UNIVERSITÀ DEGLI STUDI DI MILANO

Dipartimento di Scienze Farmacologiche e Biomolecolari

Corso di Dottorato in Scienze Farmacologiche Sperimentali e Cliniche

XXIX ciclo

Sex-dependent properties of human endothelial cells: focus on eNOS

Settore scientifico disciplinare BIO/14

Tesi di dottorato di:

Claudia Vanetti

Matricola R10550

Tutor: Dott.ssa Maria Grazia Cattaneo

Coordinatore del dottorato: Prof. Alberto Corsini

Anno Accademico 2015-2016

Index

Riassunto **4**

Abstract **7**

Introduction **10**

1. Cardiovascular diseases 11

1.1 Risk factors for CVD 11

1.2 Sex differences in CVD 12

2. The endothelium 16

2.1 Physiology 16

2.2 Nitric Oxide 18

2.3 Nitric Oxide Synthase 21

2.4 Endothelial dysfunction 26

3. Estrogens and the cardiovascular system 31

4. Angiogenesis 34

4.1 Basic aspects of angiogenesis 34

4.2 Therapeutic aspects of angiogenesis 38

4.2.1 Antiangiogenic therapy 38

4.2.2 Therapeutic angiogenesis 39

Aims of the project **41**

Results **43**

Metabolic activity in male and female HUVECs 44

Hormone deprivation induces a decrease in metabolic activity and cell number in F-ECs and M-ECs 44

Sex hormones did not prevent the decrease in metabolic activity and cell number induced by hormone deprivation in F-ECs and M-ECs 45

CS-FBS is responsible for the decreased metabolic activity and cell number in F-ECs and M-ECs 46

Thyroid hormone does not prevent the decrease in metabolic activity and cell number in F-ECs and M-ECs 47

CS-FBS impairs in vitro angiogenesis 47

Acetate and palmitic acid partially restore in vitro angiogenic phenotypes impaired by CS-FBS in F-ECs and M-ECs 48
The expression of eNOS is higher in F-ECs than in M-ECs 49
eNOS is not involved in the control of M-EC and F-EC proliferation 50
A different role for eNOS in the control of M-EC and F-EC migration 50
M-ECs strictly depend on proliferation to close the wound 51
eNOS is essential for in vitro angiogenesis in F-ECs 52
M-ECs are more dependent on proliferation also for in vitro angiogenesis 52
F-ECs form more frequently tip cells 53
Figures 54
Discussion 74
Materials and methods 87
Bibliography 94
Acknowledgements 108

Riassunto

Nonostante le malattie cardiovascolari siano percepite come malattie in prevalenza maschili, numerosi dati clinici ed epidemiologici dimostrano che riguardano anche le donne. Quello che differenzia i due generi è che nelle donne si presentano con un ritardo di almeno 10 anni rispetto agli uomini in quanto si ritiene che le donne siano protette, perlomeno fino alla menopausa, dagli estrogeni. La solidità dei dati clinici ed epidemiologici non si associa però a un'altrettanto approfondita conoscenza dei meccanismi biologici e molecolari che stanno alla base di queste differenze. Va ricordato che nella complessa serie di eventi che sfocia nell'aterosclerosi e nella malattia cardiovascolare, il primo passaggio è rappresentato da una perdita di funzionalità dell'endotelio, la cosiddetta disfunzione endoteliale. Poiché molto poco si conosce sulle proprietà innate di cellule endoteliali (EC) maschili e femminili ci è parso particolarmente interessante indagare le caratteristiche di EC umane in coltura primaria derivate da cordoni ombelicali di donatori di sesso maschile o femminile (HUVECs, abbreviate rispettivamente in M-EC e F-EC).

Nostro primo obiettivo è stato lo studio dell'influenza degli estrogeni sulle proprietà delle M-EC e F-EC. A tale scopo, abbiamo utilizzato un terreno di coltura privo di ormoni contenente siero fetale bovino sottoposto a procedura di *stripping* (CS-FBS). In queste condizioni, sia le M-EC che le F-EC hanno mostrato una riduzione della crescita e delle proprietà angiogeniche *in vitro* che la riaggiunta degli ormoni sessuali nel mezzo di coltura non è stata in grado di prevenire. Poiché l'effetto inibitorio di CS-FBS è revertito dall'aggiunta di FBS normale al terreno di coltura, ci siamo focalizzati sugli acidi grassi (FA) per due ragioni: *i*) non sono presenti nel CS-FBS; *ii*) la loro ossidazione è necessaria per la crescita delle EC e per l'angiogenesi. Infatti, l'aggiunta di acetato, un donatore di acetil-CoA, o di acido palmitico è stata in grado di revertire significativamente in entrambi i sessi l'inibizione della crescita e dello *sprouting* indotta da CS-FBS. Questi risultati confermano il ruolo centrale degli FA nella fisiologia delle EC sia

maschili che femminili, e suggeriscono cautela nell'utilizzo di terreni contenenti CS-FBS per lo studio delle EC.

In una successiva serie di esperimenti, abbiamo rivolto la nostra attenzione all'espressione e all'attività di eNOS in M-EC e F-EC, considerato il ruolo cruciale dell'enzima e del suo prodotto NO nella fisiologia dell'endotelio. Abbiamo innanzitutto osservato che le F-EC esprimono costitutivamente una maggiore quantità di eNOS, a livello sia di mRNA che di proteina, rispetto alle M-EC. Alla ricerca del significato biologico di questa differenza, abbiamo osservato che le F-EC hanno maggiori capacità migratorie rispetto alle M-EC, e soprattutto che la motilità di F-EC e M-EC differisce in maniera significativa nella dipendenza da eNOS. La migrazione delle F-EC è infatti ridotta dal silenziamento e dall'inibizione farmacologica di eNOS, che lasciano invece pressoché invariata la capacità migratoria delle M-EC. Abbiamo poi paragonato il contributo di migrazione e proliferazione al processo di *sprouting* delle M-EC e F-EC in un saggio 3-D di angiogenesi *in vitro* in cui è stata confermata la stretta dipendenza delle F-EC dall'attività di eNOS e dalla migrazione. Al contrario, le M-EC mostrano una stringente dipendenza dalla proliferazione che non coinvolge l'attività di eNOS.

In conclusione, a differenza delle M-EC, le F-EC sembrano essere strettamente dipendenti dall'attività di eNOS per la migrazione e lo *sprouting*. La maggiore espressione costitutiva di eNOS nelle F-EC potrebbe contribuire alla protezione contro le malattie cardiovascolari che caratterizza la popolazione femminile prima della menopausa, e suggerire lo sviluppo di nuove strategie *sex-oriented* per la prevenzione e la terapia delle malattie cardiovascolari.

Abstract

Atherosclerosis and cardiovascular disease (CVD) are classical examples of diseases where sex/gender differences have been described. A significant body of evidence suggests that CVD is less prevalent in women than men until midlife, and the female advantage has been attributed to estrogens, which are lost with menopause. The earliest event in the onset of atherosclerosis and CVD is endothelial dysfunction (ED). For this reason, many *in vitro* studies have been performed in endothelial cells (ECs). However, the sex of ECs has not been consistently reported. Therefore, we thought of interest to compare the behavioral and molecular features of human primary male and female ECs obtained from umbilical cords (HUVECs, abbreviated as M-ECs and F-ECs, respectively) .

To study the involvement of estrogens in the sex-related properties of ECs, we started by culturing HUVECs in a nominally hormone-free medium supplemented with charcoal-stripped serum (CS-FBS) commonly used for *in vitro* studies on hormone activities. Surprisingly, we found that both M-ECs and F-ECs underwent a significant decrease in cell number which was not reverted when sex hormones were added back to the culture medium. In addition, the presence of CS-FBS abrogated the ability of M-ECs and F-ECs to sprout in an *in vitro* 3-D angiogenesis assay. Since the inhibitory effect of CS-FBS was fully reverted by the re-addition of normal FBS, we focused our attention on fatty acids (FAs), that are lost in CS-FBS and that are crucially involved through their oxidation in the control of EC behavior. Reconstitution experiments performed by re-adding the acetyl-CoA precursor acetate or palmitic acid partially prevented the CS-FBS-induced inhibition of EC growth and sprouting. Therefore, a lack of FAs seems to be partly responsible for the impairment in the *in vitro* proliferative and angiogenic properties of M-ECs and F-ECs cultured in CS-FBS containing media.

A reduced activity of eNOS plays a key role in CVD onset and development through the reduced formation of nitric oxide (NO) in ED. Therefore, we focused on the expression and the role of the enzyme in M-ECs and F-ECs. Interestingly, we found that F-ECs constitutively expressed an higher amount of eNOS both at mRNA and protein levels. Moreover, F-ECs possessed greater migratory capabilities in comparison to M-ECs. Importantly, the increased female migratory properties were counteracted by the treatment with the eNOS inhibitor L-NAME or by the silencing of the enzyme. At variance, M-EC motility was independent of eNOS activity.

Since angiogenesis is the result of a coordinated process where the migration of specialized cells – the tip cells – and the proliferation of the so-called stalk cells both contribute to the growth of new vessels, we evaluated in an *in vitro* 3-D spheroid assay the relative contribute of motility and proliferation to the M-EC and F-EC sprouting process. Notably, F-ECs confirmed their strictly dependence on migration and eNOS activity, whereas M-ECs showed a more important reliance on proliferation which was eNOS-independent. The different migratory behavior between M-ECs and F-ECs was confirmed in mixed spheroids from GFP-carrying M-ECs and Cherry Red-expressing F-ECs where the analysis of sprouts showed that F-ECs were located as tip cells in most of the sprouts.

In conclusions, our results show that F-ECs have a higher migratory phenotype in comparison to M-ECs and are strictly dependent on eNOS activity for their migration and sprouting. The constitutive higher expression of eNOS in F-ECs, and the crucial requirement of its activity for motility and *in vitro* angiogenesis, might contribute to the protection against CVD characteristic of the younger female population, and might propose the development of novel sex-oriented strategies for the prevention and therapy of CVD.

Introduction

1. Cardiovascular disease

Cardiovascular disease (CVD) is a group of disorders of the heart and blood vessels that includes coronary artery diseases, cardiomyopathy, stroke, hypertensive heart disease, congenital heart disease, rheumatic heart disease, heart arrhythmia, valvular heart disease, aortic aneurysms, peripheral artery disease and venous thrombosis.

CVD is globally the most common cause of death. It accounts for 31.5% of all deaths, more than twice than the deceases caused by cancer[1]. It has been estimated by the Global Burden of Disease (GBD) that CVD has been responsible for 17.3 million deceases worldwide in 2013.

CVD and stroke globally produce huge health and economic burdens. For 2011 to 2012, the estimated annual costs for CVD and stroke in the United States were $316.6 billion, including direct (hospital services, prescribed medications, home health care) and indirect costs (in terms of lost of future productivity for cardiovascular and stroke premature deaths). CVD costs more than any other diagnostic group. By comparison, in 2011, the estimated direct cost of all cancers was half of CVD direct cost[2].

1.1 Risk factors for CVD

CVD can be prevented by addressing behavioral risk factors. Lifestyle-related risk factors are the foremost causes of death and disability in the world. The leading risk factor for CVD in the United States is the suboptimal diet quality that correlates with overweight and obesity, thus predisposing individuals to most major risk factors, including physical inactivity, hypertension, hyperlipidemia, and diabetes mellitus. Although tobacco use

has substantially declined in the United States, it remains the second-leading cause of total deaths and disability. Likewise, harmful alcohol consumption predisposes individuals to the development of CVD.

Next to behavioral risk factors, there are other important health risk factors, that include genetics, high blood pressure, diabetes, lipid levels, high blood cholesterol, and metabolic syndrome. Furthermore, vascular ageing contributes to the age-dependent rise in hypertension and atherosclerotic disease, and confers itself a greater risk for CVD than all of the other conventional risk factors[3].

A common feature of all cardiovascular risk factors mentioned above is the loss of endothelial function that precedes the development of vascular disease. Specifically, endothelial dysfunction (ED) is a systemic pathological state of the endothelium that occurs when the endothelium fails to sustain its normal physiologic mechanisms[4]. ED could be useful as a highly sensitive marker for the overall cardiac risk. Indeed, it has been reported[5,6] that patients with ED have greater chances of having a cardiac event compared to patients showing a normal endothelial function.

1.2 Sex differences in CVD

A significant body of epidemiological and clinical data emphasizes that sex differences play a major role in CVD[7]. It has been reported that the total number of deaths from CVD is higher in women (49% of all deaths) than men (40% of all deaths). However, more men (0.9 million) than women (0.5 million) die from CVD before the age of 75[8]. The observed sex differences are greater at younger ages, with CVD less prevalent in women than men until midlife (**Figure I**).

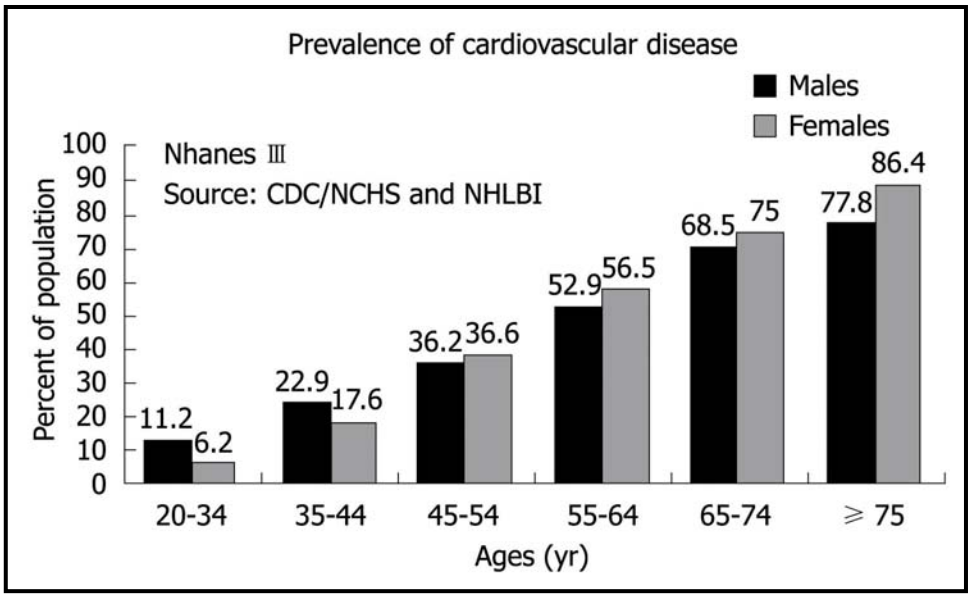

Figure I. Prevalence of CVD in men and women. From *World Journal of Cardiology (2009) 1, 295-302*

These significant sex differences also exist in the occurrence of various manifestations of CVD. It has been reported that the risk of developing coronary heart disease is higher in men than in women, particularly at younger ages. But women are more likely to develop cerebro-vascular disease or heart failure as a first event, and have greater microvascular dysfunctions relative to men, although these manifestations appear at older ages[9,10].

An important body of evidence supports the idea that the female risk for CVD increases more quickly than it does for same-aged men after the menopause[11]. The female advantage in younger women has been attributed to vascular protection by estrogens, which is lost with menopause. In premenopausal women, the high levels of endogenous estrogens have been linked to a lower risk for a number of diseases, such as hypertension, vascular disease, stroke, diabetes mellitus and obesity, compared to age-matched men[12]. During the menstrual cycle, blood pressure levels lower

when estrogen levels peak, reflecting their vasodilator activity. Similarly, the increase in endogenous estrogen production during pregnancy contributes to the maintenance of a normal blood pressure[13]. In addition, chronic estrogen treatment enhances the endothelium-dependent vasodilation of large peripheral arteries in post-menopausal women[14].

Sex differences in the manifestation of CVD have also been observed in different animal models where the protective role of estrogens has been confirmed[7]. For example, female rodent hearts are more resistant to ischemia/reperfusion-induced injury[15] and myocardial infarction[16,17], thus suggesting a cardio-protective role for estrogens in female animals. Moreover, the endothelium-dependent relaxations are more pronounced in the arteries of young female animals than in males. Ovariectomy causes blunting of such responses, whereas reintroduction of physiological levels of estrogens potentiates them[18]. Furthermore, the experimental administration of estrogens reduces the infarct size in rabbit, mice, and rat[19,20,21].

Increasing evidence suggest that differences between male and female are according to sex, regardless of their exposure to sex hormones[22,23,24]. But, despite the evidence of important sex/gender differences in both rodent and human endothelial function[10,25,26], the sex of cells has not consistently taken in account in studies involving endothelial cells (ECs), even when the effects of sex hormones have been analyzed. As a consequence, our knowledge on the molecular mechanism(s) underlying sex-based differences in cardiovascular physiology and disease is currently incomplete[7,27]. However, some inborn sex differences appear when primary cultures of human male and female ECs have been independently studied[28,29,30,31].

The presence of innate differences between male and female could have major implications for the susceptibility to many diseases, and for their diagnosis and treatment. Nevertheless, it is more likely that sex differences in cardiovascular physiology and pathology are the result of multiple factors, arising from genetic differences (*e.g.*, X and Y chromosomes, epigenetic modulation), from the action of sex steroid hormones (*e.g*., estrogen and/or testosterone), or from a combination of all these factors.

2. The endothelium

The vascular endothelium is constituted by a monolayer of thin and flat cells – the endothelial cells (ECs) – that forms the inner continuous lining of blood vessels[32]. It represents an important structural barrier between the circulation and the surrounding tissues, thus regulating the transfer of small and large molecules across them[33,34].

2.1 Physiology

The primary function of endothelium - and ECs - is the control of vascular homeostasis by modulating the blood vessel tone. To this purpose, ECs produce and release vasodilators such as nitric oxide (NO) and prostacyclin (PGI2), and vasoconstrictors, including platelet-activating factor (PAF) and endothelin (ET). Among vasodilator substances, NO is certainly the most important. Its production is modulated by a number of exogenous chemical and physical stimuli, and NO profoundly affects the function of the underlying vascular smooth muscle cells (VSMCs) to maintain an optimal blood vessel tone[33].

Another crucial physiological function of the endothelium is the maintenance of a non-thrombogenic blood-tissue interface to inhibit platelet adhesion and clotting, and to facilitate blood flow. The endothelium exerts its anticoagulant activity through the action of several factors, such as for example anti-thrombin III and heparin co-factor II. Other anticoagulant factors produced by the endothelium are the Tissue Factor Pathway Inhibitor (TFPI) - which inhibits the binding of the tissue factor-activated factor VII to the activated factor X[35] - and thrombomodulin (TM) – that, in complex with the activated protein C, can associate to the co-factor protein S, with an

increase in its anticoagulant activity[32]. A homeostatic equilibrium between anti- and pro-thrombotic factors is always present. This dynamic equilibrium is however perturbed during injuries, or when the endothelium is exposed to physical forces (shear or tensile stress) or mediators such as thrombin, endotoxin, cytokines, hypoxia, or oxidized lipids[36]. In response to these conditions, ECs undergo programmatic biochemical changes that culminate in their transformation into a pro-thrombotic surface. Importantly, ECs can easily return to the unperturbed state once that the pro-coagulant stimulus has been removed.

Mechanical stimuli are of particular importance in the endothelium homeostasis. An increase in the blood flow enhances the hemodynamic forces acting on the vessel wall with the generation of the so-called shear and tensile stress. Shear stress is a tangential, frictional force acting in the direction of blood flow. As a consequence, shear stress involves only the surface of ECs, that will be gradually elongated in the direction of the flow[37]. At variance, tensile stress acts perpendicular to the vessel wall since it is the result of the dilating force of the blood pressure. Therefore, tensile stress involves not only ECs but also the other components of the vessel wall, that are fibroblasts and VSMCs. The activation of ECs by shear stress promotes the release of vasodilatant mediators (especially NO), whereas tensile stress directly stimulates VSMCs, thus inducing their contraction and EC stretching. Therefore, the net effect of mechanic stimuli on the vascular tone results from the interactions between the pressure-induced myogenic contraction and the endothelium-dependent vasodilatation induced by blood flow.

In addition to the above-mentioned role in the regulation of blood coagulation, ECs also express cell surface-molecules that orchestrate the trafficking of circulating blood cells. These cell-associated molecules help

directing the migration of leukocytes into specific organs under physiologic conditions, and accelerate migration towards sites of inflammation. These pathways have been also implicated in platelet and erythrocyte adhesion in several common disorders associated with vascular occlusion[32].

2.2 Nitric oxide

NO is a highly lipophilic free radical gas[38] with an half-life of less than 5 seconds[38,39,40]. It can easily diffuse from its source and reach its final targets - often localized at some distance from the sites of synthesis - by crossing multiple cell membranes[38].

Experiments in isolated blood vessels and ECs suggest that the endothelial nitric oxide synthase (eNOS) (see below) is constitutively active and responsible for the so-called basal release of NO in the absence of evident external stimuli. When constitutively produced by the enzymatic action of eNOS, NO diffuses to the underlying VSMCs where stimulates the guanylyl cyclase (GC) activity, thus resulting in vaso-relaxation and dilatation[41]. Moreover, NO also reduces the proliferation of VSMCs by preventing the release of their mitogen Platelet-Derived Growth Factor (PDGF).
Other well-characterized actions of NO include the inhibition of leukocyte and platelet adhesion to the vascular wall, and the inhibition of platelet aggregation[42]. Importantly, NO also plays a crucial role in angiogenesis by promoting EC proliferation, differentiation, and migration to the perivascular space.

The main mechanism responsible for NO effects on blood vessels involves the activation of the soluble GC (sGC)-cGMP signaling pathway[43,44] (**Figure II**). This pathway mediates the NO-dependent relaxation of VSMCs and the

suppression of platelet aggregation[45]. The affinity of sGC for NO is very high, thus allowing its activation also in the presence of nanomolar concentrations of NO[46]. Once activated, sGC catalyzes the conversion of guanosine triphosphate (GTP) to cGMP which in turn activates downstream effector systems such as protein kinases, phosphodiesterases, and ion channels[47]. In VSMCs, protein kinase G (PKG) is the main downstream target of cGMP. When activated by cGMP, PKG inhibits the release of calcium from the sarcoplasmic reticulum (SR) and its influx from the extracellular space. In addition, PKG also promotes the uptake of calcium by the SR and its extrusion through the membrane calcium-ATPase. As a result, intracellular calcium is reduced, and the calcium-dependent myosin light-chain kinase (MLCK) can no longer phosphorylate myosin, thus preventing the contractile process and favoring VSMC relaxation. To the maintenance of low levels of dephosphorylated myosin also contributes the direct effect of PKG on the regulatory subunit of the myosin light-chain phosphatase (MLCP).

Also the antiaggregant effect of NO is mediated by cGMP *via* the reduction of platelet calcium levels and the negative modulation of molecules essential for platelet aggregation and activation, such as the glycoprotein IIb/IIIa[38].

Dysfunction of the sGC-cGMP pathway has been reported to contribute to the pathogenesis of many disorders, including hypertension and atherosclerosis[48,49].

