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Endothelial maintenance in health and disease: importance of sex differences

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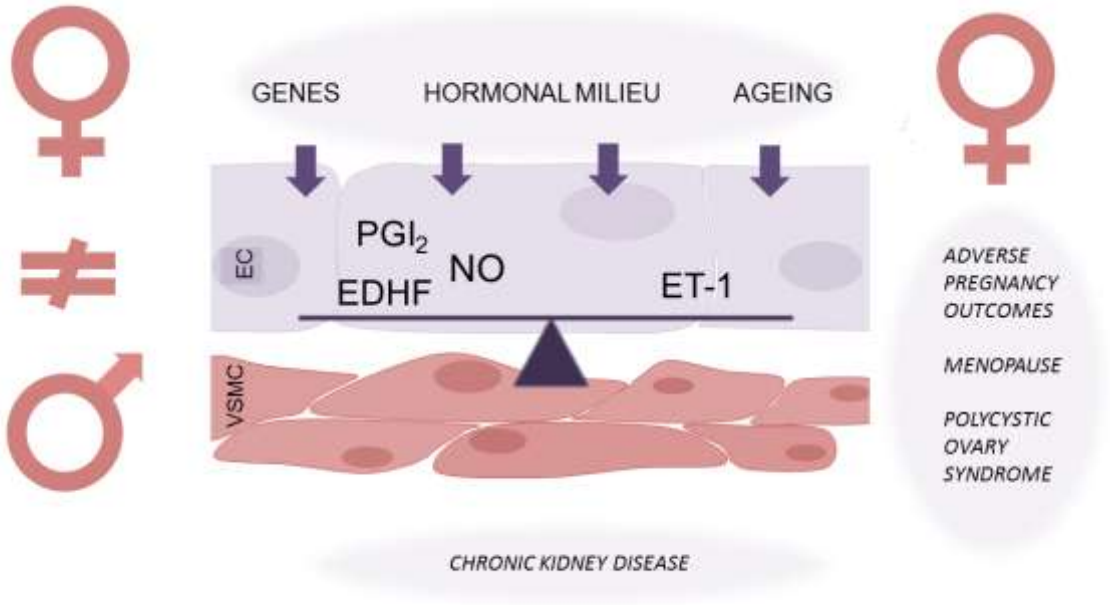
Graphical abstract



Abstract

The vascular endothelium has emerged as more than just an inert monolayer of cells lining the vascular bed. It represents the interface between the blood stream and vessel wall, and has a strategic role in regulating vascular homeostasis by the release of vasoactive substances. Endothelial dysfunction contributes to the development and progression of cardiovascular disease. Recognition of sex-specific factors implicated in endothelial cell biology is important for the identification of clinically relevant preventive and/or therapeutic strategies.

This review aims to give an overview of the recent advances in understanding the importance of sex specific observations in endothelial maintenance, both in healthy and diseased conditions. The female endothelium is highlighted in the context of polycystic ovary syndrome and pre-eclampsia. Furthermore, sex differences are explored in chronic kidney disease, which is currently appreciated as one of public health priorities.

Overall, this review endorses integration of sex analysis in experimental and patient-oriented research in the exciting field of vascular biology.

Keywords: endothelium; sex; cardiovascular; hormones; nitric oxide

1 Introduction

Sex and gender are factors associated with differences in cardiovascular (CV) morbidity and mortality. Both CV mortality rate and the prevalence of coronary heart disease (CHD) are higher in men than in women [1]. In both men and women, the risk of CHD increases with age, but shows a more prominent increase in women after the age of 50, i.e. around the menopause [2]. Age-adjusted mortality for CV disease (CVD) is currently declining, but to a lesser extent in women than in men [3].

Until the last decade, underestimation of CVD in women has been explained, not only by the lower prevalence in younger age, but also by a broad appreciation of CVD as a 'male disorder' [4]. It was anticipated that the knowledge based on studies on men is also applicable to women [4]. However, recent retrospective analyses suggest that there are clinically relevant differences between women and men in terms of prevalence, presentation, management and outcomes of CVD [5]. Thus, more insight into the influences of biological sex and gender on the development of CV disease is warranted in order to identify new preventive and therapeutic targets [5].

The endothelium has an important role in vascular homeostasis by the production and release of vasoactive substances in response to numerous stimuli. The 'healthy' endothelium promotes vasodilatation, inhibits platelet aggregation (and thus thrombus formation), and preserves vessel permeability.

It is widely appreciated that dysfunction of the endothelium is a hallmark of CVD [6]. Endothelial dysfunction (ED) is defined as an imbalance between endothelium-derived vasodilators such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2) on the one hand, and vasoconstrictors such as endothelin-1 (ET-1) and cyclooxygenase-derived prostanoids (thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2)) on the other hand. Endothelium-derived NO has an important role in dilatation of *conduit* arteries, and the combination of decreased NO bioavailability in the context of a pro-inflammatory and pro-oxidant milieu contributes to the development of atherosclerosis. EDHF primarily confers vasodilatation of the *resistance* vasculature,

with an important role in peripheral resistance and blood pressure control. Moreover, EDHF production may increase when NO production is compromised or *vice versa* [7,8].

