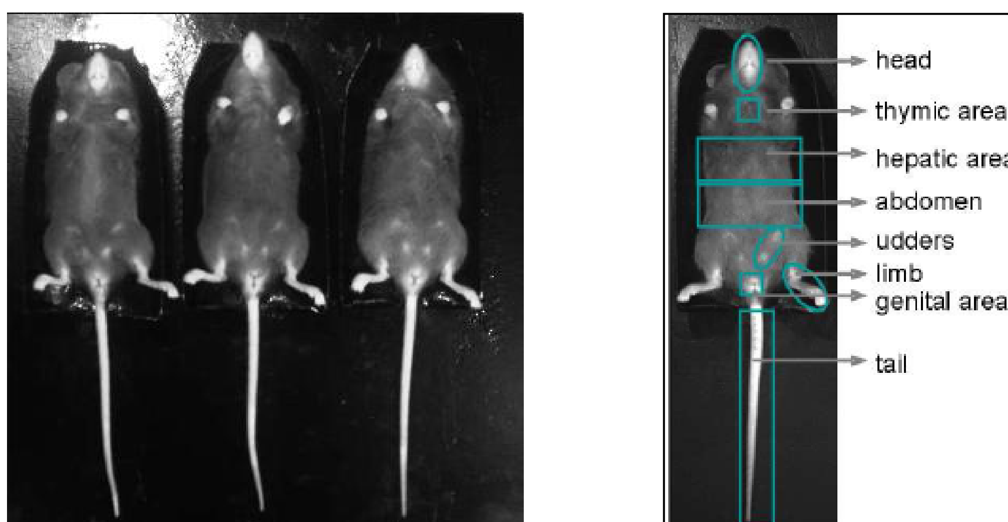


Fig. 2 Template enabling the evaluation of bioluminescence in the following areas: head, thymus, liver, intestine, gonads, limbs and tail.

Ovariectomy

Weigh and anesthetize animal with doses of anesthetics recommended in standard procedures. Confirm that the animal shows a reduced respiratory rate and no response to gentle pinching of foot pad. Shave both flanks of the animal. In the mouse, shaving is optional. Swab the shaved area with 70% ethanol. All instruments should be sterilized by dipping in 90% ethanol and then flaming in a Bunsen Burner or by other accepted methods of sterilization.

A 5mm, dorsal/ventral incision is made through the skin of the flank of the mouse below the muscles surrounding the spinal cord. The incision is centered between the bottom of the rib cage and the front of the hind limb. In the rat, a 10mm incision is

placed in a similar fashion. The skin is separated from the underlying muscle. Before making the incision through the muscle overlying the ovary, confirm the location of the ovarian fat pad which is sometimes visible under the muscle. Rather than cutting the muscle, insert the tip of double sharp iridectomy scissors just through the muscle layer, and separate the muscle fibers by opening the scissors in a dorsal ventral direction. Hold the edge of the incision open with a small rat tooth forceps and pull the ovary through the incision with a blunt forceps by grasping the fat pad surrounding it. Place a mosquito hemostat at the boundary between the oviduct and uterus, and place a ligature just below the hemostat. After removing the ovary and oviduct with a scissors, release the hemostat and make sure no bleeding occurs. Return the ovary to the abdominal cavity, and suture the muscle layer if necessary. Close the skin incision with wound clips. Turn the animal over and repeat the procedure on the other side. Return the animal to its cage and leave undisturbed in a warm, quiet place. Monitor the animal continually until it is completely recovered from anesthesia. If clear signs of pain, acute discomfort, or adverse reaction to the drug are apparent (e.g., convulsions, respiratory distress), the animal should be euthanized. Following recovery, the animal should be monitored daily for one week for signs of infection or persistent problems, in which case the animal should be euthanized.

Orchidectomy

Weigh and anesthetize animal with doses of anesthetics recommended in standard procedures. Confirm that the animal shows a reduced respiratory rate and no response to gentle pinching of food pad. Shave the abdominal region of the animal. Swab the shaved area with 70% ethanol. All instruments should be sterilized by dipping in 90% ethanol and then flaming in a Bunsen burner or by other accepted methods of sterilization. Make a 1.5 cm, transverse incision in the skin at a point level with the top of the legs. Separate the skin from the muscle layer. See diagram provided for the standard procedure for vasectomy. Make a similar incision through the abdominal muscle. Both testes can be reached through the same incision. Localize the testicular fat pad on the left side and pull it through the incision using a blunt forceps. Place a hemostat below the testes and epididymis across the testicular cord (contains blood vessels and vas deferens). Place a ligature below the hemostat

and remove the testes and epididymis with a scissors. Repeat for right testes. Suture the abdominal wall with 2-3 stitches and repeat for skin incision. (Wound clips can be used for skin incision, but sutures are recommended because the body clip can affect sexual proclivity).Return the animal to its cage and leave undisturbed in a warm, quiet place. Monitor the animal continually until it is completely recovered from anesthesia. If clear signs of pain, acute discomfort, or adverse reaction to the drug are apparent (e.g., convulsions), the animal should be euthanized. Following recovery, the animal should be monitored daily for one week for signs of infection or persistent problems, in which case the animal should be euthanized.