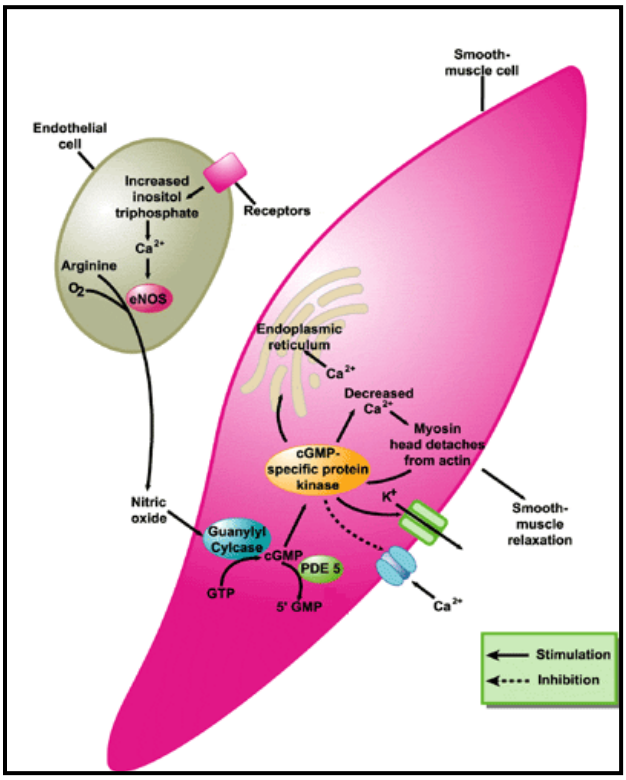

Figure II. NO and the sGC-cGMP pathway in the endothelium. Modified from *Concepts in Biochemistry*, *Wiley 2002*

Another important signaling mechanism utilized by NO is S-nitrosylation where NO (or its metabolites) reacts with cysteine residues of target proteins[38]. S-nitrosylation has been shown to impact a wide range of biological processes including apoptosis, cellular trafficking, proliferation, NO synthase activity, ion channel activity and muscle contractility, transcription factor activity, protein secretion, blood flow, as well as a wide range of patho-physiological events[50]. In contrast to the sGC-cGMP pathway, nitrosylation requires micromolar concentrations of NO, thus allowing the nitrosylation of proteins localized in close proximity to the source of NO.

2.3 Nitric Oxide Synthase

NO is synthesized by the catalytic action of enzymes termed NO Synthases (NOSs)[38,51]. It is produced from the terminal guanidine nitrogen of L-arginine and O_2 through the formation of an *NG*-hydroxy-L-arginine intermediate yielding L-citrulline.

There are three major isoforms of NOS, encoded by separate genes on separate chromosomes: neuronal NOS (nNOS), or type 1 NOS; inducible NOS (iNOS), or type 2 NOS; and endothelial NOS (eNOS), or type 3 NOS[4]. The three isoforms - that share approximately 50-60% sequence homology - have distinctive catalytic and regulatory properties, even if they show similar enzymatic and chemical properties. The common structure of NOSs contains two main domains (**Figure III**): (1) the N-terminus oxygenase domain, that exhibits homology with the other NOS isoforms and poor similarity with the cytocrome P450 mono-oxygenase enzymes; (2) the C-terminus reductase domain, that shows homology with various cytocrome P450 reductases. The N- and C-terminal domains are linked by a short sequence that binds calmodulin (CM), an allosteric modulator essential to reach full NOS activity. The N-terminus presents two binding sites: one for tetrahydrobiopterin (BH4) and heme, while the other one represents the active site where L-arginine binds to the enzyme. On the other hand, the C-terminal domain binds the co-factors NADPH, FAD and FMN, which are all essential for NOS activity. Notably, the protein eNOS is synthesized as a monomer, but it has to form dimers to bind BH4 and L-arginine to generate NO (**Figure III**). In the absence of BH4 and L-arginine, the oxygenase domain of eNOS monomers generates superoxide anions instead of NO, a condition referred to as eNOS uncoupling (see below).

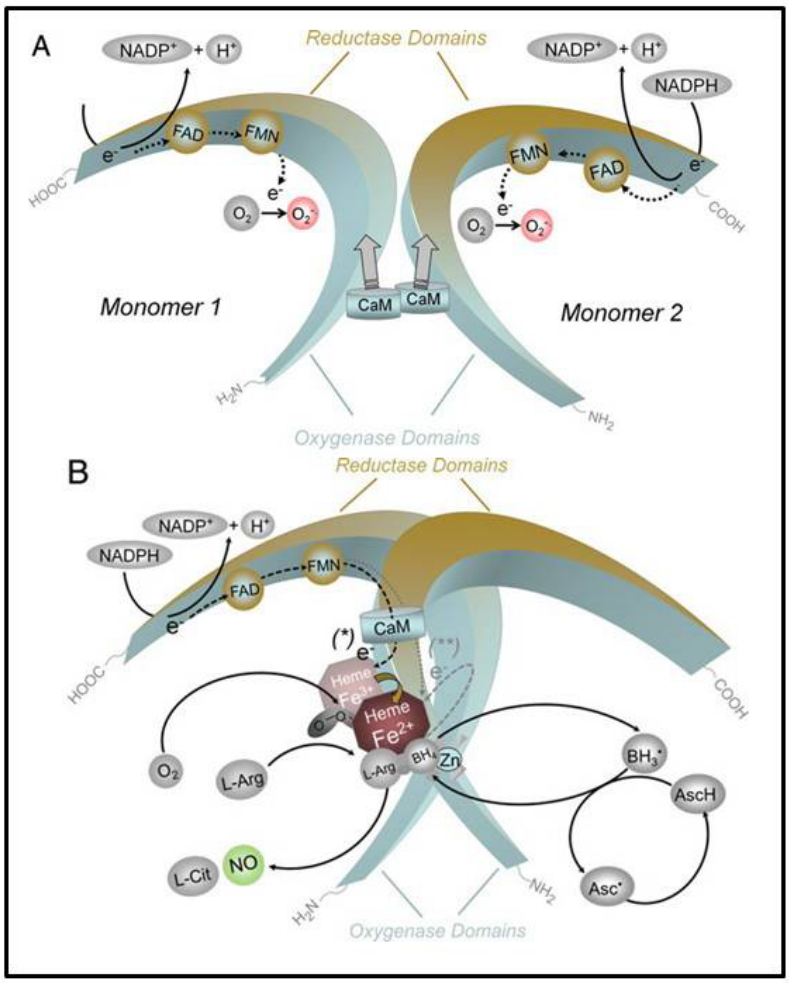

Figure III. The common structure of NOSs. **(A)** NOS monomers transfer electrons from reduced NADPH to FAD and FMN, and possess a limited ability to reduce molecular oxygen to superoxide. Monomers and reductase domains can bind calmodulin (CaM) which enhances electron transfer inside the reductase domain. NOS monomers are unable to bind the cofactor BH4 or the substrate L-arginine, thus monomers are not able to catalyze NO production. **(B)** In the presence of heme, NOS can form a functional dimer to produce NO. From *European Heart Journal (2011) 33, 829-837*

NOS enzymatic activity needs to be finely regulated to maintain NO homeostasis. A first level of regulation is represented by the tissue-specific expression of the different isoforms. nNOS is widely expressed in neurons of the central and peripheral nervous systems, but it is also found in skeletal muscle, the adventitial layer of some blood vessels, pulmonary epithelium, the gastrointestinal system, and the genitourinary system. iNOS was first

recognized in activated macrophages, but it has also been identified in various activated cell types, including monocytes, neutrophils, eosinophils, hepatocytes, VSMCs, myocytes, osteoblasts, fibroblasts, epithelium, and endothelium. eNOS is expressed in vascular endothelium as well as in blood platelets and cardiomyocytes[53].

Focusing on eNOS, its activity is controlled by a complex integration of transcriptional, post-transcriptional and posttranslational mechanisms[38]. For example, laminar shear stress modulates eNOS transcription through the activation of the transcription factor KLF2 or by the action of the Rho/Rho kinase pathway. The post-transcriptional level of regulation essentially regards mRNA stability. Although eNOS mRNA stability is notable under normal physiological conditions, it is diminished in the presence of oxidized low density lipoprotein (LDL), thrombin, inflammation, and hypoxia. The decrease in mRNA stability triggers a decrease in eNOS expression by maintaining at the same time a normal level of eNOS transcription.

Post-translational mechanism(s) represent however the most important level of regulation of eNOS activity. The membrane targeting and recruitment of eNOS in caveolae are for example essential for this modulation. Caveolae are small invaginations of the plasma membrane enriched in proteins as well as in lipids - such as cholesterol and sphingolipids - that are involved in signal transduction[54]. In caveolae, eNOS interacts with the caveolae coating protein caveolin-1 (Cav-1). This protein-protein interaction inhibits eNOS activity by sterically occupy the calmodulin (CM) binding site. Mechanistically, the recruitment of CM to eNOS displaces Cav-1 from the enzyme thereby leading to its activation. CM is a key activator of eNOS that specifically links the enzyme activity to intracellular calcium levels. Elevation of intracellular calcium - or exposure of ECs to shear stress – promotes the

dissociation of the eNOS-Cav-1 complex, thus permitting the association of eNOS with the active calcium-CM complex. The caveolar targeting of eNOS is totally dependent on its co-translational myristoylation - the irreversible addition of a myristoyl group - at the N-terminal glycine[55].

eNOS is also subject to post-translational palmitoylation on two cysteine residues (Cys15 and Cys26). This modification is reversible and requires the prior *N*-myristoylation and membrane anchoring of eNOS. In particular, palmitoylations at Cys15 and Cys26 facilitate the hydrophobic interaction of eNOS with the caveolae, where it comes in close contact with regulatory molecules including G-protein–coupled receptors (GPCRs) and Akt. Hence, palmitoylation contributes not only to the proper localization of eNOS but also permits the integration of its activity with upstream signaling generated by neuro-humoral (agonists and hormones) and physical (shear stress) stimuli.

A crucial level of post-translational regulation of eNOS activity is phosphorylation (**Figure IV**). Different sites of phosphorylation result in opposite effects on the enzyme activity: the phosphorylation of Ser1177 (in human) activates eNOS, while the phosphorylation of Thr495 inhibits it. Multiple kinases – such as the cAMP-activated protein kinase A (PKA), the phosphatidylinositol-3-kinase (PI3-K)/Akt pathway, the AMP-activated protein kinase (AMPK), the calcium-CM-dependent protein kinase II, and the extracellular regulated kinase 1/2 (ERK1/2) - are known to phosphorylate eNOS at Ser1177 in response to various stimuli (*e.g.*, the vascular endothelial growth factor (VEGF), estrogens and shear stress). The phosphorylation of Ser1177 increases the electron flow and the affinity of eNOS for the calcium-CM complex[56], thus increasing NO synthesis at lower levels of intracellular calcium. By contrast, Rho-kinase (*e.g.*, in response to angiotensin II and thrombin), and protein kinase C (PKC) phosphorylate

eNOS at Thr495, thus inhibiting the enzyme. The activation of eNOS by phosphorylation at Ser1177 is facilitated by the concurrent dephosphorylation of Thr495.

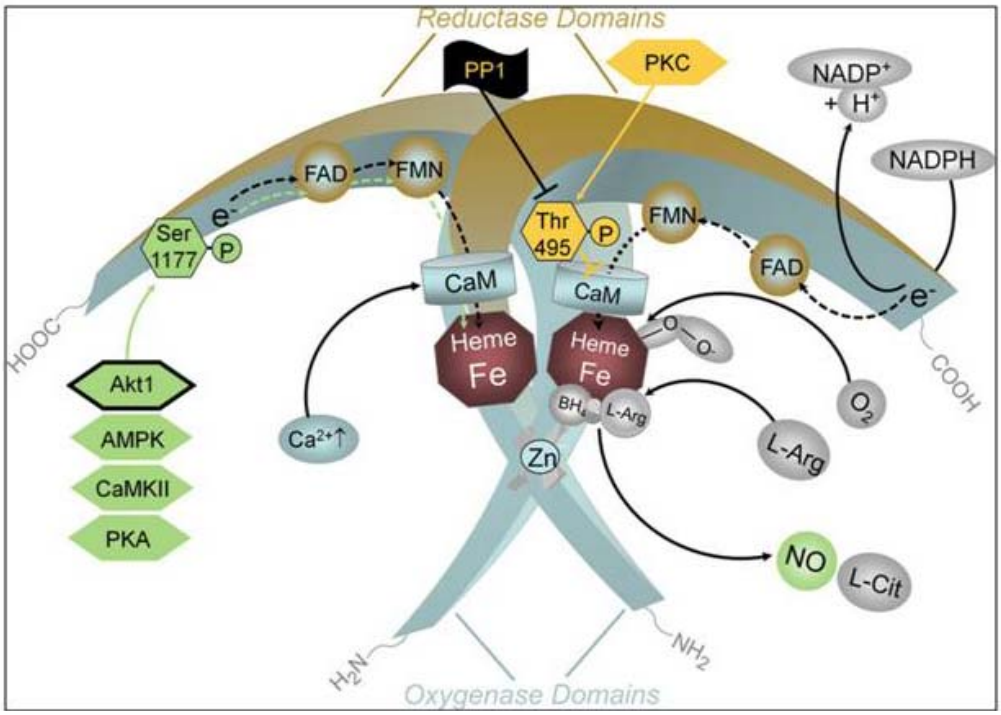

Figure IV. Regulation of eNOS activity by intracellular calcium and phosphorylation. An increase in intracellular Ca^{2+} leads to an enhanced binding of calmodulin (CaM) that facilitate the flow of electrons from NADPH (in the reductase domain) to the heme (in the oxygenase domain). The phosphorylation of Ser1177 increases the flux of electrons through the reductase domain, and finally the enzyme activity. *Vice versa*, the phosphorylation of Thr495 decreases the electron flux, and consequently the enzyme activity. Also PKC, the kinase that phosphorylates Thr495, and the protein phosphatase 1 (PP1), that dephosphorylates Thr495, are shown. From *European Heart Journal (2011) 33, 829-837*

Interestingly, eNOS is post-translationally regulated *via* S-nitrosylation by its product NO. S-nitrosylation mainly occurs on the enzyme localized in caveolae since the local hydrophobic environment favors the accumulation of NO and the formation of S-nitrosothiols. Only nitrosylation at the residues

Cys94 and Cys99 – that are located at the interface for dimer formation – results in eNOS inhibition. Importantly, S-nytrosilation is a reversible process, and denitrosylation of eNOS mainly occurs in the cytosol due to its high content of reduced thiols.

Another possible mechanism for the post-translational regulation of eNOS is acetylation. Both acetylation of Lys610 and deacetylation of Lys497 and Lys507 increase eNOS enzymatic activity[18]. Oxidative stress (*e.g.*, aging, hypertension, and diabetes) or an excessive intracellular level of NO (*e.g.*, during inflammation) impair this regulatory process.

Additional post-translational mechanisms of eNOS regulation are glycosylation - the attachment of N-acetylglucosamine by O-linked N-acetylglucosamine transferase at Ser1177, that blocks the activating phosphorylation of eNOS - and S-glutathionylation - the addition of glutathione at Cys689 and Cys908, that favors the uncoupling of eNOS by promoting its reaction with oxygen and by preventing the binding of the co-factors FAD and FMN[18].

2.4 Endothelial dysfunction

The term endothelial dysfunction (ED) refers to broad alterations in the endothelial phenotype that contribute to the development and clinical expression of atherosclerosis and CVD[57] (**Figure V**). The cardiovascular risk factors - including smoking, aging, hypercholesterolemia, hypertension, hyperglycemia - are all associated with alterations in endothelial function. ED finally leads to vessel malformation and regression, improper re-vascularization, healing and regeneration, heart and brain ischemia, neuro-degeneration, hypertension, preeclampsia, respiratory distress, and

osteoporosis[58]. Very importantly, ED is a reversible disorder, and strategies aiming at reducing cardiovascular risk factors - such as cholesterol lowering, antihypertensive therapy, smoking cessation, estrogen replacement therapy in postmenopausal women, and physical exercise - all induce an improvement in endothelial health, further supporting the proposed association between risk factors and ED. Moreover, the observation that pharmacological interventions that improve endothelial function are associated with a decrease in cardiovascular events supports the notion that cardiovascular risk factors share a common pathway that converges on ED.

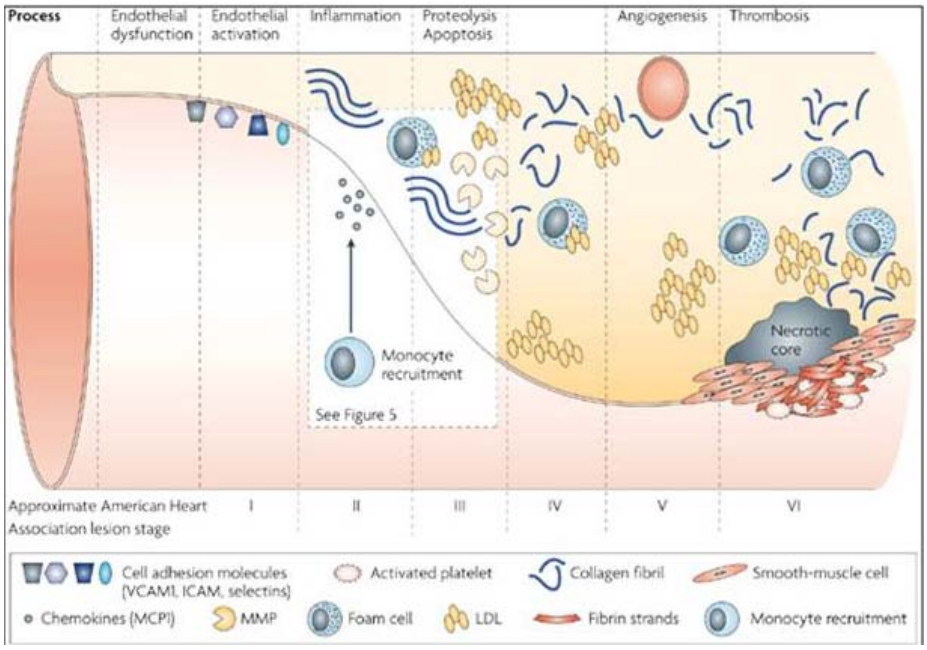

Figure 5. Processes of atherogenesis starting from endothelial dysfunction. From *Nature Reviews Drug Discovery (2008) 7, 517-529*

The patho-physiology of ED is quite complex and involves multiple mechanisms. However, the earliest event in ED is a reduced release of NO coupled with an increase in Reactive Oxygen Species (ROS) in the vascular wall. The chronic production of ROS may exceed the capacity of cellular

enzymatic and non-enzymatic anti-oxidant systems, thus contributing to the onset of vascular disease.

It is intriguing that eNOS, which normally helps to maintain the endothelium in a quiescent state, can switch to generate ROS instead of NO – the so-called eNOS uncoupling - as part of the endothelial activation process. Currently, the deficiency of the cofactor BH4 has been associated to the production of superoxide anions. Again, the deficiency of L-arginine induces the eNOS-mediated formation of hydrogen peroxide[59].

An important source of cellular ROS are mitochondria, where superoxide - produced as a by-product of oxidative phosphorylation - were rapidly converted into molecular oxygen or hydrogen peroxide by the enzymatic activity of the mitochondrial superoxide dismutase (mSOD). Hydrogen peroxide is also a damaging ROS, but it is further degraded by other enzymes, such as catalase. The balance between the metabolic production of ROS and their degradation is altered in hypoxia or in pathological conditions characterized by hyperglycemia and increased circulating free fatty acids, such as obesity-related metabolic disorder and type II diabetes. Another important source of metabolic ROS is xanthine oxidase (XO), an enzyme that generates ROS as a by-product of the purine catabolism. Finally, endothelial ROS may also be induced by external stimuli - such as inflammatory cytokines and growth factors - and by the interaction of the endothelium with leukocytes.

Regardless of their source, the interaction between ROS and NO sets up a vicious circle, which contributes to EC activation and inflammation. In fact, ROS – hydrogen peroxide, specifically – reacts with NO to form peroxynitrite, an oxidant and nitrating molecule that can damage a wide array of cellular molecules. In particular, peroxynitrite is involved in the

formation of the oxidized LDL (oxLDL) that crucially contributes to EC activation and dysfunction[60]. The binding of OxLDL to its main receptor LOX-1 supports the recruitment of circulating leukocytes, triggers foam cell formation, sustains migration and proliferation of VSMCs, and reduces NO bioavailability *via* dephosphorylation of the activating Ser1177 on eNOS[61]. Furthermore, OxLDL-LOX-1 interaction may also contribute to plaque destabilization by inducing VSMC apoptosis and the release of matrix degrading enzymes (MMPs).

ED could be considered to a some extent a kind of endothelial activation, in which ECs switch from a quiescent to an activated phenotype where the stimulation of various molecular machineries results in the expression of chemokines, cytokines, and adhesion molecules. As a matter of fact, the dysfunctioning endothelium, in concert with NO deficiency, produces some other substances and mediators that are detrimental to the vessel wall, such as for example thromboxane A2 (a pro-thrombotic agent, TXA2), and prostaglandin H2 (a potent vasoconstrictor, and also the precursor of TXA2). Up-regulation of adhesion molecules, generation of chemokines, and production of the plasminogen activator inhibitor-1 (PAI-1) – an inhibitor of the tissue plasminogen activator (tPA) and urokinase (UPA) that are required for the breakdown of blood clots – also participate to the establishment of the inflammatory and pro-thrombotic state of the activated endothelium. Vasoactive peptides, such as angiotensin-II and endothelin-1, ADMA accumulation, hypercholesterolemia, hyper-homocysteinemia, an altered insulin signaling, and hyperglycemia also contribute to the onset of ED. Among vasoactive peptides, an important element involved in ED is Endothelin-1 (ET-1). ET-1 is the strongest vasoconstrictor molecule of endothelial origin, and acts through the binding to its specific receptors ETA and ETB. ETA are expressed only on VSMCs and cause vasoconstriction and

cellular proliferation, while ETB are localized both on VSMCs and ECs, and induce vasodilatation by stimulating NO production, which in turn acts as a negative feedback on ET-1 production. When NO bioavailability is reduced, the negative feedback is compromised, and consequently the vasoconstrictor effect of ET-1 become predominant.

3. Estrogens and the cardiovascular system

Several clinical and epidemiological data support a central role for estrogens in cardiovascular physiology and disease, especially in relationship to the described premenopausal gender differences.

The effects of estrogens are mediated by both genomic and non-genomic mechanisms involving the regulation of target gene expression and the activation of non-transcriptional signaling pathways, respectively (**Figure VI**). There are two different isoforms of nuclear estrogen receptors (nERs), usually referred to as ERα and ERβ, that showed a significant overall sequence homology. Estrogens bind to the C-terminal E domain, and the resulting estrogen-ER complex undergoes dimerization, translocation to the nucleus, and binding to the estrogen response element (ERE) on target genes *via* the DNA-binding domain C[62]. Mouse knock out studies have shown that the expression of ERα is required for the protective effect of 17β estradiol (E2, the active, natural form of estrogen) against pathologic vascular injury responses and atherosclerosis. At variance, ERβ seems to be dispensable for these effects even if the ERβ-deficient mice are hypertensive when compared to wild-type animals[38].

It has also been shown that E2 reduces the production of inflammatory cytokines and neutrophil adhesion in injured vessels, although it is unknown if these effects require nER. At the cellular level, E2 inhibits VSMC proliferation - a central patho-physiologic component of the adverse vascular remodeling in response to injury - and promotes the proliferation and migration of ECs – a crucial aspect of vascular repair after injury.

