From Institution of Clinical Science,
Department of Obstetrics & Gynecology,
Karolinska Institutet, Stockholm, Sweden

Endothelial function in isolated small arteries from women at reproductive age and after menopause – possibilities for improvement

Eimantas Svedas

Stockholm 2003

All previously published papers were reproduced with permission from the publisher.

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden
© Eimantas Svedas, 2003
ISBN 91-7349-689-8

To my Family

Contents

Summary

***Background*:** Alterations in vascular function are present in patients with Preeclampsia (PE) and in women after menopause. PE is a syndrome peculiar to human pregnancy that adversely affects the mother by endothelial dysfunction and the fetus due to impaired uteroplacental blood flow. It also remains a leading cause of perinatal morbidity and mortality. A current hypothesis suggests that hypoxic placenta releases one or more unidentified factors to the maternal circulation that trigger endothelial dysfunction. Women after menopause are at increased risk for cardiovascular diseases and this could be related to changes in the hormonal environment. Estrogens are considered to provide cardiovascular protection, however the exact mechanisms of action in the resistance vasculature are far from clear.

Objective: The general aim of this thesis was to study endothelial function in isolated resistance arteries in order to clarify the possible causes of endothelial dysfunction and the potential for improvement in women with PE and after the menopause. In particular we aimed to evaluate if (1) microparticles (MP) from plasma of women with PE might induce endothelial dysfunction as characterized in vitro in arteries from women with PE; (2) Vascular Endothelial Growth Factor (VEGF) can induce signs of endothelial dysfunction in isolated resistance arteries from normal pregnant women; (3) 17β-estradiol (E_2) have beneficial effects on endothelial function in arteries from women with PE; (4) isolated arteries from healthy postmenopausal women exhibit signs of endothelial dysfunction in contrast to premenopausal women and, if so, to compare in vitro effects of estrogenic compounds for cardiovascular protection; (5) three months of Hormonal Replacement Therapy (HRT) alters resistance artery function in isolated arteries from healthy postmenopausal women.

Methodology: Normal pregnant and women with PE, pre-menopausal and post-menopausal women were recruited. Myometrial and subcutaneous resistance artery function was evaluated in-vitro using pressure- and wire-myography techniques. Endothelial function was determined by measuring flow-, endothelium-dependent and independent agonists mediated dilatation. In addition, pressure-induced myogenic responses were assessed in response to increased intraluminal pressure and vascular permeability was evaluated by applying Evans blue dye staining. Scanning electron microscopy technique was utilized for comparisons of endothelial morphology.

Results and conclusions: (1) MP, isolated from plasma of women with PE induced endothelial dysfunction after an overnight incubation in isolated myometrial arteries from healthy pregnant women. In contrast, MP derived from healthy pregnant women had no effect. MP may be of importance in the etiology of endothelial dysfunction seen in PE. (2) VEGF impaired endothelium-dependent dilatation and enhanced basal tone possibly through an endothelin-1 pathway. It also increased vascular permeability similar to that found in isolated arteries from women with PE. This might indicate a potential role for VEGF in the development of endothelial dysfunction in PE. Angiopoietin-1 (Ang-1) reversed the vascular leakage induced by VEGF, suggesting that Ang-1 may have therapeutic implications in PE. (3) E_2 improved flow-, but not agonist-mediated dilatation and reduced basal tone through a nitric oxide (NO)-mediated pathway in isolated myometrial arteries from women with PE. This suggests an important role for E_2 to improve uteroplacental circulation in PE. Morphological signs of endothelial injury seen in arteries from women with PE support the presence of endothelial malfunctions. (4) E_2 improved resistance artery function *in-vitro* an affect that was mimicked by selective estrogen receptor alpha (ER-α) agonist propyl-pyrazole triol but not by raloxifene or genistein. This suggests that ER-α might be of importance for vascular protection in the resistance circulation in women after menopause. (5) Three months supplementation with estradiol and estradiol plus medroxyprogesterone acetate, but not medroxyprogesterone acetate alone had beneficial effects on flow-mediated dilatation and endothelial morphology in isolated subcutaneous arteries from healthy postmenopausal women, suggesting combined HRT to be beneficial for resistance artery function.

Significance: Studies in isolated arteries from women with PE and healthy postmenopausal women have yielded functional and morphological signs of endothelial dysfunction. We have also shown that estrogenic compounds may improve endothelial function and therefore might be of interest from a therapeutic point of view.

List of Publications

This thesis is based on studies reported in the following papers, referred to in the text by their respective Roman numerals:

I. Marja J VanWijk, Eimantas Svedas, Kees Boer, Rienk Nieuwland, Ed VanBavel, Karolina R. Kublickiene.
Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women. (Am J Obstet Gynecol, 187: 1686-1693)

II. Eimantas Svedas, Khalid Islam, Henry Nisell, Karolina R. Kublickiene.
Vascular endothelial growth factor induced functional and morphological signs of endothelial dysfunction in isolated arteries from normal pregnant women. (Am J Obstet Gynecol, 188: 168-176)

III. Eimantas Svedas, Henry Nisell, Marja VanWijk, Yorgos Nikas, Karolina R.Kublickiene.
Endothelial dysfunction in uterine circulation in preeclampsia: Can estrogens improve it? (Am J Obstet Gynecol 187:1608-1616)

IV. Karolina Kublickiene, Eimantas Svedas, Britt-Marie Landgren, Nasrin Nahar, Milita Crisby, Henry Nisell and Lucilla Poston.
Small artery endothelial dysfunction in post-menopausal women: in-vitro function, morphology and modification by estrogen and SERMs. (Submitted)

V. Eimantas Svedas, Britt-Marie Landgren and Karolina Kublickiene.
Effects of hormonal replacement therapy on endothelial function and morphology in the isolated arteries from women after menopause. (Manuscript)

Abbreviations

ACh	Acetylcholine
A-II	Angiotensin-II
Ang-1	Angiopoietin 1
BK	Bradykinin
CEE	Conjugated equine estrogens
CHD	Coronary Heart disease
CVD	Cardiovascular disease
DP	Degree of permeability
EDHF	Endothelium-derived hyperpolarizing factor
eNOS	Endothelial Nitric Oxide Synthase
ER-α, ER-β	Estrogen receptors alpha or beta
ERT	Estrogen replacement therapy
ET-1	Endothelin-1
ET_A and ET_B	Endothelin-1 receptors A and B
Flt-1, Flt-2	fms-like-tyrosine kinase receptor 1 and 2
FMD	Flow-mediated dilatation
HDL	High density lipoprotein
HELLP	Hemolysis, Elevated Liver enzymes and Low Platelet syndrome
HRT	Hormone replacement therapy
$hrVEGF_{165}$	Human Recombinant Vascular Endothelial Growth Factor
i.d.	Internal diameter ,
IL-6, IL-8	Interleukin-6, Interleukin-8
iNOS	Inducible nitric oxide synthase
KDR	Kinase domain receptor (VEGFR-2)
KPSS	High potassium physiological salt solution
LDL	Low density lipoprotein
L-NAME	Nω-nitro-L-arginine methyl ester
Lp-a	Lipoprotein-a
MP	Microparticles
MPA	Medroxyprogesterone acetate

NO	Nitric oxide
NOS	Nitric Oxide Synthase
P	Progestagens
PE	Preeclampsia
PGI_2	Prostacyclin
PlGF	Placenta Growth Factor
PPT	Propyl-pyrazole triol
PSS	Physiological salt solution
ROS	Reactive oxygen species
SERMs	Selective Estrogen Receptors Modulators
sFlt-1	Soluble fms-like-tyrosine kinase receptor 1
STBM	Syncytiotrophoblast microvillous membranes
TF	Tissue Factor
TxA_2	Tromboxane
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular smooth muscles cells

Introduction

Cardiovascular disease (CVD) is a leading cause of death in the European Union (EU), accounting for over 1.5 million deaths for both females and males each year. Approximately 42 percent of all deaths in EU are from CVD (46 percent in women and 38 percent in men). Coronary heart disease (CHD) accounts for almost one-half and stroke for one-fourth of all deaths from CVD [1]. Evidence from middle 80's (i.e. The Nurses' Health Study) directly related increase in risk for CVD with menopause [2], since drop in estrogen levels increased cardiovascular complications in female population.

Preeclampsia (PE) is a complication of human pregnancy characterized by hypertension, proteinuria and edema. PE is highly associated with pregnancy-related risks for the mother due to general endothelial cell dysfunction and the fetus due to severely compromised uterine blood supply. Epidemiological evidence from isolated populations (e.g. Iceland) [3] or large population cohorts [4, 5] have yielded direct evidence that PE increases risk for CVD for mother and offspring later in life.

Resistance size arteries (100-400 μm) are mainly involved in the regulation of peripheral vascular resistance, blood pressure and blood flow to the target organs [6]. Vascular endothelium in this circulation primarily serves to assure blood supply to every organ according to physiological needs. The mechanisms underlying the alterations in vascular resistance and the role of vascular endothelium in human pregnancy have been investigated intensively [7], yet we still lack knowledge for primary prevention of PE. Similarly, cardiovascular effects of hormonal replacement therapy (HRT) as a tool for primary and/or secondary prevention of CVD have been studied in large cohorts and produced diverging results [8, 9]. The effects of HRT on vascular function was studied extensively in conduit arteries (brachial, femoral) from post-menopausal women *see rev* [10, 11] however we lack knowledge about mechanisms of endothelial dysfunction in the resistance arteries from post-menopausal women.

Endothelial dysfunction in Preeclampsia

Preeclampsia (PE) remains a leading cause of perinatal morbidity and mortality [12]. It is a condition peculiar to human pregnancy that adversely affects mother (by endothelial dysfunction) and fetus (by intrauterine growth restriction). The incidence of PE varies between 3% and 10% of pregnancies, and there is no evidence that it has changed appreciably during the last century [13]. Preeclampsia remains a major reason for indicated preterm birth, accounting for 40% of premature deliveries [14] and remains an enigma for contemporary medicine.

The cardinal hemodynamic features of PE are vasospasm, increased peripheral vascular resistance and thus reduced organ perfusion. Clinically this condition is characterized by an elevated blood pressure, proteinuria and edema, developing after 20 weeks of gestational age. PE can result in eclampsia characterized by development of neurological symptoms leading to seizures and risk for maternal and fetal death. PE can manifest as the HELLP syndrome, in which Hemolysis, Elevated Liver enzymes and Low Platelet count are present. These conditions

are associated with severe complications such as cerebral hemorrhage, pulmonary edema, liver hemorrhage and rupture, and disseminated intravascular coagulation. Moreover, women with PE are two and a half times more likely to die from ischemic heart disease later in life [3]. Several studies indicate that long after a preeclamptic pregnancy, women have increased insulin resistance [15], altered endothelial function [16], and an atherogenic lipid profile when compared with women who have experienced normal pregnancies [17].

It is currently accepted that poor cytotrophoblast invasion early in pregnancy results in suboptimal placentation and development of placental hypoxia, which in turn releases one or several factors detrimental to maternal endothelium. This leads to an inadequate hemodynamic adaptation to pregnancy. Genetic predisposition, immunological maladaptation to pregnancy and pre-existing vascular diseases seem to be involved in this early vascular dysfunction. Moreover, a history of preeclampsia in a previous pregnancy, multifetal pregnancy, obesity, insulin resistance and diabetes mellitus are considered to increase the risk for developing PE [18], *see rev* [19].

Maternal Endothelial Dysfunction

The endothelium, the single cell layer covering all arteries, is severely compromised in PE. In fact, endothelial dysfunction is considered to be a pathogenetic hallmark in this complication. Dysfunction of this normally protective "anti- hypertensive" and "antiplatelet" layer may explain most of the clinical findings of the maternal disorder. Endothelial dysfunction includes impaired balance between endothelium-derived vasodilators and vasoconstrictors, increased vascular permeability and enhanced expression of cell adhesion molecules and pro-coagulation factors. A classical morphological feature of endothelial dysfunction is glomeruloendotheliosis, representing endothelial cell death, denudation and obstruction in the kidney glomeruli [20].

Several markers for endothelial dysfunction, including Von Willebrand factor, fibronectin, and neurokinin B, have been shown to be increased in women with PE. In addition, there is a change in the ratio between tissue plasminogen activator and inhibitor, and between PGI_2 and TxA_2, all supporting endothelial cell activation [21-23]. Studies in our laboratory suggest ET-1 to play a pathophysiological role in PE, since plasma ET-1 concentrations are significantly increased in PE [24]. In addition, it has been demonstrated that ET-1 might contribute to the impaired uteroplacental circulation in PE, since higher ET-1 concentrations in the uterine *versus* brachial vein have been found in women with PE [25].

Investigations in isolated arteries have provided direct evidence for vascular endothelial dysfunction in PE in terms of impaired endothelium-dependent dilatation. Several studies have demonstrated impaired vasodilatory response to endothelium-dependent agonists such as bradykinin (BK) and acetylcholine (ACh) in isolated arteries from skin and uteroplacental circulation in women with PE [26-30]. Studies in our laboratory and others have clarified that impairment of shear stress responses in the isolated resistance arteries obtained from uterine and skin circulations from women with preeclampsia is due to impaired nitric oxide (NO) production [31, 32].

Oxidative stress is generally accepted to be involved in the pathogenesis of PE and is characterized by increased generation of free radicals in placenta [33], and subsequent oxidation of low-density lipoproteins, which are recognized to be detrimental to endothelial function *see*

rev [34]. Moreover, the maternal predisposition such as decreased levels of antioxidants and sensitized endothelium due to preexisting vascular disease may exaggerate the condition [35]. Administration of antioxidants in early pregnancy to women at increased risk of developing PE decreased oxidative stress, endothelial activation, and the frequency of PE, supporting the potential role of oxidative stress in PE [36].

Despite intensive research, the link between the pathophysiology of abnormal placentation and the development of generalized maternal endothelial dysfunction remains unclear. Clarification of the mechanisms that could be involved in the generation of endothelial dysfunction may provide new therapeutic options or prophylactic interventions.

Microparticles

Circulating microparticles (MP) have been suggested to be involved in the pathogenesis of cardiovascular and coagulation diseases [37, 38]. Syncytiotrophoblast microvillous membranes (STBM), a subtype of MP, have been proposed to be the link between defective placentation and endothelial dysfunction. An initial study demonstrated that incubation with STBM could suppress endothelial cell proliferation *in vitro* [39]. It has been suggested that apoptosis plays a central role in the turnover of cytotrophoblasts and the formation of cell fragmentation [40]. This could explain why STBM appear at increased concentrations in women with PE [41]. A study in our group have tested the hypothesis that STBM at concentrations similar to those found *in vivo* in women with PE might represent a pathogenetic pathway for the impaired endothelial function seen in PE. However, we failed to prove that STBM deteriorated endothelial function, at least under *in vitro* conditions [42].

Recently, MPs derived from peripheral blood cells have been proposed as a novel factor associated with endothelial dysfunction. The support for this comes from a recent investigation demonstrating that MP obtained from patients with myocardial infarction in contrast to MP from healthy participants are capable of impairing endothelial function in the isolated rat aorta [43]. In human pregnancy, the subtypes of MP derived from granulocytes, lymphocytes and endothelial cells are significantly increased in PE, and the hypothesis was raised that they might be involved in the development of endothelial dysfunction. [44, 45]. MP are capable of altering endothelial cell function through several mechanisms; they can alter cyclooxygenase expression and prostacyclin production in endothelial cells [46], up-regulate adhesion molecules on the endothelial surface [47, 48], initiate cytokine (IL-6, IL-8) and tissue factor (TF) production [49], cause platelet and neutrophil activation [50] and increase monocyte adhesiveness [47, 49].

The effects of plasma from preeclamptic *versus* normal pregnant women have been investigated extensively in cultured endothelial cells [51-54] and isolated arteries [55, 56]. It has been demonstrated that preeclamptic plasma may exert endothelial cell activation through alterations in the production of NO, PGI_2 [57, 58] or fibronectin, increased accumulation of intracellular triglycerides or increased cellular permeability *see rev* [59]. A study by Hayman et al (2000) [60] demonstrated that endothelium-dependent dilatation in isolated myometrial arteries from normal pregnant women was almost abolished after an one hour incubation with 2% plasma from women with PE. Therefore it would be of interest to investigate whether MP as an element of preeclamptic plasma, could impair endothelium-dependent dilatation in the isolated resistance myometrial arteries from normal pregnant women.

Vascular endothelial growth factor (VEGF)

Recently attention has focused on VEGF and its role in the pathogenesis of PE. This factor is also known as vascular permeability factor. VEGF is widely expressed in adult tissues and is believed to play a role in the maintenance of the endothelial function *see rev* [61]**.** It is overexpressed in a variety of pathological conditions [62], particularly solid tumors, whose growth can be prevented by the inhibition of VEGF action [63]. In animal pregnancy, VEGF has been proposed to be involved in the development of the uteroplacental vascular bed and contribute to an enhanced vasodilatation due to NO release [64, 65].

In human pregnancy VEGF is one of the pivotal factors during placental formation [66]. However, high concentrations of VEGF are related to an increase in peripheral vascular resistance and correlate with the clinical symptoms in PE [67, 68]. It is well established that hypoxia is a direct stimulus for VEGF release, and VEGF has been indicated to promote coagulation and permeability [59, 69]. The placenta in PE is hypoxic, and activation of the coagulation system and increased vascular permeability are important pathogenic features in PE. Therefore, it has been suggested that this factor may promote several clinical features of PE.

Hayman et al. (1999) [70] reported that overnight incubation with VEGF impairs endothelium-dependent dilatation to levels similar to those obtained in myometrial arteries from women with PE. This response was largely reversed by incubation with a VEGF antibody and completely reversed by a VEGF receptor (flt-1) blocker [70]. It is of importance to evaluate whether these VEGF-induced effects are specific to uterine circulation, and which mechanisms are involved. Recently it has been suggested that angiopoietin-1 (Ang-1) acts as a physiological antagonist to offset VEGF-induced vascular leakage [69]. The role of Ang-1 in the regulation of vascular permeability therefore seems to be of significance.

Estrogens

The role of estrogens in the pathogenesis of the endothelial dysfunction in PE remains unclear. Evidence for involvement comes from studies in the early 80's. Reduced supply of fetal estrogen precursors and an impaired conversion of these compounds into estrogens by the placental tissue resulting in decreased maternal plasma estrogen levels have been demonstrated in PE [71, 72]. Moreover the reduced risk for breast cancer later in life for women who experienced PE has been attributed to a reduced estrogen effect [73-75]. Estrogen combines several different actions on vasculature. It may act as an antioxidant [76, 77], up-regulate receptor mediated effects on endothelial nitric oxide synthase (eNOS) activity [78, 79], or influence calcium dynamics in vascular smooth muscle cell (VSMC) contraction [80-82]. In light of numerous studies on cardiovascular protective effects by different estrogenic compounds [81, 83, 84], it should be of importance to study the effects of 17β-estradiol (17β-E_2) on endothelial function in the isolated resistance arteries from women with PE.

Endothelial dysfunction in the Menopause

It is well recognized that pre-menopausal women in contrast to men are protected from cardiovascular diseases such as CHD, stroke and hypertension [85]. Decline in circulating levels of estrogens in women after menopause increases the risk for CVD to level similar to man [86].

Women after menopause in comparison to age-matched men also have a worse short and long-term prognosis after myocardial infarction [87-90]. Thus, the role of female ovarian hormones in the maintenance of vascular homeostasis remains of fundamental importance.

The vascular endothelium plays a central role in the regulation of vascular homeostasis by releasing paracrine factors that primarily influence vascular tone, thrombosis and inflammation. A major endothelial product responsible for homeostasis is NO, which is synthesized by the eNOS in response to a number of endothelium-dependent stimuli, including shear stress that occurs in response to increase in blood flow [91, 92]. In fact, intraluminal flow within the physiological range is the most important regulatory stimulus for NO release [93]. Menopause, progression of atherosclerosis and appearance of risk factors for its development are accompanied by an impaired release of endothelium-derived vasodilators. The magnitude of this defect in the circulation may predict later CVD events, raising the possibility of a pathogenic relationship [94]. Interventional studies suggested that hormonal replacement therapy (HRT), lipid-lowering therapy, angiotensin-converting enzyme inhibition, smoking cessation and exercise might improve endothelial function and reduce later cardiovascular events [95-97]. Thus, evaluation of endothelial function, using well-described methods for evaluation of flow- and agonist-mediated dilatation in conduit arteries *in vivo* and/or in the isolated resistance arteries *in vitro,* could be used to determine risk for CVD and for studies relevant for primary or secondary prevention [10].

Endothelial dysfunction after menopause is supported by studies in conduit arteries using high-resolution ultrasound, where evidence of a blunted flow-mediated dilatation (FDM) in post-menopausal women and the beneficial role of estrogens [98-103] was reported. In addition, utilization of venous occlusion plethysmography technique provided evidence of impaired endothelium-dependent dilatation to ACh in healthy post-menopausal women [104], further supporting a compromised endothelial function. However, up to date there is no direct evidence about flow- and pressure-mediated responses in isolated small resistance arteries from healthy post-menopausal women.

Biological effects of estrogens

Multiple effects of estrogen on vascular system, reproductive tissues, bone, liver, and brain are dependent on the distribution of the known estrogen receptors (ERs), estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β). These receptors are important targets for endogenous estrogen, estrogen replacement therapy (ERT), combine HRT and pharmacological estrogen-like compounds [105]. However, up till now distribution of these two receptors in the human vascular tree were not adequately studied in an age and gender-dependent manner. Activation of ERs prompts activation of certain genes responsible for a wide range of vascular effects, including regulation of vasomotor tone and response to injury that may be protective against development of atherosclerosis and ischemic diseases. Two general estrogen-mediated endothelium-dependent vascular effects are recognized. Rapid, when transient vasodilatation occurs within few minutes after estrogen exposure, independently of changes in gene expression. This rapid vasodilatation appears to be due to a novel ER-α–mediated activation of the eNOS, located in the plasma membrane caveola [78]. Several studies have demonstrated a significant

improvement in blood flow after acute infusion of estrogens in the coronary arteries [106-109] and increase in endothelium-dependent dilatation in the brachial artery [110-113].

Second, longer-term effects of estrogens on the vasculature, such as those related to limiting the development of increased vascular resistance, atherosclerotic lesions or vascular injury occur over hours to days after estrogen treatment and have effect on vascular gene expression *see rev* [105, 114]. It has been shown that estrogen increases the expression of the genes, responsible for crucial vasodilatory enzymes such as prostacyclin synthase and eNOS [115-117]. Studies in ovariectomized animals have yielded direct evidence about the stimulating effects of estrogens on eNOS protein expression within 2 hours after administration [84]. Supportive evidence for estrogen-induced endothelial protection comes from studies in ER-α knockouts animals [118]. The only one known individual lacking functional ER-α [119-121] suffers from CVD, further supporting a predominant role of ER-α subtype in the regulation of endothelial function by estrogens. Long-term administration of estrogens improves vascular reactivity in non-human primates [122], male-to-female transsexuals [123, 124] and in post-menopausal women [99, 125-128].