Thus, the NO signaling and/or EDHF system might represent a sex-specific target for preventive and therapeutic strategies. For example, altered EDHF responses may contribute to [9], or compensate for [10] ED in a sex-specific matter. It has been shown that EDHF contributes to sex-related differences in blood pressure control. Using specific gene-knockout technology to generate animals which lack both endothelial NO-synthase (eNOS) and cyclooxygenase (COX), i.e., the "EDHF mouse", Scotland et al (2005) has directly assessed the sex-specific involvement of EDHF in endothelium-dependent relaxation of small arteries [11]. In this animal model, EDHF-mediated response compensated the absence of endothelial NO in females, but not in males. Indeed, in female mice, the deletion of eNOS and COX did not affect blood pressure, while males became hypertensive [11]. Accordingly, EDHF is considered to be more important for endothelium-dependent dilatation in females, while NO plays predominant role in males. Additional reports of mesenteric and tail arteries in different rodent models, further stressed the importance of NO in male vasculature [12,13].

Human studies on sex-specific differences in EDHF/NO contribution are rare. Sato el al 2002 studied the influence of sex and menopausal status on endothelium-dependent dilatation induced by bradykinin (BK) of adipose arterioles. Small vessels from omental tissue and subcutaneous fat in premenopausal women were more sensitive to BK than those from postmenopausal women or men. The inhibition of eNOS erased those differences, suggesting that BK-induced dilation in peripheral small arteries from human fat tissue is predictably affected by both sex and hormonal status [14].

Although the influence of sex hormones is assumed, as reflected by majority of functional studies on changes in endothelium-dependent dilatation and altered NO contribution in animal models after ovariectomy and/or hormone supplementation therapy [15,16], endothelial cells themselves present intrinsic sex differences by means of observed sexual dimorphisms in gene- and protein- expression profiles as well as proliferative and migratory properties [17,18].

2 Maintenance of endothelial health

2.1 The role of endothelium-derived substances

The endothelium contributes to the maintenance of vascular homeostasis by releasing vasoactive substances and we will detail their action in following chapter. Importantly, we would like to stress that more detailed experimental studies in the field of vascular biology are needed to clarify the relative contribution of endothelium-derived factors in females and males. Also more experimental models assessing the intracellular pathways where sex is included as biological variable are required [19]

Nitric oxide (NO) is synthesized from L-arginine by the enzyme NO synthase (NOS). NO causes dilatation of vascular smooth muscle cells (VSMCs) through activation of soluble guanylyl cyclase receptor with generation of cyclic guanosine monophosphate [20]. Next to its vasodilatory effect, the molecule is also involved in regulation of cell growth and proliferation, and it affects transcription of certain genes implicated in the pathogenesis of atherosclerosis and hypertension [21]. Two constitutive isoforms of NOS play a vital role in NO production: endothelial NOS (eNOS) and to a lesser extent neuronal nNOS. [22]. The third isoform, inducible NOS (iNOS) is involved in inflammation [23,24]. The family of NOS enzymes (eNOS, iNOS and nNOS) all share a critical need for co-factors tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH) and the flavins and flavin mononycleotide, on top of the substrate L-arginine, to generate NO [25]. NO is released from endothelium under basal conditions, and in response to shear stress, circulating hormones and various autacoids (Figure 1)[26].

The generation of eNOS uncoupling, oxidative stress and dysregulation of signal transduction are all involved [27–29] in the molecular base of ED characterized by a decreased bioavailability of NO. ROS generated from NADPH oxidase may reduce the bioavailability of NO via reaction of NO with O_2^- generating peroxynitrite, which oxidizes B H_4 with subsequent reduction of BH_4 and eNOS uncoupling (Figure 2)[25].

Endothelium-derived hyperpolarizing factor (EDHF) is an intriguing substance released by the endothelium, whose identity has yet to be fully uncovered (for review, see [8]).

Early studies demonstrated hyperpolarization, in the presence of NO and PGI_2 blockade, monitoring membrane potentials in smooth muscle cells downstream from donor endothelial cells [30] further supporting observations that endothelium dependent relation involves the release of additional factor, which increases the membrane potential of VSMCs [31]. This additional relaxing factor has been named EDHF. EDHF-induced relaxation may be mediated simultaneously by several factors and/or pathways, depending on the species, type of vasculature bed or vessel size used, sex and physiological environment or disease status [8]. Thus, possible candidates for EDHF are epoxyeicosatrinoic acids, cannabinoids, potassium ions or myoendothelial gap junctions alone or in combination with H_2O_2 and/or cytochrome P450 2C9 (CYP2C9) products of arachidonic acid (AA) [8,32,33].

Prostacyclin (PGI_2) generation in endothelial cells occurs via stimulation by various receptor-mediated ligands [34]. Shear stress also stimulates PGI_2 release [35]. Agonists activate endothelial surface transmembrane receptors causing an increase in cytoplasmic calcium, crucial for PGI_2 synthesis and release [36]. Increase in intracellular calcium activates phospholipase A_2 (PLA2) which triggers release of AA from membrane-bound phospholipids [37]. Cyclo-oxygenase (COX) converts AA to PGH_2, which is further converted into vasoactive prostaglandins such as PGI_2 and thromboxane TXA_2 [38](Figure 1). PGl_2 easily crosses the endothelial membrane as it is lipid soluble. PGI_2 induced action in VSMCs is associated with adenylyl cyclase/ cyclic adenosine monophosphate/protein kinase A signal transduction pathway followed by relaxation [39].

