Estrogen-mediated protection against coronary heart disease: the role of the Notch pathway

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Abstract

Estrogen regulates a plethora of biological processes, under physiological and pathological conditions, by affecting key pathways involved in the regulation of cell proliferation, fate, survival and metabolism. The Notch receptors are mediators of communication between adjacent cells and are key determinants of cell fate during development and in postnatal life. Crosstalk between estrogen and the Notch pathway intervenes in many processes underlying the development and maintenance of the cardiovascular system. The identification of molecular mechanisms underlying the interaction between these types of endocrine and juxtacrine signaling are leading to a deeper understanding of physiological conditions regulated by these steroid hormones and, potentially, to novel therapeutic approaches to prevent pathologies linked to reduced levels of estrogen, such as coronary heart disease, and cardiotoxicity caused by hormone therapy for estrogen-receptor-positive breast cancer.

Keywords:

Estrogen; endothelial dysfunction; Notch; inflammation; hormone therapy; coronary heart disease

1. Introduction

Despite many successes in the treatment of cardiovascular disease, coronary heart disease (CHD) remains the leading cause of death for both women and men [1]. Pre-menopausal women are protected against CHD, in comparison to age-matched men [2]. This protection is lost after the loss of endogenous estrogen production following natural or surgical menopause, suggesting a beneficial effect of female sex steroid hormones against CHD [3, 4]. Consistently, there is evidence of an association between endothelial dysfunction, a crucial early event in the onset of atherosclerosis, and reduced endogenous production of estrogen in women after menopause [5, 6].

Reduced nitric oxide (NO) production, increased endothelium permeability and expression of proteins required for adhesion of inflammatory cells are hallmarks of endothelial dysfunction [7], and are biological processes modulated by estrogen. Estrogen promotes endothelial nitric oxide synthase (eNOS) activation, NO production [8, 9], and limits the expression of proteins involved in monocytes and neutrophils adhesion to the endothelial monolayer [10, 11], thereby preventing the migration of leukocytes to the sub-endothelial space and their subsequent production of inflammatory cytokines [12]. Specifically, in endothelial cells exposed to lipopolysaccharide (LPS) or interferon γ (IFNγ), 17β-estradiol (E2), the predominant and most biologically active form of estrogen, reduces the expression of vascular cell adhesion molecule-1 (VCAM-1) [13] and of intercellular cell adhesion molecule-1 (ICAM-1) [14]. In addition, in endothelial cell lines of brain and heart origin, estrogen strongly increases expression of the tight junction protein claudin 5, thus leading to an improvement in vascular integrity and barrier function [15] and reduced permeability to native and oxidized low density lipoproteins (LDLs) [16]. Further, estrogen promotes endothelial cell survival through the inhibition of apoptosis induced by tumor necrosis factor (TNF) α [17-20], H_2O_2 [21] or oxidized LDLs [22]. This is thought to be due to estrogen's activation of Akt [18] and of mitogen-activated protein kinases (MAPKs) [19, 20], which increase expression of anti-apoptotic proteins Bcl-2 and Bcl-XL [22]. Additionally, estrogen is able to modulate oxidative stress in the endothelium through inhibition of reactive oxygen species (ROS), produced in the mitochondria [23, 24] or in the cell membrane by NADPH (nicotinamide adenine dinucleotide phosphate oxidase) oxidases (Nox) enzymes. Estrogen is also involved in the regulation of angiogenesis, a complex process leading to the formation of new blood vessels, which requires endothelial cells proliferation, migration [25] and differentiation [26].

The molecular mechanisms by which the steroid receptors regulate all these biological processes, in the endothelium as well as in other tissues, have been the subject of a many extensive reviews [27-

30], and they will be briefly summarized here. The effects of estrogen are mainly mediated by estrogen receptors (ERs). The most characterized are ERα and ERβ, which are structurally similar and are localized in most cellular compartments, including the plasma membrane, the cytosol, the nucleus [31], and in the mitochondria [32]. For each receptor, several splice variants, mutations, post-translational modifications and interactions with others regulatory proteins have been described [33]. For both ERs, the domain structure consists of the N-terminal domain (NTD), responsible for ligand-independent activation of transcription; the DNA-binding domain (DBD), for sequence-specific binding to DNA, and the ligand-binding domain (LBD), which is the ligand-dependent activator of transcription [34]. The two ERs share about 97 % similarity in the DBD, 59 % in the LBD, and only 18 % in NTD [35]. Due to differences in the LBD, each receptor can be targeted by specific agonist/antagonist molecules [28], thus helping the investigation of differential roles of ERα and ERβ and the development of receptor-specific ligands. The molecules most commonly used to study ERs functions are: ICI 182.780, a selective estrogen receptor downregulator (SERD); 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazoledihydrochloride (MPP), an ERα-specific antagonist [36]; 4,4',4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), an ERα-specific agonist [36]; 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP), an ERβ-specific antagonist, and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN), an ERβ-specific agonist [37]. After the binding with natural or synthetic ligands, the activated ERα and ERβ can have a genomic (nuclear) or non-genomic (membrane-associated) action [3]. The genomic action corresponds to the transcription of specific target genes triggered by ERs. The two ERs regulate different set of genes in a time-, tissue- and cell-dependent manner [38-40]. These differences are due to the binding to different regulatory elements and to the recruitment of different transcription and chromatin remodeling factors, that are expressed in a cell- and tissue-specific manner [41]. The rapid non-genomic action involves instead, ERs-mediated cytoplasmatic activation of signaling pathways, such as mitogen-activated protein kinases (MAPKs), extracellular signal–regulated kinases (ERK1/2), and/or phosphoinositide 3-kinase (PI3K)/Akt pathways [33]. More recently, a G protein-coupled receptor, GPR30, has been identified [42]. GPR30 can localize both in the plasma membrane [43, 44], in the endoplasmic reticulum and in the mitochondria [45]. GPR30 has been implicated in a non-genomic estrogenic signaling [46], and its role has been studied both in cancer and cardiovascular context [47].

The above listed biological processes regulated by estrogen are also modulated, in the endothelium, by the Notch signaling pathway. The Notch pathway is a mediator of juxtacrine communication,

involved in cell fate determination during embryonic development and postnatally, for continuously renewing tissues, such as the epidermis and the endothelium [48, 49]. Specifically, Notch is a major player in the regulation of endothelial cells activation [50], survival [18, 51, 52], proliferation [53], migration and angiogenesis [54, 55].

The Notch pathway has been extensively studied for its major role in the regulation of stem cells fate [56], and since it is highly activated in many cancers types, it is still intensively investigated as a potential therapeutic target for cancer therapy [49, 57]. There is now growing evidence of a major role played by Notch in the context of vascular homeostasis [7] and crosstalk between Notch and estrogen signaling has been observed in endothelial cells [18, 58-60]. This discovery follows previous studies showing the estrogen-mediated modulation of the Notch signaling pathway in breast cancer cells [58, 61] and hippocampal neurons [62-64].

This article aims to review the existing literature on the crosstalk between Notch and estrogen in the vascular system and the role of this interplay in the protection mediated by estrogen against CHD. We will then discuss how this crosstalk could affect existing or novel therapeutic approaches involving estrogen- and Notch-mediated signaling, such as hormone replacement therapy (HRT), for the reduction of CHD risk in post-menopausal women, or anti-estrogen or anti-Notch agents for cancer therapy.