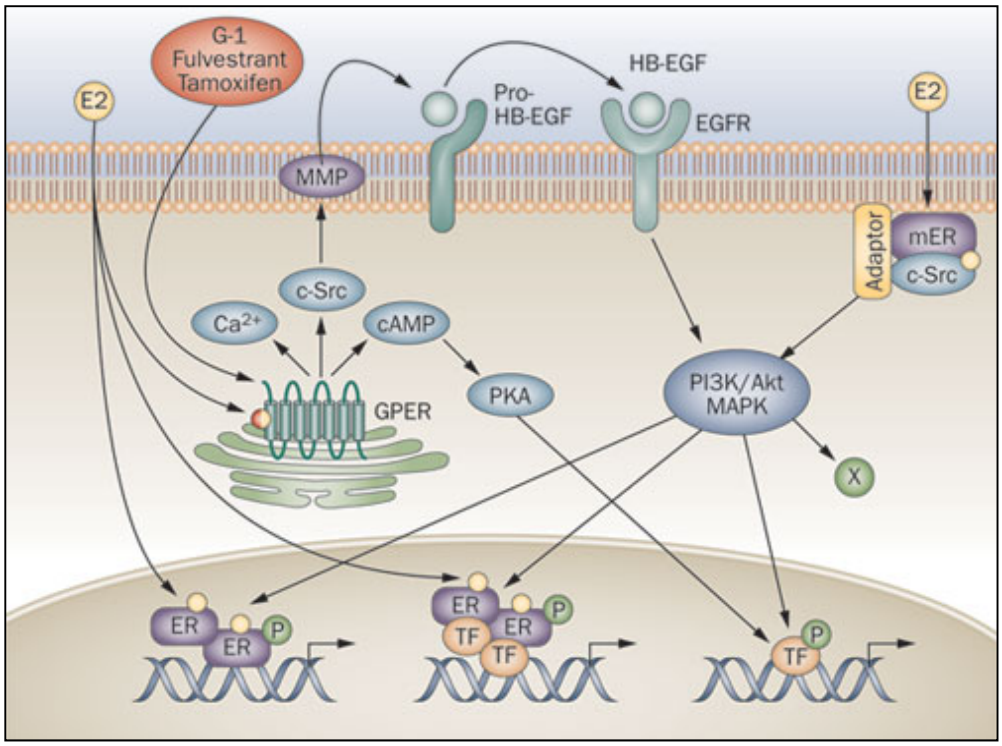

Figure 6. Genomic and non genomic estrogen signaling pathways. E2 activates nERs by inducing receptor dimerization and direct binding to the promoters of target genes, or modulation of other transcription factors through protein-protein interaction. At the plasma membrane, a subpopulation of ERs interacts with adaptor protein and signaling molecules to mediate rapid signaling *via* PI3K-Akt and MAPK pathways. E2, or the agonists G-1, also activates GPER that stimulates cAMP production, calcium mobilization and c-Src. The activation of c-Src is in turn responsible for the trans-activation of EGFR, which finally activates MAPK and PI3K-Akt pathways to induce genomic or rapid non-genomic effects. From *Nature Reviews Endocrinology (2011) 7, 715-726*

A small subpopulations of ERα is palmitoylated and localized at the plasma membrane where it forms signaling complexes in caveolae. These membrane-bound ERα elicit rapid non-genomic effects, leading to the activation of several signal transduction pathways, including c-Src, PI3-K-Akt and ERK1/2, through the interaction with specific adaptor proteins. One of the best characterized non-genomic effects of the membrane ERα is the fast activation of eNOS in ECs, resulting in NO formation and vasorelaxation.

Very recently, it has been proposed that rapid signaling through ERα is also essential for many of the transcriptional responses of ECs to E2, and that the ERα rapid signaling may be critical for the *in vivo* vasculo-protective and anti-inflammatory effects of estrogen[63].

It has been recently reported that a G protein-coupled receptor named GPR30/GPER (G protein–coupled estrogen receptor) may also contribute to estrogenic effects[64]. This receptor is physically associated with Gαs, and formation of cAMP is measured following stimulation with estrogen. Moreover, GPER promotes estrogen-mediated trans activation of the epidermal growth factor receptor (EGFR)-to-ERK1/2 signaling axis *via* a βγ-dependent mechanism. GPER is expressed in the vascular endothelium, and its stimulation induces eNOS phosphorylation and activation[65]. To the GPER-dependent vasodilatory effects also contribute the reduction of vasoconstrictor-induced changes in intracellular calcium and the inhibition of VSMC proliferation. Collectively, these findings support the hypothesis that GPER activation may dilate blood vessels by stimulating eNOS and inhibiting the proliferation and migration of VSMCs. A role for GPER in vasorelaxation is further sustain by the finding that aged female mice express reduced levels of GPER in their mesenteric arteries, and show a decrease in the vasodilatation induced by the GPER agonist G1[66].

Interestingly, GPER is strongly expressed in cardiac tissue[67], and several studies demonstrate its cardioprotective properties. Namely, GPER activation – as well as membrane ERs - limits ventricular remodeling and hypertrophy following heart attack and ischemia/reperfusion injury in preclinical models of myocardial infarction[64].

4. Angiogenesis

The term angiogenesis refers to the formation of new blood vessels from the existing ones, in opposition to vasculogenesis which is the *de novo* formation of vessels during embryogenesis[68].

Angiogenesis is mainly needed for the embryonic development and contributes to the growth of organs after birth. In the adult, vessels are quiescent for most of the time, and only rarely form new branches, since ECs are under the control of a finely tuned balance of stimulatory and inhibitory factors, including growth factors, angiopoietins, chemokines, integrins, junctional molecules, oxygen sensors, and endogenous inhibitors. However, ECs retain high plasticity, and during adulthood, angiogenesis normally occurs in selected phases of the female reproductive cycle, in the placenta during pregnancy, and in the wound healing repair process[58]. Beside physiological angiogenesis, a dysregulated angiogenic signaling leads to an uncontrolled vessel growth that contributes to the pathogenesis of many disorders. Pathological angiogenesis is associated with inflammatory disorders, blindness, asthma, atherosclerosis, infectious diseases (*e.g.*, AIDS), diabetes, cirrhosis, endometriosis, autoimmune disease and cancer[58].

4.1 Basic aspects of angiogenesis

Angiogenesis is a multi-step process that strictly depends on directed migration and proliferation of ECs. Both the processes are controlled by VEGF-A, the main growth factor acting on ECs[69]. When the balance between pro- and anti-angiogenic factors is altered - *i.e.* the angiogenic switch, induced for example by hypoxia – VEGF is secreted by many types of parenchymal cells, and binds to its tyrosine kinase receptor 2 (VEGFR2) on

still quiescent ECs. As a consequence of the activation of the VEGFR2 signaling, stimulated ECs adopt a migrating "tip" cell behavior at the forefront of the vessel sprout (**Figure VII**). Long, thin cellular processes emerging from tip cells and called filopodia extend into the environment and secrete large amounts of proteolytic enzymes, which digest the extracellular matrix (ECM) to develop the sprout. When an adequate number of filopodia has anchored tip cells to the substratum, the contraction of actin filaments within filopodia themselves pulls tip cells toward the VEGF stimulus. Importantly, tip cell filopodia express high levels of VEGFR2, and "sense" differences in VEGF concentration, thus permitting EC migration and alignment all along the VEGF gradient. In the meantime, VEGF stimulates tip cells to produce Delta-like-4 (Dll4) - a trans-membrane ligand for Notch receptors - that interacts with Notch1 receptors on the adjacent ECs. The activation of the Dll4-Notch pathway leads to the down-regulation of VEGFR2 in those cells, thus creating a situation in which neighboring cells express different levels of VEGFR2, and in turn different responsiveness to VEGF[70]. In addition, Notch receptor signaling also drives a greater expression of VEGFR1 - a high-affinity and low-activity receptor that acts as a competitive inhibitor of VEGF signaling - further increasing the gap in VEGF response between adjacent cells. The cell expressing VEGFR2 and Dll4 is a non-proliferative tip cell while the Notch-expressing neighbors of the tip cell become a proliferative stalk cell that contributes to the elongation of the nascent sprout (**Figure VII**). These stalk cells finally lumenize to form a functional blood vessel. The recruitment of pericytes and the deposition of ECM are last steps for the complete maturation and stabilization of vessels.

Tip/stalk specification is a highly dynamic process in which cells undergo a continue transition between tip and stalk identities through all the growth of the vessel. However, cells with the highest VEGFR2/VEGFR1 expression ratio

– and thus the highest VEGF responsiveness - are always at the tip of the new sprout.

It has been recently demonstrated that also the VEGF-C–VEGFR3 signaling pathway controls the branching morphogenesis of blood vessels[70]. Interestingly, VEGFR3 is expressed at a high level in endothelial tip cells, while its ligand VEGF-C is mainly secreted by macrophages localized to vessel branch points.

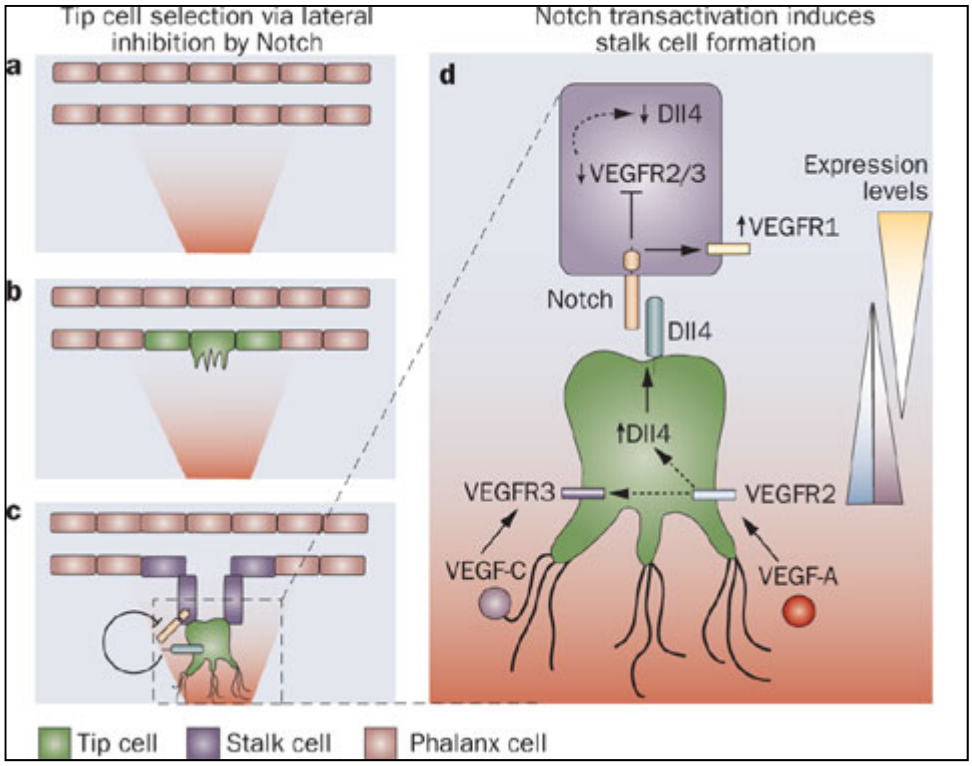

Figure VII. Branching morphogenesis **a)** Parenchymal cells up-regulate VEGF production. **b)** ECs exposed to VEGF gradient are induced to migrate becoming a future tip cell. **c)** The tip cell obtains its leading position in the growing sprout, and inhibits its neighbors ECs. **d)** VEGFR2 activation leads to up-regulation of the Notch ligand Dll4 on the tip cell. Dll4 in turn activates Notch in the neighboring cell, resulting in up-regulation of VEGFR1 and inhibition of VEGFR2 on the neighboring cell that adopt a stalk phenotype. *Modified from Nat Rev Clin Oncol (2009) 6, 315-326.*

The capability of ECs to rapidly switch from a quiescent state to a highly migratory and proliferative state during vessel sprouting is mainly regulated by the VEGF/VEGFR2 and Dll4/Notch axis. But it has recently been proposed that another crucial level of branching regulation is cellular metabolism that may drive vessel sprouting through a metabolic switch. It is well-known that ECs do not rely on oxidative metabolism for ATP production but are highly glycolytic cells, *i.e.* generate more than 80% of their ATP by aerobic glycolysis[71]. Moreover, ECs further increase their glycolytic flux when pass from the quiescent to the activated phenotype. In addition, silencing of the enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) - which stimulates glycolysis - impairs EC proliferation, migration, and vascular sprouting *in vitro*, and the genetic deficiency of PFKFB3 in ECs causes vascular hypobranching in mice[71]. All these results auggest a central regulatory role for glycolysis in vessel branching.

In addition to glycolysis, also fatty acid (FA) metabolism contribute to angiogenesis. It has been reported that ECs, in the absence of glucose, increase FA oxidation (FAO) flux in an AMPK–dependent manner to compensate for the lack in glycolytic ATP production[72]. FAO is therefore a critical bio-energetic supply pathway for ECs, especially when the availability of glucose is reduced[73,74]. Recently, it has also been shown that reduction of FAO impairs *de novo* nucleotide synthesis for DNA replication in ECs. In this manner, FAO selectively regulates EC proliferation, suggesting that distinct metabolic pathways may control distinct EC functions during vessel sprouting[75].

4.2 Therapeutic aspects of angiogenesis

4.2.1 Antiangiogenic therapy

Angiogenesis is a hallmark of cancer development that has long been considered an attractive therapeutic target. The signaling molecule VEGF plays a central role in the formation of blood vessels and is frequently highly expressed and secreted by tumor cells. Thus, clinical efforts to develop antiangiogenic therapies have largely focused on inhibiting VEGF, and several VEGF blockers – such as for example bevacizumab, a VEGF-neutralizing antibody - have been developed and authorized for medical use. However, the clinical efficacy of VEGF inhibitors is lower than that observed in preclinical cancer models. Furthermore, the efficacy of antiangiogenic therapies differs among cancer types. In general, antiangiogenic drugs are more efficient in well-vascularized tumors (*e.g.*, clear cell renal cancer), and less effective in poor vascularized cancers (*e.g.*, pancreatic adenocarcinoma and gastric cancer). Additionally, VEGF blockade show promising effects in neo-adjuvant trials, but largely negative results have been obtained in the adjuvant setting. Nonetheless, the USA Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the use of bevacizumab – in combination with classic chemotherapic drugs - in metastatic colorectal cancer, glioblastoma (GBM), non-squamous non-small cell lung carcinoma (NSCLC), metastatic renal cell carcinoma, and persistent, recurrent, or metastatic carcinoma of the cervix. Treatment with VEGF inhibitors combined with chemotherapy generally prolongs the overall and progression-free survival of responsive cancers patients. Unfortunately, the benefit is generally limited to a few months[76].

4.2.2 Therapeutic angiogenesis

The stimulation of angiogenesis may be a successful therapeutic approach in vascular insufficiency or ischemia where the goal is bypassing diseased or occluded vessels to enhance the development of compensative collateral vascular circuits[77]. Again, VEGF is in the focus, along with fibroblast growth factor (FGF) that is also involved in the control of vessel formation and differentiation. The therapeutic angiogenesis approach is supported by an impressive body of preclinical evidence that demonstrate significant improvements in blood flow, re-vascularisation, and myocardial function after angiogenic cytokine delivery in different animal models of myocardial and limb ischemic disease. But larger trials are needed to translate pre-clinical results into an established benefit for patients.

Different ways have been tested to promote therapeutic angiogenesis. The first example is protein therapy, that implies the direct use of growth factor proteins, mainly VEGF and FGF. Conflicting outcomes have however been reported in the literature on the VIVA (VEGF in Ischemia for Vascular Angiogenesis) therapy - that relies on the intracoronary and intravenous recombinant human VEGF-A infusion - and on the intracoronary infusion of basic FGF (bFGF or FGF-2). Critical topics are the short serum half-life of these proteins and the requirement of high doses and of their frequent administration. Overall, the results of clinical trials of angiogenic cytokine protein treatment were disappointing

Gene therapy is an alternative way of delivering therapeutic cytokines to achieve a more sustained therapeutic effect after a single application. Gene therapy abolishes the requirement for repeated administrations of short-lived proteins, and reduces risks correlated to systemic exposure at high

concentrations of cytokines. Preclinical studies on VEGF and FGF gene delivery by using plasmid or adenoviral vectors have been shown notable improvements in heart function and perfusion in different animal models of myocardial ischemia[77]. But, again, most of the phase II/III trials of therapeutic angiogenesis using plasmid vectors in coronary artery or peripheral arterial disease have yielded negative results. The delivery of VEGF by adenoviruses - that display a high transduction efficiency in ECs and VSMCs, and that can transduce quiescent, non-proliferating cells – also failed to demonstrate any improvement in peak walking time, or in other measures of the quality of life, in a phase II trial engaging 105 patients.

Other alternative approaches could be combination therapies (*e.g.*, VEGF plus angiopoietin or FGF), or delivery *via* stem cells. Studies in animal models suggest that bone marrow-derived or circulating endothelial progenitor cells (EPC) promote endothelial regeneration in arterial injury and collateral angiogenesis in the ischaemic heart[78].

Collectively, the lack of significant therapeutic effects in trials based on angiogenic cytokine treatments is probably due to the failure of existing approaches. Progress in the bench-to-bedside will permit advances in the delivery, expression, and biological efficacy of pro-angiogenic therapies.

Aims of the project

An impaired endothelial function represents the first step in the onset and development of cardiovascular disease (CVD). It has been demonstrated that CVD is less prevalent in women than men until midlife, and the female advantage has been attributed to estrogens, which are lost with menopause. Although these well-established epidemiological and clinical data, very little is known about innate sex properties of male and female endothelial cells (ECs).

In the present study, we investigated the *in vitro* properties of human primary male and female ECs. To this aim, we used human umbilical vein ECs (HUVECs) deriving from pooled male and female newborn donors. We started by searching for an involvement of hormones – in particular estrogens - and of other biological active molecules in male and female EC behavior. Then, in view of the importance of nitric oxide (NO) in the EC physiology, we focused on the contribution of eNOS - the enzyme responsible for its formation - to the proliferation, migration, and *in vitro* angiogenesis of male and female ECs.

Knowledge of the molecular mechanism(s) underlying sex-based differences in male and female EC behavior might have important implications for understanding the difference in the onset and development of CVD in men and women. Furthermore, these knowledge could be helpful to the development of novel sex-oriented strategies for drug therapies.

Results

Metabolic activity in male and female HUVECs

To identify putative differences in metabolic properties of male and female HUVECs (abbreviated as M-ECs and F-ECs, respectively), we first compared their ability of reducing MTT and WST-8 through cellular NAD(P)H-dependent oxido-reductase enzymes. Results are shown in **Fig. 1**, and demonstrated that no significant differences were detectable between F-ECs and M-ECs (MTT, **Fig. 1A**; WST-8, **Fig. 1B**). Similarly, F-ECs and M-ECs contained super imposable amounts of total cellular ATP (**Fig. 1C**). It is well-known that ECs are highly dependent on aerobic glycolysis for energy production[71]. We therefore compared the effects of the glycolytic inhibitor 2-deoxyglucose (2-DG, 20 mM) and of the mitochondrial electron transport chain blocker rotenone (1 μM) on the total cellular ATP content in F-ECs and M-ECS. We found that ATP levels are equally blunted in the presence of 2-DG showing percentages of inhibition of about 80% in both F-ECs and M-ECs (**Fig. 1D**). As expected, rotenone weakly reduced ATP levels (by about 20%), but its effect is again similar in cells of both sexes (**Fig. 1D**). These results confirm the dependence on glycolysis and the restricted contribute of oxidative phosphorylation to ATP generation in ECs, showing for the first time that these bio-energetic requirements are similar for F-ECs and M-ECs.

Hormone deprivation induces a decrease in metabolic activity and cell number in F-ECs and M-ECs

A crucial issue in endothelial patho-physiology concerns estrogens and their involvement in the sex-related incidence of cardiovascular pathologies[11,13]. To study the contribution of estrogens to *in vitro* EC behavior, we set up some pilot experiments to compare metabolic activities of F-ECs and M-ECs grown in a Standard Medium (199 medium w/phenol red supplemented with normal FBS, SM) or in a nominally estrogen-free medium (199 medium w/out phenol red supplemented with charcoal-stripped FBS, CS-FBS) commonly used for *in vitro*

studies on hormone activities. We refer to this last medium as Charcoal Medium w/out phenol red (ChM w/out phenol red). F-ECs and M-ECs were cultured for 48h in SM or ChM w/out phenol red, then fresh media were added, and metabolic activities were measured after a further 48h-incubation. In the absence of estrogens, MTT absorbance (**Fig. 2A**) and ATP levels (**Fig. 2B**) were significantly reduced in comparison to SM in ECs of both sexes. To elucidate if decreases in MTT and ATP were merely dependent on a failure in metabolic activity, or were *vice versa* consequent to a reduced cell growth, the number of F-ECs and M-ECS was measured, and a significant reduction was found in cells cultured in ChM w/out phenol red (**Fig. 2C**). Again, no differences emerge between F-ECs and M-ECs in the antiproliferative effects induced by the nominally estrogen-free medium.

Sex hormones did not prevent the decrease in metabolic activity and cell number induced by hormone deprivation in F-ECs and M-ECs

By using an add-back approach, we tested if estrogens were able to counteract the inhibitory effects observed in F-ECs and M-ECs cultured in ChM w/out phenol red. The addition of a physiological concentration of 17-β2 estradiol (E2, 1 nM) - or its vehicle (ethanol) - for the last 48h of incubation left unaffected the ChM-induced decrease in MTT absorbance (**Fig. 3A**). Super imposable results were obtained by measuring the total cellular ATP (**Fig. 3B**) and the number of cells (**Fig. 3C**). Since the stripping procedure removes sex hormones other than E2 from serum, we checked the effect of ChM supplementation with dihydrotestosterone (DHT, 1 nM), and found that it did not prevent the decrease in MTT absorbance (**Fig. 3D**). Likewise, the simultaneous addition of E2 and DHT (both at 1 nM concentration) left unaffected the reduced MTT absorbance (**Fig. 3D**). Also the treatment with higher concentrations of E2 and/or DHT (10 nM) did not modify these results (data not shown). All together, these results

demonstrate that ECs of both sexes underwent a comparable and significant decrease in cell number - and consequently in metabolic activity - without any apparent cell death when cultured in a phenol red-free medium supplemented with CS-FBS. Importantly, this reduction was independent of the presence of E2 and/or DHT regardless of the described expression of their receptors in both F-ECs and M-ECs[30].

CS-FBS is responsible for the decreased metabolic activity and cell number in F-ECs and M-ECs

Our results suggest that the decrease in metabolic activity and cell number observed in both male and female ECs cultured in ChM w/out phenol red was not related to the lack of sex hormones. Moreover, since ECs cultured in 199 medium w/out phenol red supplemented with normal FBS (SM w/out phenol red) did not show any difference in MTT absorbance when compared to ECs grown in SM (**Fig. 4A**), we excluded the involvement of phenol red and of its estrogenic properties[79]. Consequently, we focused our attention on FBS by comparing cells grown in SM with cells cultured in 199 medium containing phenol red and CS-FBS. We refer to this medium as Charcoal Medium (ChM). Importantly, we submit to the stripping procedure exactly the same lot of serum present in SM. Therefore, in this experimental setting, F-ECs and M-ECs were exposed to growth media that differ only for FBS components. The presence of CS-FBS was sufficient to induce a significant decrease in MTT absorbance (**Fig. 4B**) and cell number (**Fig. 4C**). Again, the addition of E2 (1 nM) did not revert the loss in MTT and ATP induced by CS-FBS in F-ECs (**Fig. 4D**) and in M-ECs (data not shown). At variance, F-ECs and M-ECs were able to fully recover their metabolic activity when SM was added instead of hormones (**Fig. 4E**), thus confirming that one or more substances removed from FBS by stripping procedure, and different from sex hormones, are crucially required for HUVEC growth. Again, no differences

were observed between F-ECs and M-ECs, favoring the suggestion that the FBS component(s) lost in CS-FBS owns wide implications in EC physiology.