The re-endothelialization induced by estrogens after vascular injury is ER-α mediated [114, 129] and may be due to increased local expression of VEGF [130]. Estrogen also inhibits apoptosis in cultured human endothelial cells in an estrogen receptor–dependent manner [131], however other factors (e.g. free radicals in the endothelial cell at low concentrations) in the mediation of antiapoptotic estrogen activity might be of importance [132-134]. In animal model of atherosclerosis, estradiol restores Fas ligand (FasL) expression, which is suppressed by atherogenic levels of serum cholesterol. The maintenance of endothelial FasL expression by estradiol may represent a mechanism of estrogen's antiatherogenic effect [135]. Moreover, it has been demonstrated that estrogens by up-regulation of HDL and inhibition of endothelial apoptosis by high-density lipoprotein (HDL)-associated lysosphingolipids might represent a novel aspect of the anti-atherogenic activity [136]. Although apoptosis of EC contributes to atherosclerotic plaque formation in the vascular wall [137], occurrence of apoptosis in EC layer in arteries from post-menopausal women has not yet been studied.

Progesterone receptors are also expressed in the vasculature [138], although their role in the development of CVD is poorly defined. Since Progestagens (P) were widely introduced into HRT regimens to provide endometrial protection [139] several studies suggested that P might offset the beneficial effects of estrogens [96]. In animal studies, beneficial effects of estrogens on vascular injury models were blocked by high but not low doses of progesterone [140-142]. In addition to estrogen-induced vascular effects, oral estrogen administration reduces low-density lipoprotein (LDL) cholesterol and lipoprotein-a (Lp-a) and increases HDL cholesterol. Concomitant progestagens therapy had little impact on the overall magnitude of estrogens effect on Lp-a [143].

The effects of combined HRT on several recognized risk markers for CVD have been reviewed by Mosca (2000) [144]. Fibrinogen, plasma viscosity, plasminogen activator inhibitor-1, tissue plasminogen activator, insulin sensitivity, homocysteine, and markers of platelet aggregation and endothelial cell activation are favorably affected by estrogens therapy [144]. On the other hand, HRT increases C-reactive protein levels, which suggest a possible pro-

inflammatory effect, but reduces the concentrations of soluble E-selectin, which might be considered as an anti-inflammatory effect [145].

HRT as a tool for primary and secondary prevention of CVD

Hormone replacement therapy has received considerable attention with regard to primary and secondary prevention of CVD. This interest was initially based on population-based studies linking a reduced risk of CVD up to 40-50% among users of either estrogen alone or in combination with P [126, 146-148]. Reports that questioned HRT usage for secondary and primary CVD prevention in post-menopausal women [149] were The Heart and Estrogen/progestin Replacement study (HERS) [8] and Women's Health Initiative (WHI) trials [9]. It was shown, that oral substitution with 0.625 mg conjugated equine estrogens (CEE) in combination with 2.5 mg medroxyprogesterone acetate (MPA) in the HERS and WHI trials did not reduce the overall rate of CHD, it also increased thromboembolic events and gallbladder disease. In addition, the WHI trial demonstrated hazardous effects on stroke, endometrial and invasive breast cancers. Both trials were limited to one hormonal regimen and could not distinguish the effects of estrogen from those of progestagen. Participants mean age at the point of recruitment was 67 and 63, respectively, and, as suggested by others [150, 151], it could be too late for prevention of CVD, whilst being at risk more than 10 years. Importantly, the arm of the trial that is evaluating the risks and benefits of unopposed 0.625 CEE in hysterectomized women is ongoing, with results expected 2005. More specific analyses of combined HRT regimens on the incidence of breast cancer in the Million Women study [152] strengthen the adverse relationship between the combined HRT and women health. However, the increased risk of breast cancer in association with use of combined (estrogen plus progestagen – both continuous and sequential regimens) HRT was substantially higher than with oestrogen-only therapy. Furthermore, the risk of breast cancer started to decline when HRT was stopped and by five years reached the same level as in women who have never taken HRT.

Several studies have demonstrated that ERT alone has beneficial effect on FMD in conduit arteries in post-menopausal women [98-103]. There is also evidence that estrogen therapy improves ACh-mediated responses in post-menopausal women, as assessed by single point Laser Doppler technique [153] and it has been suggested that beneficial effects of ERT are due to its influence on NO bioavailability [154]. It is important to notice that unopposed estrogens show almost 40% greater vasodilator response to flow in healthy post-menopausal women, who have not yet developed atherosclerotic vascular disease, but there is no effect of ERT in women with established CVD [155]. In contrast, concomitant administration of progestagens attenuated the stimulating effect of estrogen on bioavailability of NO [154] and eliminated the effect on FMD in healthy post-menopausal women [103, 156]. However, these are not universal findings, since other studies demonstrated that combination of estrogen with progestagens improved FMD [99, 100, 157, 158].

The results of these studies raise several specific questions; e.g. which HRT regimen could be favorable to reduce CVD and safe in terms of thromboembolic events and cancers? Which are the mechanisms of vascular protection in resistance vasculature by estrogenic compounds? Current evidence suggests that shear –mediated signaling pathways involved in the activation of eNOS and NO release share similar signaling pathways with estrogen-induced up-regulation of

NO release [78, 159-163]. Could shear-mediated response therefore become a particular target for estrogen action resulting in cardiovascular protection? The importance of optimal timing of HRT initiation, type and duration of treatment regime and the study population involved for CVD prevention should also be emphasized.

Selective estrogen receptor modulators (SERMs)

Due to a failure to confirm that conventional HRT regimens are beneficial for primary and/or secondary CVD prevention in women after menopause, new substances have been introduced. The ideal alternative for HRT should reduce risk for CVD preserve bone density without side effects on incidences of breast and endometrial cancer or venous tromboembolism. Selective estrogen receptor modulators (SERMs) currently are launched as a fast growing group of alternative pharmacological substances for conventional HRT [164] and naturally occurring isoflavones (phytoestrogens such as genistein) that reveal ER-agonist activity in some tissues but oppose estrogen action in others have become of interest [165]. Meta-analysis of several studies suggested a positive effect of dietary intake of soy protein on lipid profile [166-169], a reduction of systolic and diastolic blood pressure [170] and an improvement in biomarkers of lipid peroxidation [171]. The relative agonist/antagonist activities of SERMs differ between tissues. For example, Raloxifene demonstrates agonistic activities only on bone, but not in the uterus or breast tissue [172]. The tissue specificity is dependent on ER-α and ER-β distribution [173, 174], which varies between tissues; for example, uterus, vagina, and hypothalamic arcuate nucleus of the brain express predominantly ER-α, whereas others lung, prostate, ovarian granulosa cells, and hypothalamic paraventricular nucleus of the brain express predominantly ER-β. Other tissues, such as bone and the pituitary, express both ER-α and ER-β.

It is clear, that vascular endothelium selective compound would be of highest importance in terms of cardiovascular protection. However, up to date there is limited knowledge about distribution of ERs in cardiovascular system, and which ER subtype is predominant in the wide-ranging estrogen action on the vasculature. Whether well-characterized estrogenic compounds such as soy isoflavones (genistein), raloxifene may offer alternatives to conventional treatment with HRT is under investigation. For example, the ongoing Raloxifene Use for the Heart (RUTH) trial is designed to answer questions associated with the risks for CHD and breast cancer in healthy post-menopausal women [175]. Experimental evidence indicates that both raloxifene and genistein may enhance NO release in arteries and cultured endothelial cells via increasing eNOS mRNA expression [176, 177] and preserve endothelial function in ovariectomized animals [178]. Dietary phytoestrogens reduce aortic cholesterol content to a similar extent as HRT [179]. They may also reverse endothelial dysfunction after ovariectomy [180], and restore NO-mediated dilatation after chronic hypoxia [181]. In healthy post-menopausal women, a few reports exist demonstrating that prolonged (6 month) administration of raloxifene and genistein may improve flow-mediated vasodilation in the brachial artery [182, 183], however short-term (2 weeks) oral soy isoflavones supplements did not [184]. Up till now, however, there is no experimental evidence on the effects of these ligands on resistance vasculature in healthy post-menopausal women.

Aims

The general aim of the thesis was to study *in vitro* functional and morphological properties of resistance arteries obtained from women with PE and after the menopause. The particular aims of the studies upon which this thesis is based were:

1. To establish if microparticles, as a component of plasma from women with PE, may mimic the endothelial dysfunction that occurs in isolated myometrial arteries from women with preeclampsia.

2. To evaluate whether prolonged incubation with human recombinant vascular endothelial growth factor ($hrVEGF_{165}$) affects basal tone, endothelium-dependent dilatation, permeability features and morphology of the endothelium in isolated subcutaneous arteries from normal pregnant women.

3. To determine whether prolonged incubation with 17β-estradiol improves endothelial function in isolated resistance arteries from the uterine vasculature in women with PE, and to evaluate the role that NO may play in these effects.

4. To evaluate subcutaneous resistance artery function in healthy post-menopausal women *in vitro* using pressure-myography technique and to link these findings to the morphology of endothelial cell layer using scanning electron microscopy. Furthermore, to determine whether 17β-estradiol, ER-α-selective ligand propyl pyrazole triol (PPT), raloxifene and genistein might be beneficial in respect to cardiovascular protection after menopause.

5. To evaluate whether 3 months treatment with combined HRT based on estradiol and/or medroxyprogesterone acetate (MPA) alters endothelial function and morphology in isolated subcutaneous resistant arteries from healthy post-menopausal women.

Materials and methods

Study population

Four major groups of participants were included in to the studies: normal pregnant women, women with PE, non-pregnant pre-menopausal women and healthy women after menopause. Local Ethics Committee approval was obtained for the collection of subcutaneous fat, myometrial biopsies and plasma samples from women at the Department of Obstetrics and Gynecology at Huddinge University Hospital, Sweden and Department of Obstetrics and Gynecology, Academic Medical Center, Amsterdam, The Netherlands (paper I). In all cases, informed consent was obtained prior to tissue biopsy or plasma collection. Table 1 summarizes the number of participants included in each study.

Table 1. Summary of the numbers of women included in the papers (I-V).

	Paper I		Paper II	Paper III	Paper IV	Paper V	Unpublished data
	Tissue sample	Plasma sample *					
NP	22	6	41	27			
PE		16	6	20			4
Post-menopausal					66#	55#	
Pre-menopausal					21		

*Sampling was done at the Department of Obstetrics and Gynecology, Academic Medical Center, Amsterdam, The Netherlands.
Arteries from the same healthy post-menopausal volunteers were studied in Paper IV and Paper V.
NP-normal pregnant, PE-Preeclampsia.

All normal pregnant women from whom biopsies were obtained had singleton pregnancies. They were delivered at term by elective cesarean section due to previous cesarean delivery (n=31), psychosocial indications (n=28), breech presentation (n=27) or previous sphincter rupture (n=4). Normal pregnant (n=6) and women with PE (n=16) were matched for age and gestational age before obtaining the plasma samples used for incubation experiments in Paper I.

PE was defined as blood pressure ≥ 140/90 mm Hg and proteinuria exceeding > 300 mg/24 hours in the absence of urinary tract infection after 20 weeks of gestation in previously normotensive, non-proteinuric women. Thirty woman with PE were included from whom myometrial or subcutaneous fat biopsies were collected. Any women with a previous history of chronic hypertension, renal disease or diabetes mellitus were excluded from the study. Women with preeclampsia who had received anti-hypertensive agents were also excluded.

Subcutaneous fat biopsies (approximately 2x1.5x1.5 cm) were obtained from pre-menopausal women either during laparoscopic surgery (n=6), elective abdominal surgery (n=10) or under local anesthesia (n=5). 1% prilokain (Citanest®) solution was used for local anesthesia and biopsies were obtained from the lower left abdominal region. None of pre-menopausal women used hormonal contraception for last three-month prior the study. Serum estradiol and

progesterone levels were measured for confirmation of the phase of the menstrual cycle in pre-menopausal women.

Healthy post-menopausal women were recruited to the study. Inclusion criterions were amenorrhea for at least 1.5 year and serum concentrations of follicular- stimulating hormone (FSH>15 IU/ml) and estradiol (E_2<40 pg/ml). Cigarette smokers and women with hypertension, diabetes mellitus, clinical manifestations of arteriosclerosis (CHD, peripheral artery disease, or cerebrovascular disease), venous thromboembolic disease, liver disorders, unexplained vaginal bleeding, and a personal or family history of breast cancer were excluded. Before enrollment in the study, each subject underwent a physical examination, including gynecologic evaluation and mammography.

Sixty-six post-menopausal women were randomly assigned to the one of four treatment groups for 3 months (Paper V): the first group received continuous therapy with estradiol (E_2, Femanest®, 2mg/day, n=16), second group- MPA (Gestapuran®, 5mg/day, n=18), third group-combined treatment (E_2 + MPA, n = 16) and fourth - control group did not receive HRT (n = 16). Two subcutaneous fat biopsies were obtained from post-menopausal women from the lower left (pre-HRT) and right (post-HRT) abdominal region. Arteries obtained from the first biopsy were used for experiments in Paper IV in order to evaluate functional properties of resistance arteries from post-menopusal women and to evaluate the effects of E_2 and SERMs *in vitro* as well as endothelial layer morphology. The same measurements as 3-months earlier were repeated in the arteries obtained from the second biopsy in order to assess the effect of continuous HRT on endothelial function and morphology. Due to several dropouts from the study, 55 women were included in the final analysis of the data in the Paper V.

Experimental procedures and protocols

Human subcutaneous fat and myometrial biopsy handling

Following delivery of the placenta at Cesarean section, a full thickness biopsy of the myometrium was taken from the upper margin of the incision. Subcutaneous fat biopsies were taken after uterine suture. The biopsies were collected into ice-cold physiological salt solution (PSS). Resistance arteries of approximately 200μm in diameter and 2mm length were immediately dissected from the biopsy using a stereomicroscope. Surrounding tissue and adventitia were mechanically and carefully removed using microsurgery instruments. The angle of arterial branches and decrease in lumen diameter were two main criteria for identifying *in vivo* blood flow direction in the arterial path.

Biopsy tissues obtained from women using local anesthesia underwent similar routines, except that it was additionally flushed several times with fresh PSS in order to wash-out residuals of local anesthetic. Time course between biopsy and isolation of subcutaneous arteries under dissection microscope was no longer than 2 hours.

Collection of blood samples

A special protocol for blood sample collection was used in Paper I. Blood samples were taken from the antecubital vein without tourniquet using a butterfly needle and a vacutainer system

into a 4.5 ml tube containing 0.105 M citrate. Blood samples were processed immediately at room temperature to prevent any cell activation. Cells were removed by centrifugation for 20 minutes at 1550 × g at room temperature to obtain MP-containing plasma. Plasma samples were divided in aliquots of 1 ml, snap frozen in liquid nitrogen to preserve the MP structure, and stored at -80°C. Before the start of the experiments the frozen plasma aliquots were thawed on melting ice. Two tubes, containing 250 µL plasma were centrifuged at 17570 × g for 30 minutes to pellet the MP. Subsequently, 225 µL of the MP-free plasma was removed from each tube and the MP pellet was re-suspended in the remaining 25 µL of plasma and 225 µL of calcium-free PSS. As previously confirmed, the fraction isolated from the plasma according to the above-described protocol contains MP [44, 185-187]. The MP-containing plasma, MP-free plasma and isolated MP were dissolved in calcium-free PSS to the desired concentration and used immediately. Calcium-free PSS was used in order to prevent fibrin generation in the solutions. Heparin (1 IU/mL) was added to the solutions that were used for overnight incubation.

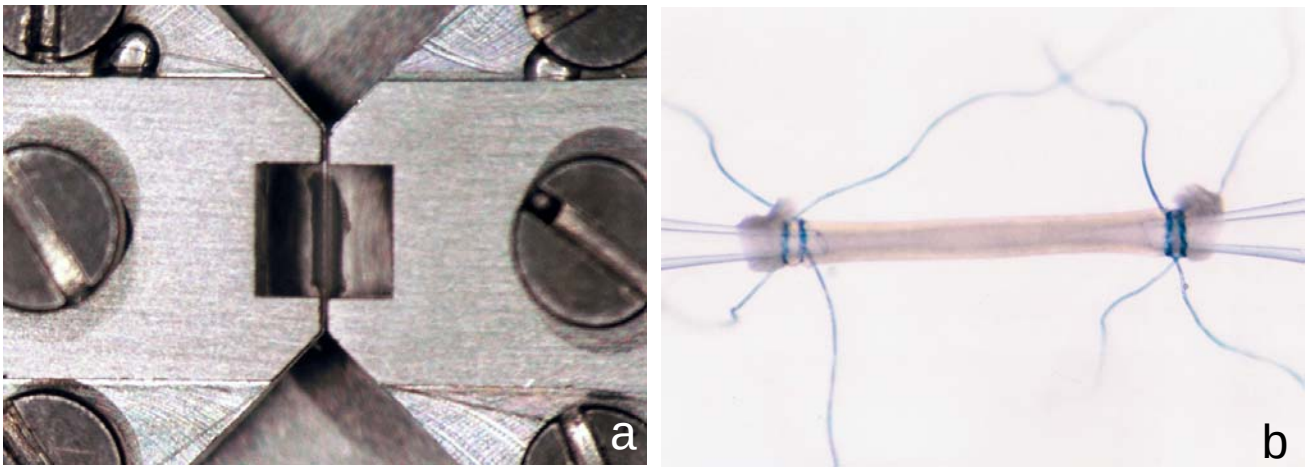
Venous blood samples in Papers IV&V were taken after a 12-h overnight fast at baseline and 3 months of follow-up. After 20 min of rest, 20 ml of blood were collected into two EDTA (1 mg/ml) tubes and kept on ice. The plasma separated by centrifugation at 2500 rpm for 20 min was stored in several aliquots at -70°C until the time for hormone analysis. Total plasma cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides were measured by enzymatic methods using general routines in the laboratory of clinical chemistry, Huddinge University Hospital, Stockholm.

Serum concentrations of 17β-E_2 and Progesterone (P) in Paper IV were determined by radioimmunoassay using commercials kits obtained from Diagnostic Product Corp., Los Angeles, CA. For E_2 the "Estradiol Double Antibody" kit was used with an additional extraction step prior analysis. The serum was extracted with diethyl ether, the ether phase was evaporated to dryness and the residue was dissolved in zero calibrator supplied by the manufacturer of the kit. After that the samples were analyzed according to the original protocol. The extraction step eliminated possible influence of matrix differences at low E_2 levels as well as influence of high circulating concentrations of estrogen conjugates in pregnant women and in women with HRT [188]. For P the "Coat-a-Count®Progesterone" kit was used without any modifications.

Wire-myography

Isolated resistance-size myometrial artery segments of approximately 2mm in length were mounted on stainless steel wires (Ø 40µm) which were attached to a force transducer and micrometer in the chambers of a Mulvany's type 4-channel Multi Myograph (Model 610, version 2.1, Danish Myo Technology, Denmark, Picture 1). Vessel length was measured with a calibrated eyepiece micrometer under the stereomicroscope. Following the mounting of all arteries, they were allowed to equilibrate for 30 min at 37˚C with continuous oxygenation with 5% CO_2. A standardized normalization procedure was then performed to allow the calculation of the artery diameter at which the *in vivo* transmural pressure of the relaxed artery would have been 100 mm Hg. Arteries were then set at 0.9 times this diameter, since it is generally accepted that this is the diameter that enables optimal contractile ability for arteries with low resting tension. All solutions (PSS: mmol/L; NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, NaH_2PO_4 1.18, EDTA 0.026 and glucose 5.5; pH 7.4, 37^0C, gassed with 5 % CO_2 in O_2)

Picture 1. Wire myography mounted artery (a) and pressurized artery (b).



including incubation solutions were refreshed every 30 minutes. Myodaq software was used for these calibrations and data registration (version 2.1, Danish Myo Technology, Denmark).

Pressure myography

Dissected arteries were mounted in a pressure arteriograph (Living Systems Instrumentation Inc., Burlington, Vt. USA) and were orientated in the *in vivo* direction of flow on a pair of opposing glass microcannulae matched for flow resistance (Picture 1). The organ bath was perfused at 7 ml/min with PSS. A servo-controlled pump maintained the required intraluminal pressure and the internal diameter (i.d.) of the artery diameter was recorded continuously using a video dimension analyzer. "In-line" pressure transducers monitored the proximal and distal pressure on each side of the specimen, enabling calculation of the mean intraluminal pressure. Each artery was equilibrated for 60 minutes while pressurized to 50-60 mm Hg, and only arteries demonstrating the suitable criteria for viability, constriction to high potassium PSS (KPSS) and dilatation to acetylcholine (ACh) 1 µmol/L, were included in the studies. The intraluminal application of VEGF alone or in combination with Ang-1, VEGF in combination with Ang-1 supernatant, control supernatant without Ang-1 (for details, *please see* Paper II) and Evans blue dye was achieved by connecting an additional flow pump while keeping intraluminal pressure constant. The other active substances were applied extraluminally.

Vascular permeability studies

When Evans blue dye (0.03%) reached the artery, intraluminal flow was stopped while keeping intraluminal pressure constant at 60 mm Hg. The whole arteriograph with the colored artery inside was then immediately removed from the stage of the inverted microscope and placed under a dissection microscope. An attached photo camera allowed photographs every minute during 5 minutes for a blind assessment of Evans blue staining. The Evans blue staining was evaluated on the basis of blue color distribution within the artery wall.

Immunohistochemistry

The arteries obtained from fat biopsies of subcutaneous tissue from the post-menopausal women in Paper IV were stained for ER-α and ER-β. The arteries were embedded in OCT compound (Histolab, Sweden) and frozen on dry ice and kept in –70°C. For immunohistochemistry 8 µm thick sections were done on super-frost glass slides with Cryotome. The sections were fixed in ice-cold acetone for 10 minutes. After washing in 1% PBS (Phosphate buffered solution) the sections were blocked with endogenous peroxide using 0.3% hydrogen peroxide (30 min). After washing twice in 1% PBS (5 min each) slides were blocked with bovine serum albumin (BSA) for 60 min. Then the sections were incubated with primary ER-α (Santa Cruz) and ER-β (Santa Cruz) antibodies in 1:10 and 1:20 dilution respectively overnight at 40°C. After washing twice in 1% PBS (5 min each) the sections were incubated with rabbit anti-mouse secondary antibody (Dako) and swine anti-rabbit secondary antibody (Dako) in 1:200 and 1:300 dilutions respectively for 60 minutes. After washing in 1% PBS (5 min each) the sections were incubated with Avidin-Biotin for 30 minutes. Sections were exposed to 0.001% of 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma chemical) with 0.03 % hydrogen peroxide followed by counterstaining with hematoxyline.

Scanning electron microscopy

For scanning electron microscopy arteries were cut longitudinally and rinsed in PSS, fixed and kept for at least 48 hours in a 2.5% (wt/vol) glutaraldehyde solution in PSS, and postfixed in a solution of 1% (wt/vol) osmium tetroxide in a sodium cacodylate buffer (0.15 M, pH 7.3) containing 75 mM sucrose. The artery samples were dehydrated in acetone series, dried in a critical-point drier using carbon dioxide, mounted on the specimen holder, and coated with gold palladium and examined for the morphological changes in endothelial cell layer under scanning electron microscope JEOL 820 (JEOL USA, Inc, Peabody, Mass). In order to reveal the effect of HRT on endothelial layer morphology in Paper V, arteries were fixed for scanning electron microscopy in the same way and paired comparisons were performed before and after 3 months continuous therapy with E_2, MPA, E_2+MPA or control.