Experimental animals

2 and 5 month old heterozygous ERE-Luc reporter mice were housed in plastic cages with hardwood chips bedding at Harlan (Bresso, Milan) animal facilities. Mice were fed *ad libitum* with a certified estrogen-free AIN93-M diet (Mucedola, Settimo Milanese, Milan, Italy), and had free access to filtered water. The animal room was maintained within a temperature range of 22–25°C and relative humidity of 50%±10%. There was a cycle of 12 hours light/dark (lights on, 07:00 AM). Mice were euthanized at the time described in the figure legends and the indicated tissues rapidly dissected and stored at -80°C until assayed. All animal experimentation was carried out in accordance with European guidelines for animal care and use of experimental animals, approved by the Italian Ministry of Research and University, and controlled by the panel of experts of the Department of Pharmacological Sciences, University of Milan.

Anaesthesia

Mice were anaesthetized with s.c. injection of 50 μL of a ketamine-xylazine water solution (78% ketamine (Ketavet 50 mg/mL, Intervet, Peschiera Borromeo, Italy) and 15% xylazine (Rompun 20 mg/mL, Bayer, Leverkusen, Germany) This amount corresponded to a dosage of 78 mg/kg (ketamine) and 6 mg/kg (xilazine).

Vaginal smears

The phase of the estrous cycle was established by vaginal smears using about 20 μL of fluid collected by water vaginal flush smeared onto a glass microscope slide. The

smear was air-dried and stained with the May Grunwald e Giemsa method (*MGG Quick Stain* Kit, Bio-optica, Milan, Italy) following the manufacturer's protocol. Cytological assessment was done with an AxioCam HRc and an Axioskop 2 mot plus (Zeiss, Germany) at the magnification of 100x. The smears were done at 9:00 a.m.

Bioluminescence Imaging (BLI)

Briefly, a CCD consists of a sensor for recording images, consisting of an integrated circuit containing an array of linked, or coupled, capacitors. Under the control of an external circuit, each capacitor can transfer its electric charge to one or other of its neighbours enabling to generate a digital image based on optical and UV spectroscopy. In principle, the CCD enables also to quantify the photons emitted by selected areas of a living animal.

Under anaesthesia, 20 minutes before BLI, animals were administered i.p. 50 μL of a water solution of the luciferase substrate luciferin (Beetle luciferin potassium salt, Promega, Madison, WI, USA) corresponding to 50 mg/kg for a 25 g mouse. Bioluminescence was measured by a Night Owl imaging unit (Berthold Technologies, Bad Wildbad, Germany), consisting of a Peltier cooled charge-coupled device slow-scan camera equipped with a 25 mm/f 0.95 lens. The camera was operated by WinLight32 software (Berthold Technologies). For photon emission measurement, mice were placed in a light-tight chamber, a gray-scale image were first taken with dimmed light, then luciferase signal was registered for 5 minutes. Merging of the pictures enabled to visualize the body areas where photon emission occurred (luciferase signal was transformed in pseudo-colors: blue-lowest, white-highest signal). For quantification, photon emission was measured in selected body areas using WinLight32 (Berthold Technologies) by superimposing a standardized electronic grid over the hepatic area and integrating the signals (counts per second, cts/s). Normalization was performed using an external source of photons (Glowell, Lux Biotechnology, Edinburgh, UK) enabling to measure the instrumental efficiency of photon counting.

Luciferase enzymatic assay

For luciferase enzymatic assay, tissues were homogenized in 500 µl of ice-cold lysis buffer (100 mM KPO4,1 mM DTT, 4 mM EGTA, 4 mM EDTA, pH 7.8) with a 5 mm inox bead in a Tissuelyser (Qiagen), undergone one freezing-thawing cycle, and were centrifuged for 30 minutes at 4900 x g, 4°C (Rotanta 460R Hettich Zentrifugen). Supernatants containing luciferase were collected and protein concentrations measured by Bradford assay, following reagent's manufacturer instructions (Pierce Biotech). Luciferase enzymatic activity was assessed by mixing 20 uL of tissue extracts (diluted 1:15 to prevent matrix interference) with 100 uL of a commercial luciferase assay buffer (Promega). Light intensity was measured with a luminometer (Glomax, Promega) and expressed as relative light units over 10 sec/µg protein (RLU/µg prot).

