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► **To cite this version:**

Ahmad Chehaitly, Emilie Vessieres, Anne-Laure Guihot, Daniel Henrion. Flow-mediated outward arterial remodeling in aging. *Mechanisms of Ageing and Development*, 2021, 194, pp.111416. 10.1016/j.mad.2020.111416 . hal-03096299

**HAL Id: hal-03096299**

**<https://univ-angers.hal.science/hal-03096299>**

Submitted on 2 Jan 2023

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## **Flow-mediated outward arterial remodeling in aging**

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## **Abstract**

The present review focuses on the effect of aging on flow-mediated outward remodeling (FMR) via alterations in estrogen metabolism, oxidative stress and inflammation. In ischemic disorders, the ability of the vasculature to adapt or remodel determines the quality of the recovery. FMR, which has a key role in revascularization, is a complex phenomenon that recruits endothelial and smooth muscle cells as well as the immune system. FMR becomes progressively less with age as a result of an increase in inflammation and oxidative stress, in part of mitochondrial origin. The alteration in FMR is greater in older individuals with risk factors and thus the therapy cannot merely amount to exercise with or without a mild vasodilating drug. Interestingly, the reduction in FMR occurs later in females. Estrogen and its alpha receptor (ER $\alpha$ ) play a key role in FMR through the control of dilatory pathways including the angiotensin II type 2 receptor, thus providing possible tools to activate FMR in older subjects although only experimental data is available. Indeed, the main issue is the reversibility of the vascular damage induced over time, and to date promoting prevention and limiting exposure to the risk factors remain the best options in this regard.

Keywords: blood flow, collateral growth, Arterial outward remodeling, aging, estrogens, gender, mitochondria

## Highlights

Flow-mediated outward remodeling (FMR) is involved in post-ischemic revascularization

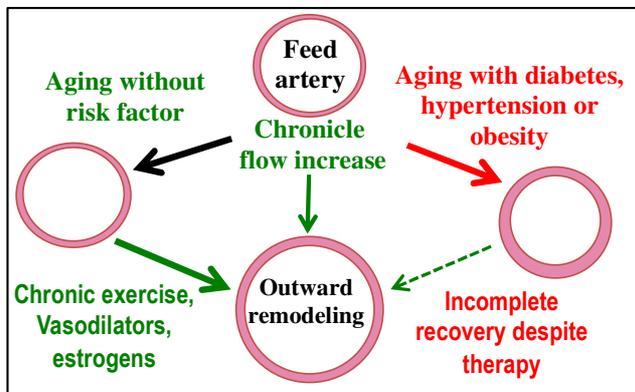
FMR is governed by shear stress and the immune system

Estrogens can activate the NO pathway involved in FMR

FMR is reduced in healthy aging, but it can be restored pharmacologically

Age and pathological risk factors abrogate FMR, often irreversibly

## Graphical abstract



## **1. Introduction**

Flow-mediated outward remodeling (FMR) promotes the growth of pre-existing arteries. It occurs in somatic growth, along with angiogenesis and arteriogenesis <sup>1</sup>. In adulthood, uterine arteries undergo regular outward remodeling during the menstrual cycle and a pronounced enlargement in diameter during pregnancy <sup>2</sup>. In pathological situations, such as diabetes <sup>3-6</sup> and hypertension <sup>7, 8</sup>, FMR and thus the growth of collateral arteries is altered. Recovery after an ischemic event is, therefore, more difficult when associated with these health risk factors. Furthermore, as has been shown in rats <sup>9</sup> and mice <sup>10</sup>, aging itself is also associated with reduced FMR or collateral growth. Similarly, aging has been reported to be a critical issue in elderly individuals for the management of revascularization <sup>11</sup>. Nevertheless, as has been shown in rats, the pathway involved in remodeling remains efficient and can be rescued by the administration of a vasodilator drug <sup>12</sup>. This suggests that while the threshold for the response to flow is shifted, the pathway itself may not be affected <sup>12</sup>. Interestingly, the extent of the decrease in FMR in old female rats is much less compared to age-matched males <sup>13, 14</sup>. Indeed, it is well known and documented that pre-menopausal women have a lower incidence of cardiovascular diseases compared to men. This low incidence of cardiovascular diseases has been largely attributed to the protective effect of estrogens, which exert many beneficial effects in arteries, including arterial dilation, acceleration of healing after arterial injury, and atheroprotection <sup>15</sup>. Thus, estrogens are thought to reduce the effects of vascular aging. Indeed, estrogens are also potent regulators of mitochondrial functions and they exert a protective effect on mitochondria <sup>16</sup>. However, endothelial cells (ECs), which sense shear stress due to blood flow, contain a low number of mitochondria and they depend mainly on glycolysis for ATP production <sup>17</sup>. This results in ECs having a low level of reactive oxygen species (ROS) as aerobic oxidation is limited in these cells. This is important as ROS play a major role in endothelial dysfunction <sup>18</sup>. In

addition, ECs are not exposed to a hypoxic environment during angiogenesis under ischemic conditions, and glycolysis produces lactate, which is a strong activator of angiogenesis <sup>19</sup>. Thus, a role for mitochondria in flow (shear stress) sensing, which is the trigger for FMR, is unlikely, although the key role of oxidative stress in FMR <sup>20</sup> could nonetheless be associated with mitochondrial functioning. On the other hand, excessive oxidative stress can also involve mitochondrial ROS production, which could impair FMR in pathological situations, such as diabetes <sup>3-6</sup> or hypertension <sup>7, 8, 21</sup>. Interestingly, mitochondria are thought to be the biological clock of cells. The American Heart Association has issued broad overview of the key role of mitochondria in cardiovascular physiology that provides insight into how mitochondrial defects contribute to cardiovascular diseases <sup>22</sup>.

