



**HAL**  
open science

## **A historical view of estrogen effect on arterial endothelial healing: From animal models to medical implication**

Rana Zahreddine, Morgane Davezac, Melissa Buscato, Natalia Smirnova, Muriel Laffargue, Daniel Henrion, Marine Adlanmerini, Françoise Lenfant, Jean-François Arnal, Coralie Fontaine

► **To cite this version:**

Rana Zahreddine, Morgane Davezac, Melissa Buscato, Natalia Smirnova, Muriel Laffargue, et al.. A historical view of estrogen effect on arterial endothelial healing: From animal models to medical implication. *Atherosclerosis*, 2021, 338, pp.30-38. 10.1016/j.atherosclerosis.2021.10.013 . hal-03758863

**HAL Id: hal-03758863**

**<https://hal.science/hal-03758863v1>**

Submitted on 5 Jan 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

## **A historical view of estrogen effect on arterial endothelial healing: From animal models to medical implication**

Rana Zahreddine<sup>1</sup>, Morgane Davezac<sup>1</sup>, Melissa Buscato<sup>1</sup>, Natalia Smirnova<sup>1</sup>, Muriel Laffargue<sup>1</sup>, Daniel Henrion<sup>2</sup>, Marine Adlanmerini<sup>1</sup>, Françoise Lenfant<sup>1</sup>, Jean-François Arnal<sup>1</sup> and Coralie Fontaine<sup>1</sup>

<sup>1</sup> I2MC, Institut National de la Santé et de la Recherche Médicale (INSERM) UMR1297, University of Toulouse3, Toulouse, France.

<sup>2</sup> MITOVASC Institute, CARFI facility, INSERM U1083, UMR CNRS 6015, University of Angers, France.

### **# Corresponding author**

Coralie Fontaine  
INSERM/UPS UMR 1048 - I2MC  
**Institut des Maladies Métaboliques et Cardiovasculaires**  
1 avenue Jean Poulhès, BP 84225  
31432 Toulouse Cedex 4  
E-mail: coralie.fontaine@inserm.fr

## **ABSTRACT**

Endothelial barrier integrity is required for maintaining vascular homeostasis and fluid balance between the circulation and surrounding tissues. In contrast, abnormalities of endothelial cell function and loss of the integrity of the endothelial monolayer constitute a key step in the onset of atherosclerosis. Endothelial erosion is directly responsible for thrombus formation and cardiovascular events in about one-third of the cases of acute coronary syndromes. Thus, after endothelial injury, the vascular repair process is crucial to restore endothelial junctions and rehabilitate a semipermeable barrier, preventing the development of vascular diseases. Endothelial healing can be modulated by several factors. In particular,  $17\beta$ -estradiol (E2), the main estrogen, improves endothelial healing, reduces neointimal accumulation of smooth muscle cells and atherosclerosis in several animal models. The aim of this review is to highlight how various experimental models enabled the progress in the cellular and molecular mechanisms underlying the accelerative E2 effect on arterial endothelial healing through the estrogen receptor (ER)  $\alpha$ , the main receptor mediating the physiological effects of estrogens. We first summarize the different experimental procedures used to reproduce vascular injury. We then provide an overview of how the combination of transgenic mouse models impacting ER $\alpha$  signaling with pharmacological tools demonstrated the pivotal role of non-genomic actions of ER $\alpha$  in E2-induced endothelial repair. Finally, we describe recent advances in the action of selective estrogen receptor modulators (SERMs) on this beneficial vascular effect, which surprisingly involves different cell types and activates different ER $\alpha$  subfunctions compared to E2.

## 1. Introduction

The endothelium is a monolayer of endothelial cells (ECs) regulating numerous physiological and pathological functions that ensure permeability and thromboresistant functions. The structure and function of endothelial cells prevent vascular diseases by acting as a physical barrier to atherogenic particles and by becoming an activated layer of migrating cells to repair injuries. Hypertension, hypercholesterolemia/hyperlipidemia, hyperglycemia in diabetes, and smoking are considered to be injurious to the endothelium and induce endothelial cell dysfunctions. Endothelial injury is also a consequence of coronary atherosclerosis treatment by percutaneous transluminal coronary angioplasty <sup>1</sup>, viral infection <sup>2</sup>, and radiotherapy used to treat a variety of cancers <sup>3</sup>. Endothelial barrier dysfunction is a key initiating event of various vascular diseases, including atherosclerosis and in-stent restenosis <sup>4-6</sup>. Indeed, endothelial denudation leads to complications associated with inflammation, increased vascular permeability to pro-atherogenic particles, and thrombosis. When endothelial structural integrity is disrupted, its repair involves restoration of a functional endothelial monolayer and reestablishment of the endothelial junctions to reform a semipermeable barrier.

A large body of evidence indicates that estrogens exert beneficial actions on the endothelium <sup>7, 8</sup>. Indeed, in addition to its well-known role on sexual development and reproduction, estrogens exert many beneficial actions on the vascular wall. In particular, chronic estrogen treatment enhances endothelial function in a number of vascular beds through structure adaptation and blood flow control in the uterine vascular system during pregnancy and endothelium-dependent vasodilation in the peripheral arteries. Estrogens also exert many beneficial effects against arterial diseases, including atheroprotection <sup>9</sup>, prevention of neointimal hyperplasia <sup>10</sup> and acceleration of endothelial healing in response to arterial injury <sup>11</sup>. This vascular protection conferred by estrogens results in a lower incidence of

cardiovascular diseases in pre-menopausal women compared to men. These different beneficial vascular effects of estrogen are mediated by estrogen receptor (ER) $\alpha$ <sup>12</sup>. Prevention of atheroma, hypertension and neointimal hyperplasia relies on nuclear ER $\alpha$ , whereas ER $\alpha$  membrane-initiated signaling (ER $\alpha$ MISS) mediates NO production and acceleration of endothelial healing in response to E2<sup>13</sup>. We propose here to summarize our current understanding of the beneficial vascular ER $\alpha$  actions focusing on arterial endothelial injury in response to E2 and various selective ER modulators.

### **1. Experimental models of endothelial healing**

Several experimental models have been developed to reproduce vascular injury and the process of re-endothelialization. In animal studies, endothelial denudation has been widely adapted for this purpose. The procedure can be carried out using small caliber guide-wires for small arteries or balloon catheters for larger arteries such as the femoral artery or carotid artery in rats, rabbits, pigs or monkeys<sup>14, 15</sup>. Using this mechanical injury induction model, significant insights have been gained regarding the cellular responses engaged during and after vascular injuries. Then, the use of genetically modified mice has significantly contributed to the investigation of the underlying molecular mechanisms. The removal of the endothelium in the mouse carotid artery with a flexible wire was first described in 1993<sup>16</sup>. However, the specific evaluation of arterial re-endothelialization appears to be difficult with this technique due to the shorter length and diameter of the vessel. In 1997, Carmeliet *et al.* developed a novel model of electric injury of the mouse femoral artery that consisted of destroying endothelial, smooth muscle, and adventitial cells and enabling subsequent quantification of both arterial neointima formation and re-endothelialization<sup>17</sup>. Then, Brouchet *et al.* adapted this model of electric injury to carotid arteries to allow the precise study of arterial reendothelialization<sup>11</sup>. Endovascular (mechanical) and perivascular (electric) injuries are similar in their efficiency to destroy the endothelium, but they differ significantly

in their effects on the underlying cell populations in the vessel wall (**Figure 1**). Endovascular injury preserves most of the adventitial cells as well as the medial smooth muscle cells (SMCs) whereas in the perivascular model, the media does not show any sign of regeneration during all the time of re-endothelialization. In both models the intima is regenerated after 1 week<sup>18</sup>. Evans blue is commonly used to quantify the re-endothelialization process but *en face* confocal microscopy allows a more precise visualization of the regenerated endothelium, as well as imaging several molecular key actors<sup>18</sup>. Using this approach, the injury limit can be precisely defined in the perivascular model at variance with the endovascular model<sup>18</sup> (**Figure 1**). In contrast to the mouse femoral artery, perivascular or endovascular injuries at carotid arteries do not induce neointimal hyperplasia. Differences in these two arterial beds are likely attributable to the difference in the vessel structures, *i.e.* to the SMC phenotype, but also potentially to EC-SMC interactions in these different localizations, demonstrating the difficulties of modelling the vascular physiopathology.

Cellular and molecular mechanisms controlling endothelial healing and the recovery of endothelial barrier function have been reviewed thoroughly by others<sup>19</sup> but remain incompletely understood and still constitute an emerging area of research. Recently, a new model of arterial denudation of abdominal aorta was developed to allow endothelial gene expression profiling and transcriptional signatures associated with vascular regeneration using next-generation RNA sequencing (RNAseq)<sup>20</sup>. Thanks to this approach, the authors identified four clearly distinct stages of endothelial regeneration that, with the exception of proliferation, have little overlap with the process of vascular expansion known as angiogenesis. Genetic lineage tracing studies provide strong evidence that the tissue-resident EC population is the major source of regenerating ECs through migration and proliferation as >95% of the regenerative ECs are derived from resident ECs<sup>21</sup>. Both bone marrow-derived and circulating progenitor cells rarely engraft and differentiate into ECs. Although circulating cells are not a

major source of regenerating ECs, these cells may favour EC regeneration and vascular repair through the release of reparative paracrine factors that act on the vascular endothelium<sup>22</sup> or conversely lead to re-endothelialization defect due to pro-inflammatory cytokine secretion<sup>23</sup>. Recently, Lupieri *et al.* generated an *ex vivo* de-endothelialization model using wire to analyse endothelial recovery independently of immune cells<sup>23</sup> (**Figure 1**). In this model, de-endothelialization was performed *ex vivo* and endothelial healing was evaluated after 5 days of culture in an EC-specific medium.  $\alpha$ -SMA immunostaining and contractile response to KCl revealed that SMCs are viable and functional at the end of the procedure<sup>24</sup>.