2. The core Notch pathway

The Notch pathway, originally discovered in Drosophila [65], is highly conserved through the evolution of Metazoan. In mammalian cells are present Notch receptors (Notch 1-4) and their ligands (Delta-like-1, 3, 4 and Jagged-1 -2), both located on the surface of cells. Notch precursor is processed into two polypeptide chains, which interact to form the functional receptor made of an extracellular (NEC) and a transmembrane subunit (NTM). Binding of ligand triggers the dissociation of NEC and the extracellular cleavage of NTM by A Disintegrin And Metalloproteases 10 and 17 (ADAM10 and ADAM17), followed by an intramembranous cleavage by γ-secretase complex, a multi-subunits membrane protease. The resulting cleaved and active form of Notch (NICD) then migrates into the nucleus. NICD modulates the transcription by binding the CSL (CBF1, Suppressor of Hairless, Lag-1), also known as recombinant signal binding protein for immunoglobulin kJ region (RBP-Jk) transcription factor, displacing co-repressors, such as SMRT (silencing mediator for retinoid and thyroid receptor)/N-CoR (nuclear receptor co-repressor), SHARP (SMRT/HDAC-1-associated

repressor protein)/MINT/SPEN and KyoT2, and recruiting transcription co-activators, such as histone acetyltransferases CBP/p300 or PCAF/GCN5 through the binding with MAML (mastermind-like) protein [49]. Notch promotes the transcription of the Hes (Hairy and Enhancer of Split) and Hey (Hairy and Enhancer of Split with YRPW) families of genes [66], which are negative regulators of transcription and of genes involved in cell cycle [67], apoptosis [68] and regulation of stemness [69]. During the past years, data have been accumulating on a non-canonical, cytoplasmic Notch signaling modulating cell proliferation and metabolism [70]. The non-canonical Notch signaling is CSL-independent, and it is based on the interaction with Wnt/β-catenin, mTORC2 (mammalian target of rapamycin complex 2)/Akt and IKKα/β pathways in the cytoplasm [71]. Non-canonical Notch signaling is also associated with mitochondria, where it has been shown that Notch/PINK1 (PTEN-induced kinase 1) interaction modulates mitochondrial function and activates mTORC2/Akt pathway, thus promoting cell survival [70, 72, 73] (Fig. 1). Notch signaling can also be activated by so-called non-canonical ligands, such as F3/contactin [74], DLK1/2 (Delta-like 1/2), and EGFL7 (epidermal growth factor-like domain 7), which lack a DSL (Delta, Serrate and LAG-2) domain, necessary for the interaction with Notch receptors in the classic Notch ligands [75]. The non-canonical ligands seem to antagonize the Notch signaling by competing with DSL ligands for Notch binding [75, 76].

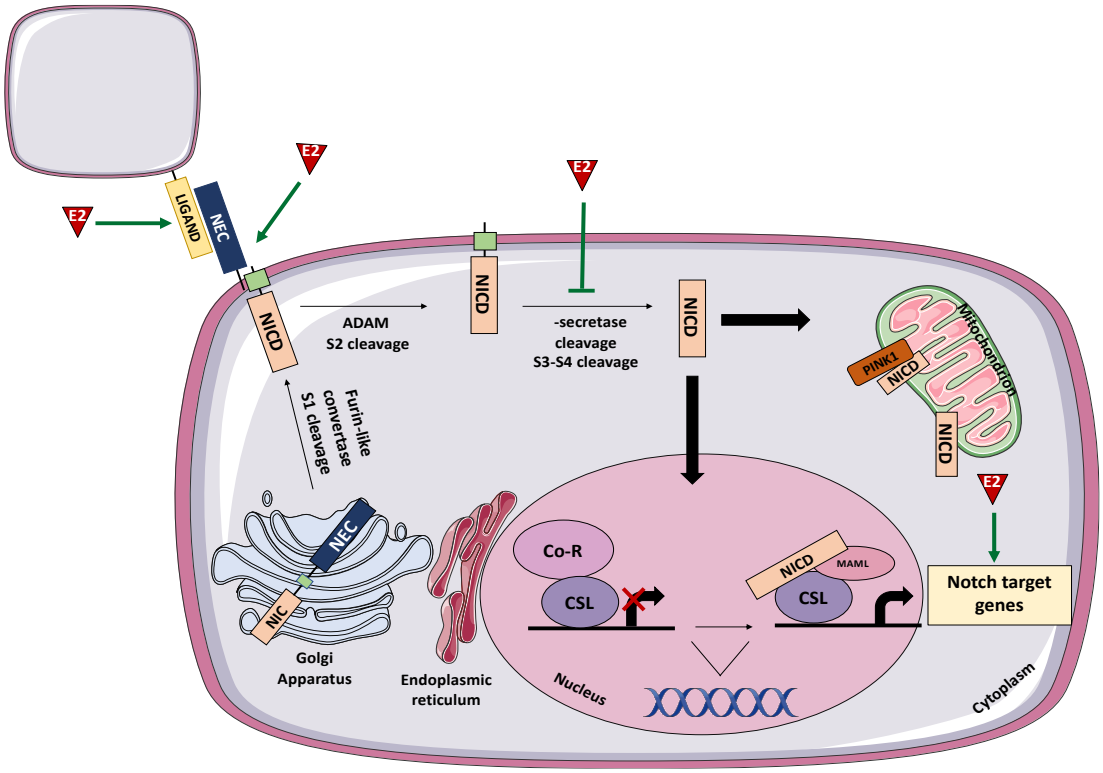

FIGURE 1. Schematic representation of the Notch signaling pathways: canonical and non-canonical. The Notch receptor is obtained after proteolytic cleavage of the newly synthesized precursor by furin-like convertase at site 1 (S1) in the Golgi apparatus. Notch receptor activation occurs after binding to a ligand present on adjacent cells, which induces a cleavage at site S2 mediated by ADAM family proteases followed by a cleavage at S3 and S4 within the transmembrane domain mediated by the γ-secretase complex. The Notch intracellular domain (NICD) translocates into the nucleus, where it interacts with the transcription factor, RBP-Jk (CSL) and the transcriptional co-activators MAML to initiate transcription of downstream target genes. In the absence of NICD, RBP-Jk (CSL) may associate with co-repressor (Co-R) proteins to repress transcription of target genes. The non-canonical Notch signaling is independent of CSL and it is also associated with mitochondria. E2 regulates the Notch signaling pathway modulating: the γ-secretase complex activity, the expression of Notch receptors and ligands, and Notch target genes. CSL indicates CBF-1/RBP-Jk/Suppressor of hairless/Lag-1; Co-R, co-repressor; Co-A, co-activator; MAML, mastermind-like; ADAM, a disintegrin and metalloprotease; GSI, γ-secretase inhibitor; PINK1, PTEN-induced kinase 1; NICD, Notch intracellular domain; NEC, Notch extracellular; E2, 17β-estradiol.

Post-translational modifications, including phosphorylation, glycosylation, acetylation and ubiquitination regulate Notch activity [77]. Furthermore, Wnt signaling [78], Sonic Hedgehog signaling [79], the cytokine transforming growth factor β (TGFβ) [80], hypoxia-inducible factor-1 (HIF-1) [81] and microRNA (miRNAs) [82] have an effect on Notch activity (a more detailed discussion of these interactions is provided in a recent review [77]). A genome-scale study in Drosophila melanogaster has shown the existence of a complex network of genes that can affect Notch activity [48]. Similarly, Notch modulates key pathways involved in the regulation of cell

194 survival and proliferation, including NF-kB (nuclear factor-kappa-light-chain-enhancer of activated
195 B-cell) [83] and ErbB2 (receptor tyrosine-protein kinase erbB-2) [84, 85].