Thyroid hormone does not prevent the decrease in metabolic activity and cell number in F-ECs and M-ECs

The stripping procedure reduces or depletes the serum concentration of many biologically active substances such as steroids, thyroid and peptide hormones, vitamins, electrolytes, enzymes, metabolites, and lipids[80,81]. Among hormones, thyroid hormone (TH, 3,5,3'-triiodo-l-thyronine, T_3) plays a central role in the regulation of angiogenesis by acting through multiple genomic and non-genomic mechanisms[82]. We therefore tested the effect of its addition by using the add-back approach described above for sex hormones. The treatment with T_3 (10 nM) was not able to prevent the inhibitory effects on MTT absorbance (**Fig. 5A**) and cell number (**Fig. 5B**) in both F-ECs and M-ECs, thus suggesting that T3 does not represent the substance lost in CS-FBS crucially required for EC growth.

CS-FBS impairs *in vitro* angiogenesis

Among growth factors, Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) are critically involved in EC physiology, being able to induce their growth, migration and *in vitro* differentiation[69]. We therefore tested if VEGF (20 ng/ml) and bFGF (20 ng/ml) were able to revert the inhibitory effect of CS-FBS. The decrease in MTT was maintained in the presence of the growth factors (**Fig. 6A**), thus demonstrating that neither VEGF nor bFGF were the substance(s) lost in CS-FBS essential for EC growth. Furthermore, we set up a 3-D assay by embedding size- and cell number-defined HUVEC spheroids in a collagen gel to study *in vitro* angiogenesis[83,84]. In this assay, spheroids from F-ECs (**Fig. 6B**, left panel) and M-ECs (data not shown) totally retained their ability to sprout when suspended in SM. At variance, the outgrowth of capillary-

like structures was significantly reduced when spheroids were suspended in ChM (**Figs. 6B**, right panel, and **6C** for quantification). Interestingly, the decrease in sprouting was more marked in spheroids from M-ECs, even if the basal capillary outgrowth was fully comparable between the two sexes (**Fig. 6C**). These results demonstrated that a crucial feature of ECs, *i.e.* their ability to undergo *in vitro* angiogenesis, is missed when they are cultured in CS-FBS.

Acetate and palmitic acid partially restore *in vitro* angiogenic phenotypes impaired by CS-FBS in F-ECs and M-ECs

Beside hormones and peptides, charcoal stripping also deprives serum of fatty acids (FAs)[85], thus limiting the ability of ECs cultured in CS-FBS to undergo FA oxidation (FAO). Since metabolic pathways are emerging as important regulators of angiogenesis[72], and especially FAO has been reported to be crucially involved in the regulation of EC proliferation[75], we focused our attention on FAs to investigate if their defeat in CS-FBS might be responsible for the alterations in the EC behavior that we observed. To this purpose, we set up reconstitution experiments by adding sodium acetate – that is metabolized to acetyl-CoA, thus substituting for the depleted FAO-derived acetyl-CoA – or palmitic acid to F-ECs and M-ECS cultured in ChM. We found that the presence of acetate (20 mM) or palmitic acid (250 μM) was able to prevent the loss in cell number induced by ChM in both F-ECs (**Fig. 7A**) and M-ECs (**Fig. 7B**). Since acetate and palmitic acid reverted the number of ECs to about the 75% of the number measured in control conditions, we also added E2 to test if its presence was able to fully restore the proliferative capability of ECs. But the addition of E2 (1 nM) did not further improve the recovery induced by acetate or palmitic acid (**Figs. 7C** and **7D**, respectively). More importantly, spheroids from both F-ECs and M-ECs partially restored their ability to sprout in the 3-D spheroid assay when acetate (20 mM) or palmitic acid (250 μM) were added to ChM (**Figs. 7E** and **7F**,

respectively). The addition of palmitic acid reverted the cumulative sprouting to about the 50% of the sprouting measured in control spheroids both in F-ECs and M-ECs. At variance, acetate apparently reversed sprouting less efficiently in M-ECs than in F-ECs (30% *vs* 70% of the control sprouting, respectively). However, when results were expressed as fold-over-ChM, acetate reversed sprouting by about 2-fold in F-ECs and 4-fold in M-ECs, due the more marked decrease in sprouting showed by M-ECs. These results suggest that the lack of FAs is involved in the reduced proliferation and sprouting observed in ECs cultured in the CS-FBS containing medium. Once more, differences between F-ECs and M-ECs were not so evident, with the exception of a higher sensitivity of M-EC sprouting to the loss of FAs.

The expression of eNOS is higher in F-ECs than in M-ECs

Cardiovascular risk factors are all associated with alterations in endothelial function that are triggered by a loss in NO production as the consequence of a reduced eNOS activity. For this reason, it is crucial to investigate if differences in the eNOS availability exist between F-ECs and M-ECs. We started our experiments by analyzing eNOS expression, confirming our previous results[30] in another set of donors. The eNOS protein expression was about 2-fold higher in F-ECs than in M-ECs (**Fig. 8A**), and a comparable increase was observed at the mRNA level (**Fig. 8B**). To better characterize this diversity, we performed western blots on ECs obtained from male and female twin umbilical cords, showing that the different eNOS expression between M-ECs and F-ECs was conserved (**Fig. 8C**). Moreover, the relative higher amount of eNOS found in F-ECs was already present when HUVECs of both sexes were immediately lysed after the flushing from male and female twin umbilical cords *i.e.* without any *in vitro* passage (**Fig. 8D**). These results suggest that the improved eNOS

expression might be a typical feature of F-ECs, already present at birth, and not acquired during the *in vitro* culture of cells.

To explore the functional status of eNOS, we investigated the dimer-to-monomer ratio in F-ECs and M-ECs. Monomeric (130 kD) and dimeric (260 kD) forms of eNOS have been described in various tissues and cells, and the formation and stability of dimers are critically required for the enzyme to functionally produce NO[42]**.** We found a significant increase in the dimer-to-monomer ratio in F-ECs compared to M-ECs (**Fig. 8E**), suggestive of a correlation between the expression of eNOS and the amount of functional enzyme in ECs.

eNOS is not involved in the control of M-EC and F-EC proliferation

To find a biological significance for the increased female expression of eNOS, we first compared proliferative properties of M-ECs and F-Cs, and their dependence on eNOS activity by using L-NAME, a well-known NOS inhibitor. We did not observe any difference in the growth rates of M-ECs and F-ECs (**Fig. 9A**) that showed totally overlapped doubling times (47.5±5.4 and 47.6±6.0 h for M-ECs and F-ECs, respectively). The addition of L-NAME (100 μM) did not modify proliferation at any time tested neither in M-ECs nor in F-ECs (**Fig. 9B**).

A different role for eNOS in the control of M-EC and F-EC migration

Having excluded the involvement of eNOS in EC proliferation, we analyzed its contribute to migration, another crucial event characterizing EC phenotype. M-EC and F-EC migration was initially evaluated in a chemotaxis assay using FBS as attractant factor. In this assay, F-ECs showed a greater migratory capability in comparison to M-ECs (**Fig. 10A**), and the presence of L-NAME (100 μM) inhibited the chemotactic response in cells of both sexes (**Fig. 10B**). However, when the percent of L-NAME-induced inhibition was calculated, it resulted significantly higher in F-ECs than in M-ECs (32.6±3.0% *vs* 15.7±1.9%, respectively) (**Fig. 10C**).

Migration was also studied by using a 2-dimensional (2-D) wound healing test. In this assay, we did not observe any difference in the percent of wound closure – measured 15h after the scratch - between control M-ECs and F-ECs (60.2±3.0 and 56.7±4.1%, respectively) (**Figs 11A**, and **11B** for quantification). But, again, the addition of L-NAME (100 μM) was more effective in inhibiting the percent of wound closure in F-ECs than in M-ECs. Indeed, the ability to close the wound was reduced by 80% in L-NAME-treated F-ECs, and only by 20% in M-ECs, in comparison to the corresponding control cells (**Fig. 11B**).

To test if the effect of L-NAME was attributable to its ability of specifically inhibiting eNOS activity, the expression of the enzyme was silenced by using siRNA, and 48h after transfection a scratch assay was performed. The knock-down of eNOS reduced by about 50% the percent of wound closure in F-ECs whereas it left unaffected the capacity of M-ECs to close the wound (**Fig. 11C**). A representative blot showing the ability of siRNA to totally silence eNOS expression is shown in **Fig. 11D**.

M-ECs strictly depend on proliferation to close the wound

Wound healing is the result of a complex process which relies on both cell migration and proliferation. To extrapolate the contribution of cell growth to the closure of *in vitro* wounds, we performed the scratch assay on M-ECs and F-ECs mitotically arrested by the addition of the antibiotic mitomycin (500 ng/ml). Surprisingly, M-ECs became unable to close the wound when proliferation is blocked by mitomycin (**Fig. 12A**). At variance, the proliferative break did not affect the ability of F-ECs to close the gap (**Fig. 12A**). Indeed, the percent of wound closure was reduced by about 85% in mitomycin-treated M-ECs, while it remained unchanged in growth-arrested F-ECs (**Fig. 12B**).

Importantly, migration and proliferation seem to be regulated through independent pathways. In fact, the simultaneous addition of L-NAME (100 μM)

and mitomycin (500 ng/ml) induced an inhibition in the percent of gap closure that did not significantly differ from the maximal reduction induced by mitomycin or L-NAME alone, in M-ECs and F-ECs, respectively (**Figs. 13A** and **13B**).

Collectively, all the results shown in **Figs. 10-13** suggest that the ability to migrate and to close the wound significantly differs in their dependence on eNOS activity between M-ECs and F-ECs. The activity of eNOS seems to be a crucial requirement in F-ECs while M-ECs show a more relevant dependence on proliferation.

eNOS is essential for *in vitro* angiogenesis in F-ECs

Angiogenesis is the result of a coordinated process where the growth of new blood vessels relies not only on the migration of specialized cells at the forefront of vascular sprouts – the tip cells – but also on trailing cells that undergo proliferation to elongate the branch – the stalk cells[70]. Therefore, we set up a 3-D spheroid assay that recapitulates *in vitro* the angiogenic process[83,84]. In this assay, spheroids from M-ECs and F-ECs undergo sprouting in a 3-D collagen matrix as the result of the migratory and proliferative contribute of tip and stalk cells. Spheroids from M-ECs and F-ECs efficiently spread after 18h of incubation (**Fig. 14A**). But, when L-NAME (1 mM) was added, sprouting was inhibited only in F-EC spheroids (**Fig. 14A**). Quantification of the cumulative sprout length showed a reduction of about 50% in F-ECs whereas M-ECs were totally unaffected (**Fig. 14B**). Super imposable results were obtained when spheroids were formed by M-ECs and F-ECs silenced for eNOS expression (**Fig. 14C**).

M-ECs are more dependent on proliferation also for *in vitro* angiogenesis

We also performed the sprouting assay on spheroids from M-ECs and F-ECs mitotically arrested by the addition of mitomycin (500 ng/ml) to elucidate the

contribution of cell growth to *in vitro* angiogenesis. At variance with the scratch assay, sprouting was impaired in both M-ECs and F-ECS (**Fig. 15A).** However, the inhibitory effect on sprouting was significantly higher in spheroids from growth-arrested M-ECs (about 70%) in comparison to spheroids from mitomycin-treated F-ECs (about 30%) (**Fig. 15B)**.

All together, results shown in **Figs. 14-15** further suggest that different mechanisms are responsible for migration and sprouting in F-ECs and M-ECs. In particular, F-ECs are crucially dependent on eNOS activity not only for their migratory phenotype but also for *in vitro* angiogenesis. At variance, M-ECs show a more important dependence on proliferation in both the *phenomena*.

F-ECs form more frequently tip cells

Results obtained by studying migration and *in vitro* angiogenesis strongly suggest that F-ECs might possess a more migratory phenotype when compared to M-ECs. We therefore tested the ability of F-ECs and M-ECs to form tip cells during the *in vitro* angiogenesis process. To test this hypothesis, we performed experiments with mosaic spheroids prepared by mixing in a 1:1 ratio GFP-carrying M-ECs and Cherry Red-expressing F-ECs (**Fig. 16A**). Quantification of the number of sprouts emerging from mixed spheroids showed that the large majority of sprouts segregated only M-ECs or F-ECs. However, in sprouts where ECs of both sexes were present, Cherry-F-ECs were located as tip cells in about 73% of the sprout (**Fig. 16B**). Notably, when mosaic spheroids were incubated in the presence of L-NAME (1 mM), the treatment significantly impaired the tip cell activity showed by F-ECs that was reduced to about 50% (**Fig. 16B**). Again, our results favor the hypothesis that F-ECs show a higher migratory phenotype strictly controlled by eNOS activity.

Figures

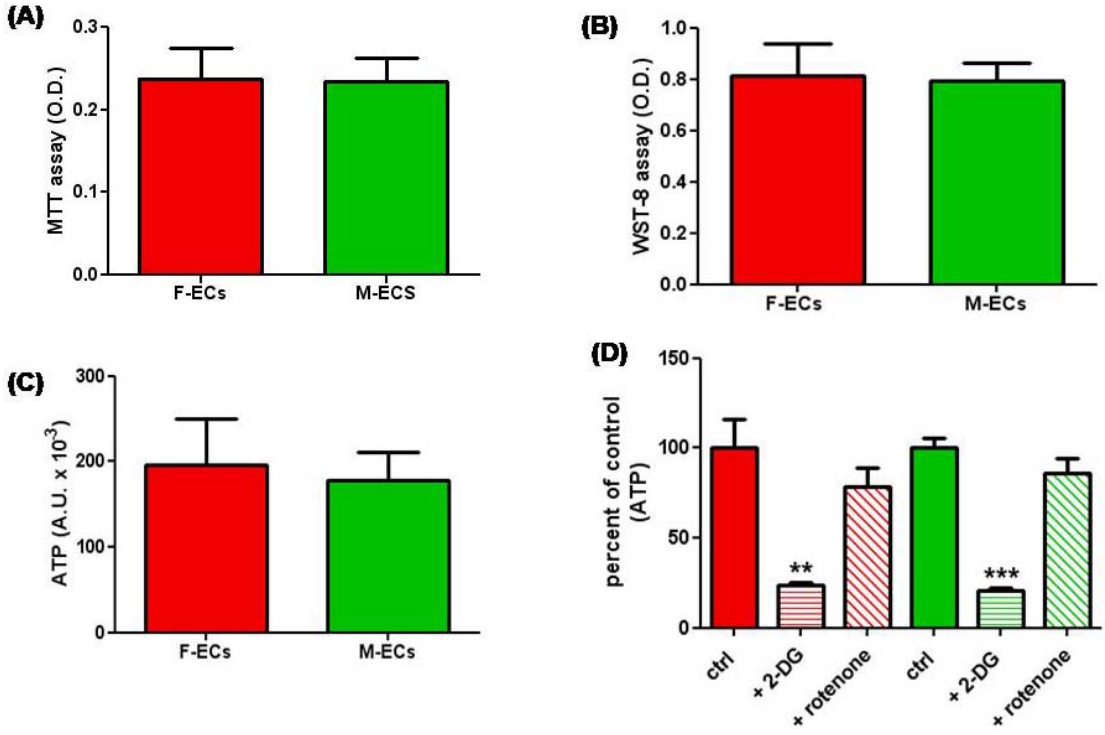

Figure 1. Metabolic activity in male and female HUVECs. The reducing ability of F-ECs (red bars) and M-ECs (green bars) was evaluated by MTT **(A)** and WST-8 **(B)** assays on cells incubated for 24 h in complete medium. Results are expressed as O.D., and no significant differences between F-ECs and M-ECs were detected (n=5 in MTT, and n=4-5 for F-ECs and M-ECs in WST-8 assays, respectively, *t* test). In **(C),** the total cellular ATP levels were measured on parallel wells. Results are expressed as A.U., and no significant differences were found (n=8-7 for F-ECs and M-ECs, respectively, *t* test). **(D)** The total ATP content was measured after 4 h of treatment with 2-DG (20 mM, horizontal bars) or rotenone (1 μM, diagonal bars). Result are expressed as percent of untreated cells (ctrl, set at 100%). **p<0.01 *vs* the corresponding control cells, One way ANOVA with Bonferroni's test, n=3.

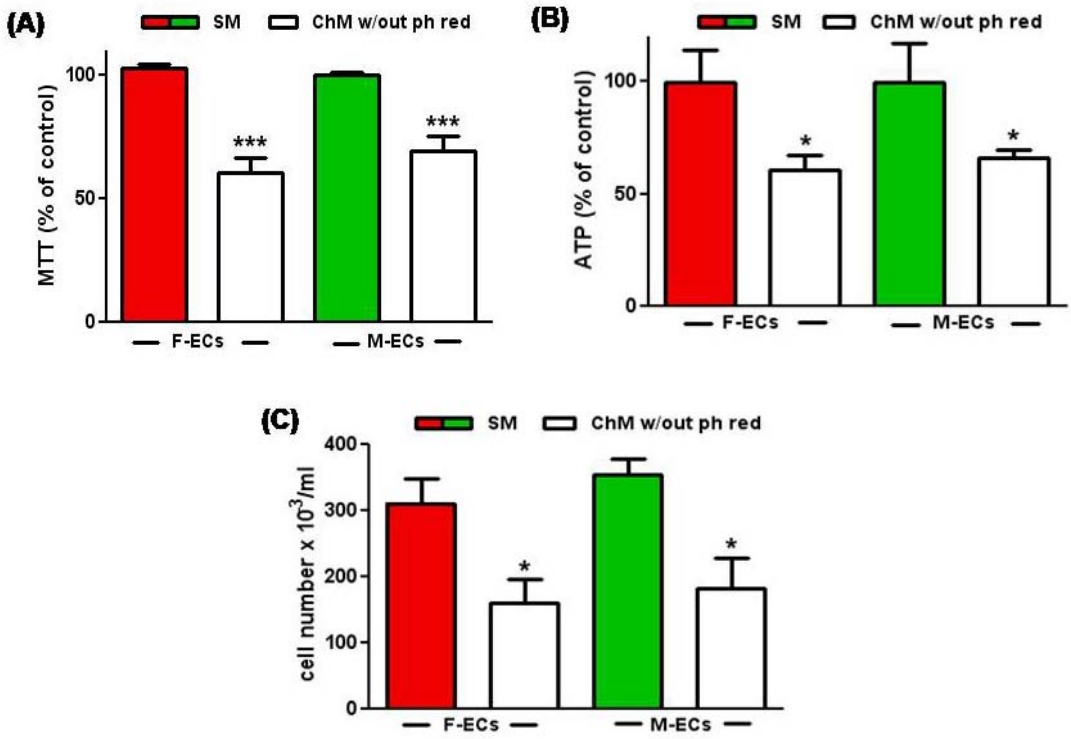

Figure 2. Hormone deprivation induces a decrease in metabolic activity and cell number in F-ECs and M-ECs. F-ECs and M-ECs were cultured in SM (SM, solid bars) or in ChM w/out phenol red (ChM w/out ph red, open bars), and 48h later media were changed. MTT absorbance **(A)**, total cellular ATP **(B)**, and cell number evaluated by cell counting with the vital dye Trypan Blue **(C)** were measured after a further 48h of incubation. Results are expressed as percent of cells cultured in SM (SM, set at 100%) in **(A)** and **(B)**, and as cell number x 10^{-3}/ml in **(C)**. ***p<0.001, n=9-7 for F-ECs and M-ECs, respectively, in **(A)**; *p<0.05, n=6-5 for F-ECs and M-ECs, respectively, in **(B)**; *p<0.05, n=3 in **(C)**. All statistical comparisons were performed *versus* the corresponding SM by using *t* test.

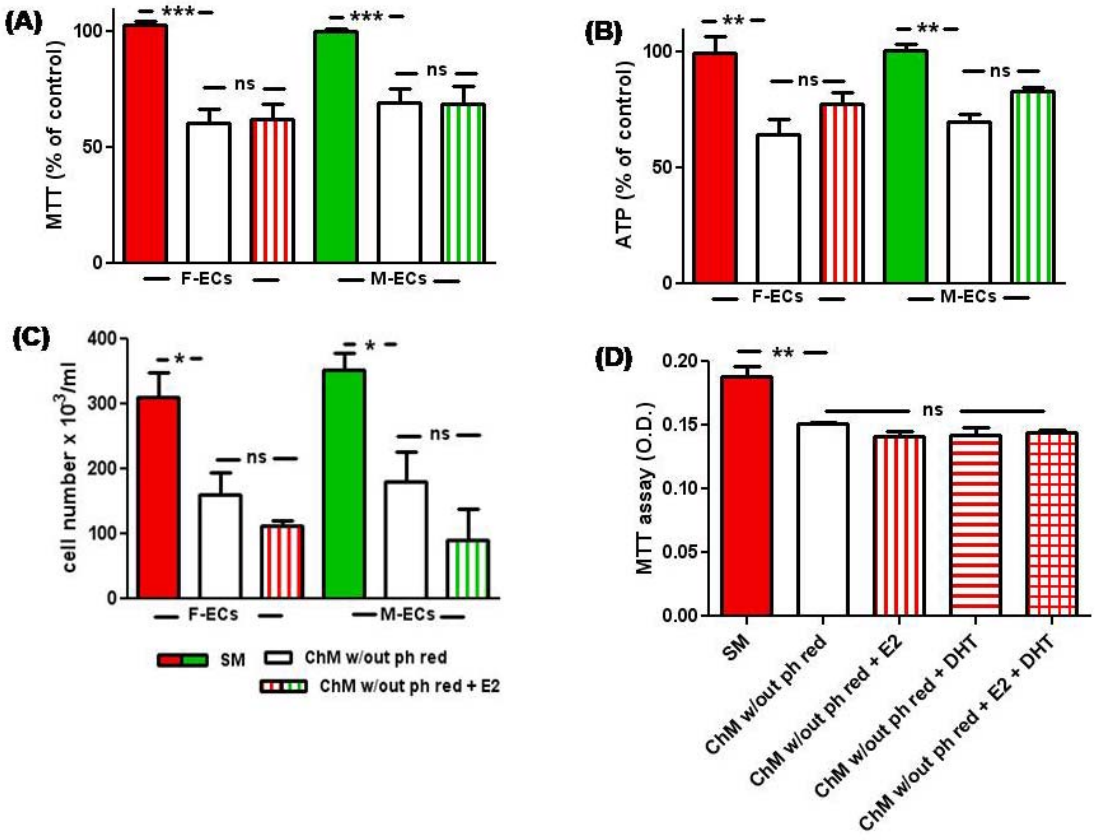

Figure 3. Sex hormones do not prevent the decrease in metabolic activity and cell number induced by hormone deprivation in F-ECs and M-ECs. Experiments were performed as described in Figure 2 on ECs cultured for 48h in SM or ChM w/out ph red. During the last 48 h of incubation, media were changed, and cells in ChM w/out ph red were treated with vehicle (ethanol, open bars) or E2 (1 nM, vertical bars) before measurements of MTT **(A)**, ATP **(B)**, and cell number **(C)**. Data are expressed as percent of cells cultured in SM (solid bar) in **(A)** and **(B),** or as cell number x 10^{-3}/ml in **(C)**. ***p<0.001, **p<0.01, *p<0.05 *vs* the corresponding SM; ns *vs* the corresponding ChM w/out ph red + vehicle; n=8-4-3 in **(A)**, **(B)**, and **(C)**, respectively, *t* test. In **(D)**, vehicle (open bar), E2 (1 nM, vertical bar), DHT (1 nM, horizontal bar), or E2 + DHT (squared bar) were added to ChM w/out ph red as described above. **p<0.001 *vs* SM, n=3, *t* test; ns *vs* ChM w/out ph red, n=3, One way ANOVA with Bonferroni's test.