Experimental protocols

1. BK-mediated dilatation in the isolated myometrial arteries from normal pregnant women was evaluated before and after 1-hour incubation with 2% and 10% solutions of 1) whole preeclamptic plasma, 2) MP-free preeclamptic plasma, 3) isolated preeclamptic MP re-suspended in PSS or 4) PSS. Separate groups of arteries were incubated overnight at 4°C in 5% solutions of 1) whole preeclamptic plasma, 2) MP-free preeclamptic plasma, 3) isolated preeclamptic MP re-suspended in PSS or 4) PSS. This overnight incubation also included a fifth group, where isolated MP from normal pregnant women were re-suspended in PSS (Paper I).
2. Changes in pressure-induced myogenic tone during incubation with VEGF, VEGF+ bosentan (ET-1 receptors blocker) or vehicle, dilatation to BK before and after 3-hours incubation with VEGF, VEGF+ bosentan or vehicle were compared using pressure myography technique. Morphology of the endothelial cell layer in the arteries from normal pregnant women after incubation with VEGF was evaluated by scanning electron microscopy. Permeability to

Evans blue dye was evaluated after incubation with VEGF, VEGF+Ang-1 or vehicle and in arteries from women with PE (Paper II).

3. Flow- and BK-mediated responses as well as changes in pressure-induced myogenic tone in the isolated myometrial resistance arteries from women with preeclampsia were evaluated before and after 3 hours incubation with 17β-E_2 (10nmol/L). The role of NO in these responses was assessed in the presence and absence of NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME 100 μmol/L) (Paper III).
4. Flow-mediated, endothelium-dependent (BK) and independent sodium nitroprusside (SNP) agonist mediated dilatation and pressure-induced responses in isolated arteries from post-menopausal women were compared to responses in arteries obtained from pre-menopausal women. The responses were compared before and after incubation (3 hours) with 17β-E_2 alone or in combination with ERs blocker ICI 182,780, with a selective ER-α agonist-PPT, with raloxifene and genistein. Two groups of women were also compared with regard to morphology of the endothelial cell layer using scanning electron microscopy. Distribution of ER-α and ER-β in the subcutaneous arteries from post-menopausal women was evaluated using immunohistochemical staining (Paper IV).
5. Flow-, endothelium-dependent agonists mediated dilatation, pressure-induced responses and endothelial cell layer morphology in the isolated arteries from post-menopausal women were compared before and after 3 months continuous therapy with estradiol (E_2, Femanest®, 2mg/day), medroxyprogesterone acetate (MPA, Gestapuran®, 5mg/day), combined treatment (E_2 + MPA) and the control group without treatment (Paper V).

Statistical analysis

Conventional parametric and non-parametric methods as well as multiple analysis of variance (MANOVA) with repeated measurements within or between groups were used. Data was analyzed using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Pressure-induced myogenic tone was calculated as the percentage decrease of the internal diameter (i.d.) of arteries in Ca^{++} -free PSS from the following equation:

$$Myogenic\ tone\ (\%) = \frac{(i.d.Ca^{2+}\ free\ \mathrm{PSS} - i.d.\ \mathrm{PSS})}{i.d.Ca^{2+}\ free\ \mathrm{PSS}} \times 100$$

Results are presented as mean ± standard error of the mean (SEM). The degree of permeability was calculated blindly by two independent investigators using a semi-quantitative scale from 0 to 3+. Categorical data were compared with Chi-squared test. One-way ANOVA was used to compare baseline clinical and biochemical characteristics between groups in Paper V. Quantitative analysis of the morphological findings using scanning electron microscopy was performed by calculating the number of endothelial cells (EC) per 50μm^2 area, which corresponds to the area of an original 1000 fold magnification picture of arterial sample. Similarly, EC death (EC death index) was scored from + to ++++ in each sample. EC number and EC death were calculated from samples before and after HRT and were compared with non-parametric statistics (Papers IV-V). A p-value < 0.05 was considered significant.

Results and discussion

Isolated microparticles in plasma from women with preeclampsia are detrimental to endothelial function

Incubation for one-hour with 2% and 10% solutions of isolated MPs, the whole plasma or MP-free plasma from individual women with preeclampsia had no effect on BK-mediated dilatation in isolated myometrial arteries from normal pregnant women. However, after an overnight incubation with 5% solution of isolated preeclamptic MPs only, but not whole or MP-free plasma, BK-mediated relaxation was abolished (*see* Figure 1). This impairment of endothelium-dependent dilatation was peculiar to the action of preeclamptic MPs, since MPs (5%) from normal pregnant women did not cause any alterations in endothelium-dependent dilatation after overnight incubation (*see* Figure 1). Thus, circulating MPs from women with preeclampsia in contrast to MPs from normal pregnant women are able to impair endothelial function after prolonged exposure, while other preeclamptic plasma constituents seems to protect the endothelium from such damage.

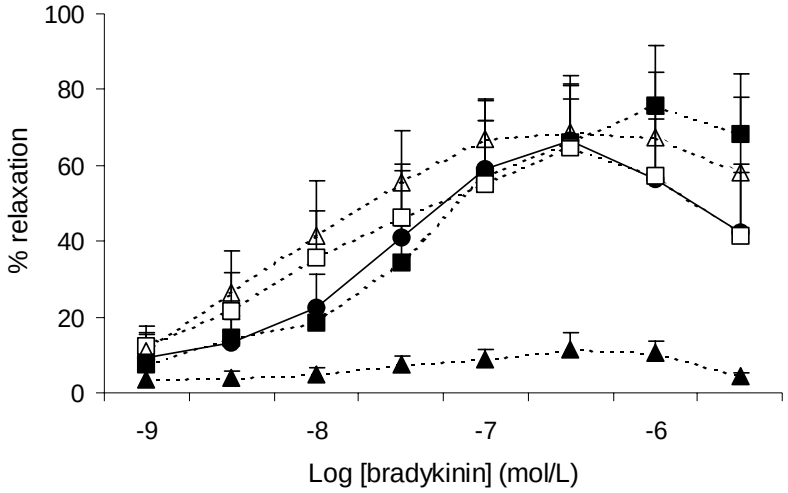


Figure 1. Bradykinin-mediated relaxation after overnight incubation with 5% whole preeclamptic plasma (--■--, n = 6), microparticle-free preeclamptic plasma (--□--, n = 6), isolated preeclamptic microparticles in physiological saline solution (···▲···, n = 6), isolated normal pregnant microparticles in physiological saline solution (--Δ--, n = 6) or with physiological saline solution (—●—, n = 6).

The pathways by which preeclamptic MP induce endothelial dysfunction in isolated arteries are presently unknown. Oxidative stress in preeclampsia could act as an important mediator in the formation of biologically active MPs. Whether preeclamptic MPs contain oxidized phopholipids that are detrimental to vascular endothelium is an issue that needs further investigations [189, 190].

Surprisingly, we did not find any harmful effect of the individual plasma from women with preeclampsia on endothelial function in myometrial arteries from normal pregnant women. This finding contrasts with previous studies demonstrating a detrimental effect of pooled plasma from preeclamptic patients on endothelium-dependent dilatation in isolated myometrial arteries from normal pregnant women even after one-hour incubation [70]. The possible explanation for this discrepancy may be related to some important differences in experimental approach between the two studies. As mentioned above, we used individual plasma samples instead of pooled plasma to evaluate if damaging factors are present in all patients. Although the preeclamptic patients included in our study were severe cases, with a high incidence of complications, we did not observe any effect on endothelial function by the plasma samples from the 16 preeclamptic patients included. Moreover, we used paired observations (before *versus* after incubation), which should be an advantage in this context. Finally, the preconstriction level, which is crucial for vasodilatory capacity, differed between the studies. The protective effects of preeclamptic plasma as suggested from the results of the present study, might be in agreement with previous observations regarding stimulatory effect of preeclamptic plasma on nitric oxide and prostacyclin production [52, 53]. It is possible that whole plasma protein composition, especially albumin, may shield the endothelial cell layer [21].

It is possible that vascular endothelium may be susceptible or predisposed to develop endothelial dysfunction in preeclampsia. This is supported by the fact that genetic factors or preexisting vascular disorders such as hypertension and diabetes increase the risk of developing PE [35, 191]. Whether normal endothelial cells contain enough defensive power to resist factors inducing dysfunction remains unknown.

VEGF induced functional and morphological signs of endothelial dysfunction

We found that prolonged incubation with VEGF enhanced pressure-induced myogenic tone (*see* Figure 2), and increased vascular permeability and impaired endothelium dependent dilatation (*see* Figure 3) in isolated resistance arteries from subcutaneous fat obtained from normal pregnant women. These findings are similar to those obtained in isolated arteries from women with preeclampsia [7] and may support the involvement of VEGF in the pathogenesis of this disease. It has been previously demonstrated that VEGF may be detrimental for endothelial function in isolated myometrial arteries from normal pregnant women [70]. Our similar findings in subcutaneous arteries exclude the possibility that VEGF could be harmful only in specific vascular beds (e.g. uterine) and speak in favor for a generalized effect. Indeed, increased VEGF concentrations have been related to an increase in uteroplacental and peripheral vascular resistance in women with preeclampsia [67, 68]. It should be emphasized however, that it is unlikely that VEGF is solely responsible for alterations in endothelial function *in vivo* in women with PE, since cytokines, lipids, metabolic and hypoxic products are all thought to affect vascular function.

In our study we also evaluated the possible mechanisms by which VEGF exerts its action on isolated arteries. The effects of VEGF appear to involve the potent vasoconstrictor ET-1, since addition of bosentan, a combined ET-A/B receptor blocker, normalized VEGF-induced myogenic tone (*see* Figure 2) and BK-mediated dilatation (*see* Figure 3). We chose bosentan to

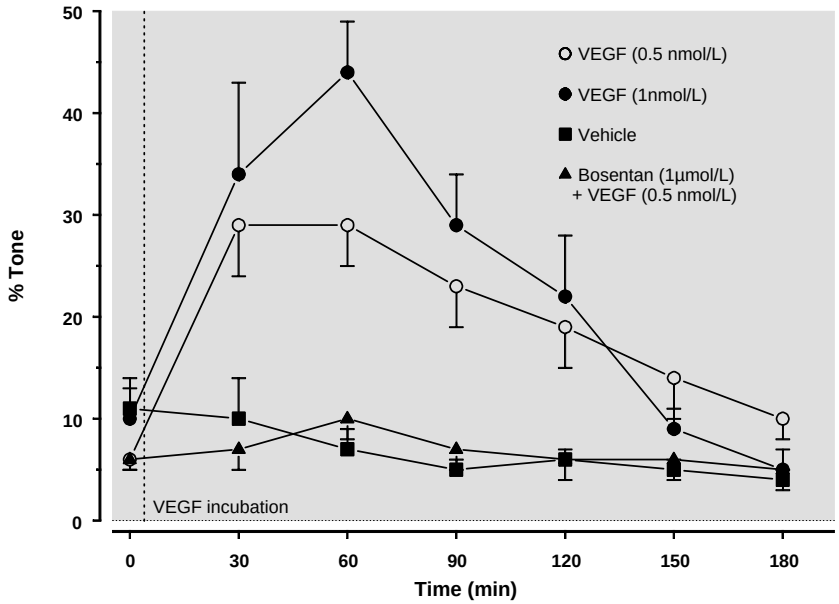


Figure 2. Percentage (%) of tone developed during 3 hours incubation with Vascular Endothelial Growth Factor (VEGF), vehicle and combination of VEGF + bosentan in small subcutaneous arteries from normal pregnant women.

evaluate this interplay, since we have previously reported that ET-1 contributes to the regulation of resistance artery tone in pregnancy [192]. Therefore VEGF-induced endothelium-dependent inhibition of vasodilatation and enhancement of pressure-induced myogenic tone in isolated arteries could be mediated through this mechanism, since VEGF has an up-regulatory effect on endothelin-1 synthesis [193, 194]. This could also help explain why preeclampsia is associated with increased levels of ET-1, known as a marker of endothelial dysfunction.

By applying a scanning electron microscopy technique we found that VEGF initiates morphological changes in the endothelial cell layer even after three hours incubation. An enhanced formation of intercellular gaps and changes in cellular shape (i.e. increase in size, intracytoplasmic edema) was observed. These changes resemble those found in the endothelial cell layer in arteries from preeclamptic women (*see* Paper II and Paper III) and further support a role for VEGF in the development of endothelial dysfunction.

It should be noted that interaction between VEGF and other factors in the cascade of endothelial activation in preeclampsia might be of therapeutic importance. The opposed effect of Ang-1 on VEGF-induced permeability features as demonstrated in Paper II, in combination with growing appreciation of gene therapy techniques is of interest. VEGF is a ligand for several receptors such as kinase domain receptor (KDR), fms-like-tyrosine kinase receptors 1 and 2 (flt-1, flt-2), which regulate biological effects of VEGF. The soluble form of flt-1 receptor (sflt-1) seems to be crucial in offsetting the biological action of VEGF on the maternal endothelium *in vivo* [70, 195-197] and the presence of such naturally occurring antagonism appears essential for endothelial maintenance. This issue needs further investigations in order to suggest novel therapeutical strategies.

Due to the risk for adverse perinatal and maternal outcome, the identification of predictors for the development of PE is of importance. Several studies have shown decreased levels of placenta growth factor (PlGF), another member of the large family of vascular growth factors in

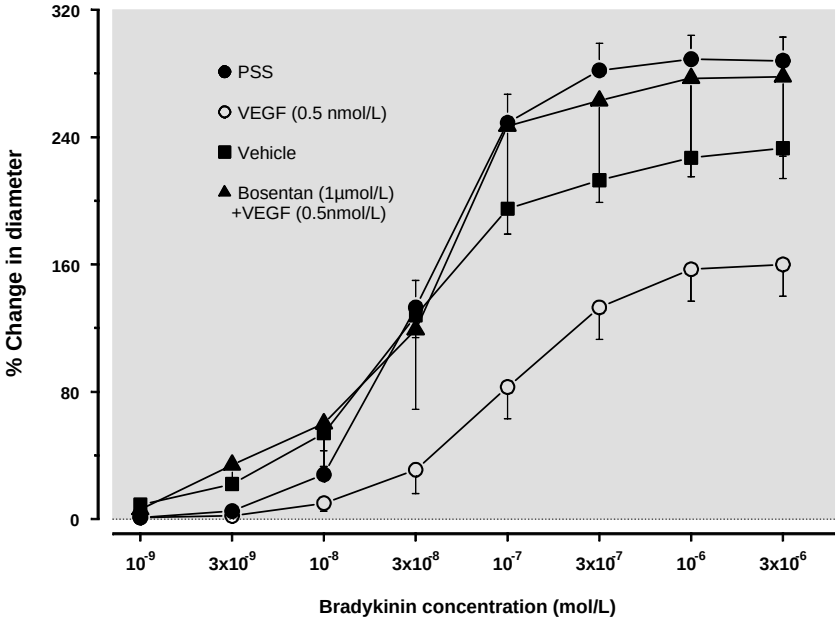


Figure 3. Bradykinin-mediated dilatation, expressed as percent (%) change in diameter, in physiological salt solution (PSS) and after incubation with VEGF, vehicle and VEGF + bosentan in subcutaneous arteries from normal pregnant women.

women with PE [197-199]. It was suggested that PlGF might be used as a predictive marker in women who subsequently developed preeclampsia [200]. VEGF may also prove a suitable marker for future investigations.

17β-estradiol improves endothelial function in arteries from women with PE

Impairment of BK-mediated dilatation in wire-mounted arteries from various vascular beds in PE is a well-documented phenomenon [27, 28, 30]. In our study we have confirmed that endothelium-dependent dilatation in response to flow-mediated shear stress and bradykinin is impaired in small arteries from the uterine circulation in PE. 17β-E_2 restored impairment of flow-mediated responses in myometrial resistance arteries to levels similar to those seen in arteries from normal pregnant women [201]. However, 17β-E_2 had no effect on BK-mediated dilatation in these arteries. In addition, 17β-E_2 reduced pressure-induced myogenic tone known to be enhanced in this disease [29]. 17β-E_2-induced effects on flow- and pressure-mediated responses were NO-mediated, since endothelial nitric oxide synthase (eNOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) reversed the effects (*see* Figures 4, 5, 6).

In the *in vivo* situation this could reflect an increase in blood flow due to reduction in uteroplacental vascular resistance. Our findings are in agreement with several studies in animal experiments *in vivo* where an increase in the uterine blood flow [83, 84] was observed after 2 hours of 17β-E_2 administration. Our results also agree with other studies *in vitro* demonstrating estradiol-induced reduction in basal tone and up-regulation of flow-mediated dilatation in small arteries from hypertensive male and female rats [160, 202] or in small subcutaneous arteries from post-menopausal women [201]. These studies imply that NO is involved in the effects of

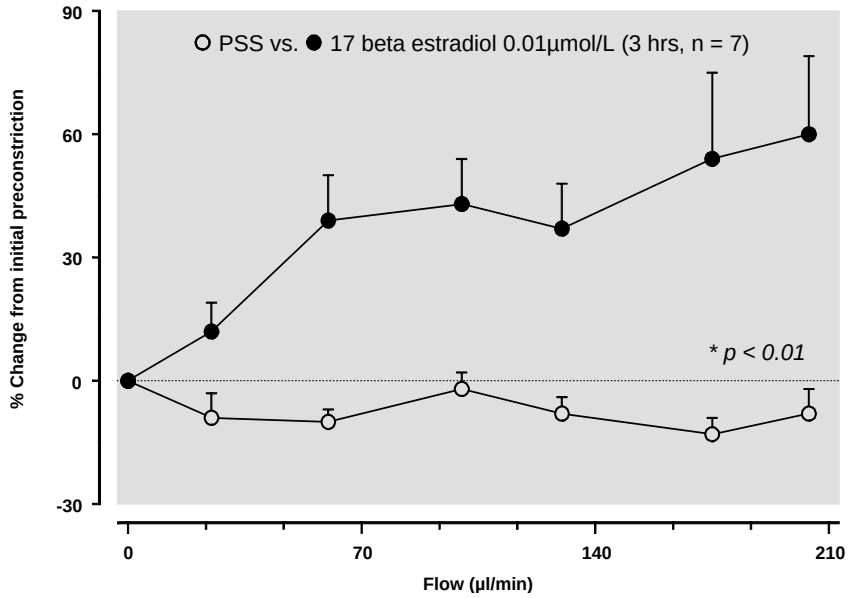


Figure 4. Flow-mediated vasodilatation in myometrial arteries from women with preeclampsia; in physiological salt solution (PSS) *versus* after incubation with 17β-estradiol.

estrogen on vascular function, however several peculiar mechanisms in this respect remain to be discussed.

Initial studies suggested that the effects of estrogen on vascular tone are related to calcium dynamics in smooth muscle cells [203]. However, the pharmacological concentrations used were far above those achieved under *in vivo* conditions. At physiological levels, the main effect of estrogens on pressure-induced tone is to open vascular smooth muscle calcium-activated potassium channels, which occurs via a NO/cGMP-dependent pathway as demonstrated in pressurized small arteries obtained from ovarectomized animals [204].

Proposed mechanisms of estrogen action on the up-regulation of NO release include the direct activation of eNOS [202], activation of eNOS via tyrosine kinases or mitogen-activated protein kinase-dependent mechanisms through heat shock protein 90 binding [205], and activation of eNOS involving phosphatidylinositol 3-kinase-Akt [79]. Existence of several pathways peculiar to certain stimuli may explain different effects of estrogen treatment on shear stress as compared with BK-mediated responses in isolated arteries from woman with PE.

Several different signaling pathways involved in BK- mediated dilatation in isolated arteries from humans have been extensively investigated. There is no agreement whether BK-mediated response is entirely dependent on the release of specific endothelial factors such as NO, endothelium derived hyperpolarizing factor (EDHF) and PGI_2 [27, 206, 207], or whether certain conditions such as PE and hypertension would determine which factors will be released in response to activation [208]. It is possible that the preference of endothelial factor involved in BK-mediated dilatation will depend on the specific vascular bed or type of artery studied [27, 206, 207]. The absence of estrogen-induced effect on BK-mediated dilatation in isolated myometrial arteries from PE may be relevant in this respect, since estradiol significantly improved BK-mediated dilatation in small subcutaneous arteries from post-menopausal women (Paper IV).

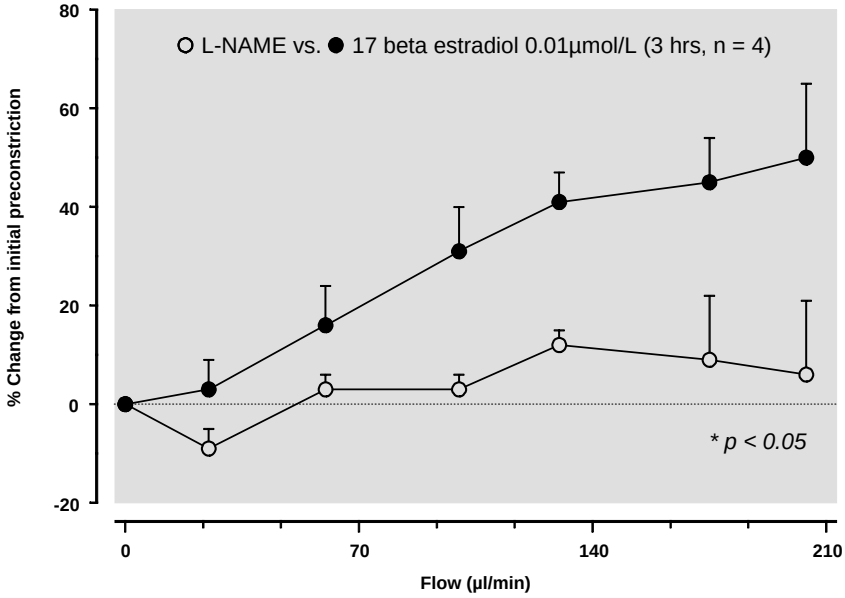


Figure 5. Flow-mediated dilatation in myometrial arteries from women with preeclampsia; after incubation with 17β-estradiol *versus* Nω-nitro-L-arginine methyl ester **(**L-NAME).

Recently, it has been demonstrated that reactive oxygen species (ROS) are involved as physiological signaling mediators in activation of eNOS and NO release in response to shear stress [132, 133], although the biological effects are critically dependent on ROS concentration. Whether estrogens in the presence of oxidative stress in PE may act, as a stabilizer between pro- and antioxidant reactions to regulate production of ROS remains an interesting issue.

Our initial intention was to investigate the longer-term genomic effect. We cannot, however, exclude the possibility that the achieved effects in our study are rapid in origin or a combination of both. Several studies have suggested a predominant role for estrogen receptor alpha (ER-α) in estrogen-induced up-regulation of NO release [160, 209]. There is evidence for the presence of a rapid acting membrane ER-α localized in the endothelial cell caveolae, where they are coupled to eNOS in a functional signaling module [159, 161]. It has been suggested that the rapid effect caused by physiological levels of estrogen is mediated, at least in part, by receptor also acting as a transcription factor to mediate the genomic effects of estrogen on vascular gene expression [159]. In pilot experiments (unpublished data) we used PPT, a highly selective agonist for ER-α in order to evaluate which receptor plays a predominant role in the action of 17β-E_2 on flow-mediated dilatation. Flow responses were obtained in myometrial arteries from women with PE before and after 3 hours incubation with PPT (10^{-8}M). This selective ER-α agonist stimulated flow-mediated relaxation in arteries from women with PE (e.g. dilatation at flow rate of 60µl/min: 9±1% PSS vs. 48±9% after PPT, n=4, p=0.04) and it seems that selective stimulation of ER-α mimics the effects of 17β-E_2 as observed in Paper III. Treatment alternatives with estrogenic-like compounds with particular selectivity on endothelial ER-α receptor might be of interest to improve endothelial function in PE.