## **2. A historical view of E2 effect on arterial endothelial healing**

The facilitating effect of the main estrogen, 17 $\beta$  estradiol (E2), on re-endothelialization was first reported in ovariectomized female rats after balloon denudation<sup>25, 26</sup> to the acceleration of endothelial anatomic recovery, estrogen administration was associated with functional recovery and decrease of intimal hyperplasia<sup>25-27</sup> (**Figure 2**). Then, the development of methods to analyse endothelial healing in mice allowed to gain mechanisms insights into E2-induced re-endothelialization acceleration. E2 accelerates endothelial healing after both endovascular or perivascular carotid injuries in mice. Indeed, both basal re-endothelialization and the accelerative effect of E2 were similar in the two models<sup>18</sup>. The use of perivascular injury allowed to precisely define the injury limit (**Figure 1**) and demonstrated that proliferation of uninjured endothelial cells precedes migration<sup>18</sup>. In addition, estrogen administration was associated with decreased intimal hyperplasia after endovascular injury of femoral arteries<sup>28</sup>. However, the contribution of the E2-induced re-endothelialization on the prevention of neointimal hyperplasia remains unclear and could be dependent on the anatomic origin (elastic *versus* muscular artery) of the vessel and/or the cholesterolemic/inflammatory context<sup>28</sup>. Direct inhibition of smooth muscle proliferation by E2 was also reported to contribute to the prevention of neointimal hyperplasia<sup>29</sup>. Numerous *in vitro* approaches also

concluded to demonstrate that E2 increases migration, proliferation of ECs and prevents cell death. These E2 effects have been particularly studied in human umbilical vein endothelial cells (HUVECs)<sup>30, 31, 32</sup> but also using bovine aortic endothelial cells (BAECs)<sup>33</sup> and human aortic endothelial cells (HAECs)<sup>34</sup>, suggesting a conserved estrogen action on endothelial healing between species.

Estrogen actions can be mediated by two nuclear receptors, ER $\alpha$  and ER $\beta$ , and by the G protein-coupled estrogen receptor (GPER). Mice deficient for ER $\alpha$  or ER $\beta$  specifically allowed to demonstrate that ER $\alpha$  mediates most of the vascular effects of estrogens<sup>12</sup>. In particular, E2 accelerates re-endothelialization in ovariectomized female ER $\beta$  knockout mice but not in ER $\alpha$  knockout female mice<sup>11</sup>. Then, mouse models with cell-specific loss of function of ER $\alpha$  (using the Cre/Lox strategy) indicated that ER $\alpha$  expression is required in both endothelium and hematopoietic cells for the accelerative effect of E2 on re-endothelialization<sup>35</sup>. However, which cell type and/or circulating factors are required from the hematopoietic compartment remains unknown. More recently, GPER emerged as the third receptor for estrogens and has been reported to mediate several beneficial vascular effects<sup>36, 37</sup>. However, these results are still controversial due to conflicting data obtained with different mouse models targeting this receptor<sup>38</sup>. Identification of GPER selective agonists and antagonists such as G-1, G15, and G36, allowed to elucidate more specially its biological effects. In particular, the use of G-1 *in vivo* demonstrated that activation of GPER induced endothelium-dependent vasodilation and NO production<sup>39</sup>. *In vitro*, GPER agonist G-1 was reported to increase proliferation and migration of ECs<sup>40, 41</sup> suggesting a potential role of this receptor on endothelial repair. Different experimental approaches allowed to show that GPER can act as an autonomous receptor but can also interact with ERs. To which extent, E2 effect on endothelial healing is dependent on GPER and ER $\alpha$  cross-talk remains unanswered. Thus, evaluating directly the impact of GPER1 agonist G-1 on endothelial healing *in vivo* would be

of great interest. In addition, biochemistry and cell biology analysis could be used to explore potential interaction between GPER and ER proteins in endothelial cells.

### **3. Membrane-initiated signalling, but not nuclear ER $\alpha$ , is involved in endothelial healing in response to E2.**

As a member of the nuclear receptor superfamily, ER $\alpha$  is composed of six modular domains, namely A to F, and is primarily considered as a ligand regulated transcription factor <sup>42</sup>. Schematically, binding of the full agonist E2 to its ligand binding domain (LBD) leads to ER $\alpha$  binding to DNA through its DNA binding domain (DBD) and recruitment of transcriptional coactivators to the activation functions AF-1 and/or AF-2 of ER $\alpha$  initiating transcription and RNA elongation of the target genes <sup>43</sup> (**Figure 3**). Besides these nuclear actions, estrogens also mediate rapid signalling through a pool of ER $\alpha$  localized at the plasma membrane <sup>44</sup> leading to membrane-initiated steroid signals (MISS) (**Figure 3**). In 1997, two different laboratories reported independently the rapid regulation of nitric oxide (NO) release by estrogens in endothelial cells in an ER-dependent manner <sup>45, 46</sup>. Short-term effects of estrogen on the vasculature were also highlighted in humans since acute ethinylestradiol treatment was shown to attenuate the abnormal coronary vasomotor responses to acetylcholine in postmenopausal women <sup>47</sup>. In 1999, Shaul and coworkers demonstrated that these short-term effects of estrogen are mediated by ER $\alpha$  functioning in a novel, non-genomic manner through MAP kinase-dependent mechanisms <sup>48</sup>. Membrane ER $\alpha$  has emerged as the primary mediator of rapid E2 responses <sup>49, 50</sup> and fractionation of endothelial cell plasma membranes revealed that ER $\alpha$  protein localizes in caveolae, where it induces stimulation of eNOS in response to E2 <sup>51, 52</sup>. Human Cystein-447 (or Cys 451 in mice) was then identified as the site of palmitoylation necessary for plasma membrane association of the receptor <sup>53, 54</sup>. Different studies reported that ER $\alpha$  is anchored at the cell membrane by scaffold proteins such as caveolin-1 promoting membrane association of ER $\alpha$  and bridging the receptor to other

membrane proteins, which then can activate MISS, including G protein-dependent signaling in endothelial cells <sup>55, 56</sup>. In particular, striatin directly binds to amino acids 176-253 of ER $\alpha$  and serves as a scaffold for the formation of an ER $\alpha$ -G $\alpha$ i complex <sup>55</sup>. Overexpression of a peptide (amino acids 176–253 of ER $\alpha$ ) that disrupts ER $\alpha$ /striatin complex formation blocks estrogen-induced rapid activation mitogen-activated protein kinase, Akt kinase, and eNOS, but has no effect on ER-dependent regulation of an estrogen response element-driven reporter plasmid <sup>55</sup>. In addition, disruption of ER-striatin interactions eliminates the ability of estrogen to stimulate cultured endothelial cell migration <sup>33</sup>. Direct interaction between ER $\alpha$  and G $\alpha$ i has also been reported with amino acids 251–260 of the receptor <sup>57</sup> and some point mutations within this domain, such as R256A, K257, D258A and R260A, all abrogate the activation of eNOS and ERK phosphorylation in response to E2 *in vitro* <sup>58</sup>.

Several knock-in mouse models with selective loss of function of membrane ER $\alpha$  action were developed to investigate the role of membrane-initiated ER $\alpha$  actions *in vivo*: (1) the disrupting peptide mouse (DPM), in which rapid signaling is blocked by overexpression of a peptide that prevents ER-striatin interaction <sup>33</sup>, (2) the C451A-ER $\alpha$  mouse, which is not able to localize ER $\alpha$  to the plasma membrane due to the point mutation of its palmitoylation site <sup>59</sup>, and (3) the R264A-ER $\alpha$  mouse in which ER $\alpha$ -G $\alpha$ i interaction is abrogated <sup>60</sup> (**Figure 4**). These 3 mouse models conclusively demonstrated the importance of ER $\alpha$ MISS on endothelial healing since *in vivo* experiments demonstrated loss of estrogen-mediated protection against vascular carotid injury in DPM, C451A-ER $\alpha$  and R264A-ER $\alpha$  mouse models following electric carotid artery injury <sup>33, 59, 60</sup>. By contrast, acceleration of re-endothelialization in response to E2 was totally preserved in complementary mutant mouse models lacking nuclear ER $\alpha$  actions through inactivation of its activation function AF-1 (designated ER $\alpha$ -AF1<sup>0</sup>) or AF-2 of ER $\alpha$  (designated ER $\alpha$ -AF2<sup>0</sup>) <sup>61, 62</sup>. In parallel, pharmacological tools have been designed to specifically activate membrane, but not nuclear,

ER $\alpha$  (**Figure 5**). To this aim, estradiol (E2) was first linked to a non-degradable poly(amido)amine (PAMAM). This estrogen-dendrimer conjugate (EDC) is excluded from the nucleus, and is highly effective in stimulating non-nuclear signalling but ineffective in stimulating nuclear ER target gene expression <sup>63</sup>. Secondly, pathway preferential estrogens (PaPEs) were also developed to activate ER $\alpha$ MISS over the nuclear pathway through a process of structural alteration of estrogenic ligands. PaPEs were designed to preserve their essential chemical and physical features but have greatly reduced binding affinity for ERs <sup>64</sup>. *In vitro*, EDC was shown to stimulate endothelial cell proliferation and migration *via* ER $\alpha$  <sup>27</sup>. In mice, E2, EDC, and PaPE equally accelerated carotid artery endothelial healing after perivascular injury in an ER $\alpha$  dependent manner <sup>27 64</sup>. Furthermore, acceleration of endothelial healing repair by EDC was also efficient in ER $\alpha$ -AF2<sup>0</sup>, further demonstrating that activation of ER $\alpha$ MISS is necessary and sufficient to induce this beneficial vascular effect. All these approaches concur to demonstrate that activation of endothelial cell migration by E2 is mediated by plasma membrane-associated ER $\alpha$ . Even if the underlying molecular mechanism of ER $\alpha$ MISS-induced endothelial healing is not totally understood, different pathways and proteins have been identified to play a major role in this process.