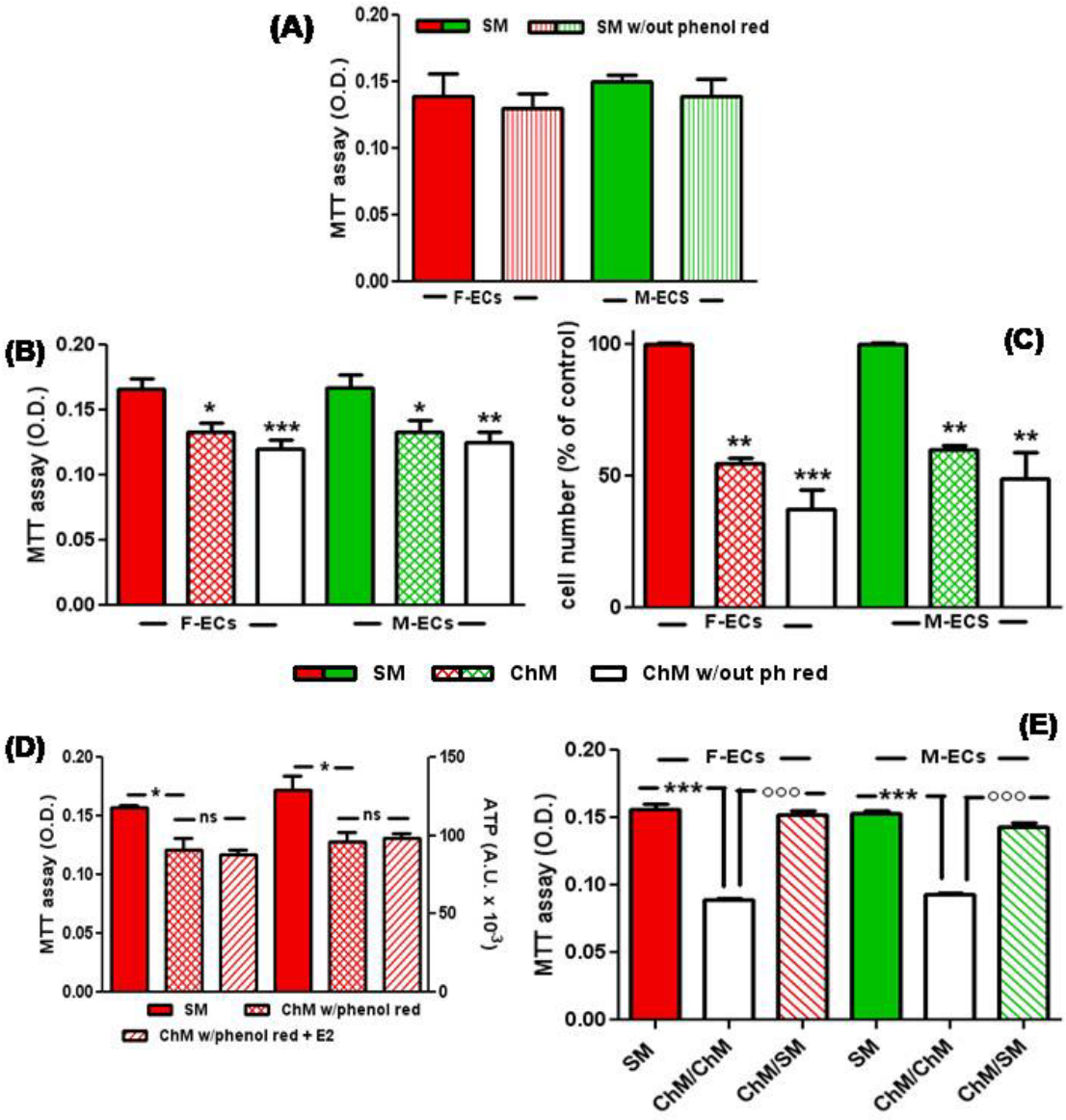

Figure 4. CS-FBS is responsible for the decreased metabolic activity and cell number in F-ECs and M-ECs. (A) MTT absorbance was measured after 48h of incubation in SM (solid bars) or SM w/out phenol red (vertical bars). MTT **(B)** and cell number **(C)** were measured after 48h of incubation in SM (solid bars), ChM (cross-hitched bars) or ChM w/out ph red (open bars). *p<0.05, **p<0.01, ***p<0.001 *vs* the corresponding SM, n=13-12 for F-ECs and M-ECs, respectively, in **(B),** and n=4 in **(C)**, One-way ANOVA with Bonferroni's test. **(D)** MTT (left axis) and ATP (right axis) were measured after 48h of incubation in SM (solid bars), ChM + vehicle (cross-hitched bars), or ChM + E2 (1 nM,

diagonal bars). *p<0.05 *vs* SM, ns *vs* ChM + vehicle, n=3, *t* test. **(E)** ChM was replaced with ChM (ChM/ChM, open bars) or with SM (ChM/SM, diagonal bars), and MTT was measured after 48h of incubation. ***p<0.001 *vs* the corresponding SM, ^{ooo}p<0.001 *vs* the corresponding ChM/ChM, n=3, *t* test.

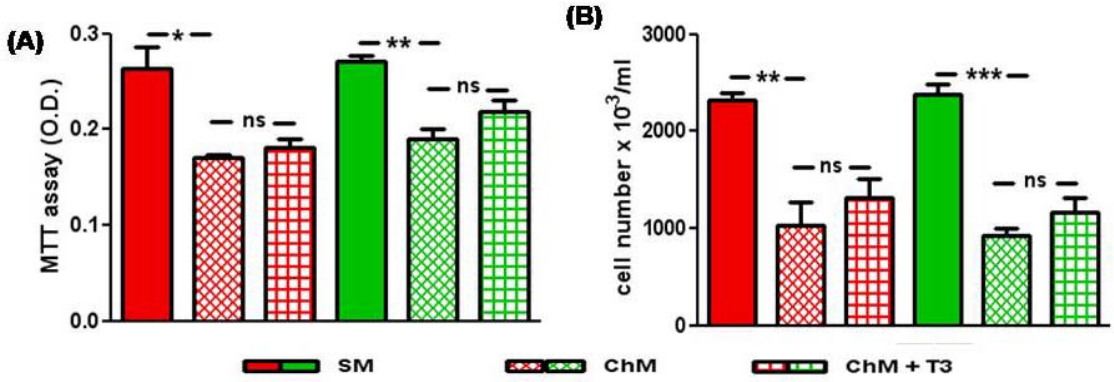

Figure 5. Thyroid hormone does not prevent the decrease in metabolic activity and cell number in F-ECs and M-ECs. MTT **(A)** and cell number **(B)** were measured after 48h of incubation in SM (solid bars), ChM (cross-hitched bars) or ChM + T3 (10 nM, squared bars). *p<0.05, **p<0.01, ***p<0.001 *vs* the corresponding SM; ns *vs* ChM, n=3, *t* test. In both graphs, F-ECs and M-ECs are red and green, respectively.

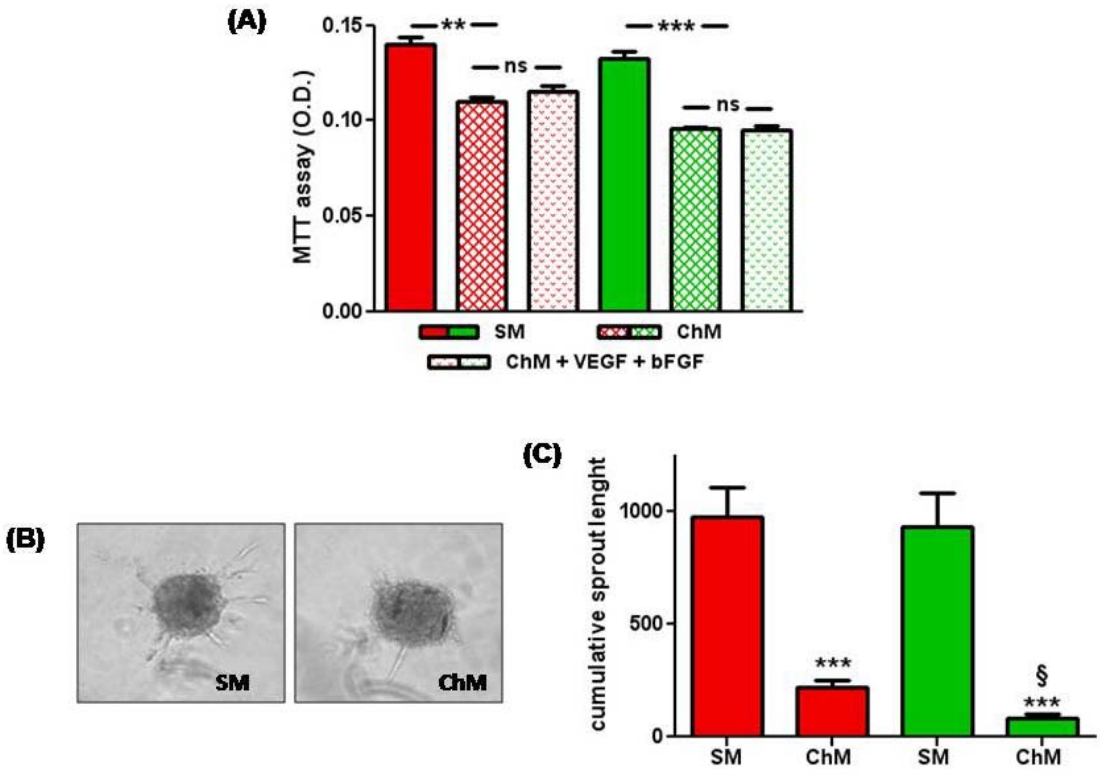

Figure 6. CS-FBS impairs *in vitro* angiogenesis. (A) MTT absorbance was measured after 48h of incubation in SM (solid bars), ChM (cross hitched bars) or ChM + VEGF + bFGF (each at 20 ng/ml, pointed bars). **p<0.01, ***p<0.001 *vs* the corresponding SM; ns *vs* ChM, n=3, *t* test. **(B)** Representative images of spheroids from F-ECs embedded in collagen gels in the presence of SM (left panel) or ChM (right panel). Photographs were taken 24 h later. **(C)** Quantification of the cumulative sprout length emerging from spheroids from F-ECs (red bars) and M-ECs (green bars). ***p<0.001 *vs* the corresponding SM, §p<0.05 *vs* female ChM, n=7, *t* test. F-ECs and M-ECs are red and green, respectively.

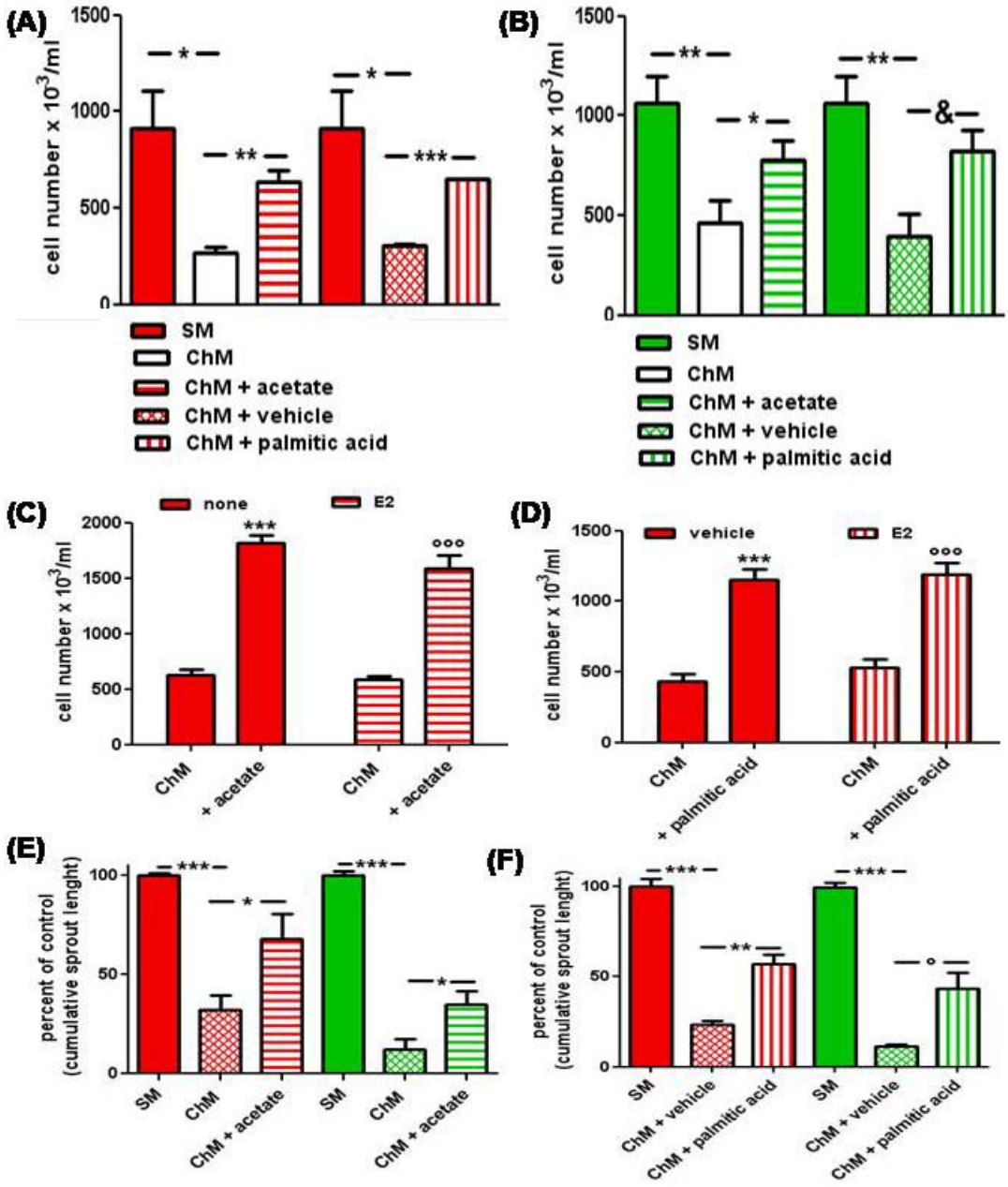

Figure 7. Acetate and palmitic acid partially restore *in vitro* angiogenic phenotypes impaired by CS-FBS in F-ECs and M-ECs. Cell number was measured in F-ECs **(A)** and M-ECs **(B)** after 48h of incubation in SM (solid bars), ChM (open bars), ChM + acetate (20 mM, horizontal bars), ChM + vehicle (ethanol/BSA, cross-hitched bars) or ChM + palmitic acid (250 µM, vertical bars). In **(A)**, *p<0.05 *vs* SM, **p<0.01 *vs* ChM, ***p<0.001 *vs* ChM + vehicle,

n=3, *t* test; in **(B),** **p<0.01 *vs* SM, *p<0.05 *vs* ChM, $^{\&}$p<0.05 *vs* ChM + vehicle, n=4, *t* test. E2 (1 nM) was added to ChM in the absence or in the presence of acetate **(C)** or palmitic acid **(D)**, and the number of cell was measured after 48 of incubation. The presence of E2 did not affect the results. In **(C)**, ***p<0.001 *vs* ChM, °°°p<0.001 *vs* ChM + E2; in **(D)**, ***p<0.001 *vs* ChM + vehicle; °°°p<0.001 *vs* ChM + vehicle + E2, n=6, Two way ANOVA. In **(E)** and **(F),** the cumulative sprout length emerging from F-ECs (red bars) and M-ECs (green bars) was measured after 24h of incubation in SM (solid bars), ChM or ChM + vehicle (cross-hitched bars), ChM + acetate (20 mM, horizontal bars), or ChM + palmitic acid (250 µM, vertical bars). Data are expressed as percent of control, *i.e.* the cumulative sprouting from cells cultured in SM. In **(E)**, ***p<0.001 *vs* SM, *p<0.05 *vs* ChM or ChM + vehicle; in **(F)**, ***p<0.001 *vs* SM, **p<0.01 *vs* ChM, °p<0.05 *vs* ChM + vehicle, n=4, *t* test.

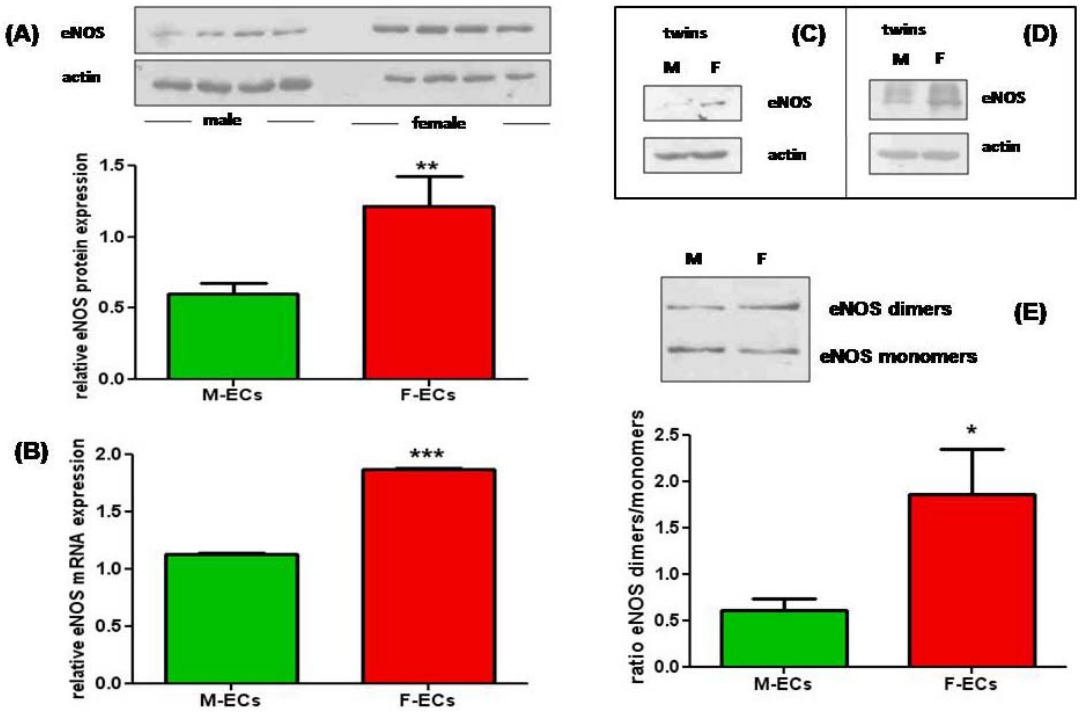

Figure 8. The expression of eNOS is higher in F-ECs than in M-ECs. (A) Total eNOS protein was evaluated by western blotting on lysates prepared from M-ECs and F-ECs. β-actin was used as a loading control. Upper panel: a representative blot out of 14 is shown. Lower panel: densitometric analysis of eNOS protein expression in M-ECs (green bars) and F-ECs (red bars). **p<0.001; n=14. **(B)** eNOS RNA levels were measured by RT-qPCR and normalized to the level of the housekeeping gene 18S. ***p<0.001, n=3. In **(C)** and **(D)**, eNOS protein was evaluated on lysates from twin M-ECs and F-ECs at P1 passage **(C)** or immediately collected after the flushing from cords **(D). (E)** eNOS dimers and monomers were separated by LT-PAGE as described in the "Materials and Methods" section. Upper panel: a representative blot out of 6 is shown. Lower panel: densitometric analysis of the dimer-to-monomer ratio in M-ECs (green bars) and F-ECs (red bars). *p<0.05; n=6.

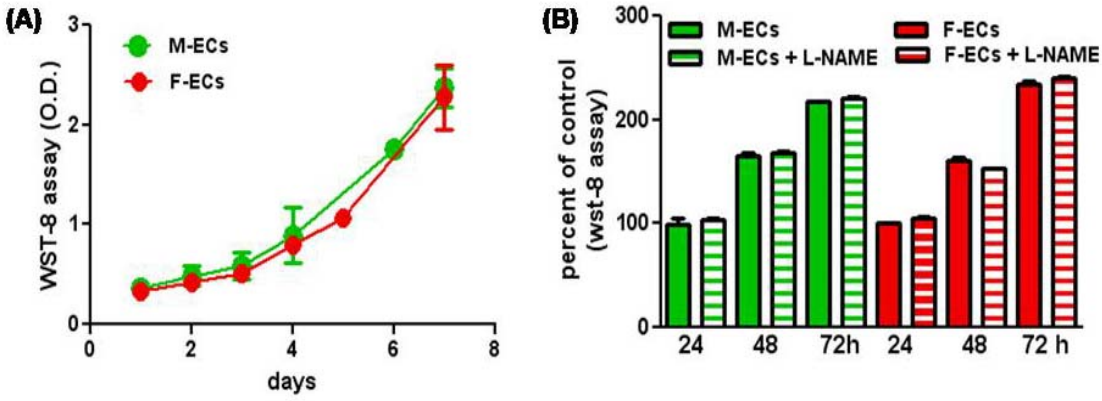

Figure 9. eNOS is not involved in the control of M-EC and F-EC proliferation. (A) M-ECs (green symbols) and F-ECs (red symbols) were cultured in SM, and WST-8 was added for the last 4 h of incubation at the indicated times. **(B)** M-ECs (green bars) and F-ECs (red bars) were cultured in the absence (solid bars) or in the presence (horizontal bars) of L-NAME (100 μM). Data are expressed as percent of control, *i.e.* cells cultured in SM (solid bars).

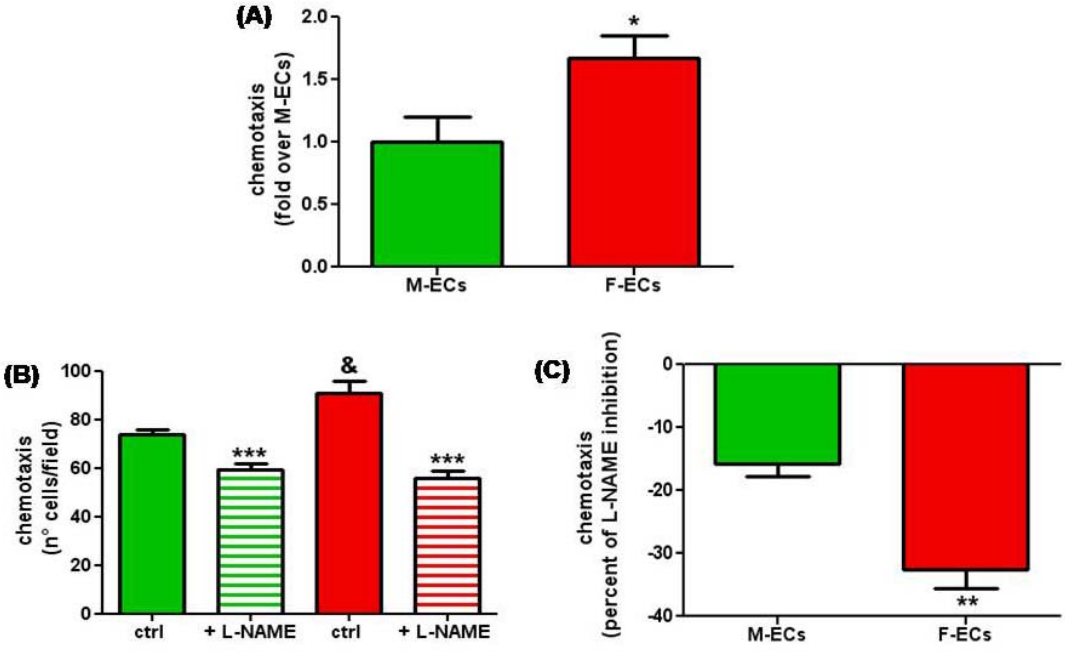

Figure 10. A different role for eNOS in the control of M-EC and F-EC migration. (A) Chemotaxis experiments were performed using 10% FBS as attractant. Results are expressed as the ratio between the number of migrating F-ECs and M-ECs, *i.e.* fold over M-ECs. *p<0.05, n=4-6 for M-ECs and F-ECs, respectively. **(B)** Chemotaxis toward FBS was performed with M-ECs and F-ECs in the absence (solid bars) or in the presence of L-NAME (100 μM, horizontal bars). Results are expressed as the number of migrating cells, and are the mean ± s.e.m of 25 counts from a representative experiment, repeated 3 times with similar results. ***p<0.001 *vs* the corresponding untreated control cells (ctrl), $^{\&}$p<0.05 *vs* M-ECs, *t* test. **(C)** Percent of L-NAME-induced inhibition of M-EC and F-EC migration. **p<0.001, n=3. In all graphs, M-ECs and F-ECs are green and red, respectively.