Scanning electron microscopy of the endothelium in arteries from women with PE provided direct morphological evidence for endothelial dysfunction in the uterine circulation. Morphological evidence for endothelial dysfunction (shrunken, detached, degenerated thin cell membranes, signs of endothelial cell death, cell adhesion, enlarged intercellular junctions) partly

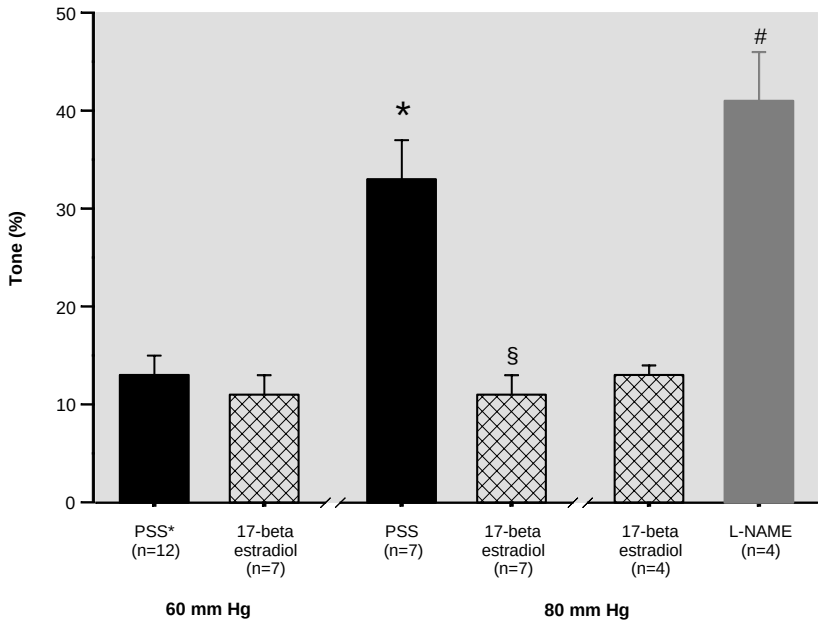


Figure 6. Pressure-induced tone in isolated myometrial arteries from women with preeclampsia. Asterisk, significance between developed tone in physiological salt solution (PSS, n = 7) at 60 mm Hg versus 80 mm Hg; section mark, significance between the tone that was developed at 80mm Hg in PSS versus after incubation with 17β-estradiol (n = 7); number sign, significance between the tone that was developed at 80 mm Hg in 17β-estradiol versus L-NAME (n = 4).

explains the enhanced vascular permeability through enlarged intercellular junctions. This is in accordance with an activated coagulation system in terms of adhered platelets in PE (*see* Figure 6 in Paper III). Whether endothelial cell death in vascular endothelium found in our study is compatible with apoptosis needs to be confirmed using additional techniques. Increased apoptosis and altered expression of different mediators for apoptosis in the placenta from women with PE have been demonstrated previously [210, 211]. Whether endothelial cells death in the endothelium of myometrial arteries from women with PE may be a consequence of estrogen deficiency and/or oxidative stress requires further investigations to be answered.

Functional and morphological evidence for endothelial dysfunction after menopause

Here we present for the first time that isolated resistance arteries from post-menopausal women demonstrate impaired endothelium-dependent dilatation to a flow stimulus, as compared to pre-menopausal women. If present *in vivo* this abnormality may contribute to an increased peripheral vascular resistance. In larger conduit arteries a similar reduction of endothelium-dependent response to flow has been reported [98-103] and this in combination with our observation on severely impaired flow responses in small arteries from the subcutaneous circulation (*see* Figures 7-8) may suggest a presence of an increased atherogenic risk in healthy post-menopausal women. Previous studies have shown that the primary mediator of vasodilation to flow in the skin circulation is nitric oxide [31]. Nitric oxide not only serves as a vasodilator but also

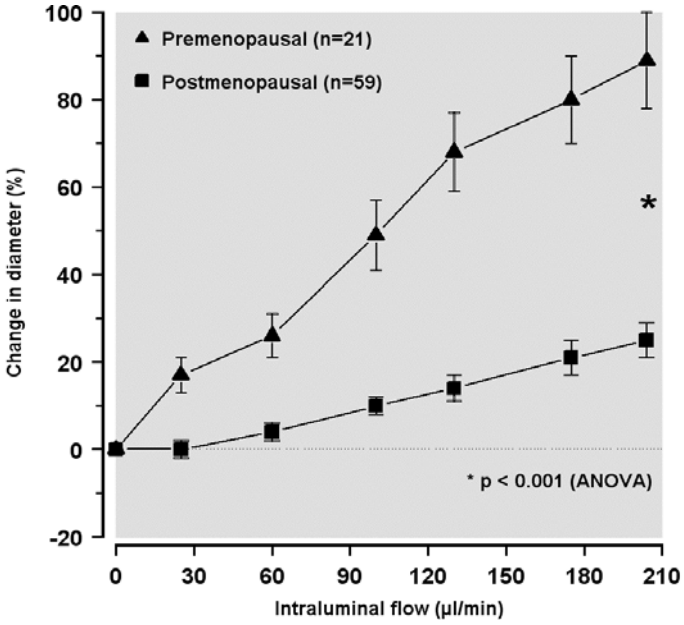


Figure 7. Flow-mediated dilatation, expressed as percent (%) change in diameter after initial pre-constriction with noradrenaline (10^{-6} M) in small subcutaneous arteries from post- and pre-menopausal women.

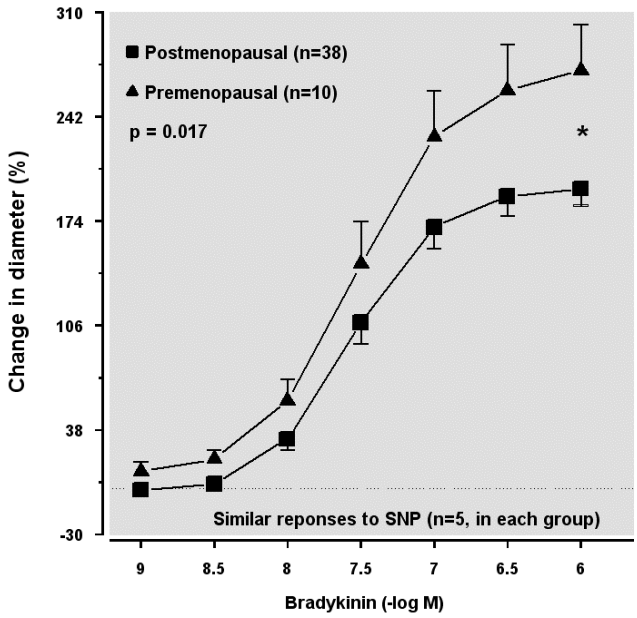
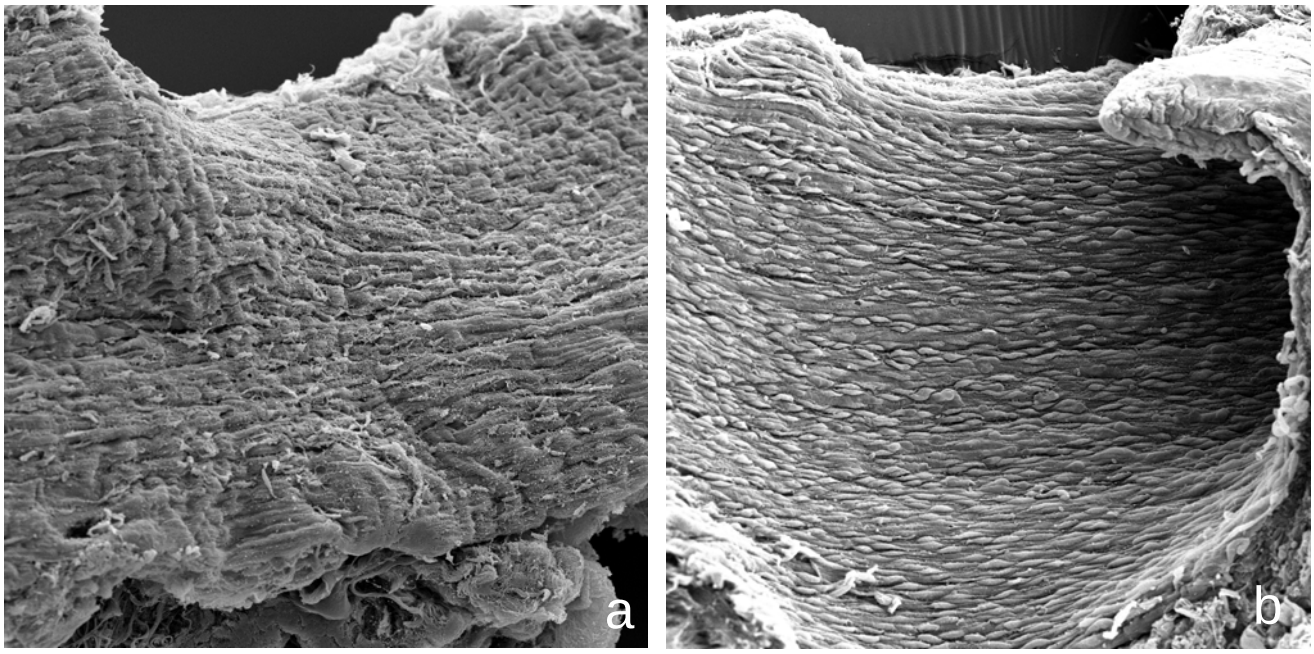


Figure 8. Bradykinin-mediated dilatation expressed as percent change from initial preconstriction with noradrenaline (1µmol/L): in physiological salt solution between arteries from post-menopausal and pre-menopausal women; Sodium nitroprusside (SNP).

contributes many other vasculoprotective pathways including anticoagulation [212], inhibition of leucocyte adhesion and smooth muscle proliferation [213, 214]. Moreover, NO inhibits the activation and expression of certain adhesion molecules [215], production of superoxide anion [216] and oxidation of LDL [217]. Absence of this important physiological response to flow will severely compromise vascular homeostasis in these women. Although differences in endothelial function have been reported in previous studies of pre-menopausal women in different phases of menstrual cycle [218, 219], we and others [220] found no association in this respect.

The observation that responses to BK, a physiological agonist for endothelium-dependent dilatation, were reduced stresses the level of endothelial dysfunction present. Similar relaxations to sodium nitroprusside in pre-and post-menopausal women in our study, and together with similar responses to nitrovasodilators using other experimental approaches (i.e. the forearm plethysmography technique) in pre- and post-menopausal women, [221-223], suggest that the defect in NO synthesis at the level of the endothelial cell is a primary mechanism of vascular dysfunction after menopause. Whether these alterations are related to an ageing process or due to the naturally occurring withdrawal of ovarian hormones is unclear. The absence of estrogens is likely to play a central role as cardiovascular complications occur more often in younger ovarectomized women [2, 224, 225] or women with early menopause [226]. Moreover, numerous *in vitro* investigations exist demonstrating a direct association between estrogen withdrawal and endothelial dysfunction in isolated arteries [227, 228].

It is now widely appreciated that the endothelium is highly important in the resistance circulation and it could be expected that endothelial dysfunction and structural changes in these arteries may modulate and exacerbate the vascular consequences of upstream events, which



Picture 2. Scanning electron microscopy pictures (×300 fold magnification) of endothelial cell layer in isolated arteries from healthy post-menopausal women (a) represents signs of endothelial cell death, activation and coagulation. Pictures of the artery from pre-menopausal women (b) represent continuous layer with tightly connected EC.

could be an initial launching step in the formation of clinical CVD in women after menopause. In our study we found no evidence for increased myogenic tone in the arteries of post-menopausal women in comparison to arteries from pre-menopausal women. It is important to notice th facilitation of pressure-induced tone by several vasoconstrictors (ET-1, AII, NE) has bee demonstrated in animal experiments suggesting that this pathway contributes to the development of hypertensive complications *see rev* [229]. Indeed, similar vasoactive factors (i.e ET-1, AII) have been associated with the development of cardiovascular complications in post-menopausal women [230-232]. Therefore it could be anticipated that in the *in vivo* situation compromised vasodilatation in combination with preserved myogenic tone under influence of increased levels of circulating vasoconstrictors or in the presence of activated sympathetic tone [233] would favour increased peripheral resistance in post- menopausal women.

Our morphological analysis of the endothelial cell layer using scanning electron microscopy have shown clear differences of endothelium morphology (for morphological details *please see* Papers IV-V) in peripheral small arteries from pre- and post-menopausal women and are likely to provide an explanation for the functional differences due to estrogen withdrawal (Picture 2). Notably, the number of endothelial cells within the vascular wall area in arteries from post-menopausal women was significantly reduced and accompanied with cellular disruption and damage. In contrast, a continuous layer of tightly connected endothelial cells with thick plasma membranes in the arteries from pre-menopausal women would assure proper vascular homeostasis. These functional and morphological abnormalities in the resistance vasculature might contribute to the increased cardiovascular risk in post-menopausal women.

Implication for improvement of endothelial dysfunction in the isolated arteries from women after menopause

17β-*Estradiol in vitro (Paper IV), HRT in vivo (Paper V) and endothelial function*

One of the main purposes of our study was to determine whether naturally occurring active form of estrogens - 17β-E_2 might reverse impairment of flow-mediated responses in the isolated arteries from women after menopause. We found that prolonged incubation *in vitro* with physiological concentrations of 17β-E_2 (10^{-7}M or 10^{-8} M) evoked a highly significant flow mediated dilatation in the second flow response in the arteries from the post-menopausal women (*see* Figure 9a-b), and these responses were in principal agreement with those obtained in subcutaneous arteries from pre-menopausal women in our study (*see* Figure 7) or pregnant women [31]. The fact that 17β-E_2 had no effect on flow-mediated dilatation in arteries from pre-menopausal (Paper IV) and pregnant women (data not shown) suggests a critical role for withdrawal of estrogens as a general pathway of endothelial dysfunction after the menopause. Our finding on the pronounced effects of 17β-E_2 being mediated by NO (*see* Figure 9c) in resistance arteries is consistent with findings obtained in our own studies and by others. Importantly, it has been shown that NO is the primary mediator of improved vasodilatation to flow after 3 hours incubation with 17β-E_2 (10^{-8}M) in myometrial arteries from women with PE (*see* Figure 3), and in shear-mediated responses in the small arteries of the skin and uterus in normal pregnant women [31, 234]. It might therefore be suggested that shear-stress signaling resulting in NO release and vasodilatation remains a particular target for estrogen action. Several mechanisms have been proposed by which estrogens may up-regulate NO release in response to shear stress, including the direct stimulation of endothelial nitric oxide (NO) synthase (eNOS) [78, 161-163, 235] or intracellular factors that could augment bioavailability of NO [236]. Recently, a stimulating effect of estrogen on inducible NOS (iNOS) gene expression in mammalian cells has been reported [117], however the physiological relevance of this finding needs to be investigated. Without measurements of NOS protein expression, however, this study cannot distinguish between an estradiol-induced increase in eNOS synthesis or a hypothesized antioxidative effect through up-regulation of antioxidant mechanisms, both of which have been proposed and substantiated in experimental investigations *in vitro* [237] and *in vivo* [84].

The findings from Paper V also demonstrated an increased flow-mediated dilatation after 3 months treatment with E_2 alone and E_2+MPA (*see* Figure 9d) supporting the role of proposed pathways described above. Thus, our results indicate that estradiol may play an important role in up-regulating shear-mediated responses after pre-treatment for 3 hours *in vitro* and are consistent with the results *in vivo*, where prolonged administration of E_2 alone or in combination with MPA, but not MPA alone, improved flow-mediated dilatation in arteries from post-menopausal women. The negligible role of MPA in shear stress mediated responses after treatment further strengthens a fundamental role for estrogen in the responses observed. Our results are in line with several other studies demonstrating that ERT alone has beneficial effect on FMD in conduit arteries in post-menopausal women [98-103]. In addition, it has been shown that unopposed estrogens increased vasodilatory response to flow *in vivo* by almost 40 % in healthy post-

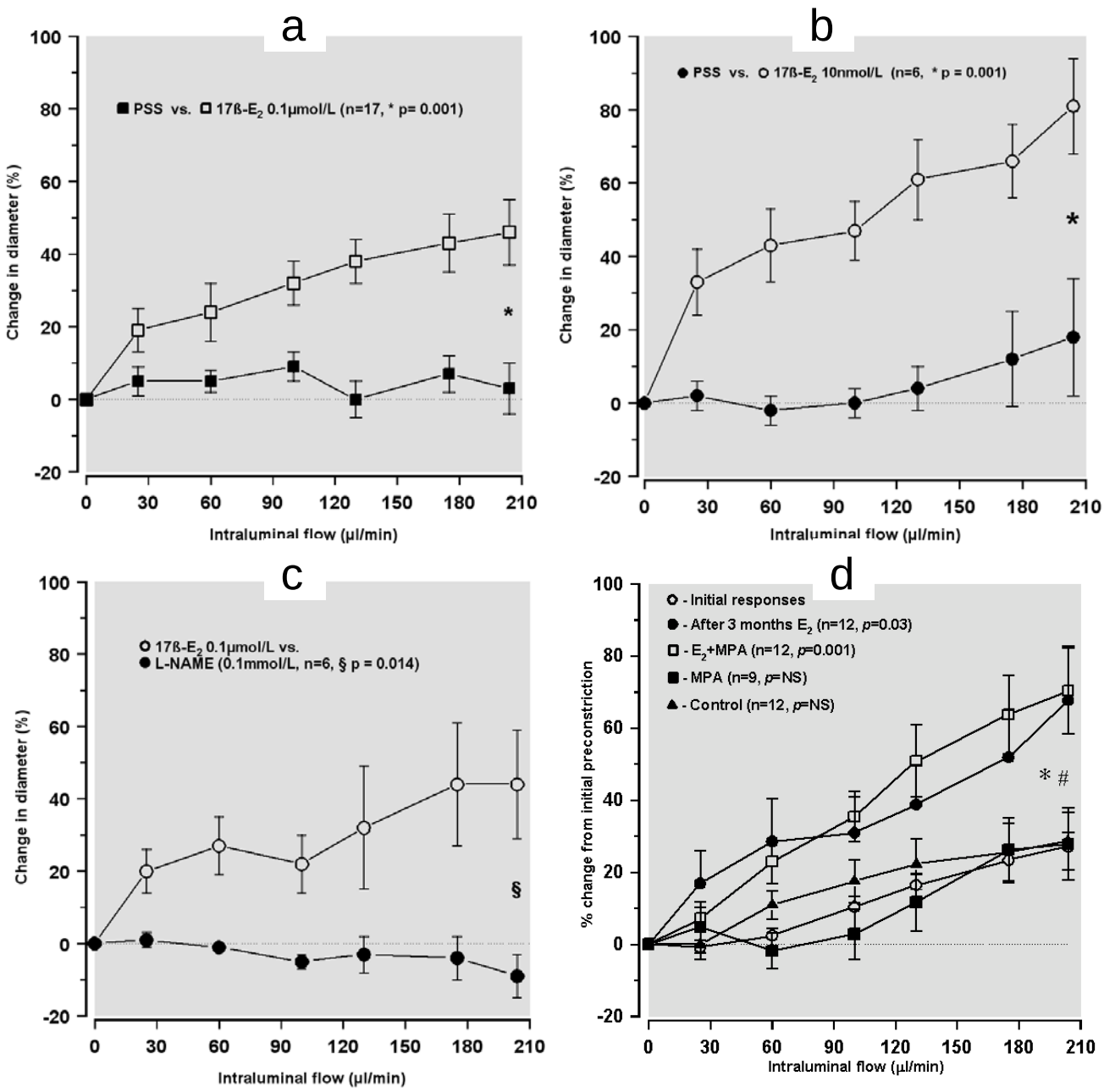


Figure 9. **a:** Flow-mediated dilatation in isolated subcutaneous arteries from post-menopausal women: in physiological salt solution (PSS) versus prolonged incubation with 17β-estradiol (17β-E_2, 10^{-7}M).

b: Flow-mediated dilatation in isolated subcutaneous arteries from post-menopausal women: in physiological salt solution (PSS) versus prolonged incubation with 17β-estradiol (17β-E_2, 10^{-8}M).

c: Flow-mediated dilatation in isolated subcutaneous arteries from post-menopausal women: prolonged incubation with 17β-E_2 versus Nω-nitro-L-arginine methyl ester (L-NAME).

d. Flow-mediated dilatation before and after 3 month treatment with Estradiol (E_2), medroxyprogesterone acetate (MPA), E_2+MPA and control in small subcutaneous arteries from healthy postmenopausal women.

menopausal women, who had not yet developed atherosclerotic vascular disease, but there was no effect of ERT in women with established CVD [155]. In contrast, some studies demonstrated that concomitant administration of progestagens attenuated the stimulating effect of estrogen on bioavailability of NO [154] and reversed the effect of estrogen on FMD in healthy post-menopausal women [103, 156]. However, these findings were not universal, since other studies

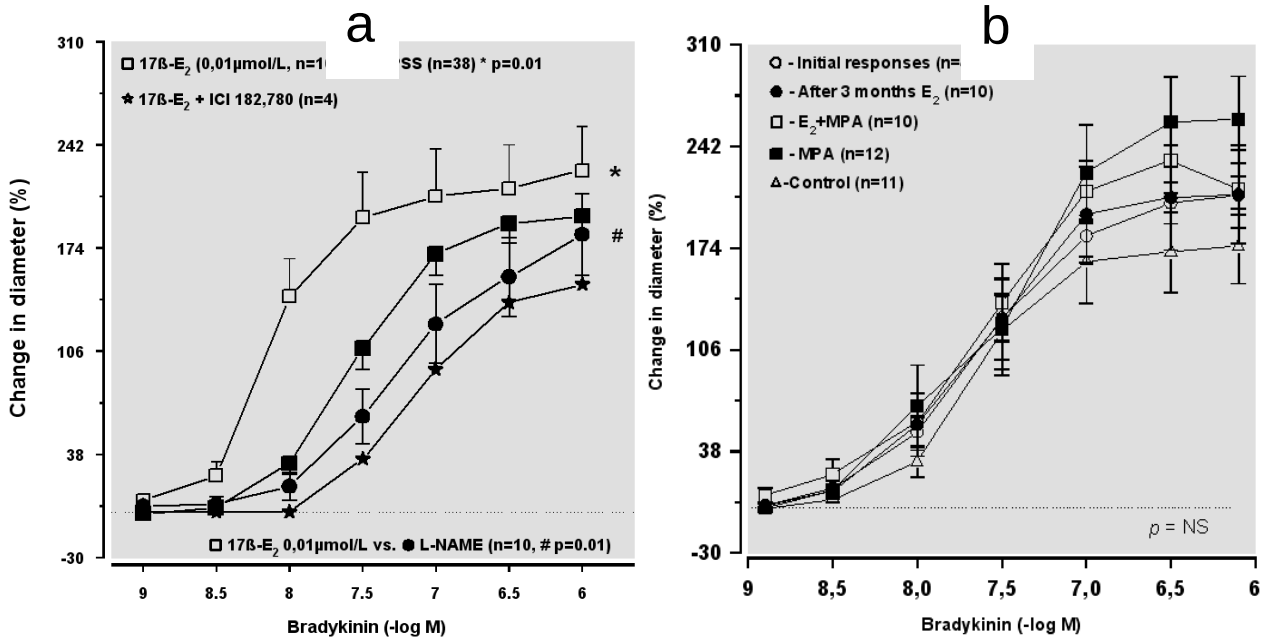


Figure 10. a: Bradykinin-mediated dilatation in arteries from post-menopausal women: in physiological salt solution (PSS) versus after prolonged incubation with 17β estradiol (17β-E_2), ∗ p = 0.01; in 17β-E_2 versus incubation with 17β-E_2 + Nω-nitro-L-arginine methyl ester (L-NAME) # p = 0.01; in 17β-E_2 plus ICI 182,780.We deliberately excluded standard errors of the means in relaxation curve to bradykinin in the presence of 17β-E_2 plus ICI 182,780 to simplify the presentation of the figure.

b: Bradykinin-mediated dilatation before and after 3 month treatment with estradiol (E_2), medroxyprogesterone acetate (MPA) , E_2+MPA and control in small subcutaneous arteries from healthy postmenopausal women.

in line with our observations have demonstrated that combination of estrogen with progestagens improved FMD [99, 100, 157, 158].