196 During the last twenty years, evidence has been accumulating of a regulation of Notch by steroid
197 hormones. Crosstalk between estrogen and Notch have been investigated in ER-positive breast cancer
198 cell lines, MCF7 and T47-A18 [58, 61] and hippocampal neurons [62-64]. Specifically, in breast
199 cancer cells, Rizzo *et al.* have shown that E2 inhibits the processing of Notch1, as indicated by
200 unchanged levels of Notch1 mRNA, reduced levels of active Notch1 and Notch target genes, and by
201 the accumulation of the inactive form of Notch1 on the cell membrane [61]. At least in part, this effect
202 of E2 seems to be due to inhibition of Notch1 cleavage by γ-secretase complex [61]. In contrast with
203 these results, Soares *et al.* reported that, in MCF7 cells, E2 induced Jagged1 and Notch1 genes and
204 Notch transcriptional activity [58]. In breast cancer cells the crosstalk between estrogen and Notch is
205 bidirectional since Notch1 is able to activate the transcription of ERα-target genes in the presence or
206 absence of E2 [86]. In hippocampal slice cultures, E2 reduces the levels of the active form of Notch1
207 [62-64] with mechanisms not thoroughly investigated that could involve, as suggested by the authors,
208 inhibition of γ-secretase, as reported for breast cancer cell lines [61, 62] (Fig.1). In human uterine
209 fibroblasts, progesterone, together with chorionic gonadotropin, induces expression of Notch1 and
210 up-regulates its activity [87], whereas, in the male reproductive system, Notch signaling (testis,
211 cremaster muscle and Wolffian duct) is regulated by testosterone [88-90]. Notch signaling is
212 subjected to regulation by testosterone in prostate [91], in which androgen receptor downregulates
213 the expression of Notch1 and Jagged1, while upregulating Sel1L, a negative regulator of Notch [92]

214 As a result of this multitude of interactions, the effects of Notch signaling are exquisitely dose-, time-
215 and cell context-dependent and the output of activation/inhibition of this pathway difficult to predict.

216

217 **3. Estrogen-mediated regulation of endothelial Notch**

218 The vascular endothelium expresses three isoforms of Notch receptors: Notch 1, 2, 4 [52, 93], and
219 four ligands: Delta-like 1, 4 (Dll1, Dll4) and Jagged1, 2 (Jag1, Jag2) [94, 95]. Similarly, to other
220 tissues, Notch activity in the endothelium is regulated by interaction with other proteins, such as
221 VEGF [96], inflammatory cytokines, such as TNFα [18, 97, 98] and interleukin 1β (IL-1β) [99, 100],
222 β-catenin [101], KRIT1 [102], and bone morphogenic protein receptor 2 (BMPR2) [53].

223 In the endothelium, Notch plays a major role in the regulation of angiogenesis [54]. Furthermore,
224 Notch prevents endothelial cells dysfunction induced by inflammation, dyslipidaemia [7, 103, 104],

and disturbed blood shear stress [105]. An *in vivo* study by Schober *et al.* has reported that microRNA-126-5p, which is required for the repair of the endothelium damaged by lipids, activates Notch1, through the downregulation of Delta-like 1 homolog (DLK1), a Notch1 inhibitor [106]. Consistently, Briot *et al.* have demonstrated, *in vitro* and *in vivo*, that endothelial Notch1 is repressed by inflammatory lipids and pro-inflammatory cytokines, and this reduction increases the expression of inflammatory molecules and binding of monocytes [99]. More recently, studies conducted by us in human umbilical vein endothelial cells (HUVECs) have shown that Notch1 protects against TNFα-induced endothelial cells apoptosis [18]. The atheroprotective role of Notch1 has been confirmed by Mack *et al.* that demonstrated that Notch1 integrates responses to laminar shear stress, thus regulating junctional integrity, cell elongation, and suppression of proliferation in the endothelium [107]. Furthermore, Polacheck and colleagues reported that the non-canonical Notch1 signaling activated by shear stress plays a crucial role in maintaining endothelial barrier function [108]. However, there are *in vitro* and *in vivo* studies showing that Notch1 causes endothelial dysfunction [100, 109-111]. These contradictory findings could be due to: a) the use of different animal models of atherosclerosis [112]; b) different origin of endothelial cells used for the *in vitro* studies (aortic or umbilical); c) different modality of endothelium damage (TNFα or IL-1β, high glucose, disturbed shear stress); d) the focus of each study being on only one of the two modalities of Notch signaling (canonical or non-canonical); e) results obtained with overexpression or endogenous Notch1.

We [60] and others [58, 59] have shown that treatment with E2 activates Notch signaling in HUVECs. Our laboratory provided evidence that E2 treatment increased the levels of the active form of Notch1 and Notch4 proteins, even though no changes in the expression levels of the genes for these receptors or their ligands were observed, suggesting an effect of E2 on Notch mRNA translation or on the processing of the protein. Treatment with the selective estrogen receptor downregulator (SERD), ICI 182.780, inhibited the activation of Notch1, suggesting a role for ERs in this context. In our study, only a small induction of Notch target gene Hey2 was observed following E2 treatment, suggesting either an involvement of non-canonical Notch signaling or that other target genes could be affected by E2 [60]. In contrast with these results, Soares *et al.* had previously reported an increase in expression levels of Notch1 and Jagged1 mRNA and the induction of RBP-Jk transcriptional activity in E2-treated HUVECs [58]. Sobrino *et al*. also reported induction of Notch signaling by E2 in HUVECs, as indicated by increased levels of mRNA for Notch4, Furin, Jagged2 and radical Fringe (glycosyltransferase that modulates Notch) detected by microarray analyses [59]. Despite clear evidence of activation of Notch signaling in HUVECs by E2, there are discrepancies in the molecular

details of this activation, likely due to different technical approaches, such as the source of HUVECs, different cell culture conditions, cells passage number and/or the technique employed for the molecular studies (semiquantitative RT-PCR, qRT-PCR, microarrays). The molecular mechanisms by which E2 regulates Notch in the endothelium need further studies (Fig. 2). Based on current knowledge about pathways regulated both by Notch and E2, in the following paragraphs we will discuss potential mechanisms by which E2, bound to either ERα and ERβ, could affect the Notch signaling.