## **2. Revascularization: angiogenesis, arteriogenesis, collateral growth, and the role of flow-mediated outward remodeling**

Post-ischemic revascularization is the result of several processes that occur simultaneously or sequentially <sup>1</sup>. First, angiogenesis is triggered by the ischemia and starts with the sprouting of preexisting capillaries. Arteriogenesis then allows capillaries to form arterioles after stabilization of the sprouts by smooth muscle cells and pericytes. Arteriogenesis hence increases the arteriolar density, which then results in more blood being available to feed the new capillaries. In parallel, collateral artery growth results from the outward remodeling of preexisting arteriolar anastomoses, which had little flow. Collateral growth increases the number of conducting arteries. Finally, upstream arteries also become subjected to increased blood flow and undergo outward remodeling, leading to an increase in their diameter. Ultimately, blood flow is restored, and the tissue recovers normal functioning.

An increase in blood flow is the main trigger for the outward remodeling that occurs in arteriogenesis, collateral growth, and for the increase in diameter of larger feeding arteries. The effect of age and the associated risk factors are described in several review articles <sup>1, 23, 24</sup>

### **3 - Flow-mediated remodeling in somatic growth and other physiological processes**

During somatic growth, the diameter of blood vessels increases along with vessel wall hypertrophy so that mechanical strength and vasomotor tone allow proper control of blood flow to tissues with growing needs <sup>25</sup>. As in pregnancy, this remodeling has a number of features in common with the remodeling that occurs in post-ischemic revascularization. It is, however, mainly governed by growth factors and hormones at a level not observed later in life.

Remodeling of the uterine arteries allows for the pronounced increase in uterine blood flow that occurs during pregnancy (for a review, see Mandala & Osol <sup>2</sup>). This is three-dimensional remodeling with enlargement in the diameter along with a substantial axial elongation and changes in vessel biomechanical properties, although wall thickening remains limited. Both local and systemic humoral systems regulate uterine artery remodeling, with a major role for shear stress and steroid-induced upregulation of endothelial NO production <sup>26</sup>. The placenta also activates this remodeling by producing VEGF or PlGF, with venoarterial communication as a plausible pathway for the placenta to control the outward remodeling of afferent arteries <sup>2</sup>. Although estrogens control outward remodeling in peripheral arteries <sup>27</sup>, their role in the remodeling of uterine arteries and veins remains uncertain.

Exercise induces a strong angiogenic stimulus in the active muscles where the capillaries are functionally recruited due to the increase in blood flow <sup>28</sup>. Indeed, exercise

increases blood flow (shear stress), blood pressure, and wall stress, thereby leading to outward hypertrophic remodeling. Numerous studies have demonstrated that the direct hemodynamic impact of exercise training on the health of the artery wall decreases cardiovascular incidents (for a review, see Green et al. 2017<sup>29</sup>). A degree of caution is warranted, however, as excessive exercise has been shown to have deleterious effects on the cardiovascular system<sup>30</sup>, with a reversal in the balance between NO and superoxide and inflammatory cytokines production<sup>31</sup>. Excessive exercising has an impact on estrogen production in women thus inducing vascular changes that are usually seen in post-menopausal women with reduced FMD<sup>32</sup> and pro-inflammatory profile<sup>31</sup>.

#### **4 – Mechanism of flow-mediated remodeling**

##### **4.1. Shear stress sensing**

Many well-documented review articles are available on flow (shear stress)-mediated signal transduction<sup>33,34</sup>, including reviews on disturbed flow, which alters large artery walls and induces atherosclerosis<sup>35,36</sup>. Nevertheless, the dilation induced by an acute increase in flow involves a rapid response of the ECs, leading to immediate relaxation of the adjacent smooth muscle cells or flow-mediated dilation (FMD). FMD mainly involves the production of NO and a range of COX-derivatives and other hyperpolarizing factors (EDHF) depending on the vessel type, the species, and the disease. Interestingly, FMD can be assessed in patients using a non-invasive technique and it correlates well with endothelial dysfunction and risk factors, although a number of limitations have recently been highlighted<sup>37</sup>.

FMR starts with FMD, which then becomes chronic and changes the arterial wall structure. A first approach to understand the effect of age on FMR is hence to analyze the effect of age on FMD. The main effect of aging on FMD is a reduction in the production of

NO in response to increased shear stress and overexpression of inflammatory factors associated with excessive oxidative stress<sup>38</sup>. These alterations also characterize the changes in endothelial function observed in cardiovascular and metabolic diseases, which are common, and present as premature vascular (endothelial) aging.