PGI2 inhibits platelet aggregation through inhibition of the platelet-activating factor [40] and to a lesser extent, by controlling the smooth muscle tone [41]. As a vasodilatory factor, PGI_2 serves as a back-up mechanism for endothelium-dependent dilatation in the absence of eNOS contribution [42]. PGI_2 also preserves endothelial cell survival and protects VSMCs from apoptosis via peroxisome-proliferator activated receptors [43].

The endothelium is also a source for endothelins. Three structurally different isoforms have been described: ET-1, 2 and 3. ET-1 is a potent vasoconstrictive peptide produced primarily by endothelial cells and to a lesser extent by VSMCs [44,45]. Important stimuli such as hypoxia, ischemia, thrombin, insulin, inflammatory cytokines or shear stress induce the transcription of ET-1 mRNA and the synthesis and the rapid secretion of ET-1 [45,46]. The biological effects of ET-1 are mediated by two receptor subtypes, ETA and ETB [47]. When ET-1 binds to ETA receptor, it is followed by vasoconstriction, cell proliferation, cell growth and cell adhesion [48]. On the contrary, binding to the ETB receptor on endothelial cells results in the release of NO and vasodilatation [49]. ET-1 may also induce indirect vasoconstrictor effects due to the generation of endothelium-derived TXA_2 [50]. ET-1 plays a key role in maintenance of vascular tone in healthy humans [51,52]. And it has been shown that ET-1 increases mean arterial blood pressure, reduces cardiac output, heart rate and stroke volume and causes long-lasting vasoconstriction in the pulmonary, myocardial, and skeletal muscle vasculature [53–55]. Based on the biological effects induced by ET-1, including profound vasoconstriction, mitogenic and proliferative effects, pro-inflammatory actions, stimulation of platelet activation and free radical formation, ET-1 has been implicated as an important factor in the development of vascular dysfunction, CVD and ageing [56].

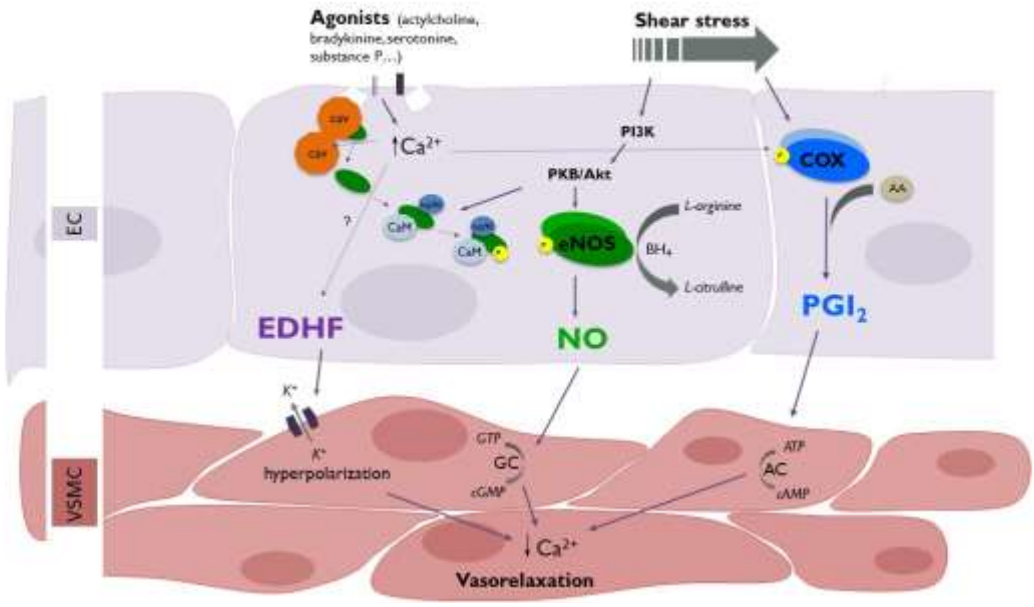


Figure 1 Mechanism of endothelium mediated vasodilatation: healthy state

Schematic presentation of endothelium-derived factors involved in dilatation: Nitric oxide (NO) is synthesized from L-arginine by endothelial NO synthase (eNOS). NO causes dilatation of vascular smooth muscle cells (VSMCs) through activation of soluble guanylyl cyclase receptor with generation of cyclic guanosine monophosphate (cGMP). Number of physiological agonists and physical forces (i.e. shear stress) activates the production of endothelium-derived factors. PGI_2 induced dilatation involves adenylyl cyclase(AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A(PKA) signal transduction pathway. Endothelium Derived Hyperpolarizing Factor (EDHF) induces relaxation which could involve several factors and/or pathways, resulting in changes of VSMCs membrane potential. Further abbreviations: endothelial cell (EC); Cav= caveolin; calmodulin (CaM); heat shock protein 90 (hsp90); protein kinase B (PKB/Akt); phosphatidylinositol-3 kinase (PI3K); cyclooxygenase (COX); guanylyl cyclase (GC); guanosine triphosphate (GTP); cyclic guanosine monophosphate (cGMP); tetrahydrobiopterin (BH_4); adenosine triphosphate (ATP); arachidonic acid (AA).