We also took an opportunity in this thesis, afforded by the use of perfusion myography technique, to evaluate other mechanisms, which may contribute to estrogen modulation of vascular tone. We were able to show that in addition to profound effects on flow-mediated dilatation, the pre-incubation with 17β-E_2 increased basal release of NO (*see* Figure 11), which then reduced the level of pressure-induced myogenic tone in arteries from post-menopausal women. Physiologically this implies that estrogens are capable of affecting resting as well as haemodynamically modified resistance artery tone. This is in accord with widely reported evidence *in vivo* and *in vitro* for an increase in basal release/availibility of NO in a wide range of physiological, pathological and experimental situations [118, 123, 222].

Although results from Papers IV-V and experimental evidence from others [157, 222, 238] suggested a significant role of E_2 alone *in vitro* or in combination with MPA *in vivo* in the regulation of pressure-induced tone, we could not confirm that 3 months intervention with E_2 alone in contrast to E_2+MPA affects myogenic tone in the resistance arteries from healthy post-menopausal women (*see* Figure 3 in Paper V). It cannot be excluded, however, that the lack of E_2 effect *in vivo* on myogenic tone is due to relative small number of women involved.

In paper IV we also showed as demonstrated previously in forearm circulation of post-menopausal women [223], that responses to endothelium-dependent agonists were reduced as compared to pre-menopausal women (*see* Figure 8) and enhanced by 17β-E_2 in arteries from post-menopausal women (*see* Figure 10a). Interestingly this response was only partially mediated through NO, as evidenced by incomplete inhibition with L-NAME, strongly suggesting

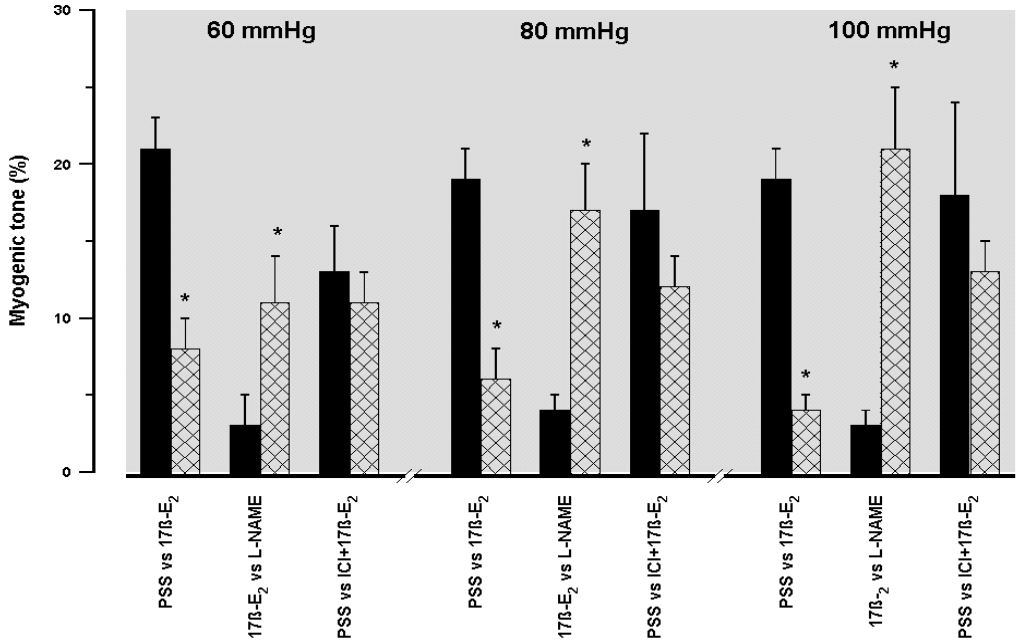
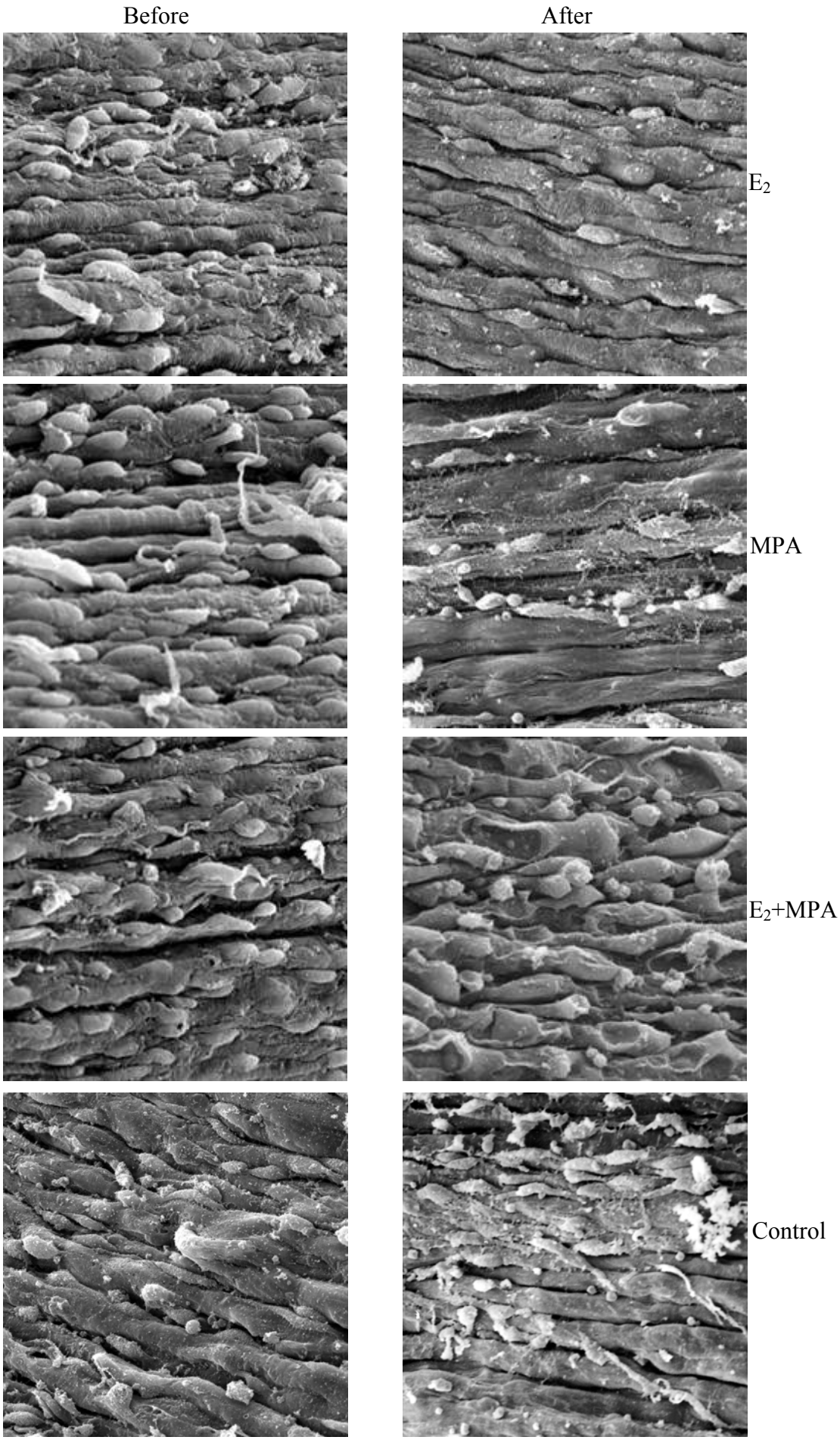


Figure 11. For the simplicity we present pressure-induced mygenic tone developed in response to changes in intraluminal pressure of 60 mmHg, 80 mmHg and 100 mmHg under different experimental conditions (solid versus crosshatched bars) in different arteries: physiological salt solution (PSS) versus 17 beta estradiol (17β-E_2, 0.01 µmol/L) n=15, * p = 0.001; 17β-E_2 versus Nω-nitro-L-arginine methyl ester (L-NAME, 0.1mmol/L) n=8, * p = 0.001; PSS versus ICI 182,780 (ICI) + 17β-E_2 (n=8).

an important up-regulation through other pathways, perhaps cyclooxygenase or EDHF-mediated. In fact, several studies implicated that NO and EDHF equally contributes to BK-mediated dilatation in subcutaneous arteries from women in reproductive age [27, 239], however contribution of EDHF increases significantly with age [240]. Moreover, data from ovarectomized animals provided direct evidence that estrogen deficiency specifically impairs EDHF-mediated vascular actions [241]. Involvement of classical estrogen receptor in our study was indicated by reversal of estradiol induced increment in BK-mediated relaxation by ICI 182,780, however selective ER-α agonist failed to increase BK-mediated dilatation (*see* Figure 4 in Paper IV) suggesting but not proving the relative importance of ER-β. In contrast to our *in vitro* observation that 17β-E_2 up-regulates BK-mediated dilatation, prolonged supplementation with combined HRT (*see* Figure 10b) had no effect on agonist-mediated dilatation irrespective of treatment regime used. The reason for this discrepancy remains unknown. However, it might be speculated that prolonged treatment with combined HRT might influence ion channel function and calcium dynamic responsible for the contribution of EDHF to BK-mediated dilatation. In fact, it has been shown that estrogen-induced dilatation *per se* depends on activation of Ca dependent K channels (BK_{ca} channels) [81] and these channels indeed are directly involved in the EDHF signaling. It could be anticipated therefore that prolonged *in vivo* exposure to circulating estrogens alone or in combination with progestagens may down-regulate the signaling of EDHF due to a possible competitive effect between similar signaling pathways. Finally, recent unpublished data from our laboratory using ER-β knockout animals suggests that ER-β plays an important role in the regulation of EDHF involvement to endothelium-dependent dilatation. Therefore, the relative contribution of ER-α *versus* ER-β to vascular function before and after prolonged treatment with combined HRT might be of importance in this respect.



Picture 3. Scanning electron microscopy (1000 fold magnification) of endothelial cell layer in isolated arteries from the same postmenopausal women before hormonal replacement therapy (HRT) and after treatment with E_2, MPA, E_2+MPA and controls. For details *see* Figure 4 in Paper V.

In addition to combined HRT-induced vascular protective effects in the resistance vasculature of healthy post-menopausal women as demonstrated in our study, long-term administration of these treatment regimes have also effects on markers of endothelial dysfunction and inflammation. It has been shown, that combination of E_2 with progestagens might positively influence levels of powerful vasoconstrictor ET-1, as well as E-selectin, trombomodulin and von Willebrand factor. Increased concentrations of these markers are known to be associated with increased cardiovascular risk [242].

In Paper V we evaluated the effect of conventional HRT regimens on clinical and biochemical parameters in healthy post-menopausal women. As expected, combined HRT (i.e E_2+MPA) favorably influenced the levels of LDL and the ratio of LDL/HDL (*see* Table 1 in Paper V). This observation is in line with many other studies indicating that these alterations could favor vascular protection [8, 243]. Although the E_2 treatment group had a tendency for favorable changes in lipid profile, it did not reach significant levels. Several studies have reported beneficial effects of E_2 on lipid profile [143, 244], and it is possible that in our study the absence of significance is due to a relatively small number of women. In contrast, MPA alone had no effect on plasma lipid composition, further supporting the importance of estrogenic compounds to alter lipid profile.

Finally, our morphological analyses of vascular endothelium in arteries obtained from the same women before and after certain HRT regimen offers additional evidence that 17β-E_2 provides cardiovascular protection in resistance vasculature in healthy post-menopausal women. The protective effects of 17β-E_2 on resistance artery vascular parameters emphasizes the degree of endothelial dysfunction present and this is further enforced by the morphological malfunctions ofsevere endothelial disruption, which was appreciably, reversed after 3 month E_2 supplementation in healthy post-menopausal women. It was apparent that treatment regime with E_2 alone reversed endothelial disruption by promoting the re-endothelialization (Picture 3), decreasing the number of apoptosis-like cells and reducing the quantity of attachments as markers for endothelial cell activation. In contrast, treatment with MPA alone or in combination with E_2 (Picture 3) had no effects (fore details please *see* Paper V). To our knowledge such morphological alterations of endothelial layer in resistance vasculature from healthy post-menopausal women after estrogen therapy have not yet been reported. However estrogens are known to be potent stimulators of cellular growth, they can provide rapid re-endothelialization in animal vascular injury models [114, 129] or protect from hypoxia [245] or oxidative stress-induced [134] endothelial cell apoptosis.

Receptor-dependence, SERMs and endothelial function in vitro (Paper IV)

In our studies we have demonstrated that 17β-E_2 increases endothelium-mediated responses in the isolated arteries from women after menopause *via* ERs, since application of a nonselective ER blocker ICI 182,780 prior incubation with 17β-E_2 (10^{-8}M) diminished the second responses, indicating that in our experimental set-up hormone receptors are required for the action of estrogens. This was a case for 17β-E_2 effects on flow, BK- and pressure induced responses in small subcutaneous arteries from healthy post-menopausal women (*see* Figures 10a and 11). In order to support our functional results, the distribution of ER-α and ER-β within the vascular

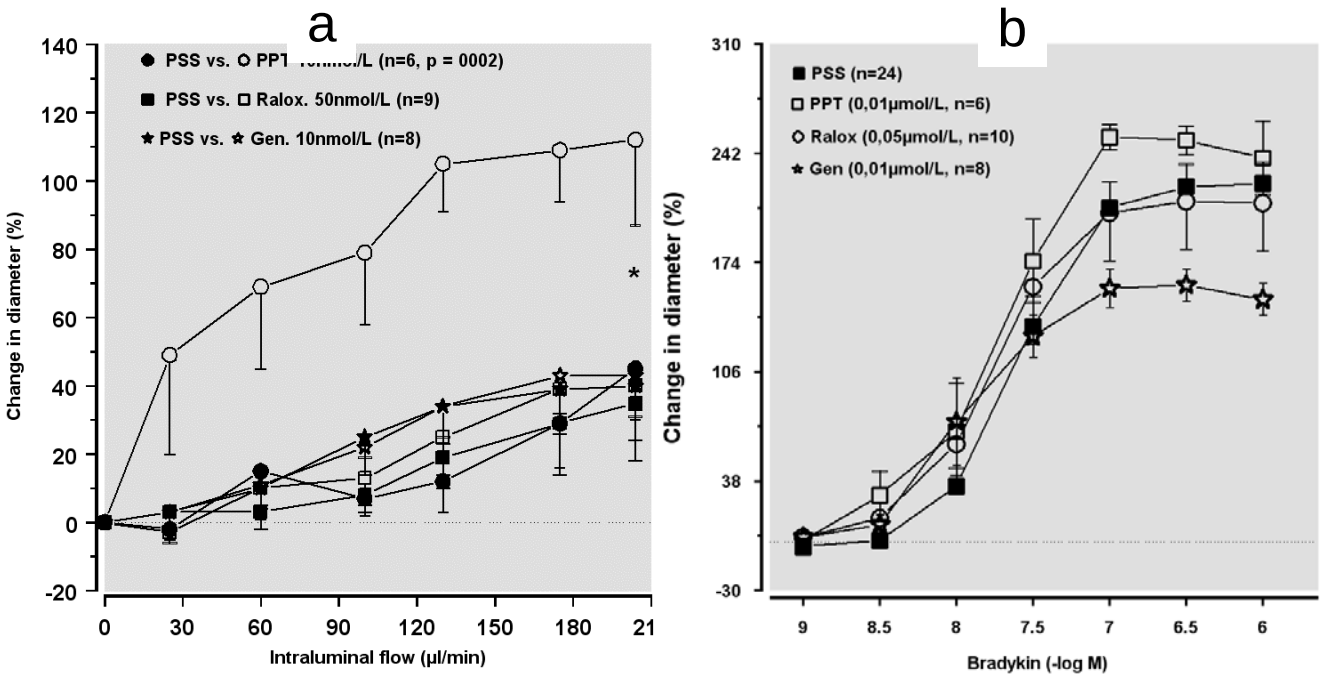


Figure 12. a: Flow-mediated dilatation in isolated subcutaneous arteries from post-menopausal women: in physiological salt solution (PSS) versus prolonged incubation with 4,4', 4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT); in PSS versus prolonged incubation with raloxifene (Ralox); in PSS versus prolonged incubation with genistein (Gen).

b: Bradykinin-mediated dilatation expressed as percent change from initial preconstriction with noradrenaline (1µmol/L): in physiological salt solution (PSS) in arteries from post-menopausal women before and after prolonged incubation with incubation with 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT); after raloxifene (Ralox); after genistein (Gen).

wall was investigated (*see* Figure 7 in Paper IV). We found that both ERs are present in small subcutaneous arteries from post-menopausal women. The expression of ER-α and ER-β in vascular smooth muscle cells was similar, however higher expression of ER-α then ER-β was found in the endothelial cells indicating a predominant role for this receptor in 17β-E_2–mediated effects. Moreover, the selective ER-α agonist PPT stimulated flow-mediated relaxation (*see* Figure 12a), decreased pressure-induced myogenic tone (*see* Figure 13) and had a tendency to improve BK-mediated dilatation in arteries from post-menopausal women (*see* Figure 12b), findings which were similar to those obtained after prolonged incubation with 17β-E_2 (*see* Figure 9a, 10a, 11). In contrast, other SERMs like genistein and raloxifene, known to be more specific stimulators of ER-β had no effect on flow-(*see* Figure 12a) and BK-mediated responses (*see* Figure 12b). The absence of beneficial effects of raloxifene and genistein in contrast to 17β-E_2 and PPT could be explained by the relative importance of ER-α *versus* ER-β for 17β-E_2–mediated up-regulation of endothelium-dependent responses. There is some evidence for favorable ER-β -mediated effects of genistein [246], ER-β-mediated effects of 17β-E_2 on the arterial response to injury [247] and on endothelium-dependent arterial responses in aging animals [248]. However, our findings of a predominance of ER-α in the endothelium from post-menopausal women and the pronounced dilatory effects of the ER-α selective agonist PPT further supports a critical role of ER-α in mediating atheroprotective effects in the resistance vasculature from post-menopausal women.

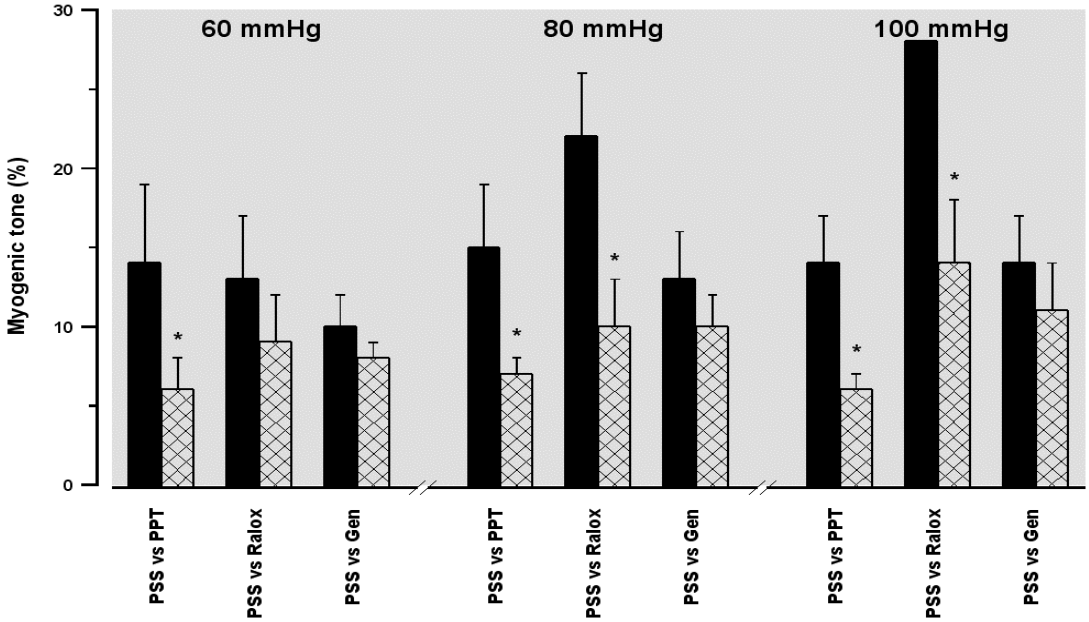


Figure 13. For the simplicity we present pressure-induced mygenic tone developed in response to changes in intraluminal pressure of 60 mmHg, 80 mmHg and 100 mmHg under different experimental conditions (solid vs crosshatched bars) in different arteries: physiological salt solution (PSS) versus 4,4',4"-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT , 0.01 μmol/L, n=6, * $p < 0.05$); PSS versus raloxifene (Ralox, 0.05 μmol/L, n=10, * $p = 0.05$); PSS versus genistein (GEN, 0.01 μmol/L, n=6).

Raloxifene stimulates basal NO release in animal and cell culture models [176, 178]. These findings may explain a decrease in pressure-induced myogenic tone after prolonged incubation with raloxifene in arteries from our post-menopausal women (*see* Figure 13). It has also been shown that Raloxifene attenuates intimal thickening in animal models in response to arterial injury and prevents migration of vascular smooth muscle cells in culture [249, 250]. Recently, however, it has been demonstrated that flow-mediated dilatation in brachial artery in healthy post-menopausal women was unaffected by prolonged treatment with raloxifene [251]. This is in line with our observation in small subcutaneous arteries where raloxifene did not stimulate flow-mediated response. It is therefore unlikely to suggest that treatment alternatives including raloxifene would be of importance for endothelial protection in healthy post-menopausal women. A long-term, ongoing randomized prevention trial to determine whether raloxifene lowers the risk of cardiovascular events in post-menopausal women should give more information on this issue [175].