Interestingly, mitochondria are thought to be the biological clock of cells, and ECs are sensitive to oxidative stress of mitochondrial origin<sup>39, 40</sup>. Furthermore, the mitochondria of ECs undergo hyper-fragmentation in cardiovascular diseases<sup>41</sup>. Although vascular ECs do not depend on mitochondria for their energy demands<sup>42</sup>, it has been hypothesized that the mitochondria of ECs play a regulatory rather than an energy-producing role<sup>43</sup>. As demonstrated in HUVECs exposed to laminar shear stress<sup>40</sup>, shear stress induces a more quiescent and less oxidative phenotype in ECs. Indeed, mitochondrial fusion and fission are sensitive to shear stress in cultured human and bovine ECs<sup>44</sup>, and shear stress-dependent Ca<sup>2+</sup> mobilization in human ECs depends on mitochondria-dependent activation of endoplasmic reticulum channels<sup>45</sup>. Thus, shear stress most probably reduces EC metabolism, with a reduction in mitochondrial ROS production<sup>46, 47</sup>. It is, therefore, tempting to hypothesize that the reduction in flow sensing and FMD observed with age is associated with increased production of ROS of mitochondrial origin, as discussed below (5.1). Indeed, free radicals produced by the mitochondria in vascular cells have a major role in the peripheral vascular disorders that occur with age<sup>48, 49</sup>.

#### **4.2. Vascular response to chronic changes in blood flow**

For flow-mediated remodeling (FMR) to occur, there must first be a moderate yet also requisite inflammatory response<sup>50, 51</sup> as well as a degree of oxidative stress<sup>20</sup>. As has been shown in rat mesenteric arteries, the chronic increase in blood flow is followed by an accumulation of perivascular macrophages during the early stage (the first 2 days), as outlined in **Figure 1**. This has been demonstrated using macrophage inactivation with clodronate,

which prevented remodeling of rat mesenteric arteries subjected *in vivo* to a doubling of blood flow after surgical ligation of the adjacent arteries<sup>50, 52</sup>. Interestingly, in these studies, both arteries submitted to high flow and arteries submitted to low flow were investigated and both the outward remodeling induced by high flow and the inward remodeling induced by a low flow were prevented by clodronate<sup>50</sup>. Both type of remodeling depend on the presence of macrophage and the vascular tone determines the direction of the remodeling. **Indeed, the chronic change in flow is associated with MCP1 production<sup>27, 53</sup> which certainly increase macrophages infiltration in the perivascular space and this inflammatory state induces oxidative stress with superoxide production<sup>4, 20</sup> by both NADPH-oxidase and macrophages (Figure 1). This oxidative stress induces peroxynitrite (ONOO-) production which initiates metalloproteinases (MMPs) activation and thus extracellular matrix (ECM) digestion<sup>51</sup> (Figure 1).** Low flow-mediated inward remodeling results of an unbalanced contractility due to the reduction in flow-dependent production of vasodilator agents followed by extracellular matrix remodeling and transglutaminase-2 activation<sup>50, 54-56</sup>.

Bone marrow cells are also involved in collateral artery growth, as has been documented in the hind limb of mice<sup>10</sup>. These findings are in keeping with two studies that have shown that revascularization of the femoral artery after ligation is severely reduced in mice lacking CD4+ cells<sup>57</sup>, CD8+ cells<sup>58</sup>, or natural killer cells<sup>57</sup>, thus highlighting a possible role for T lymphocyte activation in FMR and collateral artery growth. Indeed, in mice lacking T cells (nude mice), FMR of the mesenteric arteries is abrogated<sup>51</sup>. Our analysis of the immune cells surrounding the arteries subjected to increased blood flow revealed an accumulation of T cells two days after the increase in blood flow. These cells expressed markers of Th17 and a high level of angiotensin II type-2 receptor (AT2R)<sup>51</sup>. Interleukin-17 (IL-17) has a protective effect in post-ischemic revascularization in the mouse hindlimb<sup>59</sup>, and AT2R activation induces the production of IL-17 by memory T cells<sup>51</sup> (Figure 1). This sequence of events has been confirmed by the observation that FMR does not occur in the absence of AT2R or IL-17 in mice and the finding that the injection of IL-17 into nude mice or mice lacking AT2R restores FMR in mesenteric arteries<sup>51</sup>. The involvement of AT2R in FMR provides a new therapeutic perspective in age-related vascular dysfunction (Figure 2). This could at least in part explain why the AT2R agonist C21 improves ischemic damage in stroke<sup>60</sup> and has a protective effect in neurodegenerative disorders<sup>61</sup>. Indeed, we have shown that C21 restored FMR in old mice mesenteric arteries subjected to a chronic increase in blood flow<sup>51</sup>.

Indeed, these studies could suggest that the outward remodeling depends on the production of Th1 cytokines leading to a lytic phenotype and that the inward remodeling depends on Th2 cytokines producing a fibrotic phenotype. Nevertheless, a more complex profile characterizes FMR of small arteries with a mixture of pro- and anti-inflammatory factors involved during the 2 or 3 days following the increase in blood flow with a rapid return to normal when shear stress is normalized<sup>50</sup>. The involvement of the pro-inflammatory cytokine IL-17 and of the anti-inflammatory receptor AT2R is a good example of this complex pathway involved in FMR<sup>51</sup>.

The next step after this short inflammatory phase comprises activation of matrix metalloproteinases (MMPs), which results in partial digestion of the extracellular matrix in the vascular wall. This process facilitates expansion of the diameter and the ensuing compensatory hypertrophy (**Figure 1**). MMP9 is most likely involved in FMR, as has been shown in both large<sup>62,63</sup> and small arteries<sup>51,64</sup>. An increase in diameter is hence possible and is governed by the artery tone, as demonstrated by Bakker et al.<sup>50</sup>. The main stimulus for the expansion of the diameter is NO that is produced after eNOS activation by flow, as has been shown in both large<sup>62</sup> and small arteries<sup>64,65</sup>. The expression level of eNOS is increased and, in parallel, caveolin-1 expression is reduced so that the NO pathway is more efficient. These changes are activated by IL-17 during the inflammatory phase of the remodeling<sup>51</sup>. Indeed, IL-17 has a protective effect at low concentrations and it is able to induce eNOS phosphorylation in cultured human ECs<sup>66</sup>. Thus, IL-17 could also directly activate NO production and thereby increase the arterial diameter (**Figure 1**).