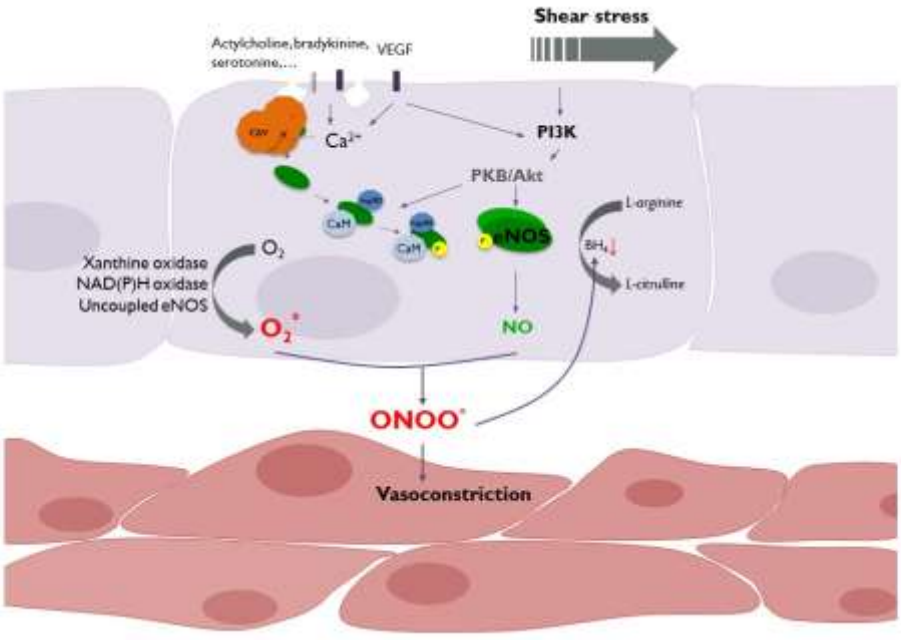


Figure 2 Endothelial signaling in the diseased state

Decreased nitric oxide (NO) bioavailability is the result of increased activation of reactive oxygen species (ROS)-generating enzymes, such as xanthine oxidase, nicotinamide adenine dinucleotide (phosphate)-oxidase (NAD(P)H)-oxidase. and in the presence of endothelial NO synthase (eNOS) uncoupling.

The largest contribution of vascular superoxide (O2-) originates from nicotinamide adenine dinucleotide (phosphate)-oxidase (NAD(P)H-oxidase), which is membrane bound. ROS generated from NAD(P)H-oxidase reduce the bioavailability of NO due to the reaction of NO with O2-- generating peroxynitrite (ONOO°). ONOO° is cytotoxic, increases platelet aggregation and induces vasoconstriction. It also oxidizes tetrahydrobiopterin (BH4), leading to reduced BH4 and eNOS uncoupling, i.e. eNOS produces O2- instead of NO. This occurs, e.g., as a result of BH4 deficiency or due to elevated levels of asymmetric dimethylarginine, or due to impaired anti-oxidative defense mechanisms.

2.2 Sex differences in endothelial cells

Although sex differences of endothelial cells are presumed to be mediated by sex hormones, recent studies in human umbilical endothelial cells (HUVECs) show that female and male endothelial cells are intrinsically sexually dimorphic. They differ in gene expression, response to shear stress, as well as migratory, morphological and proliferative characteristics [17,18]. Endothelial cells also show sex differences in expression and function of enzymes of the redox system (for review, see [57]) and in expression of phosphodiesterases (PDE) with PDE3B expressed higher in female cells and PDE1A in male cells [58].

Conflicting results are reported when it comes to expression of sex hormone receptors in HUVECs – showing either no sex differences with both female and male HUVEC expressing estrogen receptor (ER) α, ERβ and androgen receptor (AR) [17,59] or increased androgen receptor (AR) expression in male HUVECs [60].

Annibalini and co-authors [59] showed that regardless of the sex of HUVECs the components involved in androgen action are predominant to those of estrogen action. Administration of androgens had pro-inflammatory effect on HUVECs.

2.3 Endothelial progenitor cells and sex differences

Endothelial progenitor cells (EPCs) are key regulators of vascular homeostasis. About 20 years ago, the identification of bone marrow-derived progenitor cells with regenerative capacity lead to the refutation of the hypothesis that endothelial repair exclusively depends on limited migration and proliferation of local endothelial cells [61,62]. Upon endothelial damage, EPCs are mobilized from the

bone marrow, a process which is driven by the activation of eNOS in the presence of several mobilizing factors such as placental growth factor and vascular endothelial growth factor [63,64].

Bone marrow-derived circulating EPCs follow chemokine gradients to sites of endothelial injury, where they are thought to proliferate, differentiate and integrate into the endothelial cell layer. A growing body of evidence is also pointing towards their regulation of vascular repair via paracrine mechanisms. Next to the production of angiogenic growth factors, novel modes of paracrine regulation are being uncovered, such as the release of endothelial cell-derived microparticles or microvesicles that contain pro-angiogenic microRNAs [65,66]. The paradigm today is that a decline in the number or function of circulating EPCs is a major limitation to vascular regeneration which contributes to ED, the harbinger of atherosclerosis [6]. Indeed, low EPC number and/or impaired EPC function are predictive for CVD in different clinical settings [67–69], including healthy subjects [70]

The favorable CV risk profile of fertile women is reflected in higher EPC numbers and proangiogenic potential compared to age-matched men. Cyclic mobilization from the bone marrow is described corresponding with 17β estradiol concentrations [71]. Levels of circulating EPCs in postmenopausal women are comparable to those in age-matched men [72][73]. Number and migratory function of EPCs and their colony-forming capacity are higher in middle-aged women than in men [74].