Immunohistochemistry

Animals were killed under deep anesthesia. Brains were removed and hemibrain postfixed in 4% paraformaldehyde, cryoprotected, snap-frozen in liquid nitrogen, and stored at -80° C until analyzed. Using a cryostat (Microm,Walldorf, Germany) 30-µm thickness sections were collected. The distinction between resting and activated microglia and astrocytes was based on morphological analysis; for the ERE-Luc mice, the mouse antibody Mac-1 was used to specifically stain microglia cells; the mouse antibody GFAP, was used to specifically stain activated astrocytes cells. Before the immunological assay, sections were incubated in 0.05 M NH4Cl in PBS for 30 min at room temperature to saturate aldehyde residues, washed in PBS, incubated for 5 min in 1% H2O2 in PBS at room temperature to inhibit endogenous peroxidases, and washed three times with PBS.

GFAP and Mac-1 staining.

Sections were incubated with rabbit anti-GFAP antibody (1 : 1000, Dako, Carpinteria, CA, USA) or rat anti-mouse MAC-1 antibody (1 : 500, BD PharMingen, San Diego, CA, USA) at 4°C overnight. Sections were then incubated with biotinylated goat anti- rabbit antibody for GFAP (1 : 1000, Vector, Burlingame, CA,) or biotinylated rabbit anti-rat antibody for Mac-1 (1 : 500, Vector, Burlingame, CA, USA) for 60 min. Immunoreactivity was visualized using ABC elite (Vector, Burlingame, CA, USA), an avidin±biotin±horseradish peroxidise (HRP) complex

(Vector Burlingame, CA, USA), with diaminobenzidine (DAB) (Sigma, Milan, Italy) as the chromogen.

Sections mounted on slides were observed using a Zeiss Axioskop microscope (Zeiss, Germany) at magnification x 1000 and analyzed with a colorvideo image analysis system linked to the microscope.

Real-Time PCR Gene Expression Analysis.

Real-Time PCR experiments were done with total aorta, uterus, liver and hippocampus RNAs extracted with RNeasy® Mini kit (Qiagen, Hilden, Germany) as suggested by the manufacturer's instructions. For the preparation of cDNA, 1 μg RNA was denatured at 75°C for 5 min in the presence of 1.5 μg of random primers (Promega) in 15 μl final volume. Deoxynucleotide triphosphate (GE Healthcare) and Moloney murine leukemia virus reverse transcriptase (RT) (Promega) were added at 0.5 mM and 8 U/μl final concentration, respectively, in a final volume of 25 μl. The RT reaction was performed at 37°C for 1 h; the enzyme was inactivated at 75°C for 5 min. Control reactions without addition of the RT enzyme were performed for each sample. Real-Time PCR experiments were performed using TaqMan technology. The reaction mix for each sample was made up of 5 μl diluted cDNA, 12.5 μl of TaqMan 2x Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA) and 7.5 μl of primers and probe mix: 100 nM Luc forward and reverse primers (ACA-GAT-GCA-CAT-ATC-GAG-GTG-AA and GCC-AAC-CGA-ACG-GAC-ATT-T), 80 nM Luc TaqMan MGB probe 5'-TAC-GCG-GAA-TAC-TTC; pre-made TaqMan Gene Expression assays for the endogenous gene Esr1 (Mm00433149_m1, Applied Biosystems), TNFα (Mm00443258_m1, Applied Biosystems), IL1β (Mm00434228_m1 Applied Biosystems), Mcp1 (Mm00441242_m1 Applied Biosystems), Mip2 (Mm00436450_m1 Applied Biosystems), PgR (Mm00435625_m1 Applied Biosystems) and as a reference gene assay 18S rRNA VIC-MGB-PDAR (Applied Biosystems). The reaction was carried out according the manufacturer's protocol using 7900HT fast real-time PCR system (Applied Biosystems) with the following thermal profile: 2 min at 50°C; 10 min at 95°C; 40 cycles (15sec at 95°C, 1 min at 60°C), and data were analyzed using the Sequence Detection System Software v2.3 (Applied Biosystems) and the 2-ΔΔCt

method (Livak, K.J., Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt Method.

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