Adenosine monophosphate-activated protein kinase (AMPK) has also been proposed to play a role in arteriogenesis as described in a recent review article<sup>24</sup> the activation FSS and hypoxia (through reduction of the ratio of ATP to AMP) also activate 5'-Adenosine monophosphate-activated protein kinase (AMPK) phosphorylation which activates eNOS and arteriogenesis in collateral arterioles. Similarly, exercising also activates arteriogenesis through AMPK activation thus promoting FMR and arteriogenesis<sup>24</sup>.

More surprisingly, estrogens are also involved in this phase of the remodeling, as described below **and as depicted in Figure 1**. Aside from NO, other vasodilating agents are also recruited in this latter phase, such as CO produced by heme-oxygenase 1<sup>52,67</sup> and prostanoids synthesized after cyclooxygenase 2 activation, at least in obese rats<sup>68</sup>.

Finally, FMR requires an increase in wall thickness in order to normalize tensile stress, which is increased by the expansion of the diameter<sup>54, 69</sup>. The increase in diameter leads to excessive wall stress and stretching of smooth muscle cells that activates the local renin-angiotensin system and particularly the angiotensin II type 1 receptor (AT1R)<sup>70</sup>. Consequently, AT1R activation leads to ERK1/2 phosphorylation and smooth muscle hypertrophy<sup>70</sup> (Figure 1).

Aside from the chronic increase in flow, which triggers FMR, reversal of the blood flow direction often occurs in collateral vascular segments after occlusion of a feed artery. A study performed on HUVECs cultured in a flow chamber has shown that flow reversal activates a wide panel of pro-arteriogenic signaling pathways<sup>71</sup>.

#### **4.3. Role of estrogens and estrogen receptor alpha in flow-mediated remodeling**

Female sex hormones play a major role in the uteroplacental changes that occur during pregnancy, with major uterine artery outward remodeling associated with trophoblast invasion, hyperplasia, hypertrophy, and modification of the extracellular matrix<sup>2</sup>. Estrogens activate endometrial secretion of factors that promote vascular smooth muscle cell migration, which is an early step in outward arterial remodeling<sup>72</sup>. Endogenous 17-beta-estradiol (E2) is essential for increasing endothelium (NO)-mediated dilatation in rat mesenteric arteries<sup>73</sup> subjected to chronic high flow, and activation of estrogen receptor alpha (ER $\alpha$ ) is essential for FMR in the mesenteric arteries of young female rats<sup>27</sup>. Indeed, both ovariectomy of female rats and the absence of ER $\alpha$  in mice prevent FMR in mesenteric arteries<sup>27</sup>. Furthermore, endothelial ER $\alpha$  is involved in this FMR, as shown in mice that selectively lack ER $\alpha$  in ECs (Tie2Cre(+))ERf/f mice<sup>27</sup>. Furthermore, FMR is controlled by the nuclear activating function 2 (AF2) of ER $\alpha$ . Indeed, ER $\alpha$ -AF2<sup>o</sup> mice, which selectively lack the AF2 function, do not develop FMR, whereas mice lacking only the membrane-associated form of ER $\alpha$  (C451A-ER $\alpha$  mice) respond to the chronic increase in flow with outward remodeling<sup>74</sup>. Interestingly, E2 blood level control AT2R expression in blood vessel and this could provide a link between ER $\alpha$  and AT2R, both involved in FMR<sup>75</sup>.

The decline of the amplitude of FMR with age occurs later in female rats than in male rats and is correlated with a progressive decrease in E2 blood levels<sup>14</sup>. This could induce an equivalent decrease in ER $\alpha$ , the expression of which is governed by the level of E2<sup>15</sup>. While FMR did not occur in young ER $\alpha$ -/- mice, it was reduced by 50% in young ER $\alpha$ +/- mice, thus confirming the importance of gene dosage in FMR. Importantly, increasing E2 blood levels in

old female rats could restore FMR to the level observed in young animals <sup>14</sup>. The role of E2 in post-ischemic revascularization has also been demonstrated using a skin flap model in mice <sup>76</sup>. This effect of E2 on FMR could at least in part explain its protective action in central and peripheral ischemic disorders <sup>76-78</sup>. Interestingly, we also observed that E2 lost its capacity to restore FMR in female mice after a 9-month long ovariectomy, thus demonstrating nonreversible loss of the effect of E2 on FMR after prolonged estrogen deprivation <sup>14</sup>. This observation supports the previous works that highlighted the importance of the timing in treating postmenopausal women with E2. Indeed, after a long interruption, the effect of E2 may be more deleterious than beneficial on the cardiovascular system <sup>79</sup>.