The lower incidence of CVD in Asian countries has been related to phytoestrogens consumption. Therapy with genistein increases plasma nitrites/nitrates levels and decreases plasma ET-1 levels in post-menopausal women [182]. The therapy may also up-regulate eNOS activity [252]. In addition, Genistein produces acute NO-dependent dilation of human forearm vasculature with similar potency as 17β-E_2 [253]. Daily administration of genistein increased FMD of the brachial artery after 6 months of therapy [182] and one year of genistein therapy improved FMD in post-menopausal women to a similar extent as estrogen/progestin regimen [253]. These observations were made on peripheral conduit arteries in post-menopausal woman but have important implications on the possible vascular protective effects of genistein on other vascular beds, e.g. subcutaneous resistance circulation. In contrast, several studies have reported

that short-term (2 weeks) oral isoflavone supplements or soy supplementation containing primarily genistein and daidzein for 3 month had no effects on FMD in healthy menopausal women [254, 255]. Accordingly, in our *in vitro* study prolonged incubation with genistein had no overall influence on resistance artery parameters (*see* Figures 12-13). It is therefore questionable to suggest that genistein could be introduced as an alternative to improve resistance artery function in healthy pots-menopausal women.

On the other hand, it has been demonstrated that dietary soy protein supplement significantly reduces blood pressure and improves LDL/HDL cholesterol ratio and triglycerides in healthy post-menopausal women [169, 170, 254]. It might be anticipated therefore that phytoestrogens may play a role in the prevention of cardiovascular disease by primarily improving lipid profiles rather then having direct influence on the arterial function. More research is needed to specify the mechanisms of action of these compounds in hyperlipidemic and hypertensive populations, before public health recommendations can be made. New SERMs with pronounced selective ER-α or ER-β activities [164] might be a powerful tool to investigate vascular effects, since it has been suggested that ER-β can modulate ER-α activity in a response-specific and dose-dependent manner [256]. More knowledge regarding SERMs and their effects on cardiovascular events will emerge from ongoing trials.

Future perspectives

Our studies in isolated small arteries provided important evidence for endothelial dysfunction present in the maternal circulation during pregnancy complicated by PE and in women after menopause. The evidence of vascular malfunctions present in this thesis enforces the role of resistance circulation as a primary target for injury and protection. At present, there is no screening method that accurately predicts preeclampsia and currently considered or newly discovered potential predictive markers for this disorder are of critical importance. We have also substantiated our knowledge about the physiological impact of the naturally occurring female hormone 17β-E_2 and currently available SERMs on resistance artery function that is of fundamental importance for woman's cardiovascular health.

Contemporary demographic trends, including increased maternal age *per se*, fewer pregnancies and decreasing birthrate, widespread usage of hormonal contraceptives [257, 258] may negatively influence women's cardiovascular health. Retrospective analyses clearly indicate a relationship between PE and an increased risk for CVD after menopause [3-5]. It shows that the consequences of endothelial dysfunction in PE affects women's cardiovascular health even in reproductive age [16] and necessitate us to consider measures for earlier cardiovascular protection. The outcome of human pregnancy will influence not only the mother's but also the offspring's later cardiovascular health.

Although large observational trials have failed to substantiate the role for combined HRT for prevention of CVD after menopause, the role for 17β-E_2 supplementation as a powerful tool for improving vascular function in post-menopausal women is still undefeated and implies that the individual's unique personal need and risk profile must be assessed to offer optimal therapeutic alternatives. Our observations further emphasize the necessity to find endothelial-selective SERMs with ideal vasculo-protective properties and without side effects on women's health and life quality. Future research may yield better insights into the processes that occur in the vascular wall and may provide novel means to modulate cardiovascular health.

General conclusions

1. Microparticles from women with PE in contrast to those obtained from normal pregnant women cause endothelial dysfunction in isolated myometrial arteries from normal pregnant women, whilst other preeclamptic plasma constituents protects the endothelium from this effect. Microparticles might be considered as a candidate for the unknown circulating factor/s, involved in the development of endothelial dysfunction in PE.

2. VEGF impaires BK-mediated dilatation and enhances basal tone possibly through the ET-1- related pathway in the resistance vasculature from normal pregnant women. In addition, it also increases permeability similar to that obtained in arteries from women with preeclampsia. This might indicate a potential role for VEGF in the development of endothelial dysfunction in pregnancy. Ang-1 inhibited the VEGF- induced vascular leakage and may be beneficial from a preventive and/or therapeutic point of view in the management of PE. It should be of importance to evaluate the biological role of sflt-1 in the maternal circulation in normal pregnancy and PE in order to improve our knowledge about the contribution of VEGF to the pathogenesis of this disorder.

3. 17β-E improves endothelial function through up-regulation of flow-mediated dilatation and reduction of pressure-induced myogenic tone in resistance myometrial arteries from women with PE. These effects are NO mediated and suggest a possible role for estrogens in improving uteroplacental blood flow in PE. Treatment alternatives with estrogenic-like compounds with particular selectivity on endothelial ER-α might be of interest to improve endothelial function in PE.

4. Functional and morphological evidence for impaired endothelial function obtained in isolated subcutaneous resistance arteries from healthy post-menopausal women indicates that these women are at an increased risk to develop cardiovascular complications. ER-α is a predominant mediator of the beneficial effects of 17β-E_2 on resistance artery function *in vitro*, since only PPT but not raloxifene or genistein reproduced the effect.

5. Oral administration of E_2 had beneficial effects on flow-mediated responses and endothelial morphology. Combined HRT improved flow-mediated dilatation, plasma lipid composition and reduced pressure-induced myogenic tone. However, MPA alone had no effect on any of the parameters studied in isolated subcutaneous arteries from healthy post-menopausal women. HRT based on E_2 alone or in combination with MPA may be beneficial for resistance artery function in healthy post-menopausal women.

Acknowledgements

I would like to thank all the **patients, volunteers** and **personnel** at the Department of Obstetrics and Gynecology, Huddinge University Hospital for their support and assistance. In particular, I wish to express my appreciation and thanks to:

Doctor Karolina Kublickiene, my principal tutor, for her never-ending support, guidance and inspiration during my PhD studies. Also for introducing me to life in Sweden and the beginnings of my new career in research. A wonderful supervisor and friend.

Associate Professor/Docent Henry Nisell, my co-supervisor, for his trust in me, encouragement, careful reviews of the manuscripts and helpful scientific discussions.

Professor Britt Marie Landgren, for sharing her knowledge of the research world about the menopause and nice collaboration. **Margaretha Ström, Lena Ydenius and Mia Karlsson,** nurses from the Women's Health Department, for encouraging patients to volunteer in studies and helping me in the collection of samples. **Docent Aino Fianu-Jonasson**, **Doctors Jie Hu** and **Lena Edwall,** for great help when needed and **Professor Kjell Carlström,** for help with hormone analysis.

My co-authors Dr. Khalid B. Islam for sharing his knowledge of VEGF with me and our interesting and fruitful discussions. **Dr. Marja VanWijk** and Dutch colleagues at Academic Medical Center, Amsterdam, for exciting collaboration, research and pleasant time together. **Doctor Yorgos Nikas** for his help and introduction to scanning electron microscopy. **Doctor Milita Crisby and Nasrin Nahar** for important collaboration about the distribution of estrogen receptors.

Professor Lucilla Poston from the Department of Obstetrics and Gynecology, St. Thomas's Hospital, London, for her support and stimulation during our ongoing scientific collaboration and for great appreciation at the scientific meetings abroad.

Professors Outi Hovatta and **Magnus Westgren, Docent George Evaldson**, the Head of our Department, for support over the years.

Professor Ruta Nadisauskiene from the Kaunas Medical University, Lithuania for starting me off in the right direction and believing in me.

Natalia Cruz, Leonid Luksha, Lusine Aghajanova and Gillian Douglas my great fellow colleagues in the laboratory for their friendship and support, warmth atmosphere in everyday life in the lab plus many nice times spent together.

Docent Stefan Nilsson from KaroBio AB, for providing us with Raloxifene.

Doctor Marius Kublickas for always solving my problems and being there when I needed you.
Katarina Oxelbeck, secretary at out Department, for all-round assistance.

My other fellow colleagues at NOVUM and Department: **José Inzunza**, **Ami Strömberg**, **Jennifer Scott, Lev Levkov**, **Pu Zhang, Julius Hreinsson, Victoria Keros, Inger Britt Carlsson, Mia Lindeberg, Karin Gertow, Lars Ärhlund Richter, Susanne Wolbank, Anders Rehn, Heli Teerijoki, Marta Imreh, Hanna Karlsson, Gayane Avetisyan and many others,** for their friendship and support, and for great celebrations at our "Champaign and Chocolate corner".

My friends Dr. Virgilijus Rudzinskas, Vijoleta Valantiejute and others at Kaunas Family Centre for their silent, but most important support.

Inga, the most beautiful person in my entire life and our children Jorile and Benas.

The studies were supported by grants from: The Swedish Research Council, Karolinska Institutet, Heart and Lung Foundation, Åke Wiberg Foundation, Tore Nilsons Foundation, Harald Jeanssons, Harald & Greta Jeanssons Foundation, The General Maternity Hospital Memory Foundation, The Swedish Medical Society and The Swedish Society for Medical Research.

References

1. American Heart Association. 2003. International Cardiovascular Disease Statistics.1-10.
2. Colditz, G.A., Willett, W.C., Stampfer, M.J., Rosner, B., Speizer, F.E., and Hennekens, C.H. 1987. Menopause and the risk of coronary heart disease in women. *N Engl J Med* 316:1105-1110.
3. Jonsdottir, L.S., Arngrimsson, R., Geirsson, R.T., Sigvaldason, H., and Sigfusson, N. 1995. Death rates from ischemic heart disease in women with a history of hypertension in pregnancy. *Acta Obstetricia et Gynecologica Scandinavica* 74:772-776.
4. Smith, G.C.S., Pell, J.P., and Walsh, D. 2001. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129290 births. *The Lancet* 357:2002-2006.
5. Irgens, H.U., Reisater, L., Irgens, L.M., Lie, R.T., and Roberts, J.M. 2001. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study Pre-eclampsia and cardiovascular disease later in life: who is at risk? *BMJ* 323:1213-1217.
6. Mulvany, M.J., and Aalkjaer, C. 1990. Structure and function of small arteries. *Physiol Rev* 70:921-961.
7. VanWijk, M.J., Kublickiene, K., Boer, K., and VanBavel, E. 2000. Vascular function in preeclampsia. *Cardiovasc Res* 47:38-48.
8. Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., and Vittinghoff, E. 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *Jama* 280:605-613.
9. Writing Group for the Women's Health Initiative Investigators. 2002. Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results From the Women's Health Initiative Randomized Controlled Trial. *JAMA* 288:321-333.
10. Vita, J.A., and Keaney, J.F., Jr. 2001. Hormone replacement therapy and endothelial function: the exception that proves the rule? *Arterioscler Thromb Vasc Biol* 21:1867-1869.
11. Celermajer, D.S., Sorensen, K.E., Bull, C., Robinson, J., and Deanfield, J.E. 1994. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* 24:1468-1474.
12. Murphy, S.L. 2000. Deaths: final data for 1998. *Natl Vital Stat Rep* 48:1-105.
13. Robillard, P.-Y. 2002. Interest in preeclampsia for researchers in reproduction. *Journal of Reproductive Immunology* 53:279-287.
14. Meis, P., Goldenberg, R., Mercer, B., Iams, J., Moawad, A., Miodovnik, M., Menard, M., Caritis, S., Thurnau, G., Bottoms, S., et al. 1998. The preterm prediction study: Risk factors for indicated preterm births. *American Journal of Obstetrics and Gynecology* 178:562-567.
15. Laivuori, H., Tikkanen, M.J., and Ylikorkala, O. 1996. Hyperinsulinemia 17 years after preeclamptic first pregnancy. *J Clin Endocrinol Metab* 81:2908-2911.
16. Chambers, J.C., Fusi, L., Malik, I.S., Haskard, D.O., De Swiet, M., and Kooner, J.S. 2001. Association of maternal endothelial dysfunction with preeclampsia. *Jama* 285:1607-1612.
17. Hubel, C.A., Snaedal, S., Ness, R.B., Weissfeld, L.A., Geirsson, R.T., Roberts, J.M., and Arngrimsson, R. 2000. Dyslipoproteinaemia in postmenopausal women with a history of eclampsia. *Bjog* 107:776-784.
18. Caritis, S., Sibai, B., Hauth, J., Lindheimer, M., VanDorsten, P., Klebanoff, M., Thom, E., Landon, M., Paul, R., Miodovnik, M., et al. 1998. Predictors of pre-eclampsia in women at high risk. *American Journal of Obstetrics and Gynecology* 179:946-951.
19. Dekker, G., and Sibai, B. 2001. Primary, secondary, and tertiary prevention of pre-eclampsia. *Lancet* 357:209-215.
20. Kuller, J., D'Andrea, N., and McMahon, M. 2001. Renal biopsy and pregnancy. *American Journal of Obstetrics and Gynecology* 184:1093-1096.
21. Roberts, J.M. 1998. Endothelial dysfunction in preeclampsia. *Semin Reprod Endocrinol* 16:5-15.
22. Taylor, R.N., de Groot, C.J., Cho, Y.K., and Lim, K.H. 1998. Circulating factors as markers and mediators of endothelial cell dysfunction in preeclampsia. *Semin Reprod Endocrinol* 16:17-31.
23. Myers, J.E., and Baker, P.N. 2002. Hypertensive diseases and eclampsia. *Curr Opin Obstet Gynecol* 14:119-125.
24. Wolff, K., Nisell, H., Carlstrom, K., Kublickiene, K., Hemsen, A., Lunell, N.O., and Lindblom, B. 1996. Endothelin-1 and big endothelin-1 levels in normal term pregnancy and in preeclampsia. *Regul Pept* 67:211-216.
25. Nisell, H., Wolff, K., Hemsen, A., Lindblom, B., Lunell, N.O., and Lundberg, J.M. 1991. Endothelin, a vasoconstrictor important to the uteroplacental circulation in pre-eclampsia. *J Hypertens Suppl* 9:S168-169.
26. McCarthy, A.L., Woolfson, R.G., Raju, S.K., and Poston, L. 1993. Abnormal endothelial cell function of resistance arteries from women with preeclampsia. *Am J Obstet Gynecol* 168:1323-1330.
27. Knock, G.A., and Poston, L. 1996. Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. *Am J Obstet Gynecol* 175:1668-1674.
28. Ashworth, J.R., Warren, A.Y., Baker, P.N., and Johnson, I.R. 1997. Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. *Br J Obstet Gynaecol* 104:1152-1158.
29. Kublickiene, K.R., Grunewald, C., Lindblom, B., and Nisell, H. 1998. Myogenic and endothelial properties in myometrial resistance arteries from women with preeclampsia. *Hypertension in Pregnancy* 17:271-281.
30. Pascoal, I.F., Lindheimer, M.D., Nalbantian-Brandt, C., and Umans, J.G. 1998. Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. *J Clin Invest* 101:464-470.
31. Cockell, A.P., and Poston, L. 1997. Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. *Hypertension* 30:247-251.
32. Kublickiene, K.R., Lindblom, B., Kruger, K., and Nisell, H. 2000. Preeclampsia: evidence for impaired shear stress-mediated nitric oxide release in uterine circulation. *Am J Obstet Gynecol* 183:160-166.
33. Sikkema, J.M., van Rijn, B.B., Franx, A., Bruinse, H.W., de Roos, R., Stroes, E.S.G., and van Faassen, E.E. 2001. Placental Superoxide is Increased in Pre-eclampsia. *Placenta* 22:304-308.
34. Hubel, C.A. 1999. Oxidative stress in the pathogenesis of preeclampsia. *Proc Soc Exp Biol Med* 222:222-235.
35. Roberts, J.M., and Cooper, D.W. 2001. Pathogenesis and genetics of pre-eclampsia. *Lancet* 357:53-56.
36. Chappell, L.C., Seed, P.T., Briley, A.L., Kelly, F.J., Lee, R., Hunt, B.J., Parmar, K., Bewley, S.J., Shennan, A.H., Steer, P.J., et al. 1999. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. *Lancet* 354:810-816.
37. Horstman, L.L., and Ahn, Y.S. 1999. Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol* 30:111-142.
38. Joop, K., Berckmans, R.J., Nieuwland, R., Berkhout, J., Romijn, F.P., Hack, C.E., and Sturk, A. 2001. Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thromb Haemost* 85:810-820.
39. Smarason, A.K., Sargent, I.L., Starkey, P.M., and Redman, C.W. 1993. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. *Br J Obstet Gynaecol* 100:943-949.

40. Nelson, D.M. 1996. Apoptotic changes occur in syncytiotrophoblast of human placental villi where fibrin type fibrinoid is deposited at discontinuities in the villous trophoblast. *Placenta* 17:387-391.
41. Knight, M., Redman, C.W.G., Linton, E.A., and Sargent, I.L. 1998. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *British Journal of Obstetrics and Gynaecology* 105:632-640.
42. Van Wijk, M.J., Boer, K., Nisell, H., Smarason, A.K., Van Bavel, E., and Kublickiene, K.R. 2001. Endothelial function in myometrial resistance arteries of normal pregnant women perfused with syncytiotrophoblast microvillous membranes. *British Journal of Obstetrics and Gynaecology* 108:967-972.
43. Boulanger, C.M., Scoazec, A., Ebrahimian, T., Henry, P., Mathieu, E., Tedgui, A., and Mallat, Z. 2001. Circulating Microparticles From Patients With Myocardial Infarction Cause Endothelial Dysfunction. *Circulation* 104:2649-2652.
44. VanWijk, M.N.R., Boer K, van der Post JAM, van Bavel E, Sturk A. 2002. Microparticle subpopulations are increased in preeclampsia: possible involvement in vascular dysfunction? *Am J Obstet Gynecol* in press.
45. Gonzalez-Quintero, V.H., Jimenez, J.J., Jy, W., Mauro, L.M., Hortman, L., O'Sullivan, M.J., and Ahn, Y. 2003. Elevated plasma endothelial microparticles in preeclampsia. *Am J Obstet Gynecol* 189:589-593.
46. Barry, O.P., Pratico, D., Lawson, J.A., and FitzGerald, G.A. 1997. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 99:2118-2127.
47. Barry, O.P., Pratico, D., Savani, R.C., and FitzGerald, G.A. 1998. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest* 102:136-144.
48. Mesri, M., and Altieri, D.C. 1998. Endothelial cell activation by leukocyte microparticles. *J Immunol* 161:4382-4387.
49. Mesri, M., and Altieri, D.C. 1999. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 274:23111-23118.
50. Bizios, R., Lai, L.C., Cooper, J.A., Del Vecchio, P.J., and Malik, A.B. 1988. Thrombin-induced adherence of neutrophils to cultured endothelial monolayers: increased endothelial adhesiveness. *J Cell Physiol* 134:275-280.
51. Rodgers, G.M., Taylor, R.N., and Roberts, J.M. 1988. Preeclampsia is associated with a serum factor cytotoxic to human endothelial cells. *American Journal of Obstetrics and Gynecology* 159:908-914.
52. de Groot, C.J., Davidge, S.T., Friedman, S.A., McLaughlin, M.K., Roberts, J.M., and Taylor, R.N. 1995. Plasma from preeclamptic women increases human endothelial cell prostacyclin production without changes in cellular enzyme activity or mass. *American Journal of Obstetrics and Gynecology* 172:976-985.
53. Baker, P.N., Davidge, S.T., and Roberts, J.M. 1995. Plasma from women with preeclampsia increases endothelial cell nitric oxide production. *Hypertension* 26:244-248.
54. Wellings MB, R.P., Brockelsby PhD, J.C., and Baker BM, B., DM, Philip N. 1998. Activation of Endothelial Cells by Plasma From Women With Preeclampsia: Differential Effects on Four Endothelial Cell Types. *Journal of the Society for Gynecologic Investigation* 5:31-37.
55. Ashworth, J.R., Warren, A.Y., Johnson, I.R., and Baker, P.N. 1998. Plasma from pre-eclamptic women and functional change in myometrial resistance arteries. *British Journal of Obstetrics and Gynaecology* 105:459-461.
56. Hayman, R., Warren, A., Johnson, I., and Baker, P. 2001. Inducible change in the behavior of resistance arteries from circulating factor in preeclampsia: an effect specific to myometrial vessels from pregnant women. *Am J Obstet Gynecol* 184:420-426.
57. Davidge, S.T., Baker, P.N., and Roberts, J.M. 1995. NOS expression is increased in endothelial cells exposed to plasma from women with preeclampsia. *Am J Physiol* 269:H1106-1112.
58. Baker, P.N., Davidge, S.T., Barankiewicz, J., and Roberts, J.M. 1996. Plasma of Preeclamptic Women Stimulates and Then Inhibits Endothelial Prostacyclin. *Hypertension* 27:56-61.
59. Hayman, R., Brockelsby, J., Kenny, L., and Baker, P. 1999. Preeclampsia: the endothelium, circulating factor(s) and vascular endothelial growth factor. *J Soc Gynecol Investig* 6:3-10.
60. Hayman, R., Warren, A., Brockelsby, J., Johnson, I., and Baker, P. 2000. Plasma from women with pre-eclampsia induces an in vitro alteration in the endothelium-dependent behaviour of myometrial resistance arteries. *Bjog* 107:108-115.
61. Ferrara, N. 2001. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280:C1358-1366.
62. Jelkmann, W. 2001. Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem* 47:617-623.
63. Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., and Ferrara, N. 1993. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362:841-844.
64. Ni, Y., May, V., Braas, K., and Osol, G. 1997. Pregnancy augments uteroplacental vascular endothelial growth factor gene expression and vasodilator effects. *Am J Physiol* 273:H938-944.
65. Storment, J.M., Meyer, M., and Osol, G. 2000. Estrogen augments the vasodilatory effects of vascular endothelial growth factor in the uterine circulation of the rat. *Am J Obstet Gynecol* 183:449-453.
66. Zhou, Y., McMaster, M., Woo, K., Janatpour, M., Perry, J., Karpanen, T., Alitalo, K., Damsky, C., and Fisher, S.J. 2002. Vascular Endothelial Growth Factor Ligands and Receptors That Regulate Human Cytotrophoblast Survival Are Dysregulated in Severe Preeclampsia and Hemolysis, Elevated Liver Enzymes, and Low Platelets Syndrome. *Am J Pathol* 160:1405-1423.
67. Simmons, L.A., Hennessy, A., Gillin, A.G., and Jeremy, R.W. 2000. Uteroplacental blood flow and placental vascular endothelial growth factor in normotensive and pre-eclamptic pregnancy. *BJOG* 107:678-685.
68. Bosio, P.M., Wheeler, T., Anthony, F., Conroy, R., O'Herlihy, C., and McKenna, P. 2001. Maternal plasma vascular endothelial growth factor concentrations in normal and hypertensive pregnancies and their relationship to peripheral vascular resistance. *Am J Obstet Gynecol* 184:146-152.
69. Thurston, G., Rudge, J.S., Ioffe, E., Zhou, H., Ross, L., Croll, S.D., Glazer, N., Holash, J., McDonald, D.M., and Yancopoulos, G.D. 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 6:460-463.
70. Brockelsby, J., Hayman, R., Ahmed, A., Warren, A., Johnson, I., and Baker, P. 1999. VEGF via VEGF receptor-1 (Flt-1) mimics preeclamptic plasma in inhibiting uterine blood vessel relaxation in pregnancy: implications in the pathogenesis of preeclampsia. *Lab Invest* 79:1101-1111.
71. Rosing, U., and Carlstrom, K. 1984. Serum levels of unconjugated and total oestrogens and dehydroepiandrosterone, progesterone and urinary oestriol excretion in pre-eclampsia. *Gynecol Obstet Invest* 18:199-205.
72. Zeisler, H., Jirecek, S., Hohlagschwandtner, M., Knofler, M., Tempfer, C., and Livingston, J.C. 2002. Concentrations of estrogens in patients with preeclampsia. *Wien Klin Wochenschr* 114:458-461.
73. Ekbom, A., Trichopoulos, D., Adami, H.O., Hsieh, C.C., and Lan, S.J. 1992. Evidence of prenatal influences on breast cancer risk. *Lancet* 340:1015-1018.
74. Innes, K.E., and Byers, T.E. 1999. Preeclampsia and breast cancer risk. *Epidemiology* 10:722-732.
75. Cohn, B.A., Cirillo, P.M., Christianson, R.E., van den Berg, B.J., and Siiteri, P.K. 2001. Placental characteristics and reduced risk of maternal breast cancer. *J Natl Cancer Inst* 93:1133-1140.