Both number and function of EPCs have intrinsic sex differences, partly mediated by sex steroids. There is increasing evidence that estrogen plays a beneficial role on EPCs. Indeed, it induces EPC mobilization from the bone marrow in a NO dependent way [75]. Moreover, estrogen induces proliferation and migration of EPCs and inhibits their apoptosis [75]. Recently, it has been shown that, although the expression of ER is comparable in male and female EPCs, in vitro response to estrogenic compounds such as bisphenol A, was significantly different between sexes [76].

Similar protective effects were found for androgens. Circulating EPC levels correlate with plasma androgen concentrations, and treatment of hypogonadal men with testosterone results in normalization of the observed circulating EPC numbers [77]. The expression of the androgen

receptor (AR) was also confirmed in EPC, and androgens via AR are able to stimulate EPC proliferation, migration and colony formation [77,78].

3 Assessing endothelial function in vivo and in vitro

Endothelial function is diverse, varying from anticoagulant, permeability properties to regulation of vascular tone. Both *in vivo* and *in vitro* studies of endothelial function mainly address the latter.

Initial *in vivo* studies of human endothelial function were performed in the coronary circulation, evaluating the endothelium-dependent vasodilation in response to acetylcholine (ACh) infusion. Blunted responses appeared to be prognostic for CV events, even in low-risk groups [79]. Since peripheral vasculature reflects systemic ED [80] and is more easily accessible, several less invasive techniques were explored during the last decade. Currently, the most established method to study conduit artery endothelial function is brachial artery flow-mediated dilation (FMD). This method measures changes in brachial artery diameter by ultrasound before and after increase in shear stress, which is induced by reactive hyperemia. The changes in artery diameter predominantly occur as a result of local endothelial release of NO [81]. However, a main drawback of FMD is its high operator dependency, as well as a disregard for potential measurement-induced changes in autonomous nervous system tone.

Venous occlusion plethysmography studies forearm circulation, including the resistance vasculature. It measures changes in forearm volume thought to be proportional to blood flow before and after infusion of vasoactive substances into a cannulated brachial artery. The vasoactive substances may include both endothelium-dependent and -independent agonists. As such, the influence of different pathways can be dissected. The major disadvantage of the method is invasiveness of arterial cannulation [82]. Moreover, whereas it can be a useful technique for study of responses to different agonists in a single patient, comparing results between groups can be problematic due to differing basal flows, arterial pressures and forearm size [83].

A less invasive method for measuring resistance artery endothelial function is peripheral arterial tonometry [PAT] using the EndoPAT technique. Similar as FMD, it relies on inducing reactive hyperemia but uses finger plethysmograph to detect pulse wave amplitude changes. Reactive hyperemia index (RHI) is then calculated as the ratio of the digital pulse volume during reactive hyperemia divided by that at baseline. Measurements on the contralateral arm are used to control for concurrent non-endothelium-dependent changes in vascular tone. However, while it reflects ED [84,85] and has been shown to predict future CV events [86], it only partially reflects NO release [87]. Moreover, brachial and digital measures of vascular function are differentially related to CV risk and do not correlate with each other in a clear manner [88,89].

In vitro studies on isolated arteries provide more comprehensive assessment of endothelial function and are frequently used in animal studies or in isolated human arteries. The majority of investigations involve isometric wire myography a technique, which has proved to be a reliable and robust method for assessing resistance artery function [33]. This method allows measurement of isometric force exerted circumferentially on the wires. The relaxation of each vessel to incremental doses of endothelium-dependent and independent agonists is studied using a pharmacological inhibition of NO, prostaglandins, EDHF and ET-1 contribution, as well as for screening of compounds with suggested cardiovascular benefit. Perfusion myography, a more laborious and technically challenging method, requires cannulating the vessel to allow, in addition to pharmacological activation, measurements of changes in artery diameter after stimulation of flow-induced shear stress or changes in intraluminal pressure [90,91].

3.1 Hormonal influences on endothelial function in females and males

The sex/gender difference in the development, manifestation and treatment of CVD has drawn significant attention to the role of sex hormones in endothelial cell biology. Focus so far has been placed predominantly on estrogens, and their potential cardioprotective effects in women.

Assessment of vascular function in conduit [92,93] and resistance arteries [88,89] have shown enhanced endothelium dependent dilatation in females. This can be observed during the menstrual cycle when vascular reactivity improves from a level comparable with men during the early follicular phase towards a higher level, simultaneously with the rise in serum levels of ovarian hormones. [89,94,95].

Estrogen improves endothelial dependent vasodilation through several different mechanisms. Arguably the most important is the increase in NO production due to upregulation of eNOS gene transcription [96] and non-genomic activation of eNOS via mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase–Akt kinase pathways [97][98]. Furthermore, estrogen also acts on estrogen receptor alpha (ERα) to upregulate production of PGI_2 by activation of cyclooxygenase-2 [99] and reduces superoxide anion release [100].

