Estrogens: Two nuclear receptors, multiple possibilities

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1. Introduction

Estrogens control development and cell differentiation and in adult organisms maintenance of homeostasis. They do so by binding and activating their nuclear receptors, estrogen receptor alpha (ERα) and beta (ERβ). These receptors bind the same ligands, estradiol (E2) and estrone (E1), with similar affinity and are almost ubiquitously expressed within the body, so that the tissue distribution and ratio between the two receptors determine the sensitivity and type of reactions of the target tissue to the common ligand.

Estrogen binds also to a new class of membrane G protein-coupled receptor, GPR30, also referred to as GPER. Therefore, estrogens signal not only via “classical” regulation of gene transcription in the nucleus, but also via regulation of “non-nuclear” signaling pathways on ligand binding to ERs. This topic is extensively reviewed elsewhere (Haas et al., 2009; Zimmerman et al., 2016; Feldman and Limbird, 2017; Barton et al., 2018) and we will only focus on the canonical ERα and ERβ pathways (Fig. 1).

1.1. Estrogen action

Estrogens stimulate growth, blood flow, and water retention in sexual organs and are also involved in causing breast cancer and endometrial cancer. In the liver, estrogens increase lipoprotein receptors, resulting in a decrease in serum concentrations of low-density lipoprotein cholesterol (Paganini-Hill et al., 1996). On the other hand, estrogens increase the potential for coagulation. In the gastrointestinal tract, estrogens may protect against colon cancer (Calle, Miracle-McMahill, Thun et al., 1995). In aging skin, estrogens increase turgor and collagen production and reduce the depth of wrinkles (Schmidt et al., 1996) (Fig. 2).

1.1.1. Actions on breast tissue

The lobular units of the terminal ducts of the breast tissue of young women are highly responsive to estrogen. In breast tissue, estrogens stimulate the growth and differentiation of the ductal epithelium, induce mitotic activity of ductal cylindric cells, and stimulate the growth of connective tissue (Porter, 1974; Bauman, 2021). Estrogens also exert histamine-like effects on the micro-circulation of the breast. The density of estrogen receptors in breast tissue is highest in the follicular phase of...
the menstrual cycle and falls after ovulation (Soderqvist et al., 1993). Estrogens stimulate the growth of breast-cancer cells. In postmenopausal women with breast cancer (BC), the tumor concentration of estradiol is high, because of in situ aromatization, despite the presence of low serum estradiol concentrations (Yue et al., 1999). The interaction with other pathways and the dynamic ER activity enables cancer cells adaptability and might impact on clinical outcome and disease progression (Farcas et al., 2021).

1.1.2. Vascular effects

Estrogens are thought to be natural vasoprotective agents. Estrogen receptors have been detected in smooth-muscle cells of coronary arteries and endothelial cells in various sites (Venkov et al., 1996). Estrogens cause short-term vasodilation by increasing the formation and release of nitric oxide and prostacyclin in endothelial cells. They also reduce vascular smooth-muscle tone by opening specific calcium channels through a mechanism that is dependent on cyclic guanosine monophosphate. (White et al., 1995). A protective role of estrogens against atherosclerosis is suggested by the finding that estrogen treatment reduced the progression of coronary-artery atherosclerosis in oophorectomized monkeys. There was, however, no effect on preexisting plaques (Clarkson et al., 1996). On the cellular level, estrogens inhibit apoptosis of endothelial cells (Spyridopoulos et al., 1997) and promote their angiogenic activity in vitro (Morales et al., 1995).