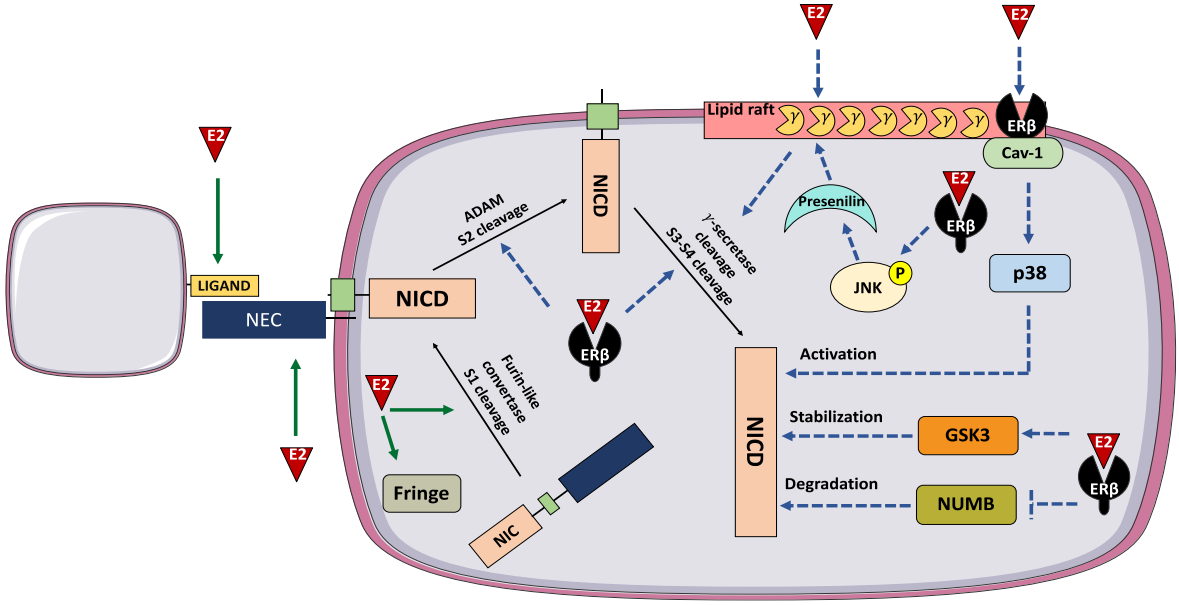

FIGURE 2. Possible molecular mechanisms involved in the crosstalk between estrogen and Notch in the endothelium. (A) Green arrows indicate mechanisms of E2-mediated regulation of Notch signaling reported in literature: E2 is able to induce the expression of Notch receptors, Notch ligands [58, 59], radical fringe and furin [59]. Dashed blue arrows indicate potential mechanisms of interaction: a) E2/ERβ could modulate proteins involved in Notch1 processing, such as ADAM or γ-secretase complex; b) E2/ERβ could inhibit the synthesis of Numb, which degrades Notch1; c) E2/ERβ could induce GSK3, which stabilizes Notch1; d) E2/ERβ could promote the access of Notch1 to γ-secretase-rich membrane lipid rafts; e) in the presence of E2, caveolin-1 binds ERβ, activating p38 kinase, which is involved in Notch1 activation; f) E2/ERβ could induce the phosphorylation of JNK, which stabilizes presenilin, a subunit of γ-secretase complex. E2, 17β-estradiol; ER, estrogen receptor ; GSK3, glycogen synthase kinase 3; N1ICD, Notch1 intracellular domain; NEC, Notch extracellular; Cav-1, caveolina-1; JNK, c-jun NH2-terminal kinase; P, phosphorylation; γ, γ-secretase.

4. Crosstalk between Notch and estrogen: effects on angiogenesis

Angiogenesis occurs during development and in adult life, for physiological processes, such as endometrial regeneration during the menstrual cycle, corpus luteum formation in the ovary [113] and wound healing [114], and under pathological conditions, such as cancer [115] and ischemic disease

[116, 117]. It has been reported that angiogenesis may influence the clinical outcome in patients with heart failure (HF) [118, 119] and, consistently, we found that HUVECs treated with sera from advanced HF patients show increased sprouting angiogenesis associated to reduced Notch4 and Jagged1 [120].

E2 promotes proliferation, migration, tubular structure formation, and VEGF secretion in cardiac microvascular endothelial cells [25], and induces angiogenesis in ischemic heart by enhancing the capillary density [121]. Among the molecular mechanisms and biological processes regulated by E2 during angiogenesis there is the induction of VEGF [122], the major angiogenic factor, which, in turn, stimulates eNOS and NO production [123] and endothelial cell proliferation and migration [124]. Furthermore, estrogen induces the expression of basic fibroblast growth factor (bFGF), vascular adhesion molecules and integrins, which have an important role in mediating endothelial cell attachment, migration and growth [125, 126].

The Dll4/Notch1 axis controls angiogenesis by regulating the formation of endothelial "tip" cells, which determines the number of new sprouts: specifically, Dll4/Notch1 inhibition promotes the formation of "tip" cells, whereas its activation leads to "stalk" cells, needed for the elongation of the newly formed vessel [55, 94]. Based on these observations, it would be expected that inhibition of Dll4/Notch1 could be used to increase new vessels formation. Instead, existing studies show that the effects of Notch inhibition on angiogenesis is context dependent. In tumors, endothelial Dll4/Notch1 axis inhibition, obtained by using anti-Dll4 antibody, causes the formation of a high number of new vessels that are not perfused or functional [127]. Under inflammatory conditions, TNFα induces Jagged1 and reduces Dll4 [18, 98], and sprouting angiogenesis is stimulated by the switch from Dll4- to Jagged1–Notch1 activation. This is thought to be due to the fact that Jagged1 is a less potent activator of Notch1, which therefore leads to reduced Notch1 activation and increased sprouting [98]. We have shown that E2-induced activation of endothelial Notch1 has an effect on angiogenesis: specifically, E2 inhibits the strong induction of endothelial tubes formation, a measure of sprouting angiogenesis, caused by Notch inhibition with DAPT [N-(N- [3,5-difluoro-phenacetyl]-l-alanyl)-S-phenylglycine t-butyl ester], an inhibitor of the γ-secretase [60] (Fig. 3A). These results indicate that the effect of E2 on Notch activity could be physiologically relevant when Notch is inhibited. Endothelial Notch inhibition occurs under inflammatory conditions [98] and after myocardial infarction (MI), in which ischemia/reperfusion (H/R) heart damage blocks endothelial tube formation [128].