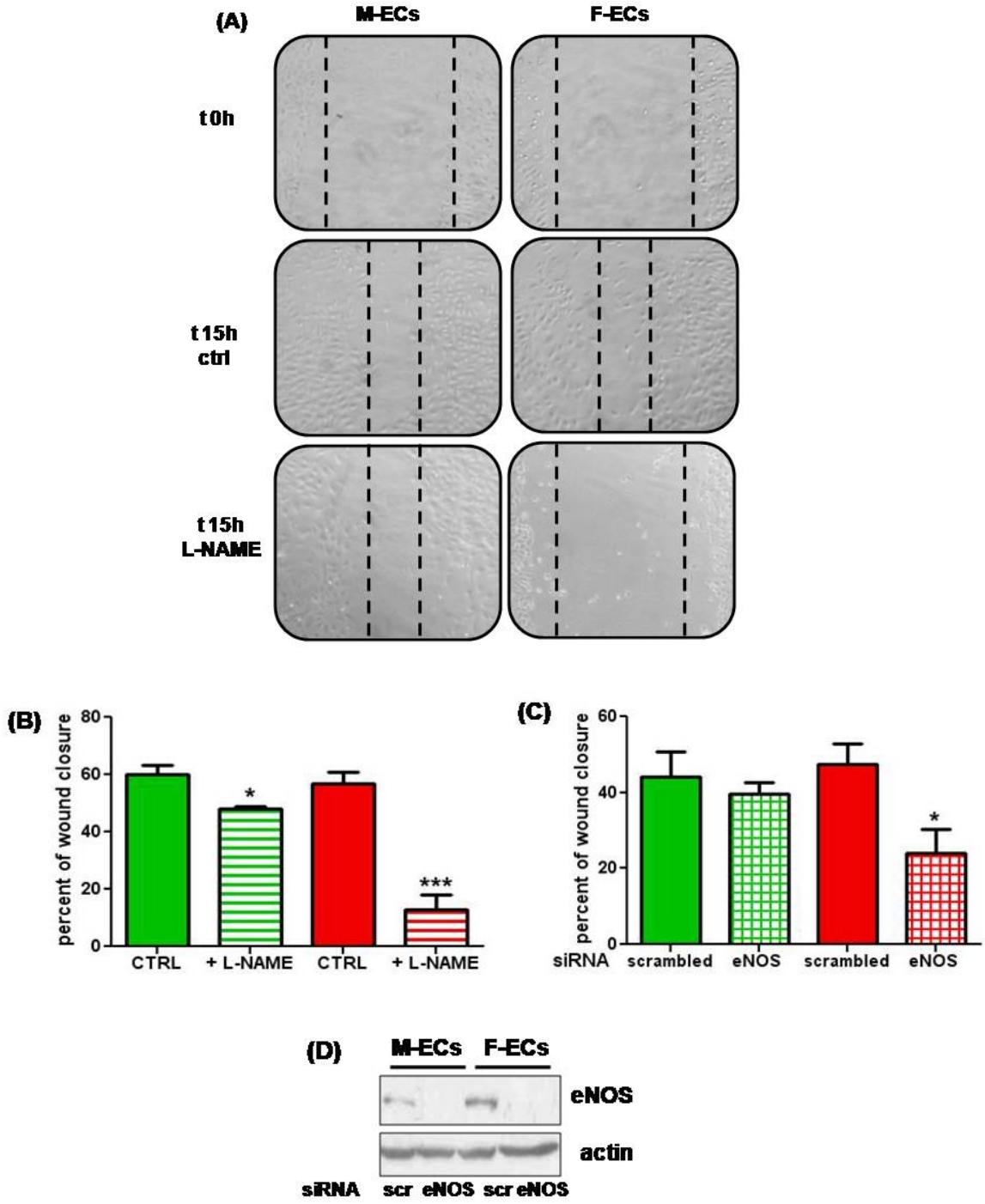

Figure 11. A different role for eNOS in the control of M-EC and F-EC migration. (A) Representative photographs of M-ECs and F-ECs were taken at the time of wounding (t 0h) and after a 15-h incubation in 20% FBS (ctrl) or in 20% FBS supplemented with L-NAME (100 μM). **(B)** The graph represents the mean ± s.e.m. of the percent of wound closure at 15 h. CTRL cells, solid bars; L-

NAME-treated cells, horizontal bars. *p<0.05, ***p<0.001 *vs* the corresponding CTRL cells, n=3-4 for M-ECs and F-ECs, respectively, *t* test. **(C)** Quantification of the percent of wound closure measured 15 h after the wound on M-ECs and F-ECs transfected with scrambled (solid bars) or eNOS siRNA (squared bars). *p<0.05 *vs* the corresponding scrambled siRNA, n=3, *t* test. In **(D)**, a representative blot out of 3 showing the degree of eNOS silencing in M-ECs and F-ECs.

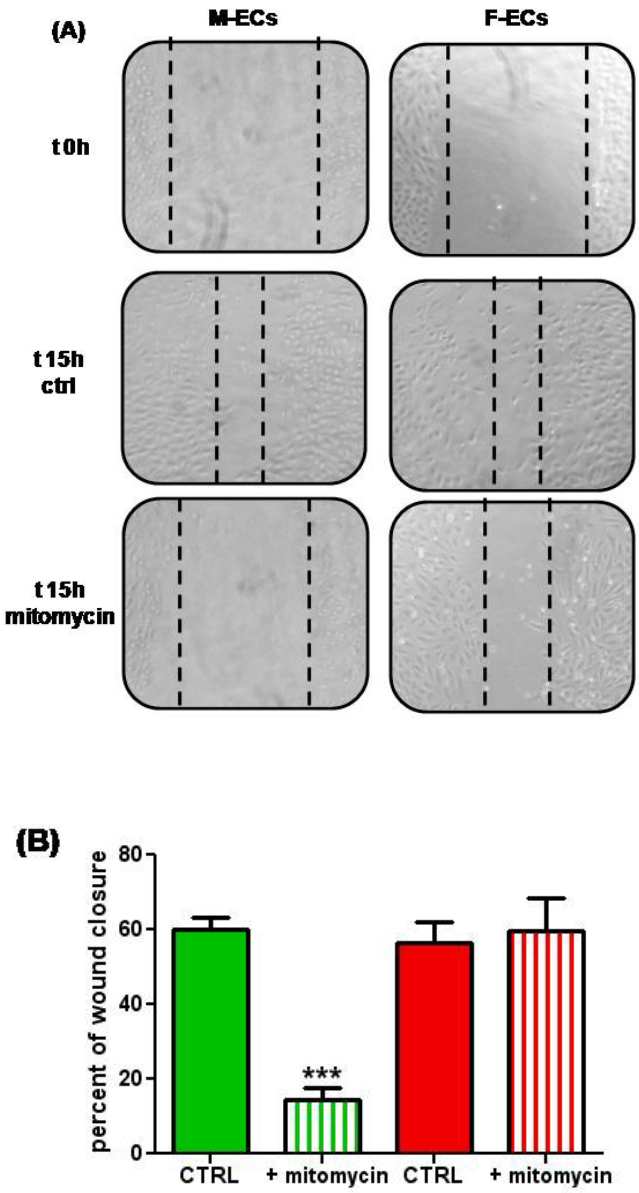

Figure 12. M-ECs strictly depend on proliferation to close the wound. (A) Representative photographs of M-ECs and F-ECs were taken at the time of wounding (t 0h) and after a 15-h incubation in 20% FBS (ctrl) or 20% FBS containing mitomycin (500 ng/ml). **(B)** The graph represents the mean ± s.e.m. of the percent of wound closure at 15 h. CTRL cells, solid bars; mytomicin-treated cells, vertical bars. ***p<0.001 *vs* the corresponding CTRL cells, n=3, *t* test.

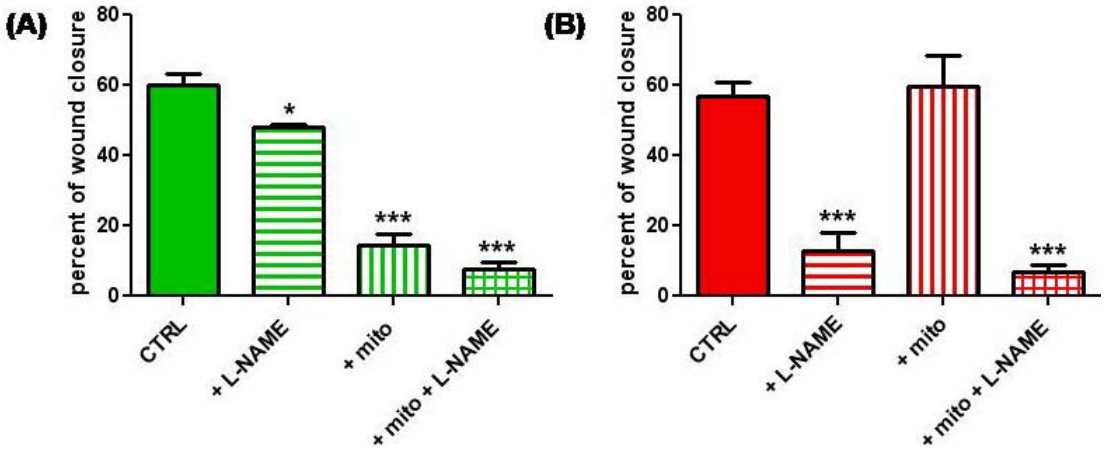

Figure 13. Proliferation and migration are independent pathways in M-ECs and F-ECs. Graphs represent the mean ± s.e.m. of the percent of wound closure measured after a 15-h incubation in 20% FBS (CTRL, solid bars), 20% FBS containing L-NAME (100 μM, horizontal bars) or mitomycin (500 ng/ml, vertical bars), and 20% FBS containing L-NAME + mitomycin (squared bars). *p<0.05, ***p<0.001 *vs* the corresponding CTRL cells, One way ANOVA with Bonferroni's test, n=3. M-ECs **(A)** and F-ECs **(B)** are green and red, respectively.

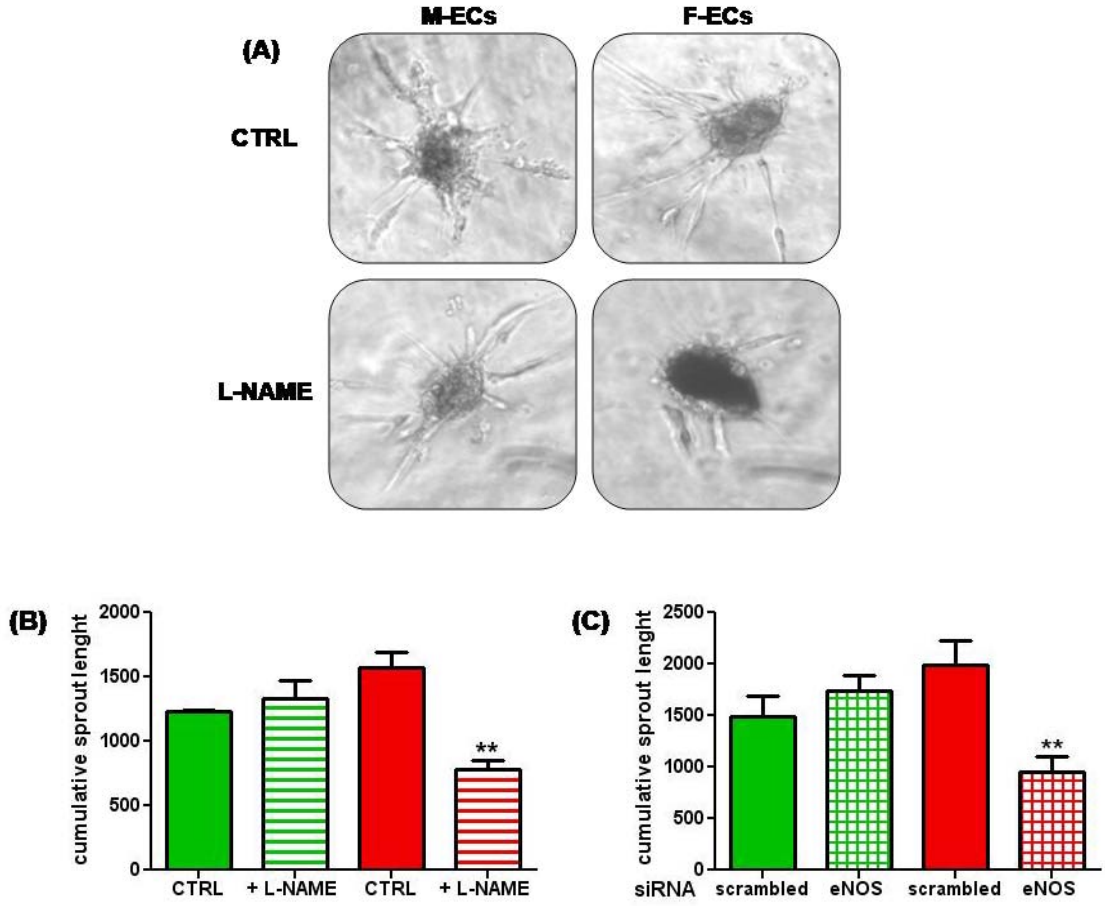

Figure 14. eNOS is essential for *in vitro* angiogenesis in F-ECs. (A) Representative images of spheroids from M-ECs and F-ECs embedded in collagen gels in the absence (CTRL) or in the presence of L-NAME (1 mM). Photographs were taken 18h later. **(B)** Quantification of the cumulative sprout length from untreated M-EC and F-EC spheroids (CTRL, solid green and red bars, respectively) or from L-NAME-treated spheroids (horizontal bars, green and red for M-ECs and F-ECs, respectively). **p<0.01 *vs* the corresponding CTRL, n=3, *t* test. **(C)** Quantification of the cumulative sprout length from M-EC and F-EC spheroids obtained from cells transfected with scrambled (solid bars) or eNOS siRNA (squared bars). M-ECs and F-ECs are green and red, respectively. **p<0.01 *vs* scrambled, n=3, *t* test.

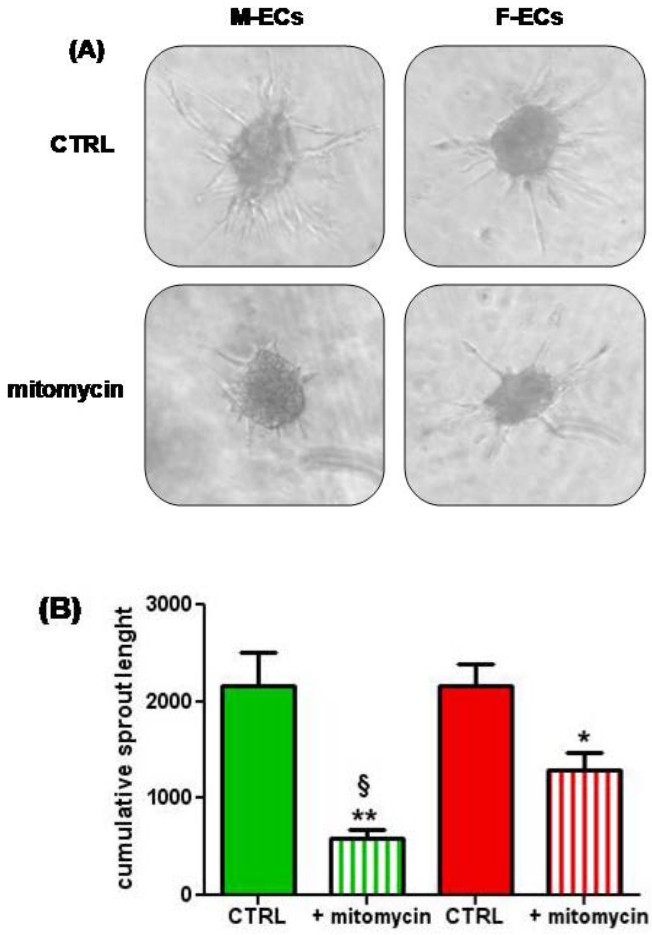

Figure 15. M-ECs are more dependent on proliferation also for *in vitro* angiogenesis. (A) Representative images of spheroids from M-ECs and F-ECs embedded in collagen gels in the absence (CTRL) or in the presence of mitomycin (500 ng/ml). Photographs were taken 18h later. **(B)** Quantification of the cumulative sprout length from untreated M-EC and F-EC spheroids (CTRL, solid green and red bars, respectively) or from mitomycin-treated spheroids (vertical bars, green and red for M-ECs and F-ECs, respectively). *$p<0.05$, **$p<0.01$ *vs* the corresponding CTRL; $^{\S}p<0.05$ *vs* mitomyicin-treated F-ECs, n=5-4 for M-ECs and F-ECs, respectively.

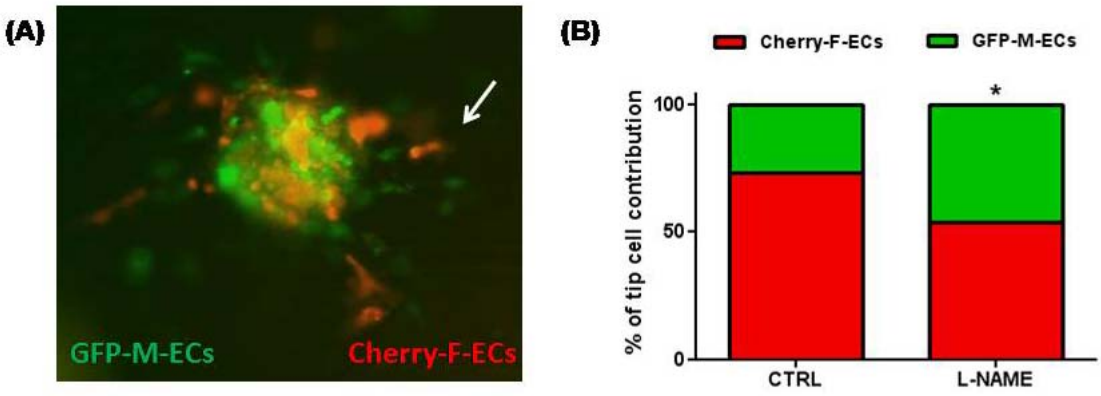

Figure 16. F-ECs form more frequently tip cells. (A) Representative fluorescence photographs of a mosaic spheroid containing a 1:1 mixture of GFP-M-ECs and Cherry-F-ECs embedded in collagen gels. Photographs were taken 18h later. The arrowhead indicates a F-EC located as tip cell in a sprout containing both GFP-M-ECs and Cherry-F-ECs. **(B)** Quantification of the percent of tip cell contribution in sprouts containing both GFP-M-ECs and Cherry-F-ECs emerging from control spheroids (CTRL) or from L-NAME-treated spheroids. *$p<0.05$, $n=17$.

Discussion

Atherosclerosis and CVD are classical examples of diseases where sex/gender differences have been described. A significant body of evidence suggests that CVD is less prevalent in women than men until midlife, and the female advantage in younger women has been attributed to estrogens, which are lost with menopause[10,25,26]. Since the earliest event in the onset of atherosclerosis and CVD is endothelial dysfunction (ED), many studies have been focused on endothelium. But, despite well-established epidemiological and clinical evidence of a different prevalence of CVD in young men and women, studies involving endothelial cells (ECs) do not frequently identify the sex of cells (even when the effects of sex hormones are analyzed), and very little is known about innate sex properties of male and female ECs (M-ECs and F-ECs, respectively). Therefore, we started our study with the aim to investigate the *in vitro* properties of human primary M-ECs and F-ECs, focusing on their metabolism and the expression and activity of eNOS.

Almost all of our knowledge about biochemical and signaling pathways, and more generally about biological processes, has been provided by established cell lines. However, sex has been rarely considered as a relevant biological variable[23], and this asexual approach has limited the understanding of potential sex-based differences not only in basic biological functions but also in patho-physiological mechanisms[22,23,24]**.** Moreover, increasing evidence suggest that cells differ according to sex, regardless of their exposure to sex hormones. For that reason, researchers are always more encouraged to pay close attention to sex as a relevant biological variable in their studies. With this aim, the National Institutes of Health (NIH) has announced that sex will be taken into account in the research designs, analyses, and reporting in vertebrate animal and human studies. Similarly, the European Research program Horizon2020 asks in most of its calls for the inclusion of sex/gender aspects.

Along with these general comments, the knowledge of the sex of cells becomes decisive when they are used for *in vitro* studies on sex/gender-dependent pathologies. As a matter of fact, some differences are reported when primary cultures of human M-ECs and F-ECs have independently been studied. Prostacyclin and prostaglandin E2 synthesis is for example more efficiently stimulated by thrombin in M-HUVECs than in F-HUVECs[28]. Another study reports that M-HUVECs express higher levels of autophagic markers than F-HUVECs[30]. A whole genome expression analysis recently demonstrates a higher expression of immune-related genes and a stronger transcriptional response to shear stress in F-ECs compared to M-ECs[31]. Differences have also been reported in other cells of the vascular system, *i.e.* in VSMCs, where RLIP76 - a cell surface protein that catalyzes the extrusion of reduced glutathione (GSH) conjugates – alters more efficiently the intracellular redox balance, C-Jun kinase signaling activation, and apoptosis in F-VSMCs than in M-VSMCs[29].

With the aim of further discovering putative differences between M-ECs and F-ECs, we started our experiments by comparing their metabolic properties. Some phenotypic metabotype differences between males and females have been described by a genome-wide analysis performed on serum of more than 2.000 subjects[86]. No data are currently available on a possible dimorphism in EC metabolic genes and bio-markers. Until now, it is well-known that ECs are strictly dependent on aerobic glycolysis[84,87,88], and only partially rely on oxidative metabolism, for ATP production. Indeed, it has been demonstrated that HUVECs maintain unchanged levels of ATP even at very low O2 concentrations, and that the EC production of ATP is significantly affected only by interfering with the glucose metabolism[88]. We have fully confirmed these data, finding that aerobic glycolysis contributes for about the 80%, and oxidative metabolism for about the 20%, to the maintenance of steady-state cellular ATP levels. However,

we do not find any difference in the bio-energetic requirement of F-ECs and M-ECs, thus showing for the first time that ECs of both sexes mainly depend on glycolysis for ATP generation. It should be pointed out that the measurement of a steady-state parameter as the total cellular ATP content might conceal some sexual dimorphic differences possibly existing between M-ECs and F-ECs. More detailed metabolic analyses, such as the measurement of the energy charge, *i.e.* the ADP/ATP ratio, or the capacity of consuming glucose over time, will be performed to further deepen the knowledge about putative sex-specific differences in EC metabolism.

The greater incidence of CVD in men and post-menopausal women - compared with pre-menopausal women - implies the existence of a vaso-protective phenotype in the young female population. Since sex hormones – especially, estrogens - are believed to be responsible for these defensive effects, we decided to study their regulatory role on metabolic properties in human M-ECs and F-ECs. Surprisingly, we consistently observed a significant decrease in cell number - and consequently in metabolic activity - when M-ECs and F-ECS have been cultured in a medium supplemented with charcoal-stripped serum (CS-FBS) commonly used for *in vitro* studies on hormone activities. Furthermore, the presence of CS-FBS totally abrogates the ability of F-EC and M-EC spheroids to sprout in an *in vitro* angiogenesis assay, thus affecting a functional property distinctive of ECs.