## **5. Flow-mediated remodeling in aging**

### **5.1. Vascular aging**

While the probability of an ischemic event increases with age, the capacity of the vascular tree to undergo proper revascularization is reduced. Indeed, aging is usually associated with a progressive pro-inflammatory state which render the elderly more prone to develop metabolic, cardiovascular and neurovascular disorders. Endothelial cells senescence is the hallmark of vascular aging with increased production of pro-inflammatory cytokines and adhesion molecules <sup>80, 81</sup>. This slowly developing pro-inflammatory state facilitates the occurrence of metabolic and cardiovascular diseases which frequency increases with aging <sup>80, 81</sup>. Estrogens provide a protective effect in women before menopause against cardiovascular disorders in part through anti-inflammatory effects <sup>82</sup>.

First, flow-mediated dilation (FMD), which initiates the remodeling, is early affected by aging. Ultrasound measurement of FMD in brachial arteries has revealed that both gender and age are independent risk factors for reduced endothelial function <sup>83</sup>. Thus, the initial stimulus involved in FMR induces less of a dilatory response with age. Indeed, no FMR of first-order mesenteric arteries subjected to a chronic increase in blood flow was noted in healthy male rats that were 12 <sup>9</sup> or 24 months <sup>12</sup> old. Aging also induced a progressive decrease in the collateral number, the diameter, and remodeling after femoral ligation in mice aged 3 to 31 months <sup>84</sup>. In this study, aging was accompanied by reduced eNOS signaling, with more nitrosylation and less phosphorylation of eNOS. However, this age-dependent reduction in collateral growth was not associated with a decrease in T cell or macrophage recruitment <sup>84</sup>.

Another study has shown that aging in mice (3 versus 18 months) reduces bone marrow cell recruitment and homing<sup>10</sup>. However, transplantation of young bone marrow into old mice did not result in recovery of blood flow in the leg subjected to ischemia after femoral ligation. A similar result was obtained by another group using an ischemic skin flap model<sup>85</sup>.

Importantly, aside from the absence of enlargement of the diameter of the arteries subjected to high flow in old male rats, the wall to lumen ratio, which is a key index of the hypertrophy associated with cardiovascular disorders<sup>86, 87</sup>, was greater than in young animals. Thus, the consequences are two-fold, with a hypertrophied artery without normalization of shear stress and, above all, no increase in flow, which makes it impossible to compensate for ischemia.

Nevertheless, in relatively healthy aging, despite the absence of an increase in the diameter of arteries subjected to enhanced blood flow, endothelium-mediated dilation in these arteries is enhanced by the chronic increase in flow, as shown in 2-year-old male rats<sup>88</sup>. In addition, treatment with the vasodilator hydralazine at a dose that does not reduce blood pressure<sup>89</sup> restored FMR in the mesenteric arteries of 2-year-old male rats<sup>12</sup>. These observations suggest that the pathways involved in FMR remain efficient, with possibly a shift of the threshold for flow (shear stress) sensitivity of the flow-sensors. This issue remains to be further investigated in order to better define which element could be targeted to improve tissue perfusion in the elderly. One possible explanation could be that the bioavailability of the NO necessary for the increase in diameter in FMR is impaired in aging and that a higher stimulus is needed, as this is can be obtained with hydralazine. In old mice subjected to femoral artery ligation, the level of eNOS and phosphorylated eNOS in collateral arteries is lower than in young mice<sup>10</sup>. Furthermore, in old male rats, treatment with an antioxidant drug or with an AT1R blocker can restore FMR of the mesenteric artery to the level obtained in young rats<sup>13</sup>, which suggests that excessive oxidative stress counteracts the NO pathway involved in FMR. Indeed, oxidative stress increases with age and reduces FMR in aged rats<sup>90</sup>. Oxidative stress increases with age through several mechanisms such as increased NADPH-oxidase levels and activity<sup>90</sup> and through an increase in ROS production by mitochondria<sup>91</sup>. In old mice treated with the mitochondrial superoxide scavenger MitoTEMPO, the blood flow recovery after femoral occlusion was equivalent to that in young animals<sup>92</sup>. These studies performed in mice are in keeping with the finding that patients with peripheral artery disease exhibit mitochondrial dysfunction and increased oxidative stress-induced damage<sup>93, 94</sup>.

Interestingly, E2 has been shown to delay human vascular EC senescence induced by hydrogen peroxide of mitochondrial origin<sup>95</sup>, thus suggesting that estrogens could also

prevent the loss of FMR with age, possibly through a reduction in oxidative stress and inflammation.

## 5.2. Vascular aging and female hormones

Numerous epidemiological studies suggest that women are better protected than men against cardiovascular diseases and that this effect is lost after menopause<sup>96</sup>. Indeed, the decline in ovarian function is associated with a decrease in NO production<sup>15</sup>. FMR has been shown to occur in 12-month-old female but not in male rats of the same age. Compared to females, the NO-dependent pathway was more efficient, and the levels of ERK1/2 phosphorylation, oxidative stress, and vascular contractility were higher in old male rats and in ovariectomized female rats, thus suggesting that excessive oxidative stress opposes NO-dependent enlargement of the arterial diameter. Importantly, in male rats and in ovariectomized female rats, treatment with hydralazine, the antioxidant TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), or candesartan (an inhibitor of the angiotensin II type 1 receptor) normalized FMR and reduced arterial contractility, thus suggesting that estrogens balance oxidative stress and vascular contractility to preserve FMR in mature female rats.<sup>13, 14</sup> Indeed, estrogen maintain NO bioavailability and thus the capacity of arteries to maintain FMR in response to a chronic increase in blood flow. Estrogens also lower endothelin-1 production by the endothelial cells and thus its vasoconstrictor and proinflammatory effects. Indeed, circulating T lymphocytes are more abundant in premenopausal women than in men<sup>97</sup>.