Functional ERs are expressed in vascular endothelial cells (EC), vascular smooth muscle cells (VSMC), and cardiomyocytes in humans and animals (Mendelsohn and Karas, 1999). Some cardiovascular effects might be mediated by a transmembrane G-protein coupled receptor GPR30 (Haas et al., 2009) Various ERs are expressed in most cardiovascular cell types, including cardiomyocytes, fibroblasts, vascular EC, and VSMC. Each ER is involved in protective effects of estrogen in multiple animal disease models, including ischemic heart disease, cardiac hypertrophy, heart failure, vascular injury, and atherosclerosis. Emerging evidence has indicated the potential importance of the non-nuclear ER signaling on diverse aspects of the cardiovascular systems (Feldman and Limbird, 2017), supporting the potential opportunity to design pathway specific selective ER modulators capable of regulating nonnuclear and nuclear effects, assisting with the development of personalized therapies for preventing and treating cardiovascular disease (Ueda et al., 2019).

1.1.3. Effects on bone

Estrogens are important for maintaining bone mineral density in both mice and humans. While we have long known the beneficial effects

![Estrogen receptor type](Fig. 1. Location of ERs in male (baby blue, left) and female (pink, right). In case of differential expression, the predominant receptor is depicted in red and bigger in size.)
of estrogen in bone, surprisingly the molecular mechanism for the role of estrogen in bone cells is only beginning to be unraveled. The skeleton is constantly being remodeled. Osteoblasts lay down the matrix for bone and osteoclasts degrade bone. If there is an increase in osteoblast number and/or activity, especially if coupled with a decrease in osteoclast activity, there is overall building of bone, such as occurs during E2-driven acquisition of bone mass during puberty. On the other hand, if there is a decrease in osteoblast number or activity and that is coupled to no change or an increase in osteoclast number or activity, a decrease in bone mineral density will occur. Thus, it is the balance between osteoblast and osteoclast numbers and activity that determines the quality and quantity of bone. In human cells, the 17-β estradiol individually or in the presence of progesterone has more effects on BM-MSCs' osteogenic differentiation compared to progesterone alone. In this study, it is indicated that the effect of the 17-β estradiol and progesterone concurrently was the same as individual 17-β estradiol on the differentiation of bone marrow derived-mesenchymal stem cells (BM-MSCs) into osteogenic lineage, although the role of the two ER types was not investigated. (Soltanyzadeh et al., 2020). The protective effects of E2 in bone are due to many mechanisms. For example, repression of pro-osteoclastic cytokines, have been well documented to promote increased bone mass (Pacifici et al., 1989; Jilka et al., 1992; Weitzmann et al., 2002). E2 exposure not only represses pro-osteoclastic cytokines, but it induces via Era apoptosis in bone resorbing osteoclasts (Kameda et al., 1997; Kousteni et al., 2002). A third mechanism of estrogen-mediated suppression of osteoclasts involves the regulation of the RANKL/(OPG) ratio. E2 also induces the transcription of alkaline phosphatase, a marker of osteoblast differentiation (Krum et al., 2008). ERα and ERβ have been detected by immunohistochemistry in osteoblasts (Bord et al., 2001; Braidman et al., 2001, Crusode de Souza, Sasso-Cerri and Cerri, 2009), osteocytes (Bord et al., 2001; Crusode de Souza et al., 2009), and osteoclasts (Bord et al., 2001; Braidman et al., 2001; Crusode de Souza et al., 2009). ERα and ERβ are also expressed in immune cells, such as T cells and monocytes (Harkonen and Vaananen, 2006; Pacifici, 2008; Kovats, 2015), which are also important in bone regulation. By immunohistochemistry, ERβ is expressed at higher levels in trabecular bone than in cortical bone. Interestingly, in this same study ERα was detected in the opposite pattern: higher in cortical bone than in trabecular bone (Bord et al., 2001). Both ERα and ERβ polymorphisms have been shown to correlate with bone mass in humans (Gennari et al., 2005). Mouse models of the function of ERα and ERβ have been generated by several labs and provided us with some insights to their individual function. For example, ERαKO mice were generated in the Korach and Smithies laboratories (K/G-ERαKO) (Couse et al., 1995) and