RHI measured by EndoPAT increases during stages of puberty for both sexes [101][102]. FMD, on the other hand, does not change during pubertal maturity [103]. Decline in endothelial function with advancing age is observed in both sexes, but is more abrupt in women at the onset of menopause [104,105]. Decrease in FMD already is observed in the perimenopause, but a further decline is accompanied by the loss of ovarian function and prolonged estrogen deficiency. [106].

Gilligan et al. (1994) showed that concomitant infusion of estrogen with ACh in the coronary circulation of postmenopausal women, acutely increased blood flow and lowered resistance [107]. Since then there have been numerous studies of the effect of hormonal replacement therapy (HRT) on endothelial function of postmenopausal women. Both short-term and long-term HRT and raloxifene, an estrogen receptor modulator, treatment improves FMD, though this effect varies by route of administration and type of HRT used [108–111].

Evidence of a potential protective effect of estrogen on the microvasculature is more controversial. There has been no consistent evidence when it comes to menstrual cycle variations of endothelial

reactivity [90,112,113] and in one study, it showed a correlation with progesterone levels rather than estrogen [89].

Short-term HRT in postmenopausal women is associated with an improvement of microvascular endothelium-dependent relaxation [114,115]. *In vitro* investigations of isolated small arteries provided supportive evidence that 17β-estradiol replacement improves endothelium-dependent dilatation to shear stress via increased production of NO and via estrogen receptor (ER) alpha on endothelial cells of healthy postmenopausal women. It also recovers endothelial cell morphology via reducing signs of apoptosis and endothelial cell activation [109]

Most recent randomized clinical trial (Kronos Early Estrogen Prevention Study (KEEPS)) studied the effect of long-term administration of either transdermal 17β-estradiol or oral conjugated estrogen therapy on RHI measured with the EndoPAT in healthy women who recently entered the menopause. No differences between treatment groups were seen in RHI. Changes in RHI inversely correlated with carotid intima medial thickness. Authors pointed out the high variability of RHI over the time as well as lower serum levels of 17 β -estradiol compared to similar studies using FMD as a marker of endothelial function [116].

Estrogen therapy in male-to-female transsexuals/transgenders improves both conduit artery as well as resistance artery endothelial function [117–119]. However, conflicting evidence exists for the effect of estrogen on endothelial function in men. In a small study of 20 healthy young men, suppression of endogenous estrogens caused a decrease in FMD [120]. Furthermore, in middle-aged men, levels of endogenous estrogens were positively associated with FMD [121]. However, in a large population study (n=5000), no correlation between estrogen and vascular function of either conduit or resistance arteries was observed in men [89].

Evidence of inconsistent outcomes of HRT has also stressed the importance of endogenous testosterone levels. It is increasingly appreciated that plasma testosterone levels, and particularly the

androgen-estrogen ratio, may play an important role in CV health, not only in males but also in females [122,123].

Androgens activate eNOS interacting with cell surface receptors. Study by Yu and co-authors [124] showed that testosterone at physiological concentrations rapidly induces NO production via PI3-kinase/Akt signaling pathway. Dihydrotestosterone (DHT) also mediates the same effect.

Men with low androgen levels have a higher risk of cardiovascular events [125] and testosterone has been shown to act as an acute coronary vasodilator in men with CHD [126]. The majority of studies support the notion that low serum levels of testosterone are associated with impaired FMD in men independent of major CV confounders both in the general population [127–129] and in different clinical settings [130,131]. In line with these findings, acute administration of high-dose testosterone enhanced endothelium-dependent FMD in brachial artery in men with CHD [132]. This study concurs with other observations that low-dose of oral testosterone augments both endothelium-dependent (FMD) and endothelium-independent vascular reactivity of brachial artery in men with CHD [133]. When looking at the microvascular endothelial function, low testosterone levels in healthy men have been shown to correlate with dysfunction even when adjusted for classical risk factors [134]. Moreover, Francomano and coauthors (2016) demonstrated an improvement of RHI following transdermal androgen replacement in severely obese hypogonadal men [135].

However, these results are in contrast to the demonstration that transdermal testosterone treatment in men with low testosterone levels did not affect endothelium-dependent dilatation [136], suggesting that the route of administration might be of importance.

In vitro studies revealed that androgens promote monocyte adhesion to endothelial cells and macrophage lipid loading in sex-specific matter. DHT acting on a novel AR/NFκ-B receptor increases binding of monocytes to the endothelium via increased expression of vascular adhesion molecule 1 (VCAM-1) only in male endothelial cells [60,137,138]. This further suggests that excessive androgen administration (doping) may have deleterious effects and be detrimental to the cardiovascular

system. However, some studies suggest that the use of androgenic anabolic steroids by male bodybuilders is not associated with impaired endothelial function or arterial structure [139,140].

Overall, even if the majority of studies suggest endothelial impairment in testosterone deficiency and improvement after testosterone treatment, some studies suggest an improvement in endothelial function in ageing men with decrease in testosterone concentrations [141,142] and further detrimental effect on endothelial function after testosterone replacement [143].

In women, low testosterone levels have been shown to be associated with ED [144,145] and one study showed an improvement of FMD following 6 weeks of testosterone therapy [146]. Excessive testosterone levels in women with polycystic ovary syndrome (PCOS) are associated with ED, and different treatment regimens associated with the normalization in testosterone levels have been also associated with the improvement in endothelial maintenance. The improvement, however, seems to be related to the positive effect on lipid profile rather than the decreased testosterone levels *per se* [147,148].