This role of E2 in FMR through AF2-ER $\alpha$  activation could explain the protective effect of E2 in ischemic disorders, as shown in myocardium, hind limb, brain, and skin<sup>76, 98-100</sup>. The protective effect of E2 against damage from myocardial ischemia/reperfusion involves the endothelial ER $\alpha$ <sup>101</sup>, while activation of the non-nuclear ER $\alpha$  pathway decreases the damage from<sup>102</sup> and the severity of stroke in the mice<sup>103</sup>. Using ER $\alpha$ -AF2<sup>0</sup> and C451A-ER $\alpha$  mice, which have selective inactivation of nuclear (AF2) and membrane ER $\alpha$ , respectively, we have shown that nuclear ER $\alpha$  plays a prominent role in the prevention of the kidney damage induced by angiotensin II-dependent hypertension in old female mice<sup>104</sup>. Interestingly, selective activation of nuclear ER $\alpha$  with estetrol, which is a fetal estrogen, prevents angiotensin II-induced hypertension and promotes FMR<sup>74</sup>. Similarly, E2 and the selective estrogen receptor modulator tamoxifen accelerate endothelial healing in a mouse

model of vascular injury<sup>105</sup>. This suggests that pharmacological targeting of the AF2 function of ER $\alpha$  warrants further investigation.

### 5.3. Flow-mediated remodeling in aging associated with risk factors

Unfortunately, aging and risk factors go hand-in-hand, often with fatal consequences. Moreover, hypertension, obesity, and diabetes are increasingly affecting young people. Damage to the vascular wall also accumulates over time, and the frequency of ischemic diseases increases with age. For example, peripheral arterial occlusive disease is mostly observed in the elderly, and its incidence is expected to increase dramatically as the population ages, as well as due to an increase in the prevalence of metabolic syndrome and type 2 diabetes. Similarly, coronary heart disease remains a major reason of death worldwide despite an improved medical management and a better quality of the treatments. The main cause of heart attack is atherosclerosis with plaques instability and rupture which induce in myocardial ischemia and then hypoxia and tissue necrosis. Besides the current therapy involving coronary artery intervention or bypass grafting it is necessary to increase to capacity of the ischemic areas to recruit new vessels and to increase arterioles diameter through activation of FMR thus improving revascularization in order to prevent the occurrence and progression of cardiac and peripheral arterial diseases<sup>106</sup>. **As depicted in Figure 2, hypertension, diabetes and obesity increase superoxide production and cellular level leading to the disruption of the physiological process involved in FMR. Thus, therapeutic tools reducing oxidative stress are likely to be efficient.**

Flow-mediated outward remodeling is severely reduced in spontaneously hypertensive rats (SHRs)<sup>9</sup>. Indeed, the kinetics of remodeling in very young SHRs is slower than in normotensive WKY rats, and SHRs reach a similar level of FMR in the mesenteric artery after 3 weeks instead of 1 week in WKY rats<sup>8</sup>. In 10-week-old SHRs, shortly after the onset of hypertension, the damage induced by high intraluminal pressure is minimal. Interestingly, the FMR amplitude decreases after reaching a plateau after 3 weeks, and this is accompanied by wall hypertrophy, hypercontractility, and excessive oxidative stress, which are thought to synergistically abrogate FMR<sup>8</sup>. In the mesenteric arteries of SHRs, the baseline H<sub>2</sub>O<sub>2</sub> and NO levels are higher than in WKY rats, whereas after an increase in chronic flow, only the level of H<sub>2</sub>O<sub>2</sub> increased in SHRs, while the level of NO was elevated in WKY rats<sup>7</sup>. This shift of

the response to flow toward the production of H<sub>2</sub>O<sub>2</sub> and ROS could participate in the decline in revascularization that occurs with age in association with hypertension <sup>107</sup>.

Obesity also impairs outward remodeling of collateral arteries. This has been shown in obese mice subjected to femoral occlusion, and it is due to excessive NADPH-oxidase activity and ROS production without a reduction in monocyte mobilization <sup>108</sup>. Surprisingly, FMR in mesenteric arteries was not reduced in obese rats despite pronounced impairment of NO-dependent relaxation <sup>109</sup>. However, COX2 inhibition suppressed FMR in old obese rats, thus suggesting that inflammation associated with obesity provides a pathway to compensate for the loss of NO-dependent dilation and to maintain FMR in the mesenteric bed <sup>110</sup>. This could be specific to the mesenteric circulation, which is overstimulated in rats with hyperphagia, and this could contribute to the weight gain in these animals.

Flow-mediated outward remodeling is severely reduced in a rat model of type 2 diabetes <sup>111</sup>. In this study, MMPs could not be activated in the arteries subjected to a chronic increase in blood flow, and this was due to the blocking effect of the **advanced glycation end-products (AGEs) generated by the hyperglycemia (Figure 2)**. The AGE-breaker drug ALT-711 reverses this effect in young diabetic rats, resulting in restoration of FMR. However, in old diabetic rats, this drug was ineffective, suggesting that long-term (1 year in the rat) diabetes induces irreversible AGE accumulation <sup>112</sup> and possibly irreversible tissue damage. In this study, performed in 1-year-old diabetic (ZDF) rats, FMR was not only absent but endothelium-mediated dilation was further decreased, although an antioxidant treatment could partially restore it <sup>4, 112</sup>. In these old diabetic rats, vasoconstrictor prostanoids and oxidative stress both reduced endothelium-mediated relaxation <sup>113</sup>.