Fig. 2. Effects of estrogen action in different organs.
Chambon laboratory (C-ER\(\text{KO}\)) (Dupont et al., 2000). The K/G E\(\text{R}_{\text{\alpha}}\)KO is not a complete knockout and expresses some E\(\text{R}_{\text{\alpha}}\) due to splicing of the Neo cassette. The female and male C-ER\(\text{R}_{\text{\alpha}}\)KO mice have a decrease in cortical bone mineral density, and an increase in trabecular bone mineral density (Sims et al., 2002). Several E\(\text{R}_{\beta}\) knockout mice have also been created. ER\(\text{K}\text{O}\) mice were first developed in the Korach, Gustafsson and Smithies laboratories (K/G-ER\(\text{K}\text{O}\)) (Krege et al., 1998) and the Chambon laboratory (C-ER\(\text{K}\text{O}\)) (Dupont et al., 2000). The female K/G-ER\(\text{K}\text{O}\) mice have an increased cortical BMD at 11 weeks of age (Windahl et al., 1999) and both cortical and trabecular BMD increases by 12 months of age (Windahl et al., 2001), whereas the BMD in the Chambon ER\(\text{R}\text{K}O\) female mice is unchanged compared to WT controls. The Chambon group claimed that the phenotype of the K/G-ER\(\text{K}\text{O}\) knockout mouse is a result of the neo selection cassette inserted into the E\(\text{R}_{\beta}\) gene and is not due to loss of E\(\text{R}_{\beta}\) itself. Furthermore, these mice express truncations in the E\(\text{R}_{\beta}\) transcript that might contribute to the phenotypes. In 2007 Antal et al., published an E\(\text{R}_{\beta}\) knockout (ER\(\text{K}\text{IST}\ L^{-/L^{-}}\)) that is not thought to have any E\(\text{R}_{\beta}\) splice forms. This mouse had reproductive abnormalities, but no phenotype in the prostate or other tissues showed to have an effect in the other ER\(\text{K}\text{O}\) strains (Antal et al., 2008). However, the bone phenotype for the ER\(\text{K}\text{IST}\ L^{-/L^{-}}\) mouse has not been published.

In humans, both E\(\text{R}\) are expressed in bones. They are involved in attaining bone density and growth/maturation. So far, experimental data seemed to show a preferential role of E\(\text{R}_{\beta}\) in the pathogenesis of osteoporosis and of E\(\text{R}_{\alpha}\) in growth and maturation processes. The results of some experimental observations are summarized and compared with the clinical observations in the patients with E\(\text{S}\text{R}_{1}\)-and E\(\text{S}\text{R}_{2}\)-variants in the session dedicated to bone phenotype in the discussion (4.2.2C).

1.1.4. Brain: estrogens and cognition

Much of what we currently know about the mechanisms for estrogen’s effects on cognitive function comes from studies of learning and memory in rodent models, the results of which parallel those observed in women. For example, the ability of E\(\text{2}\) to improve performance on memory in rodent models, the results of which parallel those observed in women. For example, the ability of E\(\text{2}\) to improve performance on memory in rodent models, the results of which parallel those observed in humans.

Most data derive form experiments in mice and the role of the E\(\text{R}\)s in the metabolic network is established and that ER\(\text{R}\) activation is beneficial in metabolic control is indisputable (Savva and Korach-Andre, 2020). The situation is not as clear in humans. The lack of metabolic disturbances in the ER\(\beta\) deficient patients (see below) suggested that further studies are necessary to clarify the actual role of ER\(\beta\) in humans.

The metabolic effects of estrogens via the two receptors are summarized in Fig. 3.