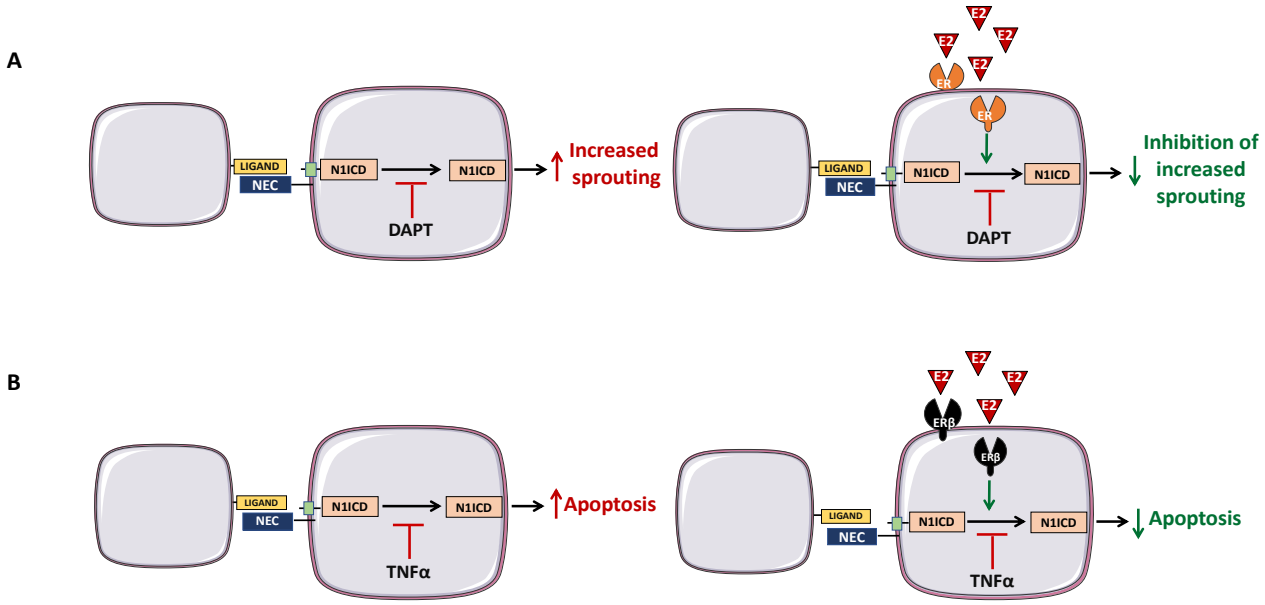

FIGURE 3. E2-mediated positive effects against endothelial Notch1 reduction. (A) DAPT inhibits the activation of Notch1 and induces sprouting angiogenesis. E2 counteracts DAPT-induced sprouting. (B) TNFα treatment reduces the active form of Notch1, determining an increase of endothelial cells apoptosis. E2 counteracts TNFα-induced reduction of active Notch1 and reduces the number of apoptotic cells through ERβ. NEC, Notch extracellular; NICD, Notch intracellular domain; E2, 17β-estradiol.

5. Crosstalk between Notch and estrogen: effects on endothelial apoptosis and atherosclerosis

CHD is caused by atherosclerosis, a chronic progressive inflammatory disease of the arterial wall that begins with formation of fatty streak in the intima, below the endothelium. Thus, endothelium integrity is crucial to prevent lipid infiltration and CHD. Inflammation disrupts endothelium integrity also by causing endothelial cells apoptosis, a marker of endothelial dysfunction associated to the progression of CHD [129-131]. Inflammation mediator TNFα dysregulates endothelial Notch by down-regulating Notch4 and up-regulating Notch2 receptors mRNA expression [97] and Notch4 knockdown [132] and Notch2–mediated down-regulation of survivin [52] have been reported to cause HUVECs apoptosis. In addition, we demonstrated that, in HUVECs, TNFα treatment reduces the levels of the active form of Notch1 [18]. In contrast with Briot *et al*. observations in human aortic endothelial cells [99], we did not observe that TNFα inhibited the transcription of Notch1, suggesting instead that, in HUVECs, TNFα inhibits the activation of the Notch1 receptor. Alternatively, since TNFα inhibits Dll4 expression while inducing Jagged1 [18, 98], the altered Jagged1/Dll4 ratio could determine the observed reduction of active Notch1 in TNFα-treated HUVECs. However, an effect of

TNFα on the stability of the active form of Notch1 cannot be ruled out. We found that E2 counteracted the TNFα-mediated inhibition of active Notch1 without affecting Jagged1 and Dll4 levels, indicating that the E2 does not increase the active form of Notch1 by the ligands modulation [18]. Further studies are required to identify the molecular mechanisms by which E2 interferes with TNFα-induced Notch1 inhibition.

Estrogen counteracts TNFα-induced endothelial cells apoptosis [17] only in the presence of active Notch1 [18] since, as shown by us, E2 did not reduce apoptosis when Notch1 was inhibited pharmacologically, by DAPT treatment, or genetically, by short interfering RNA (siRNA) (Fig. 3B). Further, the E2-mediated pro-survival effect was dependent on Akt activation, which was less pronounced when the active form of Notch1 was down-modulated [18]. The Notch pathway is well known for the enhancement of NF-kB activity [133], the latter being activated by LPS (lipopolysaccharide) and TNFα [134]. Therefore, it is possible that E2 might interfere with endothelial cells apoptosis by facilitating Notch1 cleavage, thus activating the NF-kB pathway. Further studies are needed to confirm the role of NF-kB in E2- Notch1-mediated protection against apoptosis induced by TNFα.

Atherosclerosis, and consequent CHD, is often associated with cardiometabolic syndrome (CS), a condition mainly characterized by insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, central adiposity and inflammation [135]. There is a large body of evidence showing an association between low levels of estrogen and CS, with molecular mechanism still undefined (for an exhaustive discussion of the role of E2 in cardiometabolism the reader is directed to [136]). Therefore, in addition to preventing endothelial dysfunction, E2 protects against CHD by modulating those biological processes underlying CS such as i) food intake and energy expenditure by the hypothalamus [137]; ii) the release of inflammation mediators by macrophages [138, 139]; iii) the balance between white and brown fat adipocytes, involved in fat storage or its oxidation for heat generation, respectively [140]; iv) the regulation of glucose metabolism and homeostasis [137]. Furthermore, E2 can influence the progression of CHD by regulating cardiomyocyte survival [141], proliferation of vascular smooth muscle cells (VSMCs) in blood vessels walls [142], phenotype of cardiac fibroblasts [143], and stem cells, such as endothelial progenitor cells (EPCs) and cardiac stem cells [144, 145].

Similarly to E2, there is evidence linking Notch to CS. Notch1 controls glucose metabolism by regulating insulin secretion [146] and by inhibiting the adipose expression of genes associated with

insulin sensitivity, such as adiponectin, GLUT4, C/EBPα and IRS-1 [147]. Furthermore, Notch inhibition has been shown to limit excessive FoxO1-driven hepatic glucose production [148] and results in browning of white adipose tissue [147, 149, 150] also by metabolic upregulation of mitochondrial oxidative phosphorylation and ROS [151]. Consistent with all these studies, inhibition of Notch signaling has been shown to reduce metabolic disorders and progression of atherosclerosis in mice [152, 153]. More studies are needed to establish whether Notch could be also targeted to inhibit pathological cardiac remodeling [154].

Based on the evidence of estrogen- and testosterone-mediated Notch regulation in many cell types, it would be of interest to establish whether the levels of these hormones could influence the onset and progression of CS and CHD by regulating the Notch signaling. For example, it has been reported that males, when compared with female mice, following exposure to high fat diet show higher levels of inflammatory markers in the hypothalamus only in the presence of ERα [155]. It is tempting to speculate that this could be due to ERα-mediated reduction of Notch signaling associated to a reduced inflammatory response. Similarly, ERα-mediated reduction of Notch signaling in adipocytes may promote browning of white adipose tissue [149].

6. Distinct roles of ERα and ERβ in estrogen-mediated protection of the cardiovascular system

The cardiovascular effects of estrogen are mainly mediated by the activation of ERα and ERβ [3]. Both ERs are expressed in VSMCs, vascular endothelial cells, and cardiomyocytes [156]. Studies using mice that lack functional ERα and ERβ have shown that both ERs are necessary for estrogen-mediated protection against cardiovascular injury [157], but, to date, the individual contribution of the ERs to atherosclerosis and its progression remains poorly understood. E2-mediated prevention of fatty streaks at the early stages of atherosclerosis requires ERα [158, 159]. The key role of ERα in the E2-mediated atheroprotective action has been shown also in mice deficient in both the low-density lipoprotein receptor (LDLR) and ERα, in which E2 was not able to exert its protective action [160]. The different contribution of estrogen-mediated activation of genomic and/or non-genomic ERα signaling to vascular protection is now being elucidated [161]. The estrogen-mediated activation of non-genomic (membrane) ERα signaling plays an important role in the protection against neointimal hyperplasia, a process that frequently occurs after the treatment of symptomatic atherosclerosis [142]. The non-genomic ERα signaling also mediates NO release and re-endothelialization [162]. It has also been shown that membrane ERα activation in endothelium reduces cardiac ischemic/reperfusion

(I/R) injury in mice [163]. However, the atheroprotective effects of E2 seems to be also ERα-independent. The genistein, an isoflavone with a 20-fold higher binding affinity to ERβ than to ERα, inhibits atherosclerosis development in low-density lipoprotein receptor (LDLR) KO mice [164]. Furthermore, Villablanca *et al.* reported the involvement of ERβ, but not ERα, in E2-mediated protection against atherosclerosis development [165], and selective ERβ activation by an agonist (8β-VE2) reduced atherosclerotic lesions in apolipoprotein E deficient (apoE KO) mice and it was associated with favorable modulation of vascular inflammation, as indicated by reduced serum levels of IL-1β and TNFα [166]. Furthermore, endothelial ERβ expression reduces ischemia/reperfusion-mediated oxidative burst and vascular injury [167], and treatment with an ERβ-selective agonist induces the release by macrophages of heat shock protein 27 (HSP27) [168], a protein that plays a protective role in atherosclerosis [169].