Finally, unbalanced oxidative stress with excessive superoxide and H<sub>2</sub>O<sub>2</sub> production is a common feature of the various risk factors that impair FMR and collateral growth with age. Indeed, H<sub>2</sub>O<sub>2</sub> is a potential vasodilating agent that could, at least in part, compensate for the loss of NO. For example, in human coronary arteries from coronary artery disease patients with a median age of 67, flow-mediated dilation depended on the mitochondrial production of hydrogen peroxide instead of nitric oxide <sup>114</sup>. Although this production of hydrogen peroxide allows dilation to be maintained, it creates an excessively oxidative environment that can lead to extensive tissue damage. <sup>115</sup> Of note, 17-beta-estradiol has been shown to delay human vascular EC senescence induced by hydrogen peroxide of mitochondrial origin <sup>95</sup>. Indeed,

estrogens interact not only with smooth muscle and endothelial cells but also with immune cells and thus reduced endothelial dysfunction due to inflammation, thus contributing to delay vascular or at least endothelial cells senescence <sup>82</sup>.

Ischemic heart disease is a common endpoint of the risk factors listed above and it is a main cause of death which affects now more and more younger people and women. Besides the classical surgical procedures available and the pharmacological tools available, another approach would be to better stimulate coronary arteries collateral growth and thus, FMR <sup>116</sup>. As discussed above, AMPK is a promising therapeutic target to reach this end <sup>24</sup>. Nevertheless, despite a large number of candidates that activates coronary collateral growth in preclinical models involving usually young and healthy animals, clinical trials remain deceiving. As described in detail in a recent review <sup>116</sup>, cytokines, MCP-1, growth factors such as VEGF, stem cells or ivabradine has been tested without a clear success, so far, in promoting collateral growth in patients with ischemic heart diseases. Based on the observation that exercising improves collateral growth, at least in part through an increase in shear stress, external counter pulsation has been tested in humans with promising results <sup>116</sup>.

#### **5.4. Therapeutic tools**

Exercise has been clearly shown to increase blood flow and to activate FMR, with a positive impact on arterial structure and function <sup>29</sup>. A sedentary lifestyle is usually associated with other risk factors such as obesity, diabetes, and hypertension. Inactivity per se induces inward remodeling whereas exercise has the opposite effect <sup>117</sup>. Even short-term daily exercise has been shown to induce remodeling of rat intramural coronary arterioles and to improve coronary blood flow autoregulation <sup>117</sup>. Indeed, well-controlled exercise is recommended for patients with a risk of cardiovascular events <sup>29</sup>. Unfortunately, there is an increasing trend nowadays in sedentary behavior, with more than one-third of the World population currently below the minimum physical activity requirements needed to remain healthy <sup>118</sup>. Thus, alternative solutions are needed, including the use of medications. Aside from specific treatments for diabetes, obesity, and hypertension, which have been described in detail, antioxidant treatments (Figure 2) may have the potential to rescue FMR and accelerate revascularization more generally <sup>5, 108, 112</sup>. As described above (5.1), in healthy aging, increasing blood flow is sufficient to restore FMR, as shown using a non-hypotensive dose of hydralazine in healthy old rats <sup>12</sup>. Naturally, healthy aging does not require any treatment, and

in aging associated with one or more risk factors, the dilatory machinery is not always efficient. Whereas targeting the renin-angiotensin system with an AT1R blocker and ACE inhibitors is common in cardiovascular and metabolic diseases <sup>119, 120</sup>, the AT2R is emerging as a potential new therapeutic target.

Importantly, the AT2R is essential for FMR, as shown in mouse mesenteric and skin arteries, and its stimulation restored FMR in 1-year-old male mice <sup>51</sup>. Activation of the AT2R results in opposite and beneficial pharmacological outcomes compared to activation of the AT1R. These include pronounced vasodilator, anti-fibrotic, and anti-inflammatory effects <sup>121</sup>. Stimulation of the AT2R could be an alternative to blockade of the renin-angiotensin system in some ischemic diseases that occur with age (Figure 2). Indeed, AT2R stimulation targets only the associated dilatory and anti-inflammatory pathways, without blocking the production of angiotensin II or inhibition of the AT1R, and this is now feasible in light of the development of efficient AT2R agonists <sup>122</sup>.

Finally, targeting estrogen receptors is potentially another new therapeutic option (Figure 2), as their numerous functions are now better described <sup>15</sup>. Two pathways lead to immediate vasodilatation with the potential for activation of FMR. First, membrane-associated ER $\alpha$  activates NO-dependent dilation, and selective entities are available to target this function without also stimulating the nuclear functions <sup>123, 124</sup>. The G protein-coupled estrogen receptor (GPER) also induces vasodilation without obvious nuclear effects, and a wide panel of agonists such as natural estrogens, raloxifene, fulvestrant, and a number of phytoestrogens are able to activate this receptor <sup>125</sup>. Alternatively, targeting the AF2 function of ER $\alpha$ , which is directly involved in FMR <sup>74</sup>, is another option, and pharmacological entities such as estetrol (E4) hold considerable promise in this regard <sup>126</sup>.