- As highlighted by the abrogation of endothelial healing in response to E2 in the R264A-ER $\alpha$  mouse model, the direct ER $\alpha$ -G protein interaction appears to be crucial to the functional coupling of the receptor to kinase cascades and the resulting downstream endothelial response <sup>27, 60</sup>. The inhibition of ER-mediated eNOS activation by pertussis toxin further demonstrates the crucial role of G $\alpha$ i to induce membrane-initiated steroid signals (MISS) <sup>27, 56</sup>. More recently, ER $\alpha$ -G $\alpha$ i interaction has also been involved in the rapid release of endothelial hydrogen sulfide (H<sub>2</sub>S) by E2, known to induce vasorelaxation, stimulate endothelial cell-related angiogenic properties, and protect against atherosclerosis <sup>65</sup>. In addition, through activation at the

cell membrane of  $G\alpha_{13}$ ,  $ER\alpha$  recruits the RhoA/ROCK-2/moesin cascade, leading to dynamic rearrangements of the actin cytoskeleton and cell movement which may contribute to the acceleration of endothelial migration <sup>66</sup>.

- The use of endothelial NO synthase (eNOS)-deficient mice also demonstrated the crucial role of this enzyme in the acceleration of endothelial healing after perivascular or endovascular carotid artery injuries <sup>67</sup>. Surprisingly, although E2 was shown to activate eNOS and thus to increase NO production in endothelial cells <sup>68</sup>, the stimulatory effect of E2 on re-endothelialization was abrogated in *eNOS*<sup>-/-</sup> mice, but not altered in *eNOS*<sup>+/+</sup> mice following pharmacological inhibition of eNOS enzymatic activity by *N*-nitro-L-arginine methyl ester (L-NAME) <sup>67</sup>. These data revealed an original and unexpected role of eNOS, the presence but not its enzymatic activity of which appears to be determinant for E2-induced re-endothelialization. In addition, eNOS expression was shown to be particularly important in the bone marrow to mediate E2 effect on re-endothelialization as E2 effect was restored in chimeric *eNOS*<sup>-/-</sup> mice grafted with wild-type bone marrow, although with a lesser magnitude than wild-type mice <sup>35</sup>.
- Cross-talk with growth factors have been also proposed to participate in the accelerative action of E2 on re-endothelialization. In particular, involvement of vascular endothelial growth factor (VEGF) in this process was evidenced both *in vitro* and *in vivo* with the use of a polyclonal anti-VEGF antibody. Indeed, the proliferative effect of E2 on cultured fetal bovine aortic endothelial cells (FBAEC) *in vitro*, as well as the effect of E2 on re-endothelialization after carotid injury in rats, was abolished by anti-VEGF antibodies <sup>69</sup>. The use of chimeric mice targeting fibroblast growth factor-2 (FGF2) highlighted this growth factor as another important bone-marrow-derived actor involved in this process <sup>70</sup>.

- cAMP induces some vascular protective effects on ECs such as preservation of barrier function, inhibition of inflammation and increase of proliferation/migration<sup>71, 72</sup>. In particular, Fantidis et al. reported that cAMP promotes reendothelization and attenuates SMCs proliferation using a model of carotid balloon injury. Interestingly, E2 modulates cAMP through ER $\alpha$ MISS<sup>73, 74</sup> but a direct link between these two signalling in the endothelial healing process has not been reported yet.

ER $\alpha$ MISS has particularly been described in endothelial cells<sup>75</sup> and its crucial role in endothelial healing is clearly established. However, multiple questions also remain unanswered as the underlying molecular mechanism, the topology of plasma membrane-associated ER, and how receptor trafficking to the plasma membrane *versus* nucleus is regulated. It is only through further investigations of this unique aspect of endocrinology that we will increase our understanding of how ER $\alpha$ MISS influences the physiology and the pathophysiology of the endothelium.

### **5. SERMs also accelerate endothelial healing**

ER $\alpha$  also binds to various other molecules than E2 with agonist, mixed agonist–antagonist, or full antagonist properties. Indeed, “selective estrogen receptor modulators” (SERMs) display distinct pharmacological effects, depending on the target tissues. These drugs have been intensively studied over the past decade and are widely used for the treatment of hormone responsive breast cancers, osteoporosis and for postmenopausal women’s health. In particular, tamoxifen, the prototypical SERM, is a nonsteroidal antiestrogen used as an adjuvant therapy to treat breast cancers and to prevent the occurrence of breast tumors in healthy women. In contrast to breast tissues, tamoxifen has significant estrogen-like properties in uterine, bone and vascular tissues. Tamoxifen induces specific changes in ER $\alpha$  conformation that are distinct from those induced by binding of naturally occurring forms of E2 to the receptor, and

thus selectively recruits and interacts with coactivators and corepressors. Blockade of ER $\alpha$ AF-2 activity is thought to be the major mechanism of antagonistic actions of tamoxifen<sup>76</sup>. *In vitro* and *in vivo* experiments concur to demonstrate that this agonist activity is entirely mediated by the other activation function (AF) named ER $\alpha$  AF-1. The effect of tamoxifen on ER $\alpha$ MIS remains, however, poorly described. Raloxifene is another SERM currently used in clinic to prevent and treat osteoporosis, as it is an ER agonist in bone but an antagonist in uterus and breast. Both compounds have beneficial effects on lipids, they reduce markers of cardiovascular risk, and are associated with lower rates of cardiovascular events in humans<sup>77</sup>.<sup>78</sup>. Both are also favourable in lipid-induced experimental atherosclerosis<sup>79, 80</sup>. After the publication of the results of the Women's Health Initiative citing adverse effects of conjugated equine estrogens/ medroxyprogesterone acetate association on cardiovascular risk, research on SERMs to potentially replace standard hormone therapy for the management of menopausal symptoms has emerged<sup>81</sup>. Although bazedoxifene (in combination with Premarin for the treatment of osteoporosis and vasomotor symptoms associated with menopause) and ospemifene (for the treatment of dyspareunia associated with menopause) have been approved by the Food and Drug Administration (FDA), others (such as idoxifene and levormeloxifene) have failed phase III clinical trials because of adverse impacts on pelvic floor function, with increased incidence of urinary incontinence and pelvic organ prolapse.

Experiments of balloon denudation in carotid surgery from ovariectomized rats revealed that, as E2, idoxifene inhibits SMCs proliferation through, at least in part, its accelerative action on endothelial recovery<sup>82</sup>. Several years later, another team investigated whether tamoxifen, raloxifene, and ospemifene are able to induce this estrogen's beneficial effect on the ovariectomized rat vascular wall<sup>83</sup>. They highlighted that *in vivo* treatment with tamoxifen and ospemifene post-denudation injury enhances re-endothelialization and inhibits neointima formation as estrogen, while raloxifene's long-term effects are weaker. Importantly, this study

mentions that early intervention with E2 and SERM is of particular importance in preventing the injury response. The effect of bazedoxifene on the vascular endothelium has not been explored *in vivo*, but *in vitro* experiments demonstrated that as E2, bazedoxifene induces proliferation of human umbilical artery endothelial cells<sup>84</sup>.

Molecular mechanisms behind SERMs' actions are still poorly understood. Recently, comparison of perivascular *versus* carotid endovascular injury (**Figure 2**) allowed to demonstrate that in contrast to E2, tamoxifen effect required the presence of the underlying medial smooth muscle cells (SMCs). Indeed, whereas E2 and tamoxifen both accelerate endothelial healing after endovascular injury of the carotid in mice, tamoxifen fails to accelerate endothelial healing after perivascular electric injury and even antagonizes this effect of E2 mediated by ER $\alpha$ MISS in endothelial cells<sup>24</sup>. Cre-Lox models (*Tie2Cre ER $\alpha$ <sup>lox/lox</sup>* and  *$\alpha$ SMACre ER<sup>T2</sup>ER $\alpha$ <sup>lox/lox</sup>* mice) reinforced this finding, since the use of these mice allowed to demonstrate that tamoxifen action is mediated by ER $\alpha$  in smooth muscle cells, whereas E2 action relies on endothelial/hematopoietic ER $\alpha$ <sup>24</sup>. This study highlighted an important contribution of EC-SMC cross-talk necessary for the tamoxifen-mediated endothelial regenerative effects after vascular injury. Several factors are implicated in EC-SMC communications including chemokines, cytokines, and microRNAs. Indeed, there is an increasing literature which reports the role of paracrine actions exerted by smooth muscle cells on re-endothelialization<sup>19</sup>. SMCs respond to injury with functional changes including proliferation, migration, and secretion of chemokines and cytokines that contribute to vascular repair. Recently, it was reported that SMCs promote re-endothelialization through CXCL7-mediated recruitment of ECs from uninjured endothelium<sup>85</sup>. Lupieri *et al.* also demonstrated that SMCs prevent endothelial healing through CXCL10<sup>23</sup>. Thus, depending on the context, SMC-derived paracrine factors can accelerate or slow down endothelial healing. Another recent study using SMC-EC co-cultures, revealed that SMC-EC contact is required for EC