2. Estrogen receptor structure

Estrogens exert their action by binding to specific receptors: the intracellular E\(\text{R}\)s and \(\beta\), and the membrane-bound GPER/GPR30. Fig. 4 shows the schematic structures of the two human ER\(\beta\) and ER\(\beta\) and the percentage of homology between the different domains (annotated by the letters A to F) are indicated (A). Domains involved in DNA binding (DBD), ligand binding (LBD), ligandin dependent transactivation function 1 (AF-1), and ligandin-dependent transactivation function 2 (AF2) are shown. The number of amino acids for each receptor is also indicated on the right side. E\(\beta\) mediates numerous phenotypic effects in cells by binding to and activating ER\(\beta\). E\(\beta\) enters the cell through the lipid membranes and binds ER, which can be present in the cytoplasm and the nucleus. The activated ER forms a dimer to tightly fix chromatin directly at the estrogen-responsive element (ERE) sites or indirectly at activator protein 1 (AP1) or specificity protein 1 (Sp1) sites. ER is then able to remodel chromatin by recruiting cofactors and activating RNA polymerase II (Pol II), at target genes (genomic action). Besides, E\(\beta\)s can use rapid non-genomic action through the interaction with intracellular kinases (mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and the growth factor receptor (GFR) pathways.

The two receptors are encoded by two distinct genes, E\(\text{S}\text{R}_{1}\) and E\(\text{S}\text{R}_{2}\), located on human chromosome 6 and 14 respectively.

3. Estrogen signaling

3.1. Genomic: nuclear receptors

ER\(\beta\)s and ER\(\beta\) are classical steroid receptors that typically dimerize and translocate to the nucleus after ligand binding. Once in the nucleus, the receptors bind to estrogen response elements (ERE) or interact with
Fig. 3. Location of ERα (Blue) and ERβ (Red) and metabolic effects.

Fig. 4. Schematic graphical representation of ERα and ERβ structure. See text for details.
3.2. Nongenomic: membrane associated receptor

Nongenomic signaling involves interaction between E2 and cellular membrane-associated receptors, either the ER itself or possibly via GPER (Levin, 2015; Prossnitz and Hathaway, 2015). The membrane interactions trigger rapid signaling responses (not involving transcriptional components), including activation of intracellular signaling exemplified by AKT and MAPK pathways. This mechanism seems to have been shown to play its greatest documented role in peripheral E2 physiological or transcriptional responses. (Hewitt et al., 2014, Ahlbor-y-Dieker et al., 2009).

3.3. Ligand-independent signaling

Ligand-independent responses are ER-mediated effects seen after activating other pathways, that results in ER-mediated transcriptional responses independent of estrogenic steroid ligands (reviewed in Stel-lato et al., 2016).

There are several extracellular signals able to mediate the activities of unliganded ERs including peptide growth factors (PGF) ([Ignar-Trowbridge et al., 1996], cytokines (Speirs et al., 2000), neurotransmitters (Power et al., 1991), other hormones (Catalano et al., 2004; Gonzalez et al., 2009), and even metals (Martin et al., 2003; Stoica et al., 2000) and minerals (Stoica et al., 2000; Stoica et al., 2000). Ligand-independent activation of ER lead to phosphorylation cascades (Murphy et al., 2011; Bunone et al., 1996), although also other mechanisms, such as intracellular calcium levels, play an important role (Divekar et al., 2011). A relevant cross-talk of ER signaling cross-talk takes places with peptide growth factors (PGF) pathways.

Insulin-like growth factor (IGF-1) pathway is a well-characterized example of ER-PGF cross-talk (Hewitt et al., 2017): IGF-1 activates ER in the absence of ligand and it is itself regulated by ER activity (bidirectional regulation) (Lanzino et al., 2008; Hamelers and Steenbergh, 2003; Kato et al., 1995) mostly via the mediation of Mitogen-Activated Protein Kinases (MAPKs), which led to phosphorylation and activation of ER (Kato et al., 1995).

Most of the studies described so far about activation of unliganded ERs have focused mainly on ERα subtype. Only few data are available about ERβ. This receptor subtype has been reported to be activated only by EGF (Tremblay et al., 1999), CXCL12 (Sauve et al., 2009), and 3,3a-diindolylmethane (Vivar et al., 2010).