We found that, in ERβ-silenced HUVECs, E2 was unable to increase the levels of active Notch1, both in the presence or absence of TNFα, and unable to counteract TNFα-induced apoptosis [18]. The mechanisms by which E2, through ERβ, increases Notch1 levels need further investigation. It appears plausible that E2/ERβ, could function as a transcription factor for proteins involved in Notch1 processing [170]. Another possibility is that E2/ERβ inhibits the synthesis of Numb, or related proteins, involved in active Notch1 degradation [171], or induces glycogen synthase kinase 3 (GSK3) [172], which stabilizes active Notch1 [173]: further work is needed to test these hypotheses. A non-genomic effect of estrogen could also be involved in E2/ERβ-induced Notch1 activation. Specifically, since E2 modifies the membrane lipids profile [174] and modulates caveolae formation [175] which play a role in the assembly [176] and activity of γ-secretase [177], the possibility that E2/ERβ promotes the access of Notch1 to γ-secretase–rich membrane rafts should be explored. Furthermore, ERα dissociates from caveolin-1 in the presence of E2, whereas ERβ increases association with caveolin-1, thus activating p38 kinase [178], which is known to be involved in Notch activation [179]. It is also possible that, as shown in ER-positive breast cancer cells [180], in the endothelium E2 binding ER, in particular ERβ, could induce phosphorylation of c-jun NH2-terminal kinase (JNK), which it has been shown to stabilize presenilin, a subunit of the γ-secretase complex [53]. Possible molecular mechanisms involved in the crosstalk between estrogen and Notch in the endothelium are summarized in Figure 2.

It would be of interest then to determine if ERα and ERβ act in opposite ways in the regulation of Notch activation: the opposite effect of ERα and ERβ on Notch would explain findings of E2-

mediated activation of Notch1 in endothelial cells (mainly expressing ERβ) [18, 60] and inhibition in breast cancer cells MCF7 (only expressing ERα) [61].

7. Crosstalk between Notch and estrogen: relevance for hormone replacement therapy strategies

The evidence that estrogen has cardiovascular positive effects provided the basis for the use of hormone replacement therapy (HRT) to prevent cardiovascular disease in post-menopausal women. Nevertheless, the relationship between HRT and the prevention of cardiovascular disease, in particular CHD, remains controversial. Multiple analyses of prospective cohort studies, in the 1980s, indicated that HRT was associated with a lower risk of CHD in post-menopausal women [181]. In 1990s, three large prospective clinical trials, the Women's Health Initiative (WHI), the Heart and Estrogen/progestin Replacement Study (HERS) and the Women's International Study of long Duration Oestrogen after Menopause (WISDOM) studied the role of hormone treatment with horse hormone mixtures (conjugate equine estrogens, CEEs) alone or with progestin or androgens and medroxyprogesterone (MPA), in cardiovascular disease in post-menopausal women. The results of these clinical trials showed that the formulation of HRT used was not able to prevent cardiovascular disease, such as stroke, thromboembolic events, and CHD [182-184], and it increased the risk of breast cancer [183]. These results determined a rapid decrease in the use of HRT worldwide. Afterwards, further analyses of the study population suggested that the harmful or null HRT-mediated effects seen in the previous observational studies could be due to the fact that the enrolled women initiated HRT years after menopause (timing hypothesis) [185]. Several clinical studies have shown the plausibility of the timing hypothesis. A Cochrane meta-analysis shows that women that started HRT less than 10 years after the menopause had lower CHD risk, compared to placebo or no treatment [186]. A randomized controlled trial, KEEPS (Kronos Early Estrogen Prevention Study), administrated oral or transdermal estrogen, both with cyclic progesterone treatment, to women within 6-36 months after menopause, and evaluated the progression of atherosclerosis by measuring changes in carotid artery intima-media thickness (CIMT) and in markers of cardiovascular disease (CVD) risk. The study concluded that early HRT did not influence the progression of atherosclerosis, but improved some markers of CVD risk, such as blood pressure and lipid levels, thus supporting the timing hypothesis [187]. The Danish Osteoporosis Prevention Study showed that women receiving HRT triphasic estradiol and norethisterone acetate (women with uterus) or estradiol (women without

uterus) for 10 years, beginning shortly after menopause, have a reduced risk of heart failure and myocardial infarction, without increase in risk of cancer, venous thromboembolism, or stroke [188]. The Early versus Late Intervention Trial with Estradiol (ELITE) study confirmed that HRT was associated with less progression of subclinical atherosclerosis, measured as carotid-artery intima-media thickness (CIMT), when hormone therapy was initiated within 6 years, but not 10 years or more after menopause [189]. A possible explanation of the timing hypothesis is that in the early stages of the atherosclerotic process, estrogen plays a beneficial effect on the endothelium, delaying plaque formation. Conversely, in the later stages of the atherosclerotic process, estrogen causes plaque erosion or rupture, responsible for thrombosis and acute coronary events [190]. A study showing that E2 interferes with plaques formation in an atherosclerosis mouse model expressing only ERβ [165] and our study showing that E2 bound to ERβ reduces HUVECs apoptosis, an early marker of endothelial dysfunction leading to atherosclerosis [18], are both in agreement with the timing hypothesis. The strongest support to the concept of a limited window for E2-mediated protection against atherosclerosis comes from the work of Glisic *et al*., which studied the association of endogenous estradiol with carotid plaque composition, as well as with risk of stroke, in post-menopausal women with carotid atherosclerosis. They found that endogenous estradiol levels lead to plaque instability, by increasing lipid content and intraplaque hemorrhage, which can increase the risk of stroke in women with sub-clinical atherosclerosis [191]. Based on all these results, HRT should be used with caution among post-menopausal women, especially if they have been already diagnosed with atherosclerosis.