regeneration after wire-induced carotid artery injury through the activation of Notch1 by bone morphogenic protein receptor 2 (BMP2) in both ECs and SMCs<sup>86</sup>. Future studies will be necessary to understand the specific factor(s) underlying the paracrine effect that allows endothelial healing in response to tamoxifen. In addition to different cellular cell types targeted by E2 and tamoxifen, the use of mice harbouring mutated specific functions of ER $\alpha$  revealed that tamoxifen acts through nuclear ER $\alpha$  activation, and more precisely through the AF-1 activation-function to accelerate endothelial healing, contrasting with ER $\alpha$ MISS-mediated action in response to E2<sup>24</sup>. This finding leads to revisit the mechanisms of action of tamoxifen and highlights the necessity to understand the mechanisms of each SERM to determine if their actions on vasculature do involve mechanisms similar to E2, tamoxifen, or others. It should be noted that SERM do not act only on ER $\alpha$  but are also able to modulate GPER<sup>87</sup>. In particular, in cancer cells, several SERM such as tamoxifen and raloxifene were reported to activate GPER, and thus this receptor could also potentially play a role in response to these drugs in the arterial wall<sup>88,89</sup>.

In addition to synthetic molecules, 27-hydroxycholesterol (27-HC), the most prevalent oxysterol in circulation, has been proposed as an endogenous SERM with actions reported in atherosclerosis, osteoporosis, breast cancers, and neural degenerative diseases<sup>90,91</sup>. 27-HC inhibits nuclear ER transcription and ERMISS due to direct competitive binding of 27-HC to ERs. In particular, the antagonistic role of 27-HC on endothelial healing was demonstrated in ovariectomized mice knockout for the gene encoding the catabolic enzyme CYP7B1 (*cyp7b1*<sup>-/-</sup>), which have elevations in 27-HC in both the circulation and in tissues, but entirely normal plasma cholesterol and triglyceride levels<sup>92</sup>. In these mice, E2 did not promote re-endothelialization after perivascular injury and attenuated estrogen-related atheroprotection<sup>93</sup>. This 27-HC effect is mediated by ER $\alpha$  as revealed by the use of ER $\alpha$  deficient mice<sup>94</sup>. *In vitro*, in primary bovine aortic endothelial cells, in contrast to E2, which blunts NF- $\kappa$ B

activation, 27-HC stimulates NF- $\kappa$ B activation <sup>94</sup>. 27-HC is the most abundant circulating oxysterol and its serum concentration is closely correlated with plasma cholesterol concentration. Given the high prevalence of hypercholesterolemia, it is important to consider the roles of this major cholesterol metabolite on ER signalling.

Other natural estrogens than E2 are produced during pregnancy ((estriol (E3) and estetrol (E4)) and at menopause (estrone (E1)), which display specific activities on ERs due to differential receptor affinity, metabolism (half-life) and subfunction activation <sup>95</sup>. The impact of these estrogens on the arterial wall has been poorly described. E4 antagonizes ER $\alpha$ MSS action on the acceleration of endothelial healing in a perivascular injury model of the carotid <sup>96</sup> but the action of E4 on other experimental models is lacking. This point is of clinical interest since E4 has been recently approved as a new oral contraceptive and is currently developed as a new treatment for menopause.

## **6. Conclusion and future directions**

The main estrogen, E2, exerts many beneficial effects on arteries <sup>12</sup>. Among them, acceleration of endothelial healing is of particular importance since endothelial dysfunction and denudation are considered as the first step in atherosclerosis. However, most of these studies have been performed in young mice and cardiovascular disease remains an age-related pathology. Deleterious effect of aging on the endothelial healing process was demonstrated for the first time in the rabbit iliac artery <sup>97</sup>. More recently, McDonald and coworkers demonstrated that in young (8-week-old) mice expression of the transcription factor *Atf3* was necessary to achieve endothelial repair, while in aged (18-month-old) mice, decreased *Atf3* expression was associated with the delay of endothelial regeneration <sup>21</sup>. Nevertheless, how aging could affect endothelial healing in response to E2 remains to be defined. This question is of particular importance since the vasculoprotective role of estrogens is well established before menopause, but the consequences of their abrupt decline and of the hormonal treatment

of menopause on cardiovascular risks at menopause remain debated. In addition, unanswered questions remain to be determined regarding the differential effect of hormones during aging of the arterial wall.

Several studies have also evaluated the effect of synthetic SERM on endothelial cells and revealed that as E2, these drugs are able to accelerate endothelial healing. However, whereas tamoxifen acts as an anti-estrogen on ER $\alpha$ MISS in endothelial cells, it accelerates endothelial healing through activation of nuclear ER $\alpha$  in smooth muscle cells, inviting to revisit the mechanisms of action of selective modulation of ER $\alpha$ . Indeed, SERMs' actions were mainly viewed as interfering with E2-dependent recruitment of coactivators and corepressors in a tissue-specific manner, but it is highly likely that their action on ER $\alpha$ MISS represents a novel layer of complexity. Further mechanistic studies are thus needed to elucidate the cellular and molecular pathways through which each SERM mediates its vascular protective action. This point is of particular interest since an American Heart Association scientific statement highlighted the need for comprehensive care in the treatment of women with breast cancer that minimizes the effect on cardiovascular health <sup>98</sup>.

### **Financial support**

This work was supported by the INSERM, the Université de Toulouse III, the Region Midi-Pyrénées, FEDER (Fonds Européen de Développement Régional: REGEN-EVE), the Fondation pour la Recherche Médicale, the Fondation de France (N°00086486) and the French National Research Agency (ANR-EstroShear). MD received a three-year PhD fellowship from the Fondation pour la Recherche Médicale.

### **Declaration of competing interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References

1. Minami Y, Kaneda H, Inoue M, Ikutomi M, Morita T, Nakajima T. Endothelial dysfunction following drug-eluting stent implantation: A systematic review of the literature. *International Journal of Cardiology*. 2013;165:222-228
2. Libby P, Luscher T. Covid-19 is, in the end, an endothelial disease. *European Heart Journal*. 2020;41:3038-3044
3. da Silva R. Effects of radiotherapy in coronary artery disease. *Current atherosclerosis reports*. 2019;21:50
4. Vanhoutte PM. Endothelial dysfunction: The first step toward coronary arteriosclerosis. *Circ J*. 2009;73:595-601
5. Ross R, Faggiotto A, Bowen-Pope D, Raines E. The role of endothelial injury and platelet and macrophage interactions in atherosclerosis. *Circulation*. 1984;70:III77-82
6. Cui Y, Liu Y, Zhao F, Shi D, Chen K. Neoatherosclerosis after drug-eluting stent implantation: Roles and mechanisms. *Oxidative Medicine and Cellular Longevity*. 2016;2016:5924234
7. Arnal JF, Fontaine C, Billon-Gales A, Favre J, Laurell H, Lenfant F, Gourdy P. Estrogen receptors and endothelium. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2010;30:1506-1512
8. Arnal JF, Laurell H, Fontaine C, Billon A, Calippe B, Lenfant F, Gourdy P. Estrogen receptor actions on vascular biology and inflammation: Implications in vascular pathophysiology. *Climacteric : the journal of the International Menopause Society*. 2009;12 Suppl 1:12-17
9. Billon-Gales A, Fontaine C, Douin-Echinard V, Delpy L, Berges H, Calippe B, Lenfant F, Laurell H, Guery JC, Gourdy P, Arnal JF. Endothelial estrogen receptor-alpha plays a crucial role in the atheroprotective action of 17beta-estradiol in low-density lipoprotein receptor-deficient mice. *Circulation*. 2009;120:2567-2576
10. Smirnova NF, Fontaine C, Buscato M, Lupieri A, Vinel A, Valera MC, Guillaume M, Malet N, Foidart JM, Raymond-Letron I, Lenfant F, Gourdy P, Katzenellenbogen BS, Katzenellenbogen JA, Laffargue M, Arnal JF. The activation function-1 of estrogen receptor alpha prevents arterial neointima development through a direct effect on smooth muscle cells. *Circulation Research*. 2015;117:770-778
11. Brouchet L, Krust A, Dupont S, Chambon P, Bayard F, Arnal JF. Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-beta. *Circulation*. 2001;103:423-428.
12. Fontaine C, Morfoisse F, Tatin F, Zamora A, Zahreddine R, Henrion D, Arnal JF, Lenfant F, Garmy-Susini B. The impact of estrogen receptor in arterial and lymphatic vascular diseases. *International Journal of Molecular Sciences*. 2020;21
13. Guivarc'h E, Buscato M, Guihot AL, Favre J, Vessieres E, Grimaud L, Wakim J, Melhem NJ, Zahreddine R, Adlanmerini M, Loufrani L, Knauf C, Katzenellenbogen JA, Katzenellenbogen BS, Foidart JM, Gourdy P, Lenfant F, Arnal JF, Henrion D, Fontaine C. Predominant role of nuclear versus membrane estrogen receptor alpha in arterial protection: Implications for estrogen receptor alpha modulation in cardiovascular prevention/safety. *Journal of the American Heart Association*. 2018;7
14. Haudenschield CC, Schwartz SM. Endothelial regeneration. II. Restitution of endothelial continuity. *Lab Invest*. 1979;41:407-418
15. Schwartz SM, Haudenschield CC, Eddy EM. Endothelial regeneration. I. Quantitative analysis of initial stages of endothelial regeneration in rat aortic intima. *Lab Invest*. 1978;38:568-580
16. Lindner V, Fingerle J, Reidy MA. Mouse model of arterial injury. *Circulation Research*. 1993;73:792-796