In conclusion, the controversy about testosterone treatment on vascular reactivity and endothelial function seems to be related to pre-existing CV condition, duration of treatment, age, sex and vascular bed studied, and therefore warrants further detailed investigations. The evidence on endothelial alterations associated with changes in androgenic and estrogenic environment is predominantly based on animal studies, and the evidence how steroids affect circulating plasma ED markers, morphological, anatomical and functional alterations in the function of vasculature in patients characterized by altered androgen-estrogen ratio warrants further investigations.

4 The endothelium in disease: PCOS, pre-eclampsia and chronic kidney disease

4.1 Endothelial function in women with PCOS

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of fertile age [149]. It is a multifactorial syndrome characterized by hyperandrogenism and chronic

anovulation resulting in infertility and/or miscarriage [150,151]. Women with PCOS have an increased risk to develop metabolic disorders such as insulin resistance, diabetes mellitus, dyslipidemia and early CVD [152–155]. The prevalence of PCOS is as high as 4-12 % and thus the syndrome and its metabolic complications are of great importance for women's reproductive and CV health [149].

The presence of hyperandrogenism triggers masculinized-type adiposity in PCOS women. The consequence of this is a chain of pathogenetic mechanisms such as insulin resistance, hyperinsulinism, adipocytokine dysfunction, oxidative stress, sympathetic overactivity, and ED, among others [156]. ED in PCOS women is characterized by high levels of adhesion molecules, such as soluble intercellular adhesion molecule 1 (sICAM-1), sVCAM-1, sE-selectin and ET-1, all of them well known as ED markers [154,157,158]. In addition, PCOS women have higher prevalence of subclinical signs of atherosclerosis, as reflected by decreased FMD in conduit artery [159], impaired small artery reactivity *in vitro* [160], increased carotid intimal–medial thickness (cIMT), and presence of coronary artery calcification [161–163]. The abnormal endothelial function is also observed in younger and non-obese women with PCOS [164,165].

Life-style changes are an advisable therapy since weight loss and exercise improve all parameters of PCOS [166,167], however more studies are needed to assess direct effects on vascular tone. More commonly used metformin, known to decrease insulin secretion and enhance insulin sensitivity, exerts beneficial effects on the menstrual cycle and hormone levels in PCOS [168] with subsequent deterioration of oxidative stress, improved insulin resistance, changes in lipid profile, and improved FMD [169], reduced leukocyte/endothelium interactions and changes in circulating ED markers [170–172]

4.2 History of pre-eclampsia and endothelial health

In normal pregnancy, endothelium-dependent dilatation increases via estrogen-induced upregulation of eNOS with reduction in blood pressure despite increase in cardiac output and blood volume [173].

However, up to 2-8% of pregnant women will develop pre-eclampsia (PE), a severe hypertensive complication in the second half of pregnancy [174]. PE places young women at risk for kidney, liver and heart failure, seizures and/or stroke. On a worldwide basis, the WHO estimates that over 40,000 women die from PE and eclampsia each year, and the condition has been one of the most important causes of maternal death over recent decades [175]. Epidemiological studies have demonstrated that PE also identifies mothers at an increased risk for development of hypertension, coronary heart disease, diabetes, and stroke later in life [176–178].

There is an increasing support for a pathogenic model of PE, where a failure in the trophoblast invasion of the placental bed spiral arteries reduces utero-placental blood flow. This results in secretion of factors by the placenta into the maternal circulation. These factors can cause 'activation' of the endothelium, with the clinical syndrome resulting from widespread changes in endothelial cell function in both small and large vessels [179–186]. Markers of endothelial activation including fibronectin, factor VIII antigen, tissue plasminogen activator, ET-1 and the plasminogen activator inhibitor have been reported to be increased prior to the onset or during the clinical disease [187]. This is also accompanied with increased levels of cell adhesion molecules like sICAM, sVCAM and E-selectin [188]. Functional studies on isolated small arteries from women with PE further strengthened the presence of ED by providing evidence for impaired endothelium-dependent dilatation to several endothelium dependent agonists like ACh, BK or flow induced shear stress [189–191] via reduced contribution of NO and/or EDHF. While circulating EPCs increase during normal pregnancy, their number is reduced in PE as well as in intrauterine growth restriction and gestational diabetes [192–194].

In their landmark paper, Sattar and Greer (2002) argued that the pregnancy challenge to the maternal vasculature and subsequently to endothelium could be considered as a 'stress' test [195]. In majority of pregnant women, metabolic and inflammatory changes are absorbed by physiological buffers. However, in women who develop PE, a phenotype may exist where the inflammatory and

metabolic responses to pregnancy are exaggerated and buffering mechanisms are inadequate. Therefore, in women who develop PE, the threshold for clinical CVD is breached during pregnancy and again in later life, as with increasing age, other acquired CVD risk factors are encountered. In this way, adverse pregnancy outcome may reveal women at increased risk of CVD. The pregnancy challenge-stress suggests that women with a previous history of PE have increased CV risk, which could be related to the exacerbation of underlying maternal risk factors. There is strong data to support this contention. Chambers et al 2001 demonstrated impaired endothelium-dependent dilatation to flow in brachial artery at least 3 months (median 3 years) postpartum [196]. Ramsay and co-authors in 2003 reported that microvascular function, as reflected by reduced laser Doppler imaging responses to ACh iontophoresis in the circulation of the skin, is impaired in women 15-25 years following a pregnancy affected by PE [197]. Therefore, ED could be a link between PE and later CV complications.