Preclinical and clinical studies have also shown that other than timing, the HRT effects may also vary based on formulation, dosage and route of administration [192]. Another critical point for the lack of efficacy, or switch from protective to harmful HRT vascular effect, could be the reduction of ERs expression due to aging and atherosclerosis [193]. An *in vitro* study shows that long-term exposure to E2 up-regulates ERα expression in endothelial cells, and down-regulates ERβ [194], which plays an important role in preventing endothelium dysfunction [18, 167, 195]. Consistently, the expression of ERβ is reduced in aging mice and it appears that SIRT1(Sirtuin 1)-mediated ERβ suppression in the endothelium contributes to vascular aging [196]. Continued efforts to develop an effective HRT have generated interest in the development of novel selective estrogen receptor modulators (SERMs) [197]. SERMS are able to bind to both ERα and ERβ with high affinity, and they have tissue-specific agonist, mimicking estrogen effect, or antagonist action. Tamoxifen is a SERM with predominant estrogen antagonist effect in the breast, and estrogen agonist activity in the bone [198] and uterus, in

which prolonged treatment increases the risk for endometrial cancer [199]. Clinical observations have shown that treatment with tamoxifen reduces the risk of CHD [200] and improves lipid profile, with reduction in total serum cholesterol and LDL-C (low-density lipoprotein cholesterol) [201, 202]. Raloxifene is a second generation SERM, and it is prescribed for prevention and treatment of osteoporosis in post-menopausal women, being agonist in bone tissue [203]. Raloxifene, like tamoxifen, has an ER antagonist action in breast, but without increasing the risk of endometrial cancer [204]. Furthermore, raloxifene has actions similar to estrogen on the cardiovascular system, in terms of improvement of endothelial function by the induction of vasodilation [205] and through NO synthesis in endothelial cells [206]. The evidence of the raloxifene-mediated cardioprotective effects has generated the basis for the Raloxifene Use for The Heart (RUTH) study, which however has observed a reduced risk of invasive breast cancer, but no effects on prevention of CHD [207]. Thus, more knowledge on the characteristics of the SERMs and the biological roles of ERα and ERβ in different tissues are needed for the specific treatment of various diseases, including CHD. Currently investigated SERMs target both of the ERs, but, as discussed in previous paragraphs, targeting just one subtype may lead to a more efficacious therapy with lower risk of side effects [34]. Our *in vitro* study shows that E2 protects against endothelial damage by binding ERβ, and not ERα, suggesting that specifically targeting this ER isoform may result therapeutic options to interfere with endothelial dysfunction, and consequent atherosclerosis, in post-menopausal women. Natural compounds that bind preferentially ERβ such as isoflavones protein present in soy, including S-equol, genistein, daidzein, and liquiritigenin, have been identified [208-210]. Isoflavone soy protein supplementation seems to reduce subclinical atherosclerosis in women at low-risk for cardiovascular disease, within 5 years of the onset of menopause [209]. More recently it has been shown that treatment with DPN, an ERβ-agonist, decreased cardiac fibrosis, restored angiogenesis, and significantly improved cardiac hemodynamic parameters in a mouse model of heart failure [211]. An ERβ-specific ligand could then be developed to protect against CHD, without concerns of increasing the risk of breast cancer, since it has been shown that ERβ, oppositely to ERα, has an anti-proliferative action on breast cancer cells [210, 212]. Noteworthy, the combination of tamoxifen and ERβ agonist seems to enhance anti-estrogen-mediated growth-inhibitory effects in human breast cancer cell lines [213]. In the last ten years, several studies on ERβ agonist and their potential use for the treatment of some post-menopausal symptoms, such as memory/cognitive decline and cerebral ischemia incidents/impact have been published [214-216]. Further investigations are required to assess the efficacy of these molecules in preventing CHD in post-menopausal women.

Based on our findings of E2 mediated activation of Notch1, which is necessary and sufficient for endothelial cells survival, it may be important consider that in women with an impaired Notch1 signaling, HRT could result unable to prevent endothelial dysfunction. Impairment of endothelial Notch signaling has been reported in dyslipidemic subjects [99], heart failure patients [120], and it could be also a side effect of natural [217, 218] and synthetic anti-cancer drugs [219], directed against the Notch pathway.

8. Crosstalk between Notch and estrogen: cardiotoxicity of anticancer treatment

Endocrine therapy is commonly used for the treatment of women with ER/progesterone receptor (PR)-positive breast cancer. In early stage of hormone-receptor-positive breast cancer, the current clinical practice guidelines recommend the use of SERMs, such as tamoxifen or aromatase inhibitors (AIs), such as anastrozole and exemestane, for post-menopausal women, both able to reduce cancer recurrence and improve survival [220]. Whereas tamoxifen active metabolites 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen interfere with the estrogen signaling by competing with estrogen binding to receptor, AIs block endogenous estrogen production by inhibiting the conversion of androgens to estradiol [221]. These agents are effective but intrinsic or ex novo resistance to both these agents do occur [222]. The identification of the changes underlying the resistance to apoptosis that occur in breast cancer that become unresponsive to anti-estrogen should help to overcome cancer progression and recurrence [223-225].

Our finding, showing that E2 enhances the active form of Notch1, which protects the endothelium against TNFα-induced apoptosis [18], suggests that estrogen deprivation, as in case of women on endocrine therapy, could lead to a reduction of the endothelial Notch1, thus predisposing to endothelial dysfunction. Consistently, Seruga *et al*. have shown that women with early breast cancer, who received AIs, have an increased hazard for CHD [226]. In accordance with this observation, in a cross-sectional study examining endothelial function among post-menopausal women with breast cancer on AIs treatment, there was a trend toward the increase in various biomarkers of hemostasis (plasminogen activator inhibitor-1, tissue-type plasminogen activator) and endothelial damage (VCAM-1), reduction in large and small artery elasticity and significant decrement in vascular tone compared with healthy post-menopausal women [227]. This effect seems to be caused by AIs but not tamoxifen, as reported by an observational study showing that women aged >55 years, diagnosed

with stage I-III breast cancer on AIs, have a higher risk of myocardial infarction compared with women treated with tamoxifen [228]. Additionally, a meta-analysis of randomized controlled trials has highlighted an increased risk of cardiovascular events in AIs-treated relative to tamoxifen-treated patients, and this result seems to be related to cardioprotective effects of tamoxifen rather than the harmful effects of AIs on the endothelium [229]. The molecular mechanism underlying the protective effect of tamoxifen against CHD has not yet been investigated, but based on our *in vitro* results, we can speculate that tamoxifen, by binding ERβ, could protect the vasculature endothelium by activating Notch1. Similarly, the low cardioprotective effect of raloxifene, compared to tamoxifen could be explained by its inability to increase endothelial Notch1.

Activation of the Notch pathway has been reported in every subtype of breast cancer [49, 230], including ER-positive breast cancer [231], and high level of Jagged1 have been shown to be indicators of poor prognosis [232] and progression to metastasis [233] in breast cancer patients. In ER-positive breast cancer cells, E2 inhibits the Notch pathway with an ER-dependent mechanism, and, conversely, estrogen deprivation causes reactivation of Notch, thus causing Notch-mediated breast cancer cells proliferation and survival [61]. The crosstalk between Notch and ER in breast cancer is bidirectional, as demonstrated by a study showing that Notch1 is able to activate ERα-dependent transcription, even in the absence of E2 [86]. These studies suggest the following hypotheses: i) the efficacy of anti-estrogen therapy, which would activate the pro-survival Notch, could be increased by inhibiting Notch signaling, and ii) constitutive activation of Notch could contribute to resistance to treatment with anti-estrogen. These two hypotheses are supported by studies in animal model of breast cancer [61, 234, 235] and by molecular analyses of tamoxifen–treated cell cultures established from biopsies of breast cancer [236], and of biopsies of breast cancer patients following anti-estrogen treatment [237]. A pilot phase 1 study conducted in early stage hormone responsive breast cancer patients to investigate Notch inhibitor (MK-0752) in combination with tamoxifen or letrozole showed that the treatment was safe and inhibited the expression of markers of apoptosis or cell cycle progression and metastasis in tumor biopsies [238]. Currently, Notch inhibitor LY3023414 is being investigated in combination with several anticancer agents, including fulvestrant and letrozole, in patients with advanced cancer, and in combination with abemaciclib (a CDK4/6 inhibitor) and letrozole, in patients with endometrial cancer. Additionally, a phase 2 study testing the combination of Sulindac, an inhibitor of Notch1 activation [239] and tamoxifen in patients with desmoid tumor is ongoing. A phase 1b study in post-menopausal ER+/PR+ stage I or II breast cancer testing Notch inhibitor RO4929097 in combination with letrozole was terminated because the drug become