17. Carmeliet P, Moos L, Stassen J, De Mol M, Bouché A, van den Oord J, Kocks M, Collen D. Vascular wound healing and neointima formation induced by perivascular electric injury in mice. *The American Journal of Pathology*. 1997;150:761-776
18. Filipe C, Lam Shang Leen L, Brouchet L, Billon A, Benouaich V, Fontaine V, Gourdy P, Lenfant F, Arnal JF, Gadeau AP, Laurell H. Estradiol accelerates endothelial healing through the retrograde commitment of uninjured endothelium. *The American Journal of Physiology*. 2008;294:H2822-2830
19. Evans CE, Iruela-Arispe ML, Zhao YY. Mechanisms of endothelial regeneration and vascular repair and their application to regenerative medicine. *The American Journal of Pathology*. 2021;191:52-65
20. Shirali AS, Romay MC, McDonald AI, Su T, Steel ME, Iruela-Arispe ML. A multi-step transcriptional cascade underlies vascular regeneration in vivo. *Scientific Reports*. 2018;8:5430
21. McDonald AI, Shirali AS, Aragon R, Ma F, Hernandez G, Vaughn DA, Mack JJ, Lim TY, Sunshine H, Zhao P, Kalinichenko V, Hai T, Pelegrini M, Ardehali R, Iruela-Arispe ML. Endothelial regeneration of large vessels is a biphasic process driven by local cells with distinct proliferative capacities. *Cell Stem Cell*. 2018;23:210-225 e216
22. Zhao YD, Huang X, Yi F, Dai Z, Qian Z, Tiruppathi C, Tran K, Zhao YY. Endothelial foxm1 mediates bone marrow progenitor cell-induced vascular repair and resolution of inflammation following inflammatory lung injury. *Stem Cells*. 2014;32:1855-1864
23. Lupieri A, Smirnova NF, Solinhac R, Malet N, Benamar M, Saoudi A, Santos-Zas I, Zeboudj L, Ait-Oufella H, Hirsch E, Ohayon P, Lhermusier T, Carrie D, Arnal JF, Ramel D, Gayral S, Laffargue M. Smooth muscle cells-derived cxcl10 prevents endothelial healing through PI3K $\gamma$ -dependent T cells response. *Cardiovascular Research*. 2020;116:438-449
24. Zahreddine R, Davezac M, Smirnova N, Buscato M, Lhuillier E, Lupieri A, Solinhac R, Vinel A, Vessieres E, Henrion D, Renault MA, Gadeau AP, Flouriot G, Lenfant F, Laffargue M, Metivier R, Arnal JF, Fontaine C. Tamoxifen accelerates endothelial healing by targeting ER $\alpha$  in smooth muscle cells. *Circulation Research*. 2020;127:1473-1487
25. Krasinski K, Spyridopoulos I, Asahara T, van der Zee R, Isner JM, Losordo DW. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation*. 1997;95:1768-1772
26. White CR, Shelton J, Chen SJ, Darley-Usmar V, Allen L, Nabors C, Sanders PW, Chen YF, Oparil S. Estrogen restores endothelial cell function in an experimental model of vascular injury. *Circulation*. 1997;96:1624-1630
27. Chambliss KL, Wu Q, Oltmann S, Konanah ES, Umetani M, Korach KS, Thomas GD, Mineo C, Yuhanna IS, Kim SH, Madak-Erdogan Z, Maggi A, Dineen SP, Roland CL, Hui DY, Brekken RA, Katzenellenbogen JA, Katzenellenbogen BS, Shaul PW. Non-nuclear e ER $\alpha$  signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *The Journal of Clinical Investigation*. 2010;120:2319-2330
28. Smirnova NF, Fontaine C, Buscato M, Lupieri A, Vinel A, Valera MC, Guillaume M, Malet N, Foidart JM, Raymond-Letron I, Lenfant F, Gourdy P, Katzenellenbogen BS, Katzenellenbogen J, Laffargue M, Arnal JF. The activation function-1 of ER $\alpha$  prevents arterial neointima development through a direct effect on smooth muscle cells. *Circulation Research*. 2015;117:770-778
29. Morey AK, Pedram A, Razandi M, Prins BA, Hu RM, Biesiada E, Levin ER. Estrogen and progesterone inhibit vascular smooth muscle proliferation. *Endocrinology*. 1997;138:3330-3339
30. Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation*. 1995;91:755-763

31. Oviedo PJ, Sobrino A, Laguna-Fernandez A, Novella S, Tarin JJ, Garcia-Perez MA, Sanchis J, Cano A, Hermenegildo C. Estradiol induces endothelial cell migration and proliferation through estrogen receptor-enhanced RhoA/ROCK pathway. *Molecular and Cellular Endocrinology*. 2011;335:96-103
32. Razandi M, Pedram A, Levin ER. Estrogen signals to the preservation of endothelial cell form and function. *The Journal of Biological Chemistry*. 2000;275:38540-38546
33. Bernelot Moens SJ, Schnitzler GR, Nickerson M, Guo H, Ueda K, Lu Q, Aronovitz MJ, Nickerson H, Baur WE, Hansen U, Iyer LK, Karas RH. Rapid estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury. *Circulation*. 2012;126:1993-2004
34. Cossette E, Cloutier I, Tardif K, DonPierre G, Tanguay JF. Estradiol inhibits vascular endothelial cells pro-inflammatory activation induced by C-reactive protein. *Molecular and Cellular Biochemistry*. 2013;373:137-147
35. Toutain CE, Filipe C, Billon A, Fontaine C, Brouchet L, Guery JC, Gourdy P, Arnal JF, Lenfant F. Estrogen receptor alpha expression in both endothelium and hematopoietic cells is required for the accelerative effect of estradiol on reendothelialization. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2009;29:1543-1550
36. Barton M, Filardo EJ, Lolait SJ, Thomas P, Maggiolini M, Prossnitz ER. Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. *The Journal of Steroid Biochemistry and Molecular Biology*. 2018;176:4-15
37. Yang S, Yin Z, Zhu G. A review of the functions of G protein-coupled estrogen receptor 1 in vascular and neurological aging. *European Journal of Pharmacology*. 2021;908:174363
38. Langer G, Bader B, Meoli L, Isensee J, Delbeck M, Noppinger PR, Otto C. A critical review of fundamental controversies in the field of GPR30 research. *Steroids*. 2010;75:603-610
39. Fredette NC, Meyer MR, Prossnitz ER. Role of GPER in estrogen-dependent nitric oxide formation and vasodilation. *The Journal of Steroid Biochemistry and Molecular Biology*. 2018;176:65-72
40. Xu F, Wang X, Wu N, He S, Yi W, Xiang S, Zhang P, Xie X, Ying C. Bisphenol A induces proliferative effects on both breast cancer cells and vascular endothelial cells through a shared GPER-dependent pathway in hypoxia. *Environmental Pollution*. 2017;231:1609-1620
41. Trenti A, Tedesco S, Boscaro C, Ferri N, Cignarella A, Trevisi L, Bolego C. The glycolytic enzyme PFKFB3 is involved in estrogen-mediated angiogenesis via GPER 1. *The Journal of Pharmacology and Experimental Therapeutics*. 2017;361:398-407
42. Kumar R, Thompson EB. The structure of the nuclear hormone receptors. *Steroids*. 1999;64:310-319
43. Arnal JF, Fontaine C, Abot A, Valera MC, Laurell H, Gourdy P, Lenfant F. Lessons from the dissection of the activation functions (AF-1 and AF-2) of the estrogen receptor alpha *in vivo*. *Steroids*. 2013;78:576-582
44. Arnal JF, Lenfant F, Metivier R, Flouriot G, Henrion D, Adlanmerini M, Fontaine C, Gourdy P, Chambon P, Katzenellenbogen B, Katzenellenbogen J. Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications. *Physiological Reviews*. 2017;97:1045-1087
45. Caulin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca<sup>2+</sup> mobilization. *Circulation Research*. 1997;81:885-892
46. Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, German Z, Chen Z, Shaul PW. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *The American Journal of Physiology*. 1997;273:L119-126
47. Reis SE, Gloth ST, Blumenthal RS, Resar JR, Zacur HA, Gerstenblith G, Brinker JA. Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. *Circulation*. 1994;89:52-60

48. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *The Journal of Clinical Investigation*. 1999;103:401-406
49. Pedram A, Razandi M, Levin ER. Nature of functional estrogen receptors at the plasma membrane. *Molecular Endocrinology*. 2006;20:1996-2009
50. Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERalpha and ERbeta expressed in chinese hamster ovary cells. *Molecular Endocrinology*. 1999;13:307-319
51. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. ERs associate with and regulate the production of caveolin: Implications for signaling and cellular actions. *Molecular Endocrinology*. 2002;16:100-115
52. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW. Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circulation Research*. 2000;87:E44-52
53. Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A, Visca P, Marino M. Palmitoylation-dependent estrogen receptor alpha membrane localization: Regulation by 17beta-estradiol. *Mol Biol Cell*. 2005;16:231-237
54. Acconcia F, Ascenzi P, Fabozzi G, Visca P, Marino M. S-palmitoylation modulates human estrogen receptor-alpha functions. *Biochemical and Biophysical Research Communications*. 2004;316:878-883
55. Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH. Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101:17126-17131
56. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW. Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through G $\alpha$ i. *The Journal of Biological Chemistry*. 2001;276:27071-27076
57. Kumar P, Wu Q, Chambliss KL, Yuhanna IS, Mumby SM, Mineo C, Tall GG, Shaul PW. Direct interactions with G $\alpha$ i and G $\beta$ \gamma mediate nongenomic signaling by estrogen receptor alpha. *Molecular Endocrinology*. 2007;21:1370-1380
58. Wu Q, Chambliss K, Lee WR, Yuhanna IS, Mineo C, Shaul PW. Point mutations in the ERalpha G $\alpha$ i binding domain segregate nonnuclear from nuclear receptor function. *Molecular Endocrinology*. 2013;27:2-11
59. Adlanmerini M, Solinhac R, Abot A, Fabre A, Raymond-Letron I, Guihot AL, Boudou F, Sautier L, Vessieres E, Kim SH, Liere P, Fontaine C, Krust A, Chambon P, Katzenellenbogen JA, Gourdy P, Shaul PW, Henrion D, Arnal JF, Lenfant F. Mutation of the palmitoylation site of estrogen receptor alpha in vivo reveals tissue-specific roles for membrane versus nuclear actions. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111:E283-290
60. Adlanmerini M, Febrissy C, Zahreddine R, Vessieres E, Buscato M, Solinhac R, Favre J, Anquetil T, Guihot AL, Boudou F, Raymond-Letron I, Chambon P, Gourdy P, Ohlsson C, Laurell H, Fontaine C, Metivier R, Le Romancer M, Henrion D, Arnal JF, Lenfant F. Mutation of arginine 264 on ERalpha (estrogen receptor alpha) selectively abrogates the rapid signaling of estradiol in the endothelium without altering fertility. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2020;40:2143-2158
61. Billon-Gales A, Fontaine C, Filipe C, Douin-Echinard V, Fouque MJ, Flouriot G, Gourdy P, Lenfant F, Laurell H, Krust A, Chambon P, Arnal JF. The transactivating function 1 of estrogen receptor alpha is dispensable for the vasculoprotective actions of 17beta-estradiol. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:2053-2058
62. Billon-Gales A, Krust A, Fontaine C, Abot A, Flouriot G, Toutain C, Berges H, Gadeau AP, Lenfant F, Gourdy P, Chambon P, Arnal JF. Activation function 2 (AF2) of estrogen receptor-

- alpha is required for the atheroprotective action of estradiol but not to accelerate endothelial healing. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:13311-13316
63. Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, Katzenellenbogen BS. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Molecular Endocrinology*. 2006;20:491-502
  64. Madak-Erdogan Z, Kim SH, Gong P, Zhao YC, Zhang H, Chambliss KL, Carlson KE, Mayne CG, Shaul PW, Korach KS, Katzenellenbogen JA, Katzenellenbogen BS. Design of pathway preferential estrogens that provide beneficial metabolic and vascular effects without stimulating reproductive tissues. *Science Signaling*. 2016;9:ra53
  65. Xu X, Yan Q, Liu X, Li P, Li X, Chen Y, Simoncini T, Liu J, Zhu D, Fu X. 17beta-estradiol nongenomically induces vascular endothelial H2S release by promoting phosphorylation of cystathionine gamma-lyase. *The Journal of biological chemistry*. 2019;294:15577-15592
  66. Simoncini T, Scorticati C, Mannella P, Fadiel A, Giretti MS, Fu XD, Baldacci C, Garibaldi S, Caruso A, Fornari L, Naftolin F, Genazzani AR. Estrogen receptor alpha interacts with Gα13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway. *Molecular Endocrinology*. 2006;20:1756-1771
  67. Billon A, Lehoux S, Lam Shang Leen L, Laurell H, Filipe C, Benouaich V, Brouchet L, Dessy C, Gourdy P, Gadeau AP, Tedgui A, Balligand JL, Arnal JF. The estrogen effects on endothelial repair and mitogen-activated protein kinase activation are abolished in endothelial nitric-oxide (NO) synthase knockout mice, but not by no synthase inhibition by N-nitro-L-arginine methyl ester. *The American Journal of Pathology*. 2008;172:830-838
  68. Darblade B, Pendaries C, Krust A, Dupont S, Fouque MJ, Rami J, Chambon P, Bayard F, Arnal JF. Estradiol alters nitric oxide production in the mouse aorta through the alpha-, but not beta-, estrogen receptor. *Circulation Research*. 2002;90:413-419.
  69. Concina P, Sordello S, Barbacanne MA, Elhage R, Pieraggi MT, Fournial G, Plouet J, Bayard F, Arnal JF. The mitogenic effect of 17beta-estradiol on in vitro endothelial cell proliferation and on in vivo reendothelialization are both dependent on vascular endothelial growth factor. *J Vasc Res*. 2000;37:202-208.
  70. Fontaine V, Filipe C, Werner N, Gourdy P, Billon A, Garmy-Susini B, Brouchet L, Bayard F, Prats H, Doetschman T, Nickenig G, Arnal JF. Essential role of bone marrow fibroblast growth factor-2 in the effect of estradiol on reendothelialization and endothelial progenitor cell mobilization. *The American Journal of Pathology*. 2006;169:1855-1862
  71. Maurice DH. Subcellular signaling in the endothelium: Cyclic nucleotides take their place. *Current opinion in pharmacology*. 2011;11:656-664
  72. Maurice DH, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC. Advances in targeting cyclic nucleotide phosphodiesterases. *Nature Reviews. Drug Discovery*. 2014;13:290-314
  73. Caldwell JD, Gebhart VM, Jirikowski GF. Estradiol's interesting life at the cell's plasma membrane. *Steroids*. 2016;111:4-11
  74. Szego CM, Davis JS. Adenosine 3',5'-monophosphate in rat uterus: Acute elevation by estrogen. *Proceedings of the National Academy of Sciences of the United States of America*. 1967;58:1711-1718
  75. Wu Q, Chambliss K, Umetani M, Mineo C, Shaul PW. Non-nuclear estrogen receptor signaling in the endothelium. *The Journal of Biological Chemistry*. 2011;286:14737-14743
  76. Berry M, Metzger D, Chambon P. Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. *Embo J*. 1990;9:2811-2818
  77. Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, McNabb MA, Wenger NK. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *N Engl J Med*. 2006;355:125-137

78. Matthews A, Stanway S, Farmer RE, Strongman H, Thomas S, Lyon AR, Smeeth L, Bhaskaran K. Long term adjuvant endocrine therapy and risk of cardiovascular disease in female breast cancer survivors: Systematic review. *Bmj*. 2018;363:k3845
79. Reckless J, Metcalfe JC, Grainger DJ. Tamoxifen decreases cholesterol sevenfold and abolishes lipid lesion development in apolipoprotein E knockout mice. *Circulation*. 1997;95:1542-1548
80. Fontaine C, Abot A, Billon-Gales A, Flouriot G, Berges H, Grunenwald E, Vinel A, Valera MC, Gourdy P, Arnal JF. Tamoxifen elicits atheroprotection through estrogen receptor alpha AF-1 but does not accelerate reendothelialization. *The American Journal of Pathology*. 2013;183:304-312
81. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results from the women's health initiative randomized controlled trial. *Jama*. 2002;288:321-333.
82. Yue TL, Vickery-Clark L, Loudon CS, Gu JL, Ma XL, Narayanan PK, Li X, Chen J, Storer B, Willette R, Gossett KA, Ohlstein EH. Selective estrogen receptor modulator idoxifene inhibits smooth muscle cell proliferation, enhances reendothelialization, and inhibits neointimal formation in vivo after vascular injury. *Circulation*. 2000;102:III281-288
83. Savolainen-Peltonen H, Luoto NM, Kangas L, Hayry P. Selective estrogen receptor modulators prevent neointima formation after vascular injury. *Molecular and Cellular Endocrinology*. 2004;227:9-20
84. Dudenko D, Gomez R, Garcia-Perez MA, Tarin JJ, Cano A. Bazedoxifene increases the proliferation of human arterial endothelial cells but does not affect the expression of cyclins A, B, and D1 and of p27(kip1). *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. 2021;37:269-272
85. Ren J, Zhou T, Pilli VSS, Phan N, Wang Q, Gupta K, Liu Z, Sheibani N, Liu B. Novel paracrine functions of smooth muscle cells in supporting endothelial regeneration following arterial injury. *Circulation Research*. 2019;124:1253-1265
86. Miyagawa K, Shi M, Chen PI, Hennigs JK, Zhao Z, Wang M, Li CG, Saito T, Taylor S, Sa S, Cao A, Wang L, Snyder MP, Rabinovitch M. Smooth muscle contact drives endothelial regeneration by Bmpr2-Notch1-mediated metabolic and epigenetic changes. *Circulation Research*. 2019;124:211-224
87. Pepermans RA, Prossnitz ER. ERalpha-targeted endocrine therapy, resistance and the role of GPER. *Steroids*. 2019;152:108493
88. Meyer MR, Prossnitz ER, Barton M. The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. *Vascul Pharmacol*. 2011;55:17-25
89. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology*. 2005;146:624-632
90. He S, Nelson ER. 27-hydroxycholesterol, an endogenous selective estrogen receptor modulator. *Maturitas*. 2017;104:29-35
91. Umetani M, Shaul PW. 27-hydroxycholesterol: The first identified endogenous SERM. *Trends in endocrinology and metabolism: TEM*. 2011;22:130-135
92. Li-Hawkins J, Lund EG, Turley SD, Russell DW. Disruption of the oxysterol 7alpha-hydroxylase gene in mice. *The Journal of Biological Chemistry*. 2000;275:16536-16542
93. Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL, Javitt NB, Korach KS, Shaul PW, Mangelsdorf DJ. 27-hydroxycholesterol is an endogenous serm that inhibits the cardiovascular effects of estrogen. *Nat Med*. 2007;13:1185-1192
94. Umetani M, Ghosh P, Ishikawa T, Umetani J, Ahmed M, Mineo C, Shaul PW. The cholesterol metabolite 27-hydroxycholesterol promotes atherosclerosis via proinflammatory processes mediated by estrogen receptor alpha. *Cell Metabolism*. 2014;20:172-182
95. Coelingh Bennink HJ. Are all estrogens the same? *Maturitas*. 2004;47:269-275