Some studies explored a basal and unbound action of both ERs independent from the cross-talk and activation of other signaling pathways we previously mentioned (Leung and Ho, 2011; Maggi, 2011). In breast cells (BC), unliganded ERα has been found able to regulate genes linked to development and differentiation (Caizzzi et al., 2014) while unliganded ERβ strongly affects both the genome and the proteome of BC cells by inducing changes in microRNA expression (Nassa et al., 2014; Vivar et al., 2010). In prostate cancer, the presence of unliganded ERβ seems to induce apoptosis by a KLF5 tethering mechanism (transfer of active molecules) (Nakajima et al., 2011). Despite these evidences about ERα, the major findings about ER unliganded activity concerned ERβ subtype.

Overall, the published data suggest that in unstimulated state, ERα is organized in a “prerecruitment form,” allowing a potent and rapid response to E2 by increased binding to the basal sites and recruitment to additional sites. ERβ, instead, controls the expression of specific and numerous genes in ligand-independent manner, prevalently throughout binding to sites for other transcriptional factors. “Tethering” seems as an
important mechanism throughout which both receptors exert their effects on gene expression and should be taken in account in formulating therapeutic approaches, especially if involving ERβ.

Analysis of ERα and ERβ functions using knockout (KO) mouse models has demonstrated the roles of estrogen signaling in different physiological processes. αERKO and βERKO mice are also valuable for examining the effect of ER signaling in specific target organs. Although transgenic mice do not always produce consistent results, these models are useful for evaluating the functions of genes under specific pathological conditions (for review see Hewitt and Korach, 2018).

The available data in mice suggest that estrogen receptor gene (Esr2) is a dominant-negative regulator of estrogen receptor gene 1 (Esr1)-dependent transcription in a sort of “Ying Yang” alliance between the two Esrs (Lindberg et al., 2003; korach, 1994).

4. Estrogen receptors variants and human disease

Esr1 knock-out or KO mice of both sexes are infertile, and the XX animals have hypoplastic uteri and multicystic ovaries. Heine et al. (Korach, 1994) found that male and female Esr1 knockout mice have hyperplasia and hypertrophy of adipocytes, insulin resistance and glucose intolerance. Several animal models of Esr2 deficiency have been created resulting in various phenotypes (Dupont et al., 2000; korach et al., 2003; Cacioppo et al., 2016) with different reproductive phenotypes. A recent mouse model of Esr2 knock-out with no expression of Esr2 showed infertility in both male and female animals, without sex reversal (Baetens et al., 2017).

4.1. ERα resistance

The first human ERα variant with complete estrogen insensitivity was identified in a 28-year-old male with elevated serum estrogens and gonadotropins, normal-big testes, obesity, tall stature, and delayed bone age. In addition, this patient displayed insulin resistance and glucose intolerance, recapitulating the murine phenotype. (Smith et al., 1994).

Twenty years later, the first female patient with a sequence variation in ESR1 was described. She presented at age 15 with no breast development, primary amenorrhea, infantile uterus and big cystic ovaries (Quaynor et al., 2013). The recently published 8 years follow-up confirmed her complete estrogen insensitivity (Brakta et al., 2020).

More recently, three siblings in a consanguineous family with a loss of function ESR1 variant in the ligand-binding domain were also studied. The 2 affected girls had a similar phenotype to the first female case, and 1 boy presented with a cryptorchidism, which was not present in the first male patient (Bernard et al., 2016). In none of these patients the metabolic disturbances found in the first patient (and in mice) were reported.

4.2. ERβ resistance

In patients, an association between risk of hypospadias and variants of the (CA)n polymorphism in intron 6 and with a region of intense transcription factor binding in the putative promoter region (Beleza-Meireles et al., 2007), and mono and biallelic inactivating mutation in ESR2 has been reported in male patients with syndromic and non-syndromic 46, XY DSD (Baetens et al., 2017). Also, a girl with primary amenorrhea and ESR2 mutation without ovarian dysgenesis was reported using a candidate single gene approach without functional studies that do not exclude the contribution of variants in other genes to the phenotype (Asadi et al., 2013). Furthermore, the limited clinical description (e.g., lack of information on response to estrogens) renders the comparison of this phenotype to the other cases rather difficult. We recently published the second case of ESR2-defect in a young woman.