unavailable. As far as clinical trials to test Notch inhibitors without anti-estrogens, three phase 1 studies on safety and tolerability of Notch inhibitor BMS906024 as single agent or in combination in advanced tumor and leukemias have been completed. One phase 2 study is ongoing to test AL101, a pan-Notch inhibitor in patients with adenoid cystic carcinoma bearing activating Notch mutations. Notch inhibitor RO4929097 has been tested as single agent or in combination in advanced tumor and leukemia in ten phase 2 studies that have been completed. More details on these trials can be found at www.clinicaltrials.gov.

When considering Notch inhibitors as novel therapeutic agents for cancer, it is important to consider the possible cardiotoxicity associated with endothelial Notch inhibition, which could cause endothelial dysfunction [18, 99, 107, 108] and defective expansion of the cardiac vasculature and impairment of fatty acid transport to cardiomyocytes [240]. Notch inhibition in the endothelium would also cause VSMCs loss, thus affecting vascular integrity, as shown in mice with global Akt2KO and endothelial-specific Akt1 deletion in hearts [241]. Given the major role of Notch in the estrogen-mediated protection of the vascular wall, the combined treatment with AIs and Notch inhibitors could have even more deleterious effects on the vascular system, in comparison to the effects of each agent alone. The effects of AIs and Notch inhibitors in breast cancer cells and in the vasculature are summarized in Figure 4.

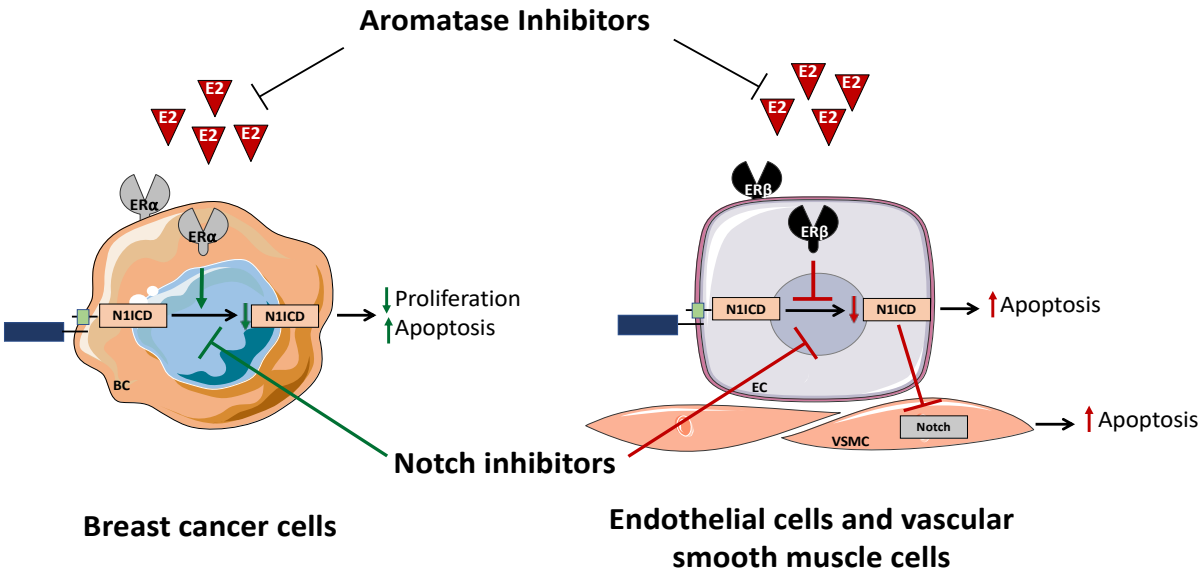

FIGURE 4. Estrogen inhibition has opposite effects on Notch signaling in breast cancer cells (BCs) and in the vasculature. In breast cancer cells, estrogen deprivation causes Notch1 activation, resulting in increased cancer cells proliferation and survival. Therefore the combined treatment with Notch1 inhibitors would be

necessary for decreased proliferation and increased apoptosis of cancer cells. In contrast, in endothelial cells (ECs), estrogen deprivation determines the reduction of Notch1 activation, which causes endothelial apoptosis. The reduction of endothelial Notch determines an inhibition of the Notch pathway in the adjacent vascular smooth muscle cells (VSMCs), which undergo apoptosis. The use of Notch inhibitors may exacerbate these vascular effects.

9. Conclusions and future perspectives

Estrogen regulates a wide set of cellular functions under physiological and pathological conditions, including cancer and cardiovascular disease. The role of estrogen in promoting cancer onset and growth in estrogen-responsive tissues (i.e. epithelium of mammary gland) has been elucidated and anti-estrogen is a "success story" in our quest for cancer drugs, since women with ER-positive breast cancer treated with tamoxifen for 5 years have a reduced risk of recurrence and of related mortality [242]. On the contrary, the molecular pathways underlying the protective effects of estrogen in the cardiovascular system, demonstrated by many studies, are still elusive, and we are still not able to use estrogen to prevent the onset and/or the progression of diseases, such as CHD that, in Europe and developed countries, kills seven times more than breast cancer [243].

Evidence has been accumulating of crosstalk between estrogen and the Notch pathway, a major determinant of cell fate. In fact, it has been shown that E2 inhibits the Notch pathway in breast cancer cells and neurons, while activating it in endothelial cells. Furthermore, Notch acts as a regulator of E2 receptor transcriptional activity in breast cancer cells. The data obtained so far may represent only the tip of the iceberg of the complex regulation of Notch by steroid hormones. First, there are many other cell types that respond to steroid hormones with an active Notch signaling, in which this crosstalk has not been investigated yet and, second, we are still beginning to understand the molecular details underlying this regulation. The molecular mechanism by which E2 modulates Notch are still unknown and it needs to be established why E2 inhibits the Notch pathway in breast cancer cells and neurons while activating it in endothelial cells. Additionally, the role of each ER in the context of Notch regulation needs to be established. Endothelial cells express equal or higher levels of ERβ in comparison to ERα [195, 244, 245], whereas breast cancer cells express mainly ERα [60, 246]. As already discussed in this review, it is possible that that the two receptors have opposite activities on Notch processing, with ERβ and ERα activating and inhibiting Notch1, respectively.

The identification of specific SERMs able to either activate or inhibit Notch could have tremendous impact on the development of a novel HRT: based on existing data, it is possible to speculate that a SERM able to selectively bind ERβ may exert a positive action on the endothelium without activating

ERα and providing a proliferation stimulus in breast cells. Similarly, an ERβ-specific SERM, by activating Notch1 only in the endothelium, could be used to limit the cardiotoxicity observed in breast cancer patients treated with anti-estrogen.

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