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Vascular Effects of Estrogenic Menopausal Hormone Therapy

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Abstract

Cardiovascular disease (CVD) is more common in men and postmenopausal women (Post-MW) than premenopausal women (Pre-MW). Despite recent advances in preventive measures, the incidence of CVD in women has shown a rise that matched the increase in the Post-MW population. The increased incidence of CVD in Post-MW has been related to the decline in estrogen levels, and hence suggested vascular benefits of endogenous estrogen. Experimental studies have identified estrogen receptor ER α , ER β and a novel estrogen binding membrane protein GPR30 (GPER) in blood vessels of humans and experimental animals. The interaction of estrogen with vascular ERs mediates both genomic and non-genomic effects. Estrogen promotes endothelium-dependent relaxation by increasing nitric oxide, prostacyclin, and hyperpolarizing factor. Estrogen also inhibits the mechanisms of vascular smooth muscle (VSM) contraction including [Ca²⁺]_i, protein kinase C and Rho-kinase. Additional effects of estrogen on the vascular cytoskeleton, extracellular matrix, lipid profile and the vascular inflammatory response have been reported. In addition to the experimental evidence in animal models and vascular cells, initial observational studies in women using menopausal hormonal therapy (MHT) have suggested that estrogen may protect against CVD. However, randomized clinical trials (RCTs) such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women's Health Initiative (WHI), which examined the effects of conjugated equine estrogens (CEE) in older women with established CVD (HERS) or without overt CVD (WHI), failed to demonstrate protective vascular effects of estrogen treatment. Despite the initial set-back from the results of MHT RCTs, growing evidence now supports the 'timing hypothesis', which suggests that MHT could increase the risk of CVD if started late after menopause, but may produce beneficial cardiovascular effects in younger women during the perimenopausal period. The choice of an appropriate MHT dose, route of administration, and estrogen/progestin combination could maximize the vascular benefits of MHT and minimize other adverse effects, especially if given within a reasonably short time after menopause to women that seek MHT for the relief of menopausal symptoms.

Keywords

sex hormones; progesterone; testosterone; phytoestrogens; estrogen receptor; endothelium; vascular smooth muscle; hypertension

INTRODUCTION

The incidence of cardiovascular disease (CVD) increases with age in both men and women, but further increases in postmenopausal women (Post-MW) possibly due to the changes in the sex hormone environment and the