4.2.1. Our case report

The patient is now a 21-year-old orphan woman from East Africa, who had arrived in our country as a refugee. The patient had presented at 16.5 years of age with absent breast development with completely infantile nipples, full developed pubic hair, hypoplastic labia majora, a non-oestrogenised infantile vulva and primary amenorrhoea. The patient’s height was 150.6 cm (3rd Percentile, -1.86 SD), her weight 48.1 kg. Eunuchoid proportions impressed us already clinically (Fig.), indeed her sitting height (SH) was 76.4 (-4 SD) and the quotient SH/SILL (Sub Ischial Leg Length) – 2.2 SD (Prader et al., 1989). Bone age was (surprisingly) 16 years old (according to Greulich & Pyle), which corresponds to adult fully grown-up biological age (Lang-Muritano et al., 2018).

The combination of infantilism, long legs and adult bone age made us immediately aware of the novel aspects of the clinical picture (Fig. 6).

Under ERT she developed adult breasts and under a combined cyclic estrogen/gestagen transdermal patch regular menstruations occurred.

The follow-up of bone mass density (BMD), measured by dual energy X-ray absorptiometry (DEXA) in our patient after 3.5 years of continuous combined transdermal estrogen/gestagen and oral vitamin D/calcium therapy is shown in Table 1. The evident lack of improvement or even decrease of BMD despite otherwise optimal breast development and prompt occurrence of regular menses could be considered a confirmation of the key role of ESR2 on the attainment of bone mass (see Table 2).

4.2.2. Discussion of the ESR2-variant phenotype

4.2.2.1. Ovarian phenotype. ESR2 is expressed in the granulosa cells of the small growing and preovulatory follicles (ESR1 much less or not at all) in various species (Couse et al., 2004; Slomczynska et al., 2001; Byers et al., 1997; Pelletier and El-Alfy, 2000), and in the granulosa cells and oocytes in human fetal ovary during the 8th to the 22nd gestational week, a time crucial for gonadal development (Zhang et al., 2018). The mutated ESR2 completely lost its function in pre-granulosa cell system, showed a dominant negative effect on the normal receptor (which may account for the heterozygosity) and was not stimulated by exogenous estradiol. These data suggest that ESR2 is necessary for ovarian determination and ESR1 is not sufficient to drive ovarian development in humans. In rats targeted disruption of Esr2 (genetic knock-out) or its selective blockade (use of specific antagonist PHTPP) increased the number of activated follicle in Esr2+/− ovary, whereas the number of primordial follicles was markedly decreased. Excessive recruitment of primordial follicles led to premature ovarian senescence in Esr2−/− rats (Chakravarti et al., 2020). It is possible to hypothesize that a similar primordial follicles “gatekeeping” defect is the basis of the early-onset primary ovarian failure in our patient, but the exact mechanism remains to be elucidated.

4.2.2.2. Breast and uterus. The impact of the ESR2 mutation on mammary and uterine development is unclear because of the inadequate estrogen production by the dysgenetic ovaries. The prompt response of breast and uterine tissue to exogenous estrogens confirms that ESR2 is sufficient and necessary to stimulate breast and endometrial growth.

4.2.2.3. Bone. The bone phenotype in our patient calls for more elaboration. In fact, our patient’s bones are severely osteoporotic similarly to the ESR1 deficient-patients and at the same time - unlike subjects with a mutated ESR1 - completed their growth (adult bone age) as expected in a young lady of 16.5 years of age. In a ESR1 deficient who was followed for many years, bone maturation was also finally reached albeit much later (chronological age 24 years) (Brakta et al., 2020).