## 6. Conclusion

FMR in collateral growth and arteriogenesis is a complex phenomenon that recruits endothelial and smooth muscle cells as well as the immune system. FMR is involved in post-ischemic revascularization, which often occurs in patients with one or several risk factors, including age. Activating FMR in older subjects requires increasing the stimulus on the endothelium by the addition of extra vasodilatation to the initial stimulus (the increase in shear stress). However, as the need for revascularization is greater in older individuals with risk factors, the therapy cannot merely amount to exercise with or without a mild vasodilating drug. Indeed, a key issue is the reversibility of the vascular damage induced over time, and

thus promoting prevention and limiting exposure to the risk factors remain the best options in this regard.

### **Funding**

This work was supported in part by the foundation for Medical Research (Fondation pour la Recherche Médicale, contract FRM - DPC20171138957, Mitoshear project) and the National Agency for Research (Agence Nationale de le Recherche, contract # ANR-18-CE14-0016-01, Estroshear project)

### **Conflict of interest**

The authors declare that there are no conflicts of interest.

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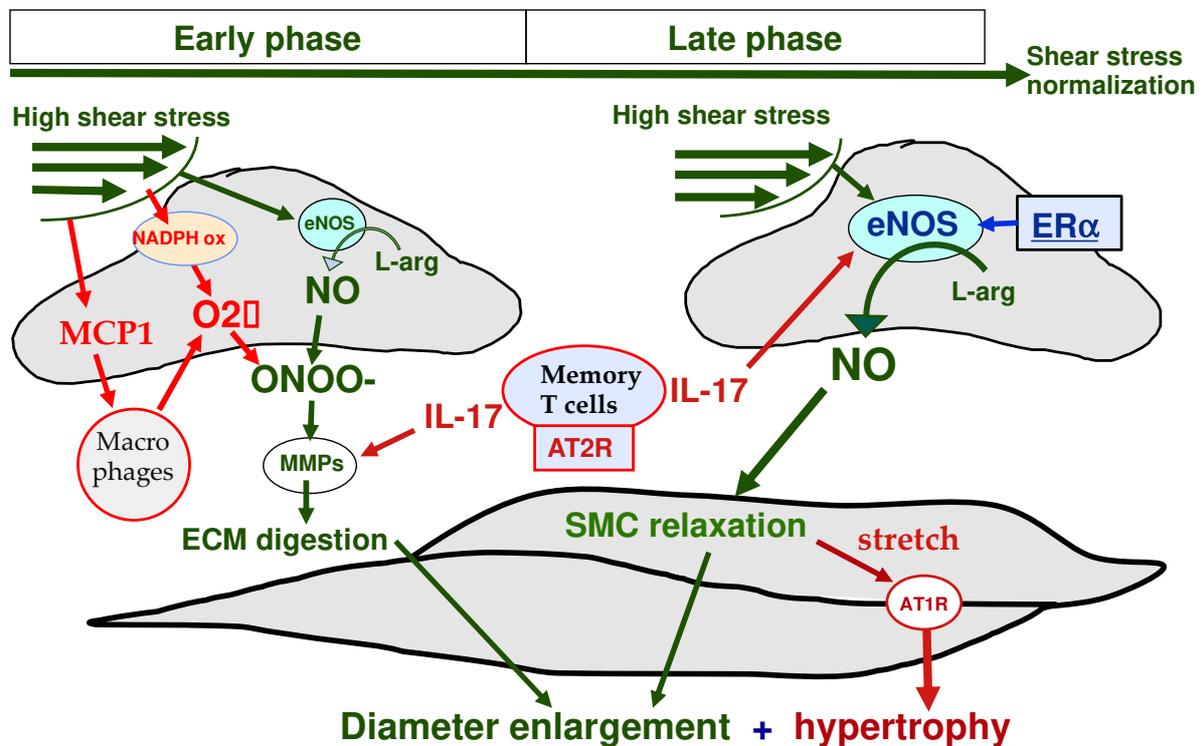
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**Figure 1: Schematic representation of the vascular response to a chronic increase in blood flow.**

In resistance arteries, a chronic increase in blood flow (high shear stress) first activates the NO-pathway and thus the production of NO (within minutes). As the stimulus continues, after several hours a rise in oxidative stress occurs, with the production of superoxide ( $O_2^\bullet$ ) and then the production of the monocyte chemo-attractant protein 1 (MCP1), which attracts macrophages to the perivascular space. The association of NO and  $O_2^\bullet$  produces peroxynitrite ion ( $ONOO^-$ ), which triggers activation of matrix metalloproteinases (MMPs) and extracellular matrix (ECM) digestion. In this early phase, between days 1 and 3 after increasing blood flow, memory T cells and preferentially Th17 cells produce interleukin-17 (IL-17) upon stimulation of the angiotensin II type 2 receptor (AT2R). IL-17 further activates MMPs and also increases eNOS expression level in endothelial cells which thus produce more NO, thereby inducing outward remodeling as an increase in arterial diameter. At this stage, activation of the estrogen receptor alpha (ER $\alpha$ ) participates in overactivation of the NO pathway. The enlargement in diameter allows normalization of the shear stress, and the increase in diameter then enhances the tensile stress (stretch) on the vascular wall, thereby leading to the stimulation of the angiotensin II type 1 receptor (AT1R) which induces hypertrophy and thus normalization of the tensile stress. Altogether, after 6 or 7 days the vessels become larger and thicker, with normalized shear and tensile stress.