76. Behl C., Widmann M., Trapp T., and Holsboer F. 1995. 17-[beta] Estradiol Protects Neurons from Oxidative Stress-Induced Cell Death in Vitro. *Biochemical and Biophysical Research Communications* 216:473-482.
77. Leal, A.M., Ruiz-Larrea, M.B., Martinez, R., and Lacort, M. 1998. Cytoprotective actions of estrogens against tert-butyl hydroperoxide-induced toxicity in hepatocytes. *Biochemical Pharmacology* 56:1463-1469.
78. Chambliss, K.L., Yuhanna, I.S., Mineo, C., Liu, P., German, Z., Sherman, T.S., Mendelsohn, M.E., Anderson, R.G., and Shaul, P.W. 2000. Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ Res* 87:E44-52.
79. 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* 87:677-682.
80. Goetz, R.M., Thatte, H.S., Prabhakar, P., Cho, M.R., Michel, T., and Golan, D.E. 1999. Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 96:2788-2793.
81. Rosenfeld, C.R., White, R.E., Roy, T., and Cox, B.E. 2000. Calcium-activated potassium channels and nitric oxide coregulate estrogen-induced vasodilation. *Am J Physiol Heart Circ Physiol* 279:H319-328.
82. Salom, J.B., Burguete, M.C., Perez-Asensio, F.J., Torregrosa, G., and Alborch, E. 2001. Relaxant effects of 17-beta-estradiol in cerebral arteries through Ca(2+) entry inhibition. *J Cereb Blood Flow Metab* 21:422-429.
83. Rupnow, H.L., Phernetton, T.M., Shaw, C.E., Modrick, M.L., Bird, I.M., and Magness, R.R. 2001. Endothelial vasodilator production by uterine and systemic arteries. VII. Estrogen and progesterone effects on eNOS. *Am J Physiol Heart Circ Physiol* 280:H1699-1705.
84. Vagnoni, K.E., Shaw, C.E., Phernetton, T.M., Meglin, B.M., Bird, I.M., and Magness, R.R. 1998. Endothelial vasodilator production by uterine and systemic arteries. III. Ovarian and estrogen effects on NO synthase. *Am J Physiol* 275:H1845-1856.
85. Celermajer, D.S., Sorensen, K.E., Spiegelhalter, D.J., Georgakopoulos, D., Robinson, J., and Deanfield, J.E. 1994. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol* 24:471-476.
86. Gordon, T., Kannel, W.B., Hjortland, M.C., and McNamara, P.M. 1978. Menopause and coronary heart disease. The Framingham Study. *Ann Intern Med* 89:157-161.
87. Greenland, P., Reicher-Reiss, H., Goldbourt, U., and Behar, S. 1991. In-hospital and 1-year mortality in 1,524 women after myocardial infarction. Comparison with 4,315 men. *CIRCULATION* 83:484-491.
88. Tunstall-Pedoe, H., Kuulasmaa, K., Amouyel, P., Arveiler, D., Rajakangas -, A.M., and Pajak, A. 1994. Myocardial infarction and coronary deaths in the World Health Organization MONICA project: Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation* 90:583-612.
89. Tunstall-Pedoe, H., Morrison, C., Woodward, M., Fitzpatrick, B., and Watt, G. 1996. Sex Differences in myocardial infarction and coronary deaths in the scottish MONICA population of Glasgow 1985-1991: Presentation, diagnosis, treatment, and 28-day case fatality of 3991 events in men and 1551 events in women. *Circulation* 93:1981-1992.
90. Marrugat, J., Anto, J.M., Sala, J., Masia, R., Albert, X., Bassaganyas, J., Guerra, J.C., Perez, G., Perez-Ayuso, M.J., Ribalta, A., et al. 1994. Influence of gender in acute and long-term cardiac mortality after a first myocardial infarction. *Journal of Clinical Epidemiology* 47:111-118.
91. Corretti, M.C., Anderson, T.J., Benjamin, E.J., Celermajer, D., Charbonneau, F., Creager, M.A., Deanfield, J., Drexler, H., Gerhard-Herman, M., Herrington, D., et al. 2002. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 39:257-265.
92. Malek, A.M., and Izumo, S. 1994. Molecular aspects of signal transduction of shear stress in the endothelial cell. *J Hypertens* 12:989-999.
93. Noris, M., Morigi, M., Donadelli, R., Aiello, S., Foppolo, M., Todeschini, M., Orisio, S., Remuzzi, G., and Remuzzi, A. 1995. Nitric oxide synthesis by cultured endothelial cells is modulated by flow conditions. *Circ Res* 76:536-543.
94. Chowienczyk, P.J., Cockcroft, J.R., and Ritter, J.M. 1994. Blood flow responses to intra-arterial acetylcholine in man: effects of basal flow and conduit vessel length. *Clin Sci (Lond)* 87:45-51.
95. Levine, G.N., Keaney, J.F., Jr., and Vita, J.A. 1995. Cholesterol reduction in cardiovascular disease. Clinical benefits and possible mechanisms. *N Engl J Med* 332:512-521.
96. Vogel, R.A., and Corretti, M.C. 1998. Estrogens, progestins, and heart disease: can endothelial function divine the benefit? *Circulation* 97:1223-1226.
97. Vita, J.A., and Keaney, J.F., Jr. 2000. Exercise--toning up the endothelium? *N Engl J Med* 342:503-505.
98. Lieberman, E.H., Gerhard, M.D., Uehata, A., Walsh, B.W., Selwyn, A.P., Ganz, P., Yeung, A.C., and Creager, M.A. 1994. Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. *Ann Intern Med* 121:936-941.
99. McCrohon, J.A., Adams, M.R., McCredie, R.J., Robinson, J., Pike, A., Abbey, M., Keech, A.C., and Celermajer, D.S. 1996. Hormone replacement therapy is associated with improved arterial physiology in healthy post-menopausal women. *Clin Endocrinol (Oxf)* 45:435-441.
100. Bush, D.E., Jones, C.E., Bass, K.M., Walters, G.K., Bruza, J.M., and Ouyang, P. 1998. Estrogen replacement reverses endothelial dysfunction in postmenopausal women. *Am J Med* 104:552-558.
101. Gerhard, M., Walsh, B.W., Tawakol, A., Haley, E.A., Creager, S.J., Seely, E.W., Ganz, P., and Creager, M.A. 1998. Estradiol therapy combined with progesterone and endothelium-dependent vasodilation in postmenopausal women. *Circulation* 98:1158-1163.
102. Koh, K.K., Cardillo, C., Bui, M.N., Hathaway, L., Csako, G., Waclawiw, M.A., Panza, J.A., and Cannon, R.O., 3rd. 1999. Vascular effects of estrogen and cholesterol-lowering therapies in hypercholesterolemic postmenopausal women. *Circulation* 99:354-360.
103. Wakatsuki, A., Okatani, Y., Ikenoue, N., and Fukaya, T. 2001. Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen. *Circulation* 104:1773-1778.
104. Mather, K.J., Norman, E.G., Prior, J.C., and Elliott, T.G. 2000. Preserved forearm endothelial responses with acute exposure to progesterone: A randomized cross-over trial of 17-beta estradiol, progesterone, and 17-beta estradiol with progesterone in healthy menopausal women. *J Clin Endocrinol Metab* 85:4644-4649.
105. Mendelsohn, M.E., and Karas, R.H. 1999. The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 340:1801-1811.
106. Gilligan, D.M., Quyyumi, A.A., and Cannon, R.O., 3rd. 1994. Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. *Circulation* 89:2545-2551.
107. Reis, S.E., Gloth, S.T., Blumenthal, R.S., Resar, J.R., Zacur, H.A., Gerstenblith, G., and Brinker, J.A. 1994. Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. *Circulation* 89:52-60.
108. Collins, P., Rosano, G.M., Sarrel, P.M., Ulrich, L., Adamopoulos, S., Beale, C.M., McNeill, J.G., and Poole-Wilson, P.A. 1995. 17 beta-Estradiol attenuates acetylcholine-induced coronary arterial constriction in women but not men with coronary heart disease. *Circulation* 92:24-30.
109. Guetta, V., Quyyumi, A.A., Prasad, A., Panza, J.A., Waclawiw, M., and Cannon, R.O., 3rd. 1997. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* 96:2795-2801.
110. Gilligan, D.M., Badar, D.M., Panza, J.A., Quyyumi, A.A., and Cannon, R.O., 3rd. 1994. Acute vascular effects of estrogen in postmenopausal women. *Circulation* 90:786-791.

111. Volterrani, M., Rosano, G., Coats, A., Beale, C., and Collins, P. 1995. Estrogen acutely increases peripheral blood flow in postmenopausal women. *Am J Med* 99:119-122.
112. Tagawa, H., Shimokawa, H., Tagawa, T., Kuroiwa-Matsumoto, M., Hirooka, Y., and Takeshita, A. 1997. Short-term estrogen augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilation in postmenopausal women. *J Cardiovasc Pharmacol* 30:481-488.
113. Al-Khalili, F., Eriksson, M., Landgren, B.M., and Schenck-Gustafsson, K. 1998. Effect of conjugated estrogen on peripheral flow-mediated vasodilation in postmenopausal women. *Am J Cardiol* 82:215-218.
114. Pare, G., Krust, A., Karas, R.H., Dupont, S., Aronovitz, M., Chambon, P., and Mendelsohn, M.E. 2002. Estrogen Receptor-{alpha} Mediates the Protective Effects of Estrogen Against Vascular Injury. *Circ Res* 90:1087-1092.
115. Weiner, C., Lizasoain, I., Baylis, S., Knowles, R., Charles, I., and Moncada, S. 1994. Induction of Calcium-Dependent Nitric Oxide Synthases by Sex Hormones. *PNAS* 91:5212-5216.
116. Binko, J., and Majewski, H. 1998. 17 beta-Estradiol reduces vasoconstriction in endothelium-denuded rat aortas through inducible NOS. *Am J Physiol* 274:H853-859.
117. Walsh, L.S., Ollendorff, A., and Mershon, J.L. 2003. Estrogen increases inducible nitric oxide synthase gene expression. *Am J Obstet Gynecol* 188:1208-1210.
118. Rubanyi, G.M., Freay, A.D., Kauser, K., Sukovich, D., Burton, G., Lubahn, D.B., Couse, J.F., Curtis, S.W., and Korach, K.S. 1997. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *J Clin Invest* 99:2429-2437.
119. Sudhir, K., Chou, T.M., Chatterjee, K., Smith, E.P., Williams, T.C., Kane, J.P., Malloy, M.J., Korach, K.S., and Rubanyi, G.M. 1997. Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man. *Circulation* 96:3774-3777.
120. Sudhir, K., Chou, T.M., Messina, L.M., Hutchison, S.J., Korach, K.S., Chatterjee, K., and Rubanyi, G.M. 1997. Endothelial dysfunction in a man with disruptive mutation in oestrogen-receptor gene. *Lancet* 349:1146-1147.
121. Rubanyi, G.M., Kauser, K., and Johns, A. 2002. Role of estrogen receptors in the vascular system. *Vascul Pharmacol* 38:81-88.
122. Williams, J.K., Honore, E.K., and Adams, M.R. 1997. Contrasting effects of conjugated estrogens and tamoxifen on dilator responses of atherosclerotic epicardial coronary arteries in nonhuman primates. *Circulation* 96:1970-1975.
123. New, M., Gishel, Timmins, B.H., Katrina L., Duffy, M., Stephen J., and Tran, B.H., Binh T. 1997. Long-Term Estrogen Therapy Improves Vascular Function in Male to Female Transsexuals. *Journal of the American College of Cardiology* 29:1437-1444.
124. McCrohon, J.A., Walters, W.A., Robinson, J.T., McCredie, R.J., Turner, L., Adams, M.R., Handelsman, D.J., and Celermajer, D.S. 1997. Arterial reactivity is enhanced in genetic males taking high dose estrogens. *J Am Coll Cardiol* 29:1432-1436.
125. Barrett-Connor, E. 1997. Sex Differences in Coronary Heart Disease: Why Are Women So Superior? The 1995 Ancel Keys Lecture. *Circulation* 95:252-264.
126. Grady, D., Rubin, S.M., Petitti, D.B., Fox, C.S., Black, D., Ettinger, B., Ernster, V.L., and Cummings, S.R. 1992. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Ann Intern Med* 117:1016-1037.
127. Grodstein, F., Stampfer, M.J., Colditz, G.A., Willett, W.C., Manson, J.E., Joffe, M., Rosner, B., Fuchs, C., Hankinson, S.E., Hunter, D.J., et al. 1997. Postmenopausal hormone therapy and mortality. *N Engl J Med* 336:1769-1775.
128. Grodstein, F., Stampfer, M.J., Manson, J.E., Colditz, G.A., Willett, W.C., Rosner, B., Speizer, F.E., and Hennekens, C.H. 1996. Postmenopausal Estrogen and Progestin Use and the Risk of Cardiovascular Disease. *N Engl J Med* 335:453-461.
129. Brouchet, L., Krust, A., Dupont, S., Chambon, P., Bayard, F., and Arnal, J.F. 2001. Estradiol Accelerates Reendothelialization in Mouse Carotid Artery Through Estrogen Receptor-{{alpha}} but Not Estrogen Receptor-{beta}. *Circulation* 103:423-428.
130. Krasinski, K., Spyridopoulos, I., Asahara, T., van der Zee, R., Isner, J.M., and Losordo, D.W. 1997. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation* 95:1768-1772.
131. Spyridopoulos, I., Sullivan, A.B., Kearney, M., Isner, J.M., and Losordo, D.W. 1997. Estrogen-Receptor–Mediated Inhibition of Human Endothelial Cell Apoptosis : Estradiol as a Survival Factor. *Circulation* 95:1505-1514.
132. Go, Y.M., Patel, R.P., Maland, M.C., Park, H., Beckman, J.S., Darley-Usmar, V.M., and Jo, H. 1999. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH(2)-terminal kinase. *Am J Physiol* 277:H1647-1653.
133. Yeh, L.H., Park, Y.J., Hansalia, R.J., Ahmed, I.S., Deshpande, S.S., Goldschmidt-Clermont, P.J., Irani, K., and Alevriadou, B.R. 1999. Shear-induced tyrosine phosphorylation in endothelial cells requires Rac1-dependent production of ROS. *Am J Physiol* 276:C838-847.
134. Sudoh, N., Toba, K., Akishita, M., Ako, J., Hashimoto, M., Iijima, K., Kim, S., Liang, Y.-Q., Ohike, Y., Watanabe, T., et al. 2001. Estrogen Prevents Oxidative Stress-Induced Endothelial Cell Apoptosis in Rats. *Circulation* 103:724-729.
135. Amant, C., Holm, P., Xu, S.-h., Tritman, N., Kearney, M., and Losordo, D.W. 2001. Estrogen Receptor-Mediated, Nitric Oxide-Dependent Modulation of the Immunologic Barrier Function of the Endothelium: Regulation of Fas Ligand Expression by Estradiol. *Circulation* 104:2576-2581.
136. Nofer, J.-R., Levkau, B., Wolinska, I., Junker, R., Fobker, M., von Eckardstein, A., Seedorf, U., and Assmann, G. 2001. Suppression of Endothelial Cell Apoptosis by High Density Lipoproteins (HDL) and HDL-associated Lysosphingolipids. *J. Biol. Chem.* 276:34480-34485.
137. Mallat, Z., and Tedgui, A. 2001. Current Perspective on the Role of Apoptosis in Atherothrombotic Disease. *Circ Res* 88:998-1003.
138. Kuhl, H. 1996. Effects of progestogens on haemostasis. *Maturitas* 24:1-19.
139. The North American Menopause Society. 2003. Role of progestogen in hormone therapy for postmenopausal women: position. *Menopause* 10:113-132.
140. Hanke, H., Hanke, S., Bruck, B., Brehme, U., Gugel, N., Finking, G., Muck, A.O., Schmahl, F.W., Hombach, V., and Haasis, R. 1996. Inhibition of the protective effect of estrogen by progesterone in experimental atherosclerosis. *Atherosclerosis* 121:129-138.
141. Oparil, S., Levine, R.L., Chen, S.-J., Durand, J., and Chen, Y.F. 1997. Sexually Dimorphic Response of the Balloon-Injured Rat Carotid Artery to Hormone Treatment. *Circulation* 95:1301-1307.
142. Levine, R.L., Chen, S.-J., Durand, J., Chen, Y.-F., and Oparil, S. 1996. Medroxyprogesterone Attenuates Estrogen-Mediated Inhibition of Neointima Formation After Balloon Injury of the Rat Carotid Artery. *Circulation* 94:2221-2227.
143. Espeland, M.A., Marcovina, S.M., Miller, V., Wood, P.D., Wasilauskas, C., Sherwin, R., Schrott, H., and Bush, T.L. 1998. Effect of Postmenopausal Hormone Therapy on Lipoprotein(a) Concentration. *Circulation* 97:979-986.
144. Mosca, L. 2000. Hormone replacement therapy in the prevention and treatment of atherosclerosis. *Curr Atheroscler Rep* 2:297-302.
145. Cushman, M., Legault, C., Barrett-Connor, E., Stefanick, M.L., Kessler, C., Judd, H.L., Sakkinen, P.A., and Tracy, R.P. 1999. Effect of Postmenopausal Hormones on Inflammation-Sensitive Proteins : The Postmenopausal Estrogen/Progestin Interventions (PEPI) Study. *Circulation* 100:717-722.
146. Adams, M.R., Kaplan, J.R., Manuck, S.B., Koritnik, D.R., Parks, J.S., Wolfe, M.S., and Clarkson, T.B. 1990. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys. Lack of an effect of added progesterone. *Arteriosclerosis* 10:1051-1057.
147. Rijpkema, A.H., van der Sanden, A.A., and Ruijs, A.H. 1990. Effects of post-menopausal oestrogen-progestogen replacement therapy on serum lipids and lipoproteins: a review. *Maturitas* 12:259-285.

148. Stampfer, M.J., and Colditz, G.A. 1991. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 20:47-63.
149. Austin, P.C., Mamdani, M.M., Tu, K., and Jaakkimainen, L. 2003. Prescriptions for Estrogen Replacement Therapy in Ontario Before and After Publication of the Women's Health Initiative Study. *JAMA* 289:3241-3242.
150. Mendelsohn, M.E., and Karas, R.H. 2001. The time has come to stop letting the HERS tale wag the dogma. *Circulation* 104:2256-2259.
151. Lemay, A. 2002. The relevance of the Women's Health Initiative results on combined hormone replacement therapy in clinical practice. *J Obstet Gynaecol Can* 24:711-715.
152. Beral, V. 2003. Breast cancer and hormone-replacement therapy in the Million Women Study. *The Lancet* 362:419-427.
153. Arora, S., Veves, A., Caballaro, A.E., Smakowski, P., and LoGerfo, F.W. 1998. Estrogen improves endothelial function. *J Vasc Surg* 27:1141-1146; discussion 1147.
154. Rosselli, M., Imthurn, B., Keller, P.J., Jackson, E.K., and Dubey, R.K. 1995. Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17 beta-estradiol and norethisterone acetate. A two-year follow-up study. *Hypertension* 25:848-853.
155. Herrington, D.M., Espeland, M.A., Crouse, J.R., III, Robertson, J., Riley, W.A., McBurnie, M.A., and Burke, G.L. 2001. Estrogen Replacement and Brachial Artery Flow-Mediated Vasodilation in Older Women. *Arterioscler Thromb Vasc Biol* 21:1955-1961.
156. Kawano, H., Motoyama, T., Hirai, N., Yoshimura, T., Kugiyama, K., Ogawa, H., Okamura, H., and Yasue, H. 2001. Effect of medroxyprogesterone acetate plus estradiol on endothelium-dependent vasodilation in postmenopausal women. *The American Journal of Cardiology* 87:238-240.
157. Koh, K.K., Jin, D.K., Yang, S.H., Lee, S.-K., Hwang, H.Y., Kang, M.H., Kim, W., Kim, D.S., Choi, I.S., and Shin, E.K. 2001. Vascular Effects of Synthetic or Natural Progestagen Combined With Conjugated Equine Estrogen in Healthy Postmenopausal Women. *Circulation* 103:1961-1966.
158. de Kleijn, M.J.J., Wilmink, H.W., Bots, M.L., Bak, A.A.A., van der Schouw, Y.T., Planellas, J., Engelen, S., Banga, J.-D., and Grobbee, D.E. 2001. Hormone replacement therapy and endothelial function: Results of a randomized controlled trial in healthy postmenopausal women. *Atherosclerosis* 159:357-365.
159. Russell, K.S., Haynes, M.P., Sinha, D., Clerisme, E., and Bender, J.R. 2000. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci U S A* 97:5930-5935.
160. Huang, A., Sun, D., Koller, A., and Kaley, G. 2000. 17beta-estradiol restores endothelial nitric oxide release to shear stress in arterioles of male hypertensive rats. *Circulation* 101:94-100.
161. Mendelsohn, M.E. 2000. Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? *Circ Res* 87:956-960.
162. Chambliss, K.L., and Shaul, P.W. 2002. Rapid activation of endothelial NO synthase by estrogen: evidence for a steroid receptor fast-action complex (SRFC) in caveolae. *Steroids* 67:413-419.
163. Goligorsky, M.S., Li, H., Brodsky, S., and Chen, J. 2002. Relationships between caveolae and eNOS: everything in proximity and the proximity of everything. *Am J Physiol Renal Physiol* 283:F1-10.
164. Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. 2003. Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. *Molecular and Cellular Endocrinology* 206:13-22.
165. Bian, Z., Nilsson, S., and Gustafsson, J.A. 2001. Selective estrogen receptor modulators and coronary heart disease. *Trends Cardiovasc Med* 11:196-202.
166. Anderson, J.W., Johnstone, B.M., and Cook-Newell, M.E. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med* 333:276-282.
167. Potter, S.M., Baum, J.A., Teng, H., Stillman, R.J., Shay, N.F., and Erdman, J.W., Jr. 1998. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am J Clin Nutr* 68:1375S-1379S.
168. Baum, J.A., Teng, H., Erdman, J.W., Jr., Weigel, R.M., Klein, B.P., Persky, V.W., Freels, S., Surya, P., Bakhit, R.M., Ramos, E., et al. 1998. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. *Am J Clin Nutr* 68:545-551.
169. Goodman-Gruen, D., and Kritz-Silverstein, D. 2001. Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women. *J Nutr* 131:1202-1206.
170. Washburn, S., Burke, G.L., Morgan, T., and Anthony, M. 1999. Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. *Menopause* 6:7-13.
171. Wiseman, H., O'Reilly, J.D., Adlercreutz, H., Mallet, A.I., Bowey, E.A., Rowland, I.R., and Sanders, T.A. 2000. Isoflavone phytoestrogens consumed in soy decrease F(2)-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. *Am J Clin Nutr* 72:395-400.
172. McDonnell, D.P., Wijayaratne, A., Chang, C.-y., and Norris, J.D. 2002. Elucidation of the molecular mechanism of action of selective estrogen receptor modulators. *The American Journal of Cardiology* 90:F35-F43.
173. Enmark, E., and Gustafsson, J.A. 1999. Oestrogen receptors - an overview. *J Intern Med* 246:133-138.
174. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. 2001. Mechanisms of estrogen action. *Physiol Rev* 81:1535-1565.
175. Mosca, L. 2001. Rationale and overview of the Raloxifene Use for the Heart (RUTH) trial. *Ann N Y Acad Sci* 949:181-185.
176. Simoncini, T., and Genazzani, A.R. 2000. Raloxifene acutely stimulates nitric oxide release from human endothelial cells via an activation of endothelial nitric oxide synthase. *J Clin Endocrinol Metab* 85:2966-2969.
177. Rahimian, R., Dube, G.P., Toma, W., Dos Santos, N., McManus, B.M., and van Breemen, C. 2002. Raloxifene enhances nitric oxide release in rat aorta via increasing endothelial nitric oxide mRNA expression. *Eur J Pharmacol* 434:141-149.
178. Pavo, I., Laszlo, F., Morschl, E., Nemcsik, J., Berko, A., Cox, D.A., and Laszlo, F.A. 2000. Raloxifene, an oestrogen-receptor modulator, prevents decreased constitutive nitric oxide and vasoconstriction in ovariectomized rats. *Eur J Pharmacol* 410:101-104.
179. Alexandersen, P., Haarbo, J., Breinholt, V., and Christiansen, C. 2001. Dietary phytoestrogens and estrogen inhibit experimental atherosclerosis. *Climacteric* 4:151-159.
180. Squadrito, F., Altavilla, D., Squadrito, G., Saitta, A., Cucinotta, D., Minutoli, L., Deodato, B., Ferlito, M., Campo, G.M., Bova, A., et al. 2000. Genistein supplementation and estrogen replacement therapy improve endothelial dysfunction induced by ovariectomy in rats. *Cardiovasc Res* 45:454-462.
181. Karamsetty, M.R., Klinger, J.R., and Hill, N.S. 2001. Phytoestrogens restore nitric oxide-mediated relaxation in isolated pulmonary arteries from chronically hypoxic rats. *J Pharmacol Exp Ther* 297:968-974.
182. Squadrito, F., Altavilla, D., Morabito, N., Crisafulli, A., D'Anna, R., Corrado, F., Ruggeri, P., Campo, G.M., Calapai, G., Caputi, A.P., et al. 2002. The effect of the phytoestrogen genistein on plasma nitric oxide concentrations, endothelin-1 levels and endothelium dependent vasodilation in postmenopausal women. *Atherosclerosis* 163:339-347.