Charcoal-stripped serum is largely used in cellular, biological, and pharmacological studies. The stripping procedure reduces or depletes the concentration of many biologically active substances such as steroids, thyroid and peptide hormones, vitamins, electrolytes, enzymes, metabolites, and lipids[80,81,85]. Some alterations in cell signaling and differentiation have been

described in cells cultured in CS-FBS in comparison to their counterpart grown in normal serum[89,90]. Furthermore, the growth of human CFU-megacaryokytes is improved when cells are cultured in CS-FBS[91] whereas the mouse osteoblastic cell line MC3T3-E1 displays a limited growth and an impaired alkaline phosphatase expression in CS-FBS[92]**.** Again, CS-FBS modifies the phospholipid metabolism in type II rat pneumocytes[85] and the cellular bioenergetics of breast cancer cells[93]. Regarding ECs, a variety of results describing the effects of sex hormones on their behavior have been obtained in cells cultured in media supplemented with CS-FBS. However, most of these studies did not refer to cell proliferation even if they involved cells grown in CS-FBS for at least 24-48 h[94,95], and also when hormones different from estrogens were studied[96,97]. To our knowledge, up to now no data are available on the direct comparison of ECs grown in normal or CS-FBS. In our experiments, we consistently observed a reduced F-EC and M-EC growth when CS-FBS was used, advising about the disregarded contribute of CS-FBS to data obtained from ECs cultured in nominally hormone-free media. More importantly, F-ECs and M-ECs also lose some of their typical properties when cultured in CS-FBS, *i.e.* the ability to sprout, thus favoring the idea that FBS component(s) lost in CS-FBS possess broad implications in EC physiology.

A crucial question arising from our results concerns the failure of sex hormones in preventing the inhibitory effects on cell growth and sprouting we described in ECs of both sexes cultured in CS-FBS. In a collaborative study, we recently found the expression of ERs, GPER, and androgen receptors (ARs) in both F-ECs and M-ECs[30], thus suggesting that lack of effect may not be due to the absence of receptor expression. However, controversial data are reported in the literature on ER and AR expression in HUVECs, since some authors report the absence[98,99] – or, at least, a weak expression[100,101] - of classical ERs. On the contrary,

Annibalini et al.[102] show that F-ECs and M-ECs express both ERβ and ARs while ERα are absent. Other events - such as a loss in ER activity due to an increase in ER methylation during *in vitro* passages of ECs[102] – may also be excluded as responsible for the inability of sex hormones to prevent reduced cell growth and sprouting, since our experiments have been performed on low passage ECs (1-4).

It should be pointed out that most of the *in vitro* studies on E2 and ECs focus on the hormone ability to improve EC functions through an increase in eNOS expression and activity. However, these findings have been frequently obtained in ECs from other species – among these, the bovine aortic ECs (BAECs) are often used for biochemical studies on eNOS expression[103] – or in immortalized model of ECs – such as the somatic cell hybrid EA.hy926, deriving from HUVECs fused with the human lung carcinoma cell line A549[104,105], or the EC.304 cells, now identified as a human bladder cancer cell line[106]. Similarly, the proliferative effect of E2 on ECs has been reported for example in BAECs[107] or in media not containing CS-FBS[108]. We can therefore propose that HUVECs cultured in CS-FBS are not the best model to study hormonal effects in ECs. This observation is also sustained by the results obtained by re-adding thyroid hormone that, despite its well-known pro-angiogenic properties[109], does not prevent the CS-FBS-induced inhibitory effects on ECs. In conclusion, our data showing that CS-FBS impairs the *in vitro* HUVEC distinctive properties - irrespective of the sex of cells and of the presence of sex and/or thyroid hormones - may be part of a more general discussion on serum starvation and on its interfering potential with experimental results and conclusions[110]. But they also support the idea that CS-FBS loses one or more component(s) showing broad implications in EC physiology. This hypothesis is strongly supported by the observation that re-addition of normal serum totally restores the metabolic activity of F-ECs and M-ECs previously grown in CS-FBS.

In addition to various hormones and peptides, charcoal stripping also removes lipids from FBS[85]. This defeat is potentially rather important since metabolism is emerging as a crucial regulator of EC physiology, especially angiogenesis[72]. In particular, FAs have been reported to be crucially involved in the regulation of EC proliferation[75]. Their oxidation to acetyl-CoA generates a carbon source required in ECs for the synthesis of nucleotides necessary for DNA replication[75]. Indeed, the genetic or pharmacological block of FAO impairs EC proliferation, vessel sprouting, and angiogenesis both *in vitro* and *in vivo*[75]. Interestingly, this central role of FAO seems to be exclusive for ECs since most cells do not use FAs for DNA synthesis[75]. Our results, showing the ability of acetate and palmitic acid to partially prevent the decrease in cell number and sprouting induced by the CS-FBS containing medium, clearly suggest that lipids are one of the crucial components of serum lost in CS-FBS and required for the maintenance of EC growth and *in vitro* morphogenetic properties. Our data also confirm the central role of FAs and FAO in the control of EC proliferation and sprouting. Again, as discussed above for the ATP-dependence on glycolysis, differences between F-ECs and M-ECs are not evident.

Collectively, all the results discussed above demonstrate that F-ECs and M-ECs do not significantly differ in their metabolic properties showing a comparable dependence on glycolysis for ATP generation and on FAs/FAO for cell growth and sprouting. The use of CS-FBS should be discouraged in ECs since the lack of lipids profoundly alters EC physiological properties, thus suggesting caution in the interpretation of results obtained in ECs cultured in CS-FBS-containing media.

Searching for differences between F-ECs and M-ECs, we focused on eNOS, the enzyme responsible for NO formation. In our previous work[30], we anticipated

that eNOS expression – both at mRNA and protein levels - is higher in F-ECs in comparison to M-ECs. Here, we confirm these results in another set of donors. But, very importantly, we demonstrate that: *i)* the difference in eNOS expression is conserved when M-ECs and F-ECs are derived from twin donors; *ii)* eNOS is more expressed in F-ECs even when cells are collected immediately after the flushing from cords, *i.e.* without any *in vitro* passage. This latter result is of particular relevance since it shows for the first time that the enhanced eNOS expression in F-ECs may be considered an inborn feature, and not an *in vitro* acquired attribute.

To investigate the biological significance attributable to the higher eNOS expression in F-ECs, we analyzed its involvement in EC physiology starting from proliferation. Our results show that FBS-induced growth is independent on eNOS activity both in F-ECs and M-ECs. This result is not surprising since a number of data in the literature exclude a role for eNOS in FBS-induced proliferation[111,112]. However, we demonstrate that the independence of FBS-induced proliferation of eNOS is unrelated to sex in ECs.

Beside proliferation, migration is another critical EC property strictly involved in the angiogenic process. At variance with proliferation, a number of papers demonstrate that eNOS-derived NO facilitates EC migration, both in chemotaxis and in the scratch assays[111,113]. In addition, it has been demonstrated that the activation of eNOS – by phosphorylation at Ser1177 - stimulates EC migration even in the absence of chemotactic gradient[113]. However, the contribution of eNOS to F-EC and M-EC motility has never been studied. Our data demonstrate for the first time that F-ECs show a greater migratory capability in comparison to M-ECs in the chemotaxis assay, and that eNOS is crucially required for migration only in F-ECs. Indeed, the pharmacological inhibition of eNOS more significantly

reduces the chemo-tactic response in F-ECs than in M-ECs. The divergence is even more dramatic in the scratch assay, where L-NAME - or eNOS silencing - totally block the ability to close the wound in F-ECs, leaving almost unaffected the capability of M-ECs of closing the gap. Therefore, we can conclude that only F-ECs are critically dependent on eNOS to migrate. This conclusion is also supported by immune-fluorescence experiments (still ongoing in our laboratory, and too preliminary to be included in this thesis) that suggest the presence of a higher amount of lamellipodia – the protrusions characterizing the leading edge of migrating cells[114] - on F-EC membranes. Moreover, we have also observed a prevalent eNOS localization at lamellipodia only in F-ECs.

Taking into account the different involvement of eNOS in the regulation of EC proliferation and migration – where proliferation is eNOS-independent - it is remarkable that M-ECs, at variance from F-ECs, are sensitive to the proliferative block induced by mytomycin treatment. Indeed, only M-ECs are significantly impaired in the wound closure when mitotically arrested. These results emphasize the almost complete independence of M-ECs on the contribution of eNOS activity and their dependence on proliferation rather than migration in the wound closure.

Collectively, these results suggest that proliferation and migration are regulated through two independent pathways - eNOS-independent and eNOS-dependent, respectively - in M-ECs and F-ECs. Moreover, these two processes show a different relevance in M-EC and F-EC behavior. M-ECs mostly depend on eNOS-independent proliferation to close the wound. At variance, F-ECs possess a more motile phenotype and require eNOS for their activities. Therefore, we can conclude that different mechanisms underlie migration in F-ECs and M-ECs.

The disparity between M-ECs and F-ECs in terms of dependence on eNOS activity is even more evident when cells are compared in an *in vitro* model of angiogenesis. It is well-established that the formation of blood vessels requires migration and proliferation of ECs. Sprouting angiogenesis is indeed the result of a coordinated process where the growth of new blood vessels relies not only on the migration of specialized cells at the forefront of vascular sprouts – the tip cells – but also on trailing cells that undergo proliferation to elongate the branch – the stalk cell[68,70,115]. Here, we show that eNOS activity is fundamental in F-ECs also for branching formation. On the contrary, M-ECs fully retain their ability to sprout even in the presence of L-NAME or when eNOS is silenced, thus confirming their eNOS-independence. Conversely, M-EC spheroids are much more impaired when proliferation is blocked in comparison to F-EC spheroids. These data may partially explain the lower sprouting observed in M-ECs in comparison to F-ECs cultured in CS-FBS. In this experimental condition, the absence of FAs - and the consequent impairment in FAO - compromises the proliferative process, leading to a more remarkable impairment in M-EC sprouting. From the analysis on mosaic spheroids from GFP-carrying M-ECs and Cherry Red-expressing F-ECs, it emerges that F-ECs are preferentially located at the forefront of the sprouts, thus showing for most of the time the tip migratory phenotype. At variance, M-ECs mostly constitute the *core* of the sprout. Notably, the frequency of F-ECs in the tip position significantly decreases in the presence of L-NAME, thus confirming again that the migratory phenotype that characterize F-ECs is crucially controlled by eNOS activity.

It is therefore possible to suggest that *in vitro* angiogenesis depends on different mechanisms in F-ECs and M-ECs. More precisely, F-ECs mainly contribute to the branching development *via* eNOS-dependent migration, whereas M-ECs participate to the process through eNOS-independent proliferation. In F-ECs, the

higher expression and activity of eNOS lead to the prevalence of a migratory phenotype. At variance, M-ECs rely on a different pathway that favors a proliferative phenotype possibly through the enhancement of FAO, thus permitting the overcoming of their lower migratory ability. In other words, F-ECs and M-ECs are able to achieve the same results, *i.e.* angiogenesis, but *via* different strategies, represented by motility for F-ECs, and proliferation for M-ECs.

In conclusions, our results demonstrate that different mechanisms – eNOS-dependent and –independent, respectively - are responsible for migration and sprouting in F-ECs and M-ECs. The constitutive higher expression of eNOS in F-ECs, and the crucial requirement of its activity for motility and sprouting, might contribute to the protection against CVD characteristic of the younger female population. Due to the critical dependence of F-ECs on its activity, any minimal loss in eNOS availability might therefore have harmful consequences on the female endothelial function.

In addition, our results - even if restricted up to now to an *in vitro* approach - make some contribution to the sex-related research in the cardiovascular field, with the aim to take part in the development of precision medicine. Therapeutic interventions have been traditionally designed to equally treat large populations of individuals – the "one-size-fits-all" approach. Conversely, precision medicine – defined as "an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person" by the NIH - is now becoming a benchmark in pharmacology. From this perspective, it is important to increase knowledge on the divergence in the onset and development of CVD between men and women, and on the gender dimorphic profiles that are emerging in terms of drug efficacy. As a

consequence, the elucidation of the biological basis for sex differences in CVD can lead the way to the development of gender-oriented preventive and pharmacological treatments.

Future progress of the project

To date, our findings are limited to an *in vitro* approach, even if our results have been obtained in a well-established and consolidated human primary EC model. Therefore, further investigations with the support of different models and/or methodological approaches are required to confirm and increase the knowledge and scientific significance of sex-based differences in male and female EC behavior.

With the aim of studying if the higher expression of eNOS - already present at born in F-ECs – is maintained during lifetime, we will extend our analyses to an *ex vivo* model represented by expanded endothelial progenitor cells (EPCs). EPCs express a variety of cell surface markers similar to those expressed by ECs and are involved in the maintenance of endothelial integrity and in the promotion of vascular repair[116]. Importantly, the number of circulating EPCs is reduced in CVD, and low EPC levels confer an increased risk of atherosclerotic disease progression and adverse cardiovascular outcome. Therefore, the study on EPCs from peripheral blood represents an innovative strategy to evaluate endothelial compartment through a non-invasive method.

EPCs will be isolated from peripheral blood samples and cultured to form colonies (endothelial colony-forming cells, ECFCs)[117] that will be used to analyze eNOS expression and function. To this purpose, we have already started the collection of ECFCs from healthy young women (age 20–40 yrs), perimenopausal women (40-55 yrs), and women in physiological menopause (55-70 yrs) assuming (or not assuming) hormone replacement therapy. EPCs from matched-age healthy

men will be concomitantly collected, expanded, and analyzed. EPCs collection, isolation, and expansion will be performed in collaboration with Prof.ssa Silvia Della Bella (Dept of Medical Biotechnology and Translational Medicine, BIOMETRA, University of Milano).

Materials and Methods

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly derived umbilical cords essentially as described by Jaffe et al.[118]. Cords were anonymously donated after informed consent according to the national ethical legislation, and gently collected by gynecologists and nurses of the Macedonio Melloni Hospital. Umbilical cord vein was cannulated with a butterfly needle (0,8 mm) and then washed with abundant sterile saline solution (0.9% NaCl) to remove blood clots. Afterwards, the vein was sealed at one end with a clamp and filled with a collagenase solution (0.125%) solubilised in Ca^{2+}/Mg^{2+} phosphate-buffered saline (PBS) buffer prepared by mixing 4 volumes of part A - containing NaCl 140 mM, KCl 3.9 mM, KH_2PO_4 2.1 mM, Na_2HPO_4 8.1 mM - with 2 volumes of part B - containing $CaCl_2$ 1.3 mM and $MgCl_2$ 0.93 mM. After 10 minutes of incubation at 37°C, the collagenase solution was collected in a 50-ml tube with an equal volume of 199 medium supplemented with 10% of fetal bovine serum (FBS). Cells were finally harvested by centrifugation and plated on 25 cm^2 flasks previously coated with 0.1% gelatin. HUVECs were routinely grown in 199 medium supplemented with 20% FBS, 25 μg/ml endothelial cell growth supplement (ECGS) and 50 μg/ml heparin, and used at passages 1-4. Notably, we always used HUVECs pooled from two or more donors to minimize the variability associated with cells derived from a single male or female newborn donor.

Charcoal stripping of FBS was performed following standard protocols[80]. Briefly, dextran-coated charcoal - obtained by overnight incubation of 2.5% w/v charcoal with 0.025% w/v dextran T-70 in PBS - was re-suspended in FBS and stirred overnight at 4°C. After centrifugation, the stripped FBS was sterilized by filtration through a 0.22 μm filter. Notably, charcoal stripping was applied to the same lot of serum present in the standard medium.

Cell metabolism assays

Viable cell metabolism was measured by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. In metabolically active cells, the MTT substrate - prepared as a stock solution of 5 mg/ml in PBS and added at a final concentration of 0.5 mg/ml - is converted into a purple insoluble formazan product that is solubilised in DMSO after 4 h of incubation at 37°C. In addition, we also performed experiments with WST-8 (Dojindo), a highly water-soluble tetrazolium salt giving a yellow-color formazan dye when reduced by cellular dehydrogenase activities. The total cellular ATP content was evaluated by means of the CellTiter-Glo® Luminescent Assay (Promega, following the manufacturer's instructions. All assays were performed on HUVECs plated at a density of $1.5x10^4$ cells/well in 0.1% gelatin-coated 96-well microplates. For the ATP assay, white plates were used to maximize light output signal. Optical density at 570 nm for MTT and 450 nM for WST-8, and luminescence for ATP were measured using a multiplate spectrophotometer (Victor™, PerkinElmer). All assays were performed in triplicate.

Evaluation of cell number and viability

Cell number and viability were assessed by the trypan blue exclusion test on HUVECs plated in 0.1% gelatin-coated 35-mm Petri dishes.

Cell proliferation assay

HUVECs were seeded in triplicate at a density of 2.000 cells/well in 0.1% gelatin-coated 96-well plates, and cell proliferation was measured by WST-8 assay, according to the manufacturer's instructions. The absorbance was measured at 450 nm after 4h of incubation at 37°C.

Cell migration assays

Cell migration was detected by chemotaxis experiments in a 48-well modified Boyden's chamber as described[119]. Briefly, after suspension in 199 medium containing 1% fatty acid free bovine serum albumin (FFA-BSA), HUVECs were

added to the upper chamber at a density of $5.0x10^4$ cells/well. Polyvinylpyrrolidine (PVP)-free polycarbonate filters (8 μm pore size, Neuroprobe) - coated with type I collagen (10 μg/ml) - were placed over a bottom chamber containing 10% FBS as attractant. After a 6-h incubation at 37°C, the cells that had migrated to the lower side of the filter were stained with Diff-Quick stain (Neuroprobe), and 5 unit fields per filter were counted by a scorer blind to the experimental conditions using a Zeiss microscope.

For the wound healing assay, a scratch wound was applied on confluent HUVECs using a sterile 200 μl pipette tip. Experiments were performed on cells plated in 0.1% gelatin-coated 24-well plates. After the scratch, HUVECs were incubated in 199 medium with 20% FBS in the presence of mitomycin (500 ng/ml) or L-NAME (100 μM), when indicated. Images were captured immediately after wounding (t_{0h}) and 15 h later (t_{15h}) at a 4x magnification with an Olympus U-CMAD3 phase contrast microscope equipped with an Olympus digital camera. The percent of wound closure (gap area at t_0 *minus* gap area at t_{15h}/gap area at t_0 x100) was measured with the National Institute of Health (NIH) Image J software package.

Three-dimensional (3-D) spheroid sprouting assay

EC spheroids of a defined cell number were embedded into collagen gels in the presence of 25 ng/ml VEGF as previously described[83,84]. Briefly, a specific number of HUVECs (750 cells/well) was suspended in 199 medium containing 10% FBS and 0.25% w/v methylcellulose, and seeded in non-adherent round bottom 96-well plates. Under these conditions, a single EC spheroid was assembled within 24 hours in every well. Spheroids (48-50 for each experimental samples) were quickly harvested and collected by a 5-min centrifugation at 500*g*. Each pellet was suspended in 199 medium with 20% FBS and 0.5% w/v methylcellulose, and the various test substances – VEGF, acetate, palmitic acid or its vehicle, and L-NAME at final concentrations of 25 ng/ml, 20 mM, 250 μM and 1 mM, respectively – were added in a minimal volume to this

medium. Finally, an equal volume of a collagen stock solution - prepared by mixing at 4°C acidic rat tail collagen I (2 mg/ml; 8 vol) with 10x 199 medium (1 vol) and 0.1 mM NaOH (approx. 1 vol) to adjust the pH to 7.4 – was added. The spheroid-containing gel was rapidly transferred into pre-warmed 96-well plates, and incubated at 37°C in 5% CO_2. After 18 h, images were acquired with a phase-contrast microscope (10x objective magnification, Olympus U-CMAD3) equipped with an Olympus digital camera. In-gel angiogenesis was quantified by measuring the length of all of the capillary-like sprouts originating from individual spheroids using the NIH Image J program. At least, 10 randomly selected spheroids per experimental group were measured in each experiment. For mitotically inactivated HUVECs, mitomycin (500 ng/ml) was added overnight during the formation of spheroids. When silenced cells were used, HUVECs were plated to form spheroids 24 h after siRNA transfection.

To assess male and female endothelial tip cell competition, M-ECs and F-ECs transduced with lentiviral vectors expressing GFP or CherryRed, respectively, were used. Mosaic spheroids were prepared by mixing in a 1:1 ratio GFP-carrying M-ECs and Cherry Red-expressing F-ECs. Lentiviral vector preparations - at a titer of 1-4x10^9 transducing units/ml - were a kind gift of Dr. Francesco Bifari, Dept of Medical Biotechnology and Translational Medicine, University of Milano. Images were obtained using a Zeiss LSM 510 Meta NLO confocal microscope.

Immunoblot analyses

For standard immunoblot analysis, HUVECs were washed with PBS and directly lysed in SDS-PAGE sample buffer (62 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.04% bromophenol blue). After SDS-PAGE electrophoresis, proteins were transferred onto nitrocellulose membranes that were blocked with 5% (w/v) non fat dried milk in Tris-buffered saline (TBS: Tris-HCl 10 mM pH 8, NaCl 150 mM) containing 0.05%

Tween-20 (TBS-T). Filters were incubated overnight with the indicated primary antibodies (anti eNOS, 1:2.000, and anti β-actin, 1:1.000), and for 1 h with the appropriate peroxidase-conjugated secondary antibody (DAKO). The immunoreactive bands were visualized by chemiluminescence (LiteAblot Plus, EuroClone). For the detection of eNOS monomers and dimers, low temperature SDS-PAGE (LT-PAGE) was performed[120]. Briefly, HUVECs were lysed for 20 min on ice in a buffer containing Tris-HCl 20 mM pH 7.4, NaCl 150 mM, EDTA 5 mM, Na_3VO_4 1 mM, 1% NP-40 supplemented with a protease inhibitor cocktail (Sigma Aldrich). After centrifugation (10.000*g*, 15 min, 4°C), lysates were mixed with SDS-PAGE sample buffer and resolved by LT-PAGE with 6% gels without prior heat denaturation. All gels and buffers were kept at 4 °C during the whole procedure. Subsequent to LT-PAGE, the gels were transferred, and the blots were probed as routine Western blot. Densitometric analyses of the immunoblots were performed using the NIH Image J program.

Total RNA extraction for reverse transcription and quantitative real time PCR (RT-qPCR)

Total RNA was extracted using the RNeasy® Mini Kit and accompanying QIAshredder™ (Qiagen) according to the manufacturer's instructions. A 15-min on-column incubation with DNase I (Qiagen) was performed, thus avoiding DNA contamination of samples. Reverse transcription was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific), again following the manufacturer's instructions. For quantitative analysis of gene expression, we used the ABI Prism 7000 Sequence Detection System, SDS software version 1.2.3 (Applied Biosystems, CA, USA) with the TaqMan® Primer and Probe assays for human eNOS (NOS3, Hs00167166_m1) and for the endogenous control 18S (Hs99999901_s1). Sequences were amplified from 50 ng of cDNA. For calculation of results, the $2^{-\Delta\Delta Ct}$ method was used allowing normalization to 18S.