96. Abot A, Fontaine C, Buscato M, Solinhac R, Flouriot G, Fabre A, Drougard A, Rajan S, Laine M, Milon A, Muller I, Henrion D, Adlanmerini M, Valera MC, Gompel A, Gerard C, Pequeux C, Mestdagt M, Raymond-Letron I, Knauf C, Ferriere F, Valet P, Gourdy P, Katzenellenbogen BS, Katzenellenbogen JA, Lenfant F, Greene GL, Foidart JM, Arnal JF. The uterine and vascular actions of estetrol delineate a distinctive profile of estrogen receptor alpha modulation, uncoupling nuclear and membrane activation. *EMBO Molecular Medicine*. 2014;6:1328-1346
97. Gennaro G, Menard C, Michaud SE, Rivard A. Age-dependent impairment of reendothelialization after arterial injury: Role of vascular endothelial growth factor. *Circulation*. 2003;107:230-233
98. Mehta LS, Watson KE, Barac A, Beckie TM, Bittner V, Cruz-Flores S, Dent S, Kondapalli L, Ky B, Okwuosa T, Pina IL, Volgman AS, American Heart Association Cardiovascular Disease in W, Special Populations Committee of the Council on Clinical C, Council on C, Stroke N, Council on Quality of C, Outcomes R. Cardiovascular disease and breast cancer: Where these entities intersect: A scientific statement from the american heart association. *Circulation*. 2018;137:e30-e66

## Figure legends

**Figure 1:** Experimental models of carotid arteries injury in mice.

Endovascular (mechanical) and perivascular (electric) injuries *in vivo* are similar in their efficiency to destroy the endothelial cells (ECs), but they differ significantly in their effects on the underlying smooth muscle cell (SMC) populations in the vessel wall. Endovascular injury preserves most of the adventitial cells as well as the medial smooth muscle cells, whereas, in the perivascular model, the media does not show any sign of regeneration during all the time of re-endothelialization. Endovascular *ex-vivo* de-endothelialization using wire allows to analyse endothelial recovery independently of immune blood cells.

**Figure 2:** A historical view of E2 effect on arterial endothelial healing.

From the demonstration of the acceleration of endothelial healing by 17 $\beta$ -estradiol (E2) to the identification of the crucial role of the membrane estrogen receptor initiated signaling pathway in this process (ER $\alpha$ MISS).

**Figure 3:** Mechanisms of ER $\alpha$  action.

As a member of the nuclear receptor superfamily, ER $\alpha$  is composed of six modular domains, namely A to F. Full agonist 17 $\beta$ -estradiol (E2) binding to its ligand-binding domain (LBD) leads to recruitment of transcriptional coactivators (CoA) to the activation function AF-1 and/or AF-2 of ER, facilitating ER binding to DNA through the DNA binding domain (DBD) and initiating transcription of its target genes. Besides these nuclear actions, estrogens mediate also membrane initiated steroid signalling (MISS) through the activation of a pool of ER $\alpha$  localized at the plasma membrane.

**Figure 4:** ER $\alpha$ MISS but not nuclear ER $\alpha$  is necessary to accelerate endothelial healing in response to E2.

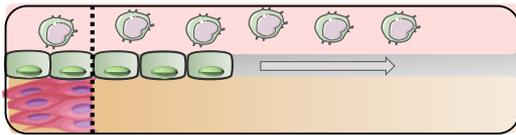
Beneficial effect of E2 on re-endothelialization is abrogated using mouse models targeting estrogen receptor (ER) membrane initiated steroid signalling (MISS) whereas it is preserved in mouse models targeting nuclear ER $\alpha$ .

**Figure 5:** Selective activation of ER $\alpha$ MISS is sufficient to induce endothelial healing.

Estrogen-dendrimer conjugate (EDC) and pathway preferential estrogens (PaPEs) are highly effective in stimulating non-nuclear signalling but ineffective in stimulating nuclear ER target gene expression. Both compounds accelerate re-endothelialization.

## Electric (perivascular) injury

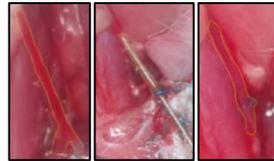
*in vivo*



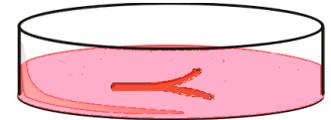
limit injury

## Mechanical (endovascular) injury

*in vivo*



*ex vivo*



Blood cells  
ECs  
SMCs

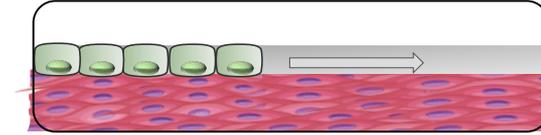
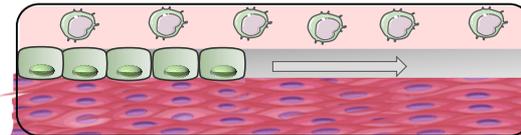