183. Saitta, A., Altavilla, D., Cucinotta, D., Morabito, N., Frisina, N., Corrado, F., D'Anna, R., Lasco, A., Squadrito, G., Gaudio, A., et al. 2001. Randomized, double-blind, placebo-controlled study on effects of raloxifene and hormone replacement therapy on plasma no concentrations, endothelin-1 levels, and endothelium-dependent vasodilation in postmenopausal women. *Arterioscler Thromb Vasc Biol* 21:1512-1519.
184. Hale, G.E., Hughes, C.L., and Cline, J.M. 2002. Endometrial cancer: hormonal factors, the perimenopausal "window of risk," and isoflavones. *J Clin Endocrinol Metab* 87:3-15.
185. George, J.N., Thoi, L.L., McManus, L.M., and Reimann, T.A. 1982. Isolation of human platelet membrane microparticles from plasma and serum. *Blood* 60:834-840.
186. Thiagarajan, P., and Tait, J. 1991. Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. *J. Biol. Chem.* 266:24302-24307.
187. Merten, M., Pakala, R., Thiagarajan, P., and Benedict, C.R. 1999. Platelet Microparticles Promote Platelet Interaction With Subendothelial Matrix in a Glycoprotein IIb/IIIa–Dependent Mechanism. *Circulation* 99:2577-2582.
188. Carlstrom, K. 1996. Low endogenous estrogen levels--analytical problems and tissue sensitivity. *Acta Obstet Gynecol Scand Suppl* 163:11-15.
189. Weerheim, A.M., Kolb, A.M., Sturk, A., and Nieuwland, R. 2002. Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal Biochem* 302:191-198.
190. Doi, H., Kugiyama, K., Ohgushi, M., Sugiyama, S., Matsumura, T., Ohta, Y., Oka, H., Ogata, N., Hirata, A., Yamamoto, Y., et al. 1999. Membrane active lipids in remnant lipoproteins cause impairment of endothelium-dependent vasorelaxation. *Arterioscler Thromb Vasc Biol* 19:1918-1924.
191. Arngrimsson, R., Sigurard ttir, S., Frigge, M.L., Bjarnad ttir, R.I., Jonsson, T., Stefansson, H., Baldursdottir, A., Einarsdottir, A.S., Palsson, B., Snorradottir, S., et al. 1999. A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13. *Hum Mol Genet* 8:1799-1805. Write to the Help Desk NCBI | NLM | NIH Department of Health & Human Services Freedom of Information Act | Disclaimer i1686-pc-linux-gnu Jul 1716 2002 1716:1734:1753.
192. Kublickiene, K.R., Nisell, H., Poston, L., Kruger, K., and Lindblom, B. 2000. Modulation of vascular tone by nitric oxide and endothelin 1 in myometrial resistance arteries from pregnant women at term. *Am J Obstet Gynecol* 182:87-93.
193. Matsuura, A., Kawashima, S., Yamochi, W., Hirata, K., Yamaguchi, T., Emoto, N., and Yokoyama, M. 1997. Vascular endothelial growth factor increases endothelin-converting enzyme expression in vascular endothelial cells. *Biochem Biophys Res Commun* 235:713-716.
194. Fernandez-Patron, C., Radomski, M.W., and Davidge, S.T. 1999. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circ Res* 85:906-911.
195. Banks, R.E., Forbes, M.A., Searles, J., Pappin, D., Canas, B., Rahman, D., Kaufmann, S., Walters, C.E., Jackson, A., Eves, P., et al. 1998. Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1. *Mol Hum Reprod* 4:377-386. Write to the Help Desk NCBI | NLM | NIH Department of Health & Human Services Freedom of Information Act | Disclaimer i686-pc-linux-gnu Jul 316 2002 2016:2034:2053.
196. Clark, D.E., Smith, S.K., He, Y., Day, K.A., Licence, D.R., Corps, A.N., Lammoglia, R., and Charnock-Jones, D.S. 1998. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod* 59:1540-1548.
197. Helske, S., Vuorela, P., Carpen, O., Hornig, C., Weich, H., and Halmesmaki, E. 2001. Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. *Mol Hum Reprod* 7:205-210.
198. Tjoa, M.L., van Vugt, J.M.G., Mulders, M.A.M., Schutgens, R.B.H., Oudejans, C.B.M., and van Wijk, I.J. 2001. Plasma placenta growth factor levels in midtrimester pregnancies. *Obstetrics & Gynecology* 98:600-607.
199. Su, Y.N., Lee, C.N., Cheng, W.F., Shau, W.Y., Chow, S.N., and Hsieh, F.J. 2001. Decreased maternal serum placenta growth factor in early second trimester and preeclampsia. *Obstetrics & Gynecology* 97:898-904.
200. Tidwell, S.C., Ho, H.N., Chiu, W.H., Torry, R.J., and Torry, D.S. 2001. Low maternal serum levels of placenta growth factor as an antecedent of clinical preeclampsia. *Am J Obstet Gynecol* 184:1267-1272.
201. Kublickiene, K.R., Nisell, H., and Poston, L. 2000. 17beta-estradiol upregulates flow-mediated nitric oxide-induced dilatation in small subcutaneous arteries from postmenopausal women. *J Soc Gynecol Investig* 7 (Supplement):186 Abstract.
202. Huang, A., Sun, D., Kaley, G., and Koller, A. 1998. Estrogen preserves regulation of shear stress by nitric oxide in arterioles of female hypertensive rats. *Hypertension* 31:309-314.
203. Belfort, M.A., Saade, G.R., Wen, T.S., and Vedernikov, Y.P. 1996. The direct action of 17 beta-estradiol in isolated omental artery from nonpregnant and pregnant women is related to calcium antagonism. *Am J Obstet Gynecol* 175:1163-1172.
204. Wellman, G.C., Bonev, A.D., Nelson, M.T., and Brayden, J.E. 1996. Gender differences in coronary artery diameter involve estrogen, nitric oxide, and Ca(2+)-dependent K+ channels. *Circ Res* 79:1024-1030.
205. Russell, K.S., Haynes, M.P., Caulin-Glaser, T., Rosneck, J., Sessa, W.C., and Bender, J.R. 2000. Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release. *J Biol Chem* 275:5026-5030.
206. Pascoal, I.F., and Umans, J.G. 1996. Effect of pregnancy on mechanisms of relaxation in human omental microvessels. *Hypertension* 28:183-187.
207. Kenny, L.C., Baker, P.N., Kendall, D.A., Randall, M.D., and Dunn, W.R. 2002. Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. *Clin Sci (Lond)* 103:67-73. Write to the Help Desk NCBI | NLM | NIH Department of Health & Human Services Freedom of Information Act | Disclaimer Jul 17 2003 2011:2042:2011.
208. Campbell, W.B., and Gauthier, K.M. 2002. What is new in endothelium-derived hyperpolarizing factors? *Curr Opin Nephrol Hypertens* 11:177-183.
209. Lantin-Hermoso, R.L., Rosenfeld, C.R., Yuhanna, I.S., German, Z., Chen, Z., and Shaul, P.W. 1997. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am J Physiol* 273:L119-126.
210. DiFederico, E., Genbacev, O., and Fisher, S.J. 1999. Preeclampsia Is Associated with Widespread Apoptosis of Placental Cytotrophoblasts within the Uterine Wall. *Am J Pathol* 155:293-301.
211. Allaire, A.D., Ballenger, K.A., Wells, S.R., McMahon, M.J., and Lessey, B.A. 2000. Placental apoptosis in preeclampsia. *Obstetrics & Gynecology* 96:271-276.
212. Radomski, M.W., Palmer, R.M., and Moncada, S. 1990. Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br J Pharmacol* 101:325-328.
213. Bath, P.M., Hassall, D.G., Gladwin, A.M., Palmer, R.M., and Martin, J.F. 1991. Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. *Arterioscler Thromb* 11:254-260.

214. Tanner, F.C., Meier, P., Greutert, H., Champion, C., Nabel, E.G., and Luscher, T.F. 2000. Nitric Oxide Modulates Expression of Cell Cycle Regulatory Proteins : A Cytostatic Strategy for Inhibition of Human Vascular Smooth Muscle Cell Proliferation. *Circulation* 101:1982-1989.
215. Takahashi, M., Ikeda, U., Masuyama, J., Funayama, H., Kano, S., and Shimada, K. 1996. Nitric oxide attenuates adhesion molecule expression in human endothelial cells. *Cytokine* 8:817-821.
216. Clancy, R.M., Leszczynska-Piziak, J., and Abramson, S.B. 1992. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J Clin Invest* 90:1116-1121.
217. Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A., and Parthasarathy, S. 1993. Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett* 334:170-174.
218. Hashimoto, M., Akishita, M., Eto, M., Ishikawa, M., Kozaki, K., Toba, K., Sagara, Y., Taketani, Y., Orimo, H., and Ouchi, Y. 1995. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation* 92:3431-3435.
219. Bungum, L., Kvernebo, K., Oian, P., and Maltau, J.M. 1996. Laser doppler-recorded reactive hyperaemia in the forearm skin during the menstrual cycle. *Br J Obstet Gynaecol* 103:70-75.
220. Bowyer, L., Brown, M.A., and Jones, M. 2001. Vascular reactivity in men and women of reproductive age. *Am J Obstet Gynecol* 185:88-96.
221. Sarabi, M., Millgard, J., and Lind, L. 1999. Effects of age, gender and metabolic factors on endothelium-dependent vasodilation: a population-based study. *J Intern Med* 246:265-274.
222. Majmudar, N.G., Robson, S.C., and Ford, G.A. 2000. Effects of the menopause, gender, and estrogen replacement therapy on vascular nitric oxide activity. *J Clin Endocrinol Metab* 85:1577-1583.
223. Higashi, Y., Sanada, M., Sasaki, S., Nakagawa, K., Goto, C., Matsuura, H., Ohama, K., Chayama, K., and Oshima, T. 2001. Effect of estrogen replacement therapy on endothelial function in peripheral resistance arteries in normotensive and hypertensive postmenopausal women. *Hypertension* 37:651-657.
224. Virdis, A., Ghiadoni, L., Pinto, S., Lombardo, M., Petraglia, F., Gennazzani, A., Buralli, S., Taddei, S., and Salvetti, A. 2000. Mechanisms responsible for endothelial dysfunction associated with acute estrogen deprivation in normotensive women. *Circulation* 101:2258-2263.
225. Ohmichi, M., Kanda, Y., Hisamoto, K., Morishige, K., Takahashi, K., Sawada, K., Minekawa, R., Tasaka, K., and Murata, Y. 2003. Rapid changes of flow-mediated dilatation after surgical menopause. *Maturitas* 44:125-131.
226. van der Schouw, Y.T., van der Graaf, Y., Steyerberg, E.W., Eijkemans, J.C., and Banga, J.D. 1996. Age at menopause as a risk factor for cardiovascular mortality. *Lancet* 347:714-718.
227. Huang, A., Sun, D., Koller, A., and Kaley, G. 1997. Gender difference in myogenic tone of rat arterioles is due to estrogen- induced, enhanced release of NO. *Am J Physiol* 272:H1804-1809.
228. Huang, A., Sun, D., Koller, A., and Kaley, G. 1998. Gender difference in flow-induced dilation and regulation of shear stress: role of estrogen and nitric oxide. *Am J Physiol Regulatory Integrative Comp Physiol* 275:R1571-1577.
229. Mombouli, J.-V., and Vanhoutte, P.M. 1999. Endothelial Dysfunction: From Physiology to Therapy. *Journal of Molecular and Cellular Cardiology* 31:61-74.
230. Schiffrin, E.L. 2001. Role of endothelin-1 in hypertension and vascular disease. *Am J Hypertens* 14:83S-89S.
231. Ylikorkala M.D., P.D., Olavi, Cacciatore M.D., P.D., Bruno, Paakkari M.D., P.D., Ilari, Tikkanen M.D., P.D., Matti J., and Viinikka M.D., P.D., Lasse. 1998. The Long-Term Effects of Oral and Transdermal Postmenopausal Hormone Replacement Therapy on Nitric Oxide, Endothelin-1, Prostacyclin, and Thromboxane. *Fertility and Sterility* 69:883-888.
232. Nogawa, N., Sumino, H., Ichikawa, S., Kumakura, H., Takayama, Y., Nakamura, T., Kanda, T., Mizunuma, H., and Kurabayashi, M. 2001. Effect of long-term hormone replacement therapy on angiotensin-converting enzyme activity and bradykinin in postmenopausal women with essential hypertension and normotensive postmenopausal women. *Menopause* 8:210-215.
233. Du, X.J., Riemersma, R.A., and Dart, A.M. 1995. Cardiovascular protection by oestrogen is partly mediated through modulation of autonomic nervous function. *Cardiovasc Res* 30:161-165.
234. Kublickiene, K.R., Cockell, A.P., Nisell, H., and Poston, L. 1997. Role of nitric oxide in the regulation of vascular tone in pressurized and perfused resistance myometrial arteries from term pregnant women. *Am J Obstet Gynecol* 177:1263-1269.
235. Mendelsohn, M.E. 2002. Genomic and nongenomic effects of estrogen in the vasculature. *Am J Cardiol* 90:3F-6F.
236. Wagner, A.H., Schroeter, M.R., and Hecker, M. 2001. 17beta-estradiol inhibition of NADPH oxidase expression in human endothelial cells. *Faseb J* 15:2121-2130.
237. Arnal, J.F., Dinh-Xuan, A.T., Pueyo, M., Darblade, B., and Rami, J. 1999. Endothelium-derived nitric oxide and vascular physiology and pathology. *Cell Mol Life Sci* 55:1078-1087.
238. Sanada, M., Higashi, Y., Nakagawa, K., Tsuda, M., Kodama, I., Kimura, M., Chayama, K., and Ohama, K. 2003. A Comparison of Low-Dose and Standard-Dose Oral Estrogen on Forearm Endothelial Function in Early Postmenopausal Women. *J Clin Endocrinol Metab* 88:1303-1309.
239. Luksha, L., Nisell, H., and Kublickiene, K. 2003. The mechanism of EDHF-mediated responses in subcutaneous small arteries from healthy pregnant women. *Am J Physiol Regul Integr Comp Physiol* Submitted.
240. Coats, P., Johnston, F., MacDonald, J., McMurray, J.J., and Hillier, C. 2001. Endothelium-derived hyperpolarizing factor : identification and mechanisms of action in human subcutaneous resistance arteries. *Circulation* 103:1702-1708.
241. Liu, M.Y., Hattori, Y., Sato, A., Ichikawa, R., Zhang, X.H., and Sakuma, I. 2002. Ovariectomy attenuates hyperpolarization and relaxation mediated by endothelium-derived hyperpolarizing factor in female rat mesenteric artery: a concomitant decrease in connexin-43 expression. *J Cardiovasc Pharmacol* 40:938-948.
242. van Baal, W.M., Kenemans, P., Emeis, J.J., Schalkwijk, C.G., Mijatovic, V., van der Mooren, M.J., Vischer, U.M., and Stehouwer, C.D.A. 1999. Long-term effects of combined hormone replacement therapy on markers of endothelial function and inflammatory activity in healthy postmenopausal women. *Fertility and Sterility* 71:663-670.
243. Herrington, D.M., Reboussin, D.M., Klein, K.P., Sharp, P.C., Shumaker, S.A., Snyder, T.E., and Geisinger, K.R. 2000. The Estrogen Replacement and Atherosclerosis (ERA) Study: Study Design and Baseline Characteristics of the Cohort. *Controlled Clinical Trials* 21:257-285.
244. 1995. Effects of estrogen or estrogen/progestin regimens on heart disease risk factors in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial. The Writing Group for the PEPI Trial. *Jama* 273:199-208.
245. Razandi, M., Pedram, A., and Levin, E.R. 2000. Estrogen signals to the preservation of endothelial cell form and function. *J Biol Chem* 275:38540-38546.
246. Makela, S., Savolainen, H., Aavik, E., Myllarniemi, M., Strauss, L., Taskinen, E., Gustafsson, J.A., and Hayry, P. 1999. Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. *Proc Natl Acad Sci U S A* 96:7077-7082.

247. Karas, R.H., Hodgin, J.B., Kwoun, M., Krege, J.H., Aronovitz, M., Mackey, W., Gustafsson, J.A., Korach, K.S., Smithies, O., and Mendelsohn, M.E. 1999. Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice. *Proc Natl Acad Sci U S A* 96:15133-15136.
248. Zhu, B.H., Ueno, M., Matsushita, T., Fujisawa, H., Seriu, N., Nishikawa, T., Nishimura, Y., and Hosokawa, M. 2001. Effects of aging and blood pressure on the structure of the thoracic aorta in SAM mice: a model of age-associated degenerative vascular changes. *Exp Gerontol* 36:111-124.
249. Kauffman, R.F., Bean, J.S., Fahey, K.J., Cullinan, G.J., Cox, D.A., and Bensch, W.R. 2000. Raloxifene and estrogen inhibit neointimal thickening after balloon injury in the carotid artery of male and ovariectomized female rats. *J Cardiovasc Pharmacol* 36:459-465.
250. Takahashi, K., Ohmichi, M., Yoshida, M., Hisamoto, K., Mabuchi, S., Arimoto-Ishida, E., Mori, A., Tsutsumi, S., Tasaka, K., Murata, Y., et al. 2003. Both estrogen and raloxifene cause G1 arrest of vascular smooth muscle cells. *J Endocrinol* 178:319-329.
251. Duschek, E.J., Stehouwer, C.D., de Valk-de Roo, G.W., Schalkwijk, C.G., Lambert, J., and Netelenbos, C. 2003. Raloxifene, conjugated oestrogen and endothelial function in postmenopausal women. *J Intern Med* 254:85-94.
252. Walker, H.A., Dean, T.S., Sanders, T.A.B., Jackson, G., Ritter, J.M., and Chowienczyk, P.J. 2001. The phytoestrogen genistein produces acute nitric oxide-dependent dilation of human forearm vasculature with similar potency to 17[beta]estradiol. *Circulation* 103:258-262.
253. Squadrito, F., Altavilla, D., Crisafulli, A., Saitta, A., Cucinotta, D., Morabito, N., D'Anna, R., Corrado, F., Ruggeri, P., Frisina, N., et al. 2003. Effect of genistein on endothelial function in postmenopausal women: a randomized, double-blind, controlled study. *Am J Med* 114:470-476.
254. Teede, H.J., Dalais, F.S., Kotsopoulos, D., Liang, Y.L., Davis, S., and McGrath, B.P. 2001. Dietary soy has both beneficial and potentially adverse cardiovascular effects: a placebo-controlled study in men and postmenopausal women. *J Clin Endocrinol Metab* 86:3053-3060.
255. Hale, G., Paul-Labrador, M., Dwyer, J.H., and Merz, C.N. 2002. Isoflavone supplementation and endothelial function in menopausal women. *Clin Endocrinol (Oxf)* 56:693-701.
256. Frasor, J., Barnett, D.H., Danes, J.M., Hess, R., Parlow, A.F., and Katzenellenbogen, B.S. 2003. Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus. *Endocrinology* 144:3159-3166.
257. Kemmeren, J.M., Tanis, B.C., van den Bosch, M.A.A.J., Bollen, E.L.E.M., Helmerhorst, F.M., van der Graaf, Y., Rosendaal, F.R., and Algra, A. 2002. Risk of Arterial Thrombosis in Relation to Oral Contraceptives (RATIO) Study: Oral Contraceptives and the Risk of Ischemic Stroke. *Stroke* 33:1202-1208.
258. Van Den Bosch, M.A., Kemmeren, J.M., Tanis, B.C., Mali, W.P., Helmerhorst, F.M., Rosendaal, F.R., Algra, A., and Van Der Graaf, Y. 2003. The RATIO study: oral contraceptives and the risk of peripheral arterial disease in young women. *J Thromb Haemost* 1:439-444.