With regard to osteoporosis, both receptors are expressed in bones. Estrogens prevent osteoporosis by promoting stemness and osteogenesis of bone marrow stroma cells (BMSCs) and prevent their senescence (Wu et al., 2018). These effects are mediated by ESR2-dependent upregulation of SATB2 (AT-rich sequence binding protein 2), without the participation of ESR1. These data would support the notion that ESR2 defects per se could explain our patient’s severe osteoporosis. Also, our
patient had a primary ovarian insufficiency and only some estrogens of adrenal or peripheral origin (estrone is in the normal range), which add to the severity of osteoporosis.

While the bone phenotype in our patient would offer a clinical correlation to the experimental evidence of the prominent positive role of $ESR2$ on BMD, the observation that in the $ESR1$ deficient patient the osteoporosis was severe and persisted despite high endogenous estrogen and other therapies suggests that the presence of both intact ESRs is essential to attain a normal BMD in humans.

Although the severe osteoporosis is compatible with estrogen deficiency (due to the gonadal failure) and/or estrogen resistance (due to $ESR2$ mutation) the epiphyseal closure in the absence of estrogen production is difficult to account for. $ESR1$ regulates bone growth (travani

\[ \text{Table 1} \]

\begin{center}
\begin{tabular}{|l|c|c|c|}
\hline
 & DEXA at & DEXA after 2 years of & DEXA after 3.5 yrs of \\
 & diagnosis & therapy & therapy \\
\hline
Z-score whole & -4.0 & -3.3 & NA \\
body & & & \\
Z-value LWS & -4.2 & -3.3 & -4 \\
& & & \\
T2 T-score & NA & NA & -4.1 \\
& & & \\
Z-value right & -2.2 & -1.8 & -1.8 \\
femur & & & \\
Z-value left & -2.2 & -1.9 & -1.8 \\
femur & & & \\
\hline
\end{tabular}
\end{center}

Fig. 6. Schematic comparison of body proportions in a normal girl, in our patient and in a girl with Turner syndrome (late diagnosis, untreated) all at 17 years of age. The eunuchoid proportions and the adult bone age in our patient were decisive in the differential diagnosis vs Turner syndrome.
et al., 2017). Experiments performed in ovariectomized mice with the selective ESR1 (PPT) and ESR2 (DPN) agonists showed that bone growth and growth plate maturation are mainly mediated via ESR1. In a ESR1 deficient female patient who was followed for many years, bone maturation was greatly delayed (despite high endogenous estrogens) but finally reached in adulthood (Brakta et al., 2020). With regard to ESR2 in our functional studies, the mutant receptor seemed to increase the transactivation potential of ESR1 in bone, perhaps via an increased availability of the coactivator NCoA1, set free by the mutant ESR2 that cannot efficiently recruit it. On the other hand, in our expression studies, normal wild type ESR2 seemed to work in bone cells at very low concentration of E2 (0.1 pmol) mirroring the estrogen levels measured in our patient and is not affected by mutant ESR2: bone maturation/epiphyseal closure in our patient might have been stimulated by low levels of estrogen derived by the peripheral conversion of adrenal androgen precursors. On the other hand, given the very low estradiol levels to be expected in this patient, a ligand-independent ESR activation (Cui et al., 2013) might be considered.

4.2.2.4. Hypothalamus/pituitary/gonadal axis. The role of ESR2 in the regulation of the hypothalamus/pituitary/gonadal axis is unclear. Although adult GnRH neurons express ESR2 but not ESR1 in all species studied, targeted deletion of ESR2 does not alter E2-mediated GnRH expression in these neurons, suggesting that a direct action of estrogens through ESR2 remains to be elucidated (Handa et al., 2012; Wintermantel et al., 2006). In our patient, the mildly elevated basal gonadotropins were due to primary hypogonadism (lack of estrogen negative feedback) and suggest that ESR2 is not necessary for FSH/LH secretion, mirroring the findings in the 46, XY DSD patients (Baetens et al., 2017). Nevertheless, LH and FSH levels were expected to be higher. It is feasible to hypothesize that, as in bone, ESR1 transactivation capacity is augmented even at low estrogens via an increased availability of the coactivator NCoA1. In ESR1 deficient female patients, LH levels are also moderately elevated despite very high levels of estrogens and a non-functional ESR1, suggesting that a partial effect of ESR2 on hypothalamus/pituitary/gonadal axis and gonadotropins secretion could be considered.