Figure 1

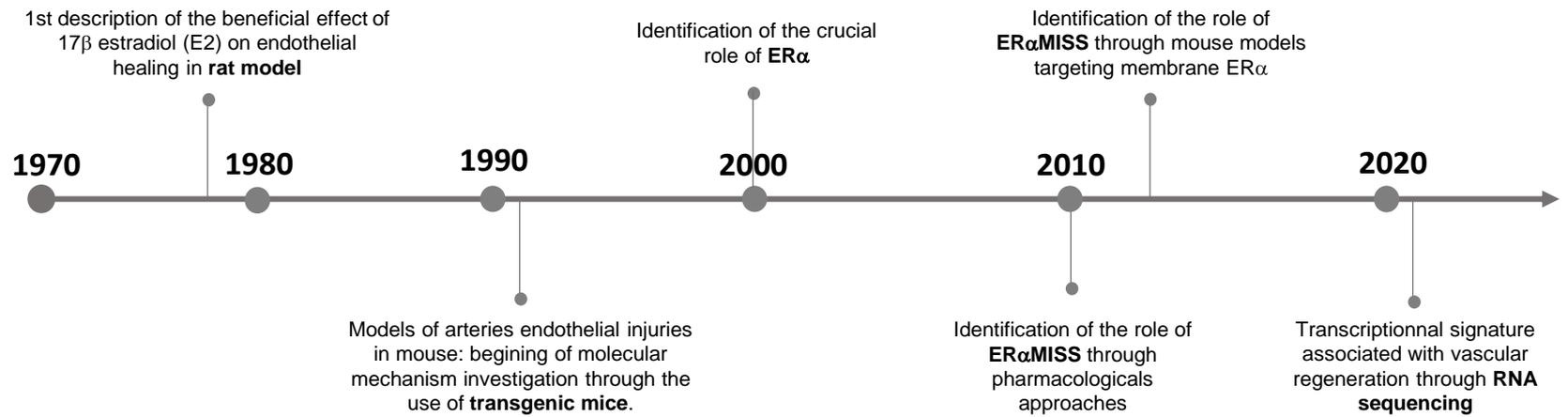


Figure 2

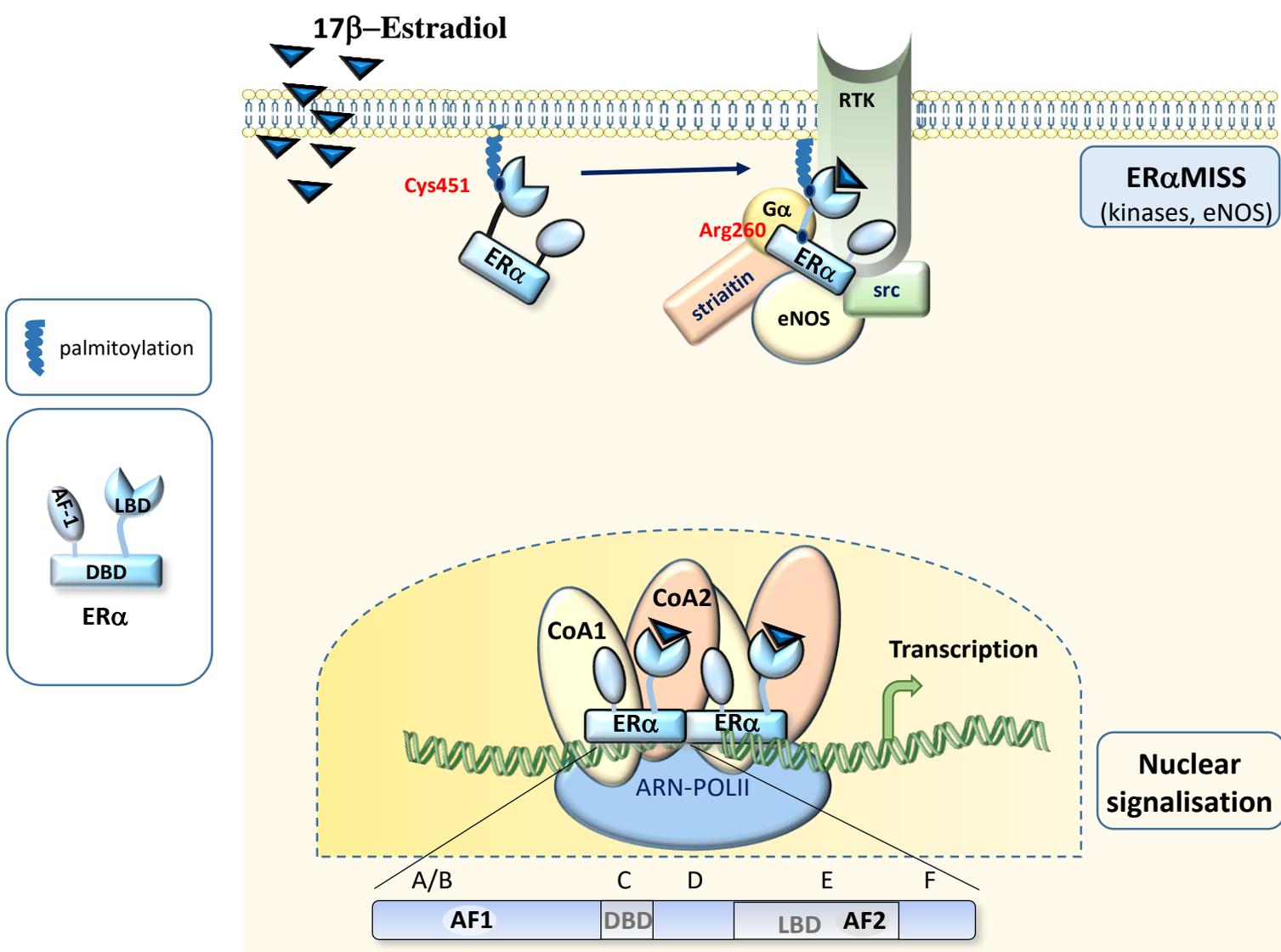


Figure 3

# Selective activation of membrane ER $\alpha$

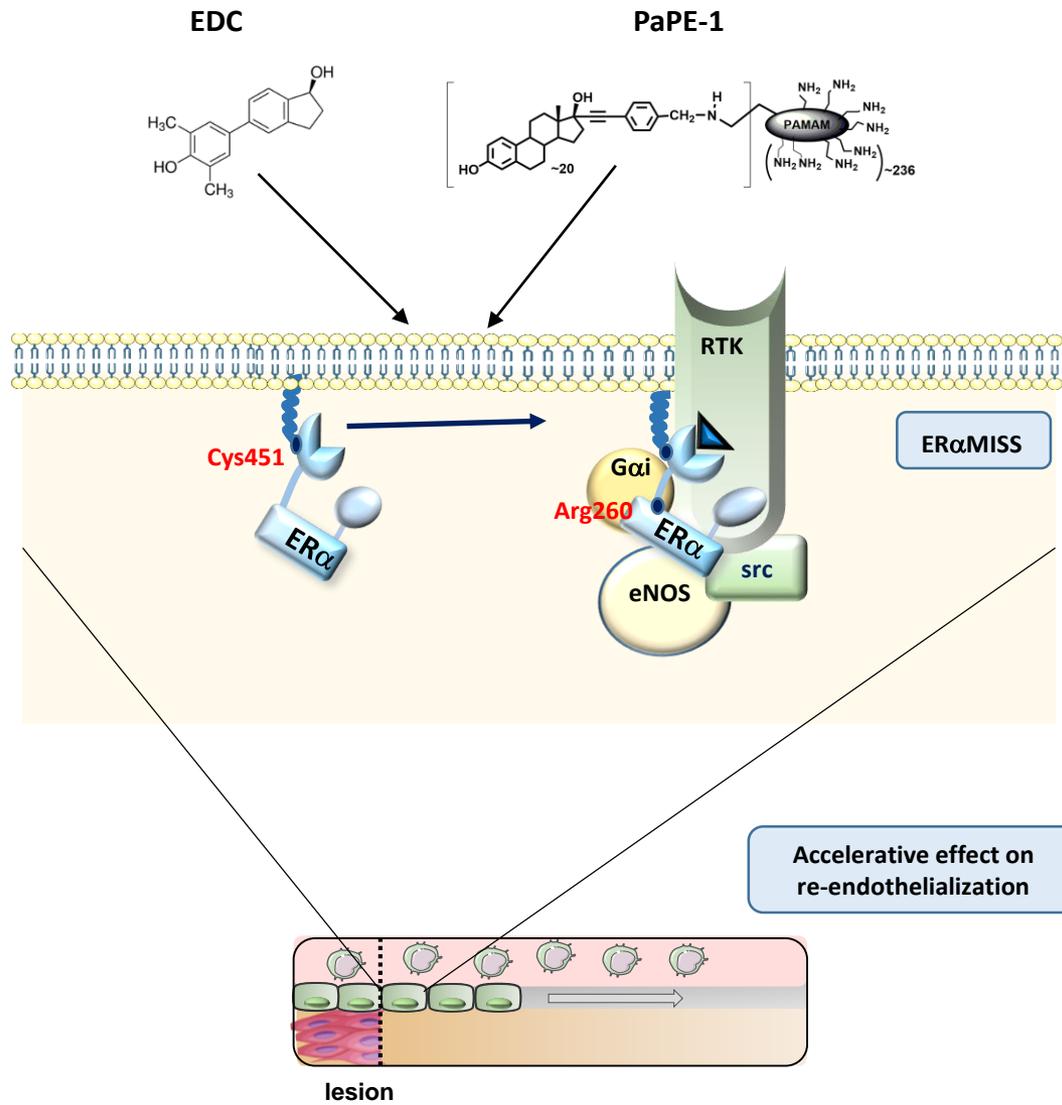
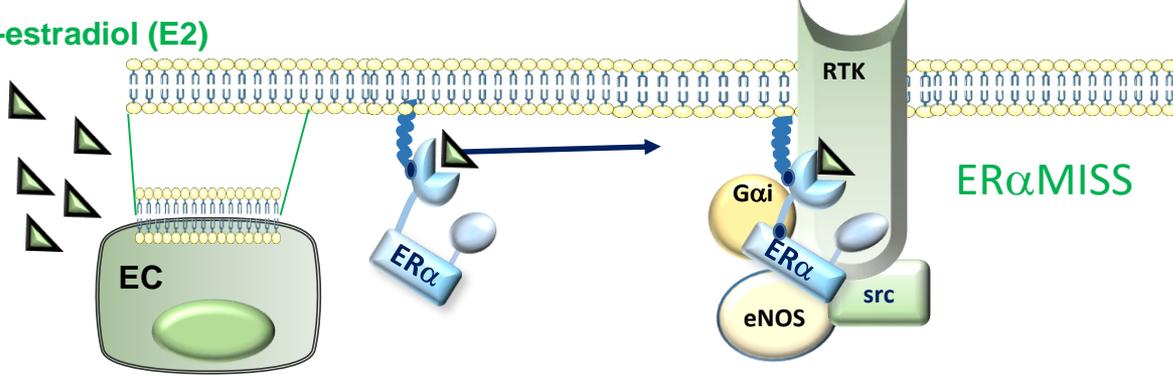


Figure 4

|   |   |   |  |
|---|---|---|--|
| <p style="writing-mode: vertical-rl; transform: rotate(180deg);">Mouse models targeting ER<math>\alpha</math>MISS</p>     | <p>C451A-ER<math>\alpha</math></p>          | <p>Loss of membrane localisation</p>                      | <p style="writing-mode: vertical-rl; transform: rotate(180deg);">Loss of E2 beneficial effect after carotid injury</p>   |
|   | <p>Disrupted peptide mouse (DPM)</p>        | <p>Disruption of ER-striatin interaction</p>              |  |
|   | <p>R264-ER<math>\alpha</math></p>           | <p>Disruption of ER-G<math>\alpha</math>i interaction</p> |  |
| <p style="writing-mode: vertical-rl; transform: rotate(180deg);">Mouse models targeting nuclear ER<math>\alpha</math></p> | <p>ER<math>\alpha</math>AF2<sup>0</sup></p> | <p>Abrogation of transcriptional response</p>             | <p style="writing-mode: vertical-rl; transform: rotate(180deg);">Preserved E2 beneficial effect after carotid injury</p> |
|   | <p>ER<math>\alpha</math>AF1<sup>0</sup></p> | <p>Partial abrogation of transcriptional response</p>     |  |

Figure 5

17 $\beta$ -estradiol (E2)



Blood  
(Hematopoietic  
cells)

Intima (ECs)

Media (SMCs)