At this stage, it is also important to stress that *in vivo* studies concentrating on FMD in brachial artery have showed impaired endothelium dependent dilatation in women with a history of PE [198–200], while *in vivo* venous occlusion plethysmography technique has provided some conflicting results likely due to different follow up periods and/or CVD risk profile [201–205]. Further investigations on vascular function are needed especially in a well-defined group of women with a history of early-onset PE and in combination of different methods to study vascular function and structure together with risk factors (known and novel) that could provide a rationale for follow up of those women and for further prospective studies. This could offer a justification for enhanced screening/monitoring of women with a previous history of PE towards prevention of CVD later in life. Most importantly however, is to identify women at risk of developing PE early and if 'at risk' women can be identified, this would allow stratified care with personalized fetal and maternal surveillance, early diagnosis, timely intervention, and significant health economic savings [206].

4.3 Chronic kidney disease and sex differences

Chronic kidney disease (CKD) is a global health issue, with worldwide an estimated prevalence of 8-16%[207]. In CKD patients, the risk for CV morbidity and mortality is unacceptably high, and the presence of CKD is considered an independent CV risk factor and a coronary artery disease - equivalent for all-cause mortality [208,209]. Indeed, in moderate to severe CKD, CV death is more likely to occur than the progression to kidney failure and the need for renal replacement therapy. Also in the dialysis stage, CVD accounts for premature death in more than 50% of the patients, with an annual mortality rate 10-20 fold higher than the general population [210].

Mechanisms underlying the increased CV risk in CKD are not fully elucidated yet. As for vascular disease, both the endothelium and medial layer of the vessel wall are affected, in part by common mechanisms, resulting in atherosclerosis and arteriosclerosis, respectively [211]. Structural and functional abnormalities of the endothelium may play a key role in CKD-associated vascular disease [212].

Clinical data clearly show that ED occurs early in the course of renal failure and that it is a predisposing factor for accelerated atherosclerosis [213,214]. A myriad of factors, including the high prevalence of traditional risk factors, but also the presence of non-traditional risk factors such as inflammation, oxidative stress, CKD-bone mineral disorder and reduced number and function of EPCs are thought to contribute to ED in CKD [215–218].

Current experimental evidence of ED in CKD is mainly based on findings in male animal models of uremic environment [219]. Clinical evidence is based on circulating plasma markers of ED and number and function of EPCs [220], as well as non-invasive assessment of conduit arteries [221,222]. However, the anticipated functional and morphological abnormalities of the resistance vessels of CKD patients are yet to be unraveled in precise detail and in a sex-specific manner. Recent studies of isolated subcutaneous arteries from patients with end-stage renal disease (ESRD) did not show a difference between female and male patients in endothelial function in response to physical and

pharmacological stimuli, or in the expression of markers for endothelial activation [91,223]. Further studies are ongoing to identify novel pathways and treatment options in a sex-specific manner for CKD patients.

Sex/gender influences on the incidence, prevalence and progression of renal disease have recently started to be appreciated in the field. Worldwide, the prevalence of mild to severe CKD is higher for women than for men, except for the age group 20-29 years [224]. On the other hand, women are less likely to start dialysis, but the underlying factor of this observation still needs to be established. As a possible consequence, 60% of the dialysis patients are males (for review, see [225]). In ESRD, cardiovascular prognosis in women is better compared to men, possibly as a result of the protective actions of estrogens [226]. However, this survival benefit is much smaller than that seen in the general population. Finally, increasing evidence suggest that adverse pregnancy outcomes in women are also associated with increased risk of developing ESRD [227].

5 Future research implications

While the importance of sex and gender perspectives are increasingly appreciated in the cardiovascular research field there is still a great lack of evidence on this topic. The current generation of experimental and clinical investigators support the encouragement, promotion and integration of sex and gender analysis in experimental and patient-orientated research and in education at different levels. This is in line with the requirements of EU FP7 program (sex and gender in research as a mark of excellence), HORIZON 2020 (sex and gender aspects should be implemented in area of health)(http://ec.europa.eu/programmes/horizon2020/en/h2020-section/promoting-gender-equality-research-and-innovation) and US National Institute of Health (to balance sex in cell and animal research)[228] and Canadian Institute of Health Research (www.cihr-irsc.gc.ca).

Sex represents an important biological variable that needs constant consideration [229] and simplified initial steps for integrating sex and gender in experimental biomedical research have been recently suggested by Ritz and coauthors (2014) [230]. Sex differences in endothelial homeostasis have been increasingly appreciated through a number of experimental and clinical studies. Our review supports the notion that inclusion of sex and gender perspectives will progress our understanding of cardiovascular pathophysiology that could lead to improved and/or novel therapies for both women and men. Being preclinical and clinical experimental researchers we strongly endorse the notion that experiments on pooled arteries from both women and men, isolated endothelial cells cultures or animal models of the disease not representing both sexes, should be considered with caution and not appreciated in modern physiology. Sex- and gender-oriented research is important to move the intriguing research field of vascular biology forward.

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