Table 2 shows the course of gonadotropin values in our ESR2 deficient patient. We could speculate that the slight elevation of LH under estrogen only in the first months of treatment might be due to dominant positive feedback of the higher estrogen levels under transdermal therapy in a naïve HPG-axis. This effect mirrors that of the positive feedback of the estrogen spike immediately preceding the LH mid-cycle peak. On the other hand, gestagens seem to exert fully normal negative feedback on the hypothalamus-pituitary feedback.

The interpretation of FSH values is more complex due to the possible/possible additional role of inhibin-A which we could not measure.

4.2.2.5. Brain. ESR1 and ESR2 are expressed in several area of the brain and estrogens are involved in numerous neurocognitive processes. They are also important for neurogenesis and clinical as well as experimental evidences support their protective effect against neurodegenerative diseases such as Alzheimer and Parkinson (Garcia-Segura et al., 2001; Wise et al., 2001; Amantea et al., 2005). In Esr2 deficient female mice

| Chromosomal sex | ESR1 | ESR2 |
|-----------------|------|------|
| Male (n = 2)     | Female (n = 3) | Male (n = 3) | Female (n = 1) |
| Gonads          | Normal or cryptorchid testes | Hyperstimulated ovaries | Dygenetic testes | Dygenetic ovaries (developmental disruption) |
| Phenotype       | Male | Female | DSF | Female |
| Bone maturation  | Delayed | Delayed | Delayed/age conform/NA | Age conform (adult) |
| Metabolic disturbances | Yes/no | No/NA | NA | No |
| Neurological disturbances | No | No | No | Yes (except bone) |
| Response to estrogen therapy | No | No | No | No |

Table 3 Gonadotropin values during follow-up in ESR2 deficient patient.

| LH | At diagnosis | Under transdermal estradiol only | Under transdermal estradiol and gestagen |
|----|--------------|-------------------------------|-----------------------------------------|
| LH (NV) 1.1–3.8 | 8.7 | 11.3 | <0.3 |
| FSH (NV) 1.4–4.2 | 24.4 | 45.1 | 0.7 |

Krezel and coworkers observed a behavior consistent with increased anxiety and concluded that, particularly in females, there is an important role for Esr2-mediated estrogen signaling in the processing of emotional behavior (Krezel et al., 2001). Furthermore, Rissman et al. (Rissman et al., 2002) showed that E2 affects learning and memory via ESR2 in mice. In our patient we did not observe any significant neurological or cognitive deficit. Accordingly, none of the other non-syndromic receptor-deficient patients showed any neurological abnormalities.

Thus, the role of ESR2 in CNS still needs further investigation.

5. Conclusions and outlook

The discovery of ESR2 by Gustafsson and coworkers (Kuiper et al., 1996) opened the way to a deeper understanding of the complex and multiple actions of estrogens, showing that is not the ligand but the relationship between the two receptors that determines the variety of estrogen effects. Comparison of the gonadotropin levels and bone phenotypes of ESR1 and ESR2 defects suggests that both receptors are necessary for the integrity of interactions within the HPG axis and for proper bone mineralization.

Although broad generalization should await the description of more patients, it appears that loss-of-function mutations in the estrogen receptor beta led to testicular and ovarian dysgenesis, suggesting that ESR2 is necessary for human gonadal determination in both sexes and that ESR1 is not sufficient to drive gonadal development. It remains to be established whether estrogen resistance due to milder defects in ESRs might account for unexplained cases of ovarian failure or infertility.

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