Small interfering RNA (siRNA) transfection

Validated Stealth™ RNAi duplexes against human eNOS (GC content 48%) were provided from Thermo Fisher Scientific. As control RNA, we utilized a Stealth™ RNAi negative control duplex (Medium GC Duplex, Thermo Fisher Scientific) with a 48% GC content, suitable for use as a control with Stealth™ RNAi duplexes containing 45-55% of GC. All sets of RNAi molecules were individually transfected into HUVECs at a 10 nM concentration using the PepMute transfection reagent according to the manufacturer's instructions (SignaGen Laboratories). The ability of the RNAi molecules to knockdown eNOS expression was analyzed by western blot analysis.

Statistical procedures

All data were expressed as mean ± s.e.m of at least 3 independent experiments. Statistical significance was determined by unpaired Student's *t*-test or by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, according to the number of groups compared. The GraphPad Prism version 5.00 software was used.

Reagents and antibodies

All tissue culture reagents were from Euroclone, except ECGS and heparin (Sigma Aldrich). Charcoal, dextran T-70, MTT, trypan blue, methylcellulose (product number M0512), L-NAME, sodium acetate, palmitic acid, and FFA-BSA were from Sigma Aldrich; rat tail collagen I from Serva; recombinant human $VEGF_{165}$ and mitomycin from Peprotech; E2, DHT, and T3 from Cayman Chemical. Antibodies used were mouse monoclonals anti eNOS (BD Biosciences) and anti β-actin (Sigma Aldrich). Palmitic acid was used as conjugate to FFA-BSA. Briefly, a 75 mM stock solution of palmitic acid dissolved in ethanol were diluted 1:10 with a 10% FFA-BSA solution prepared in 199 medium, and incubated overnight at 37°C before every experiment.

Bibliography

[1] Townsend, N., Wilson, L., Bhatnagar, P., Wickramasinghe, K., Rayner, M. and Nichols, M. (2016). Cardiovascular disease in Europe: epidemiological update 2016. Eur Heart J 37, 3232-3245.

[2] Mozaffarian, D. et al. (2016). Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation 133, e38-360.

[3] Jani, B. and Rajkumar, C. (2006). Ageing and vascular ageing. Postgrad Med J 82, 357-62.

[4] Endemann, D.H. and Schiffrin, E.L. (2004). Endothelial dysfunction. J Am Soc Nephrol 15, 1983-92.

[5] Lerman, A. and Zeiher, A.M. (2005). Endothelial function - Cardiac events. Circulation 111, 363-368.

[6] Bonetti, P.O., Lerman, L.O. and Lerman, A. (2003). Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23, 168-75.

[7] Mahmoodzadeh, S., Fliegner, D. and Dworatzek, E. (2012). Sex differences in animal models for cardiovascular diseases and the role of estrogen. Handb Exp Pharmacol, 23-48.

[8] Mosca, L., Barrett-Connor, E. and Wenger, N.K. (2011). Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes. Circulation 124, 2145-54.

[9] Leening, M.J. et al. (2014). Sex differences in lifetime risk and first manifestation of cardiovascular disease: prospective population based cohort study. BMJ 349, g5992.

[10] Bacon, S.L. et al. (2011). The research on endothelial function in women and men at risk for cardiovascular disease (REWARD) study: methodology. BMC

Cardiovasc Disord 11, 50.

[11] McDermott, B.J. and Gray, G.A. (2014). Biological sex themed section: incorporating the female dimension into cardiovascular pharmacology. Br J Pharmacol 171, 537-40.

[12] dos Santos, R.L., da Silva, F.B., Ribeiro, R.F. and Stefanon, I. (2014). Sex hormones in the cardiovascular system. Horm Mol Biol Clin Investig 18, 89-103.

[13] Barton, M. and Meyer, M.R. (2009). Postmenopausal hypertension: mechanisms and therapy. Hypertension 54, 11-8.

[14] Arnal, J.F., Fontaine, C., Billon-Galés, A., Favre, J., Laurell, H., Lenfant, F. and Gourdy, P. (2010). Estrogen receptors and endothelium. Arterioscler Thromb Vasc Biol 30, 1506-12.

[15] Bae, S. and Zhang, L. (2005). Gender differences in cardioprotection against ischemia/reperfusion injury in adult rat hearts: focus on Akt and protein kinase C signaling. J Pharmacol Exp Ther 315, 1125-35.

[16] Brown, D.A., Lynch, J.M., Armstrong, C.J., Caruso, N.M., Ehlers, L.B., Johnson, M.S. and Moore, R.L. (2005). Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. J Physiol 564, 619-30.

[17] Johnson, M.S., Moore, R.L. and Brown, D.A. (2006). Sex differences in myocardial infarct size are abolished by sarcolemmal KATP channel blockade in rat. Am J Physiol Heart Circ Physiol 290, H2644-47.

[18] Vanhoutte, P.M., Zhao, Y., Xu, A. and Leung, S.W. (2016). Thirty Years of Saying NO: Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator. Circ Res 119, 375-96.

[19] Booth, E.A., Marchesi, M., Kilbourne, E.J. and Lucchesi, B.R. (2003). 17Beta-

estradiol as a receptor-mediated cardioprotective agent. J Pharmacol Exp Ther 307, 395-401.

[20] Hale, S.L., Birnbaum, Y. and Kloner, R.A. (1996). beta-Estradiol, but not alpha-estradiol, reduced myocardial necrosis in rabbits after ischemia and reperfusion. Am Heart J 132, 258-62.

[21] Patten, R.D. et al. (2004). 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. Circ Res 95, 692-9.

[22] Pollitzer, E. (2013). Biology: Cell sex matters. Nature 500, 23-4.

[23] Shah, K., McCormack, C.E. and Bradbury, N.A. (2014). Do you know the sex of your cells? Am J Physiol Cell Physiol 306, C3-18.

[24] Ritz, S.A. et al. (2014). First steps for integrating sex and gender considerations into basic experimental biomedical research. FASEB J 28, 4-13.

[25] Rubanyi, G.M., Johns, A. and Kauser, K. (2002). Effect of estrogen on endothelial function and angiogenesis. Vascul Pharmacol 38, 89-98.

[26] Kublickiene, K. and Luksha, L. (2008). Gender and the endothelium. Pharmacol Rep 60, 49-60.

[27] Taylor, K.E., Vallejo-Giraldo, C., Schaible, N.S., Zakeri, R. and Miller, V.M. (2011). Reporting of sex as a variable in cardiovascular studies using cultured cells. Biol Sex Differ 2, 11.

[28] Batres, R.O. and Dupont, J. (1986). Gender differences in prostacyclin and prostaglandin E2 synthesis by human endothelial cells. Prostaglandins Leukot Med 22, 159-71.

[29] Matarrese, P. et al. (2011). Gender disparity in susceptibility to oxidative stress and apoptosis induced by autoantibodies specific to RLIP76 in vascular

cells. Antioxid Redox Signal 15, 2825-36.

[30] Addis, R. et al. (2014). Human umbilical endothelial cells (HUVECs) have a sex: characterisation of the phenotype of male and female cells. Biol Sex Differ 5, 18.

[31] Lorenz, M. et al. (2015). Does cellular sex matter? Dimorphic transcriptional differences between female and male endothelial cells. Atherosclerosis 240, 61-72.

[32] Cines, D.B. et al. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91, 3527-61.

[33] Galley, H.F. and Webster, N.R. (2004). Physiology of the endothelium. Br J Anaesth 93, 105-13.

[34] Gimbrone, M.A. and García-Cardeña, G. (2016). Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ Res 118, 620-36.

[35] Broze, G.J. (1995). Tissue factor pathway inhibitor. Thromb Haemost 74, 90-3.

[36] Bevilacqua, M.P., Pober, J.S., Majeau, G.R., Fiers, W., Cotran, R.S. and Gimbrone, M.A. (1986). Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci U S A 83, 4533-7.

[37] Michiels, C. (2003). Endothelial cell functions. J Cell Physiol 196, 430-43.

[38] Dudzinski, D.M., Igarashi, J., Greif, D. and Michel, T. (2006). The regulation and pharmacology of endothelial nitric oxide synthase. Annu Rev Pharmacol Toxicol 46, 235-76.

[39] Govers, R. and Rabelink, T.J. (2001). Cellular regulation of endothelial nitric oxide synthase. Am J Physiol Renal Physiol 280, F193-206.

[40] Qian, J. and Fulton, D. (2013). Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. Front Physiol 4, 347.

[41] Brunner, H. et al. (2005). Endothelial function and dysfunction. Part II: Association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension. J Hypertens 23, 233-46.

[42] Förstermann, U. and Sessa, W.C. (2012). Nitric oxide synthases: regulation and function. Eur Heart J 33, 829-37, 837a-837d.

[43] Ziche, M. and Morbidelli, L. (2000). Nitric oxide and angiogenesis. J Neurooncol 50, 139-48.

[44] Friebe, A. and Koesling, D. (2003). Regulation of nitric oxide-sensitive guanylyl cyclase. Circ Res 93, 96-105.

[45] Friebe, A., Mergia, E., Dangel, O., Lange, A. and Koesling, D. (2007). Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase. Proc Natl Acad Sci U S A 104, 7699-704.

[46] Beckman, J.S. and Koppenol, W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 271, C1424-37.

[47] Murad, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. J Clin Invest 78, 1-5.

[48] Ruetten, H., Zabel, U., Linz, W. and Schmidt, H.H. (1999). Downregulation of soluble guanylyl cyclase in young and aging spontaneously hypertensive rats. Circ Res 85, 534-41.

[49] Mizuno, Y., Jacob, R.F. and Mason, R.P. (2010). Advances in pharmacologic modulation of nitric oxide in hypertension. Curr Cardiol Rep 12, 472-80.

[50] Foster, M.W., Hess, D.T. and Stamler, J.S. (2009). Protein S-nitrosylation in health and disease: a current perspective. Trends Mol Med 15, 391-404.

[51] Albrecht, E.W., Stegeman, C.A., Tiebosch, A.T., Tegzess, A.M. and van Goor, H. (2002). Expression of inducible and endothelial nitric oxide synthases, formation of peroxynitrite and reactive oxygen species in human chronic renal transplant failure. Am J Transplant 2, 448-53.

[52] Huang, P.L. (2009). eNOS, metabolic syndrome and cardiovascular disease. Trends Endocrinol Metab 20, 295-302.

[53] Sessa, W.C. (2004). eNOS at a glance. J Cell Sci 117, 2427-9.

[54] Lisanti, M.P., Scherer, P.E., Tang, Z. and Sargiacomo, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. Trends Cell Biol 4, 231-5.

[55] Gonzalez, E., Kou, R., Lin, A.J., Golan, D.E. and Michel, T. (2002). Subcellular targeting and agonist-induced site-specific phosphorylation of endothelial nitric-oxide synthase. J Biol Chem 277, 39554-60.

[56] McCabe, T.J., Fulton, D., Roman, L.J. and Sessa, W.C. (2000). Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. J Biol Chem 275, 6123-8.

[57] Widlansky, M.E., Gokce, N., Keaney, J.F. and Vita, J.A. (2003). The clinical implications of endothelial dysfunction. J Am Coll Cardiol 42, 1149-60.

[58] Shiva Shankar, T.V. and Willems, L. (2014). Epigenetic modulators mitigate angiogenesis through a complex transcriptomic network. Vascul Pharmacol 60, 57-66.

[59] Deanfield, J.E., Halcox, J.P. and Rabelink, T.J. (2007). Endothelial function and dysfunction: testing and clinical relevance. Circulation 115, 1285-95.

[60] Pirillo, A., Norata, G.D. and Catapano, A.L. (2013). LOX-1, OxLDL, and atherosclerosis. Mediators Inflamm 2013, 152786.

[61] Zhou, J., Abid, M.D., Xiong, Y., Chen, Q. and Chen, J. (2013). ox-LDL

downregulates eNOS activity via LOX-1-mediated endoplasmic reticulum stress. Int J Mol Med 32, 1442-50.

[62] Murphy, E. (2011). Estrogen signaling and cardiovascular disease. Circ Res 109, 687-96.

[63] Lu, Q., Schnitzler, G.R., Ueda, K., Iyer, L.K., Diomede, O.I., Andrade, T. and Karas, R.H. (2016). ER Alpha Rapid Signaling Is Required for Estrogen Induced Proliferation and Migration of Vascular Endothelial Cells. PLoS One 11, e0152807.

[64] Gaudet, H.M., Cheng, S.B., Christensen, E.M. and Filardo, E.J. (2015). The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. Mol Cell Endocrinol 418 Pt 3, 207-19.

[65] Meyer, M.R., Prossnitz, E.R. and Barton, M. (2011). The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. Vascul Pharmacol 55, 17-25.

[66] Lindsey, S.H., Liu, L. and Chappell, M.C. (2014). Vasodilation by GPER in mesenteric arteries involves both endothelial nitric oxide and smooth muscle cAMP signaling. Steroids 81, 99-102.

[67] Carmeci, C., Thompson, D.A., Ring, H.Z., Francke, U. and Weigel, R.J. (1997). Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45, 607-17.

[68] Potente, M., Gerhardt, H. and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. Cell 146, 873-87.

[69] Simons, M., Gordon, E. and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol 17, 611-25.

[70] Jakobsson, L. et al. (2010). Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol 12, 943-53.

[71] De Bock, K., Georgiadou, M. and Carmeliet, P. (2013). Role of endothelial cell metabolism in vessel sprouting. Cell Metab 18, 634-47.

[72] Eelen, G., de Zeeuw, P., Simons, M. and Carmeliet, P. (2015). Endothelial cell metabolism in normal and diseased vasculature. Circ Res 116, 1231-44.

[73] Dagher, Z., Ruderman, N., Tornheim, K. and Ido, Y. (2001). Acute regulation of fatty acid oxidation and amp-activated protein kinase in human umbilical vein endothelial cells. Circ Res 88, 1276-82.

[74] Krützfeldt, A., Spahr, R., Mertens, S., Siegmund, B. and Piper, H.M. (1990). Metabolism of exogenous substrates by coronary endothelial cells in culture. J Mol Cell Cardiol 22, 1393-404.

[75] Schoors, S. et al. (2015). Fatty acid carbon is essential for dNTP synthesis in endothelial cells. Nature 520, 192-7.

[76] Welti, J., Loges, S., Dimmeler, S. and Carmeliet, P. (2013). Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. J Clin Invest 123, 3190-200.

[77] Zachary, I. and Morgan, R.D. (2011). Therapeutic angiogenesis for cardiovascular disease: biological context, challenges, prospects. Heart 97, 181-9.

[78] Dimmeler, S., Zeiher, A.M. and Schneider, M.D. (2005). Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 115, 572-83.

[79] Berthois, Y., Katzenellenbogen, J.A. and Katzenellenbogen, B.S. (1986). Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 83, 2496-500.

[80] Leake, R.E., Freshnet, R.I., Munir, I., in: Green, B., Leake, R.E., (1987). Steroid hormones – a practical approach. Irl Press, Oxford, 213-214.

[81] Cao, Z., West, C., Norton-Wenzel, C.S., Rej, R., Davis, F.B. and Davis, P.J. (2009). Effects of resin or charcoal treatment on fetal bovine serum and bovine calf serum. Endocr Res 34, 101-8.

[82] Mousa, S.A., Lin, H.Y., Tang, H.Y., Hercbergs, A., Luidens, M.K. and Davis, P.J. (2014). Modulation of angiogenesis by thyroid hormone and hormone analogues: implications for cancer management. Angiogenesis 17, 463-9.

[83] Cappellini, E., Vanetti, C., Vicentini, L.M. and Cattaneo, M.G. (2015). Silencing of Eps8 inhibits in vitro angiogenesis. Life Sci 131, 30-6.

[84] Heiss, M., Hellström, M., Kalén, M., May, T., Weber, H., Hecker, M., Augustin, H.G. and Korff, T. (2015). Endothelial cell spheroids as a versatile tool to study angiogenesis in vitro. FASEB J 29, 3076-84.

[85] Viscardi, R.M., Ullsperger, S. and McKenna, M.C. (1991). Carbon stripping extracts serum free fatty acids: implications for media supplementation of cultured type II pneumocytes. Lab Invest 65, 250-7.

[86] Mittelstrass, K. et al. (2011). Discovery of sexual dimorphisms in metabolic and genetic biomarkers. PLoS Genet 7, e1002215.

[87] Culic, O., Gruwel, M.L. and Schrader, J. (1997). Energy turnover of vascular endothelial cells. Am J Physiol 273, C205-13.

[88] Quintero, M., Colombo, S.L., Godfrey, A. and Moncada, S. (2006). Mitochondria as signaling organelles in the vascular endothelium. Proc Natl Acad Sci U S A 103, 5379-84.

[89] Tso, C.L. et al. (2000). Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-

dependent cancer cells. Cancer J 6, 220-33.

[90] Dang, Z.C. and Lowik, C.W. (2005). Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway. Mol Cell Biochem 268, 159-67.

[91] Lindquist, D.L. and de Alarcon, P.A. (1987). Charcoal-dextran treatment of fetal bovine serum removes an inhibitor of human CFU-megakaryocytes. Exp Hematol 15, 234-8.

[92] Yohay, D.A., Zhang, J., Thrailkill, K.M., Arthur, J.M. and Quarles, L.D. (1994). Role of serum in the developmental expression of alkaline phosphatase in MC3T3-E1 osteoblasts. J Cell Physiol 158, 467-75.

[93] Radde, B.N., Ivanova, M.M., Mai, H.X., Salabei, J.K., Hill, B.G. and Klinge, C.M. (2015). Bioenergetic differences between MCF-7 and T47D breast cancer cells and their regulation by oestradiol and tamoxifen. Biochem J 465, 49-61.

[94] Simoncini, T., Fornari, L., Mannella, P., Varone, G., Caruso, A., Garibaldi, S. and Genazzani, A.R. (2005). Differential estrogen signaling in endothelial cells upon pulsed or continuous administration. Maturitas 50, 247-58.

[95] Sobrino, A. et al. (2009). Estradiol stimulates vasodilatory and metabolic pathways in cultured human endothelial cells. PLoS One 4, e8242.

[96] Goglia, L., Tosi, V., Sanchez, A.M., Flamini, M.I., Fu, X.D., Zullino, S., Genazzani, A.R. and Simoncini, T. (2010). Endothelial regulation of eNOS, PAI-1 and t-PA by testosterone and dihydrotestosterone in vitro and in vivo. Mol Hum Reprod 16, 761-9.

[97] Lee, T.S., Lin, J.J., Huo, Y.N. and Lee, W.S. (2015). Progesterone Inhibits Endothelial Cell Migration Through Suppression of the Rho Activity Mediated by cSrc Activation. J Cell Biochem 116, 1411-8.

[98] Jensen, I., Rinaldo, C.H., Nordbø Berge, L., Seternes, O.M. and Moens, U. (1998). Human umbilical vein endothelial cells lack expression of the estrogen receptor. Endothelium 6, 9-21.

[99] Tatsumi, H., Kitawaki, J., Tanaka, K., Hosoda, T. and Honjo, H. (2002). Lack of stimulatory effect of dienogest on the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 by endothelial cell as compared with other synthetic progestins. Maturitas 42, 287-94.

[100] Hervé, M.A., Meduri, G., Petit, F.G., Domet, T.S., Lazennec, G., Mourah, S. and Perrot-Applanat, M. (2006). Regulation of the vascular endothelial growth factor (VEGF) receptor Flk-1/KDR by estradiol through VEGF in uterus. J Endocrinol 188, 91-9.

[101] Evans, M.J., Lai, K., Shaw, L.J., Harnish, D.C. and Chadwick, C.C. (2002). Estrogen receptor alpha inhibits IL-1beta induction of gene expression in the mouse liver. Endocrinology 143, 2559-70.

[102] Annibalini, G. et al. (2014). Effects of sex hormones on inflammatory response in male and female vascular endothelial cells. J Endocrinol Invest 37, 861-9.

[103] Hayashi, T., Yamada, K., Esaki, T., Kuzuya, M., Satake, S., Ishikawa, T., Hidaka, H. and Iguchi, A. (1995). Estrogen increases endothelial nitric oxide by a receptor-mediated system. Biochem Biophys Res Commun 214, 847-55.

[104] Kleinert, H., Wallerath, T., Euchenhofer, C., Ihrig-Biedert, I., Li, H. and Förstermann, U. (1998). Estrogens increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. Hypertension 31, 582-8.

[105] Haynes, M.P., Sinha, D., Russell, K.S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W.C. and Bender, J.R. (2000). Membrane estrogen receptor

engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. Circ Res 87, 677-82.

[106] Brown, J. et al. (2000). Critical evaluation of ECV304 as a human endothelial cell model defined by genetic analysis and functional responses: a comparison with the human bladder cancer derived epithelial cell line T24/83. Lab Invest 80, 37-45.

[107] Concina, P. et al. (2000). 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 37, 202-8.

[108] Oviedo, P.J. et al. (2011). Estradiol induces endothelial cell migration and proliferation through estrogen receptor-enhanced RhoA/ROCK pathway. Mol Cell Endocrinol 335, 96-103.

[109] Liu, X., Zheng, N., Shi, Y.N., Yuan, J., Li, L. (2014). Thyroid hormone induced angiogenesis through the integrin αvβ3/protein kinase D/histone deacetylase 5 signaling pathway, J Mol Endocrinol 52, 245-254.

[110] Pirkmajer, S. and Chibalin, A.V. (2011). Serum starvation: caveat emptor. Am J Physiol Cell Physiol 301, C272-9.

[111] Murohara, T., Witzenbichler, B., Spyridopoulos, I., Asahara, T., Ding, B., Sullivan, A., Losordo, D.W. and Isner, J.M. (1999). Role of endothelial nitric oxide synthase in endothelial cell migration. Arterioscler Thromb Vasc Biol 19, 1156-61.

[112] Papapetropoulos, A., Fulton, D., Mahboubi, K., Kalb, R.G., O'Connor, D.S., Li, F., Altieri, D.C. and Sessa, W.C. (2000). Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem 275, 9102-5.

[113] Dimmeler, S., Dernbach, E. and Zeiher, A.M. (2000). Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced

endothelial cell migration. FEBS Lett 477, 258-62.

[114] Krause, M. and Gautreau, A. (2014). Steering cell migration: lamellipodium dynamics and the regulation of directional persistence. Nat Rev Mol Cell Biol 15, 577-90.

[115] Arima, S., Nishiyama, K., Ko, T., Arima, Y., Hakozaki, Y., Sugihara, K., Koseki, H., Uchijima, Y., Kurihara, Y. & Kurihara, H. (2011). Angiogenic morphogenesis driven by dynamic and heterogeneous collective endothelial cell movement, Development. 138, 4763-76.

[116] Yoder, M.C. (2012). Human endothelial progenitor cells. Cold Spring Harb Perspect Med 2, a006692.

[117] Colombo, E. et al. (2012). Fast reduction of peripheral blood endothelial progenitor cells in healthy humans exposed to acute systemic hypoxia. J Physiol 590, 519-32.

[118] Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 52, 2745-56.

[119] Cattaneo, M.G., Chini, B. and Vicentini, L.M. (2008). Oxytocin stimulates migration and invasion in human endothelial cells. Br J Pharmacol 153, 728-36.

[120] Eid, A.A., Lee, D.Y., Roman, L.J., Khazim, K. and Gorin, Y. (2013). Sestrin 2 and AMPK connect hyperglycemia to Nox4-dependent endothelial nitric oxide synthase uncoupling and matrix protein expression. Mol Cell Biol 33, 3439-60.

Acknowledgements

I would like to express my deepest gratitude to my tutor Maria Grazia Cattaneo, PhD, and to Professor Lucia Vicentini, for the support, the dedication and the opportunity they gave me to work in their lab, joining exciting projects. A big thank you also to Professor Alberto Corsini for his unconditioned helpfulness. Moreover, I am grateful to Francesco Bifari, MD, PhD, for his ability to motivate and to encourage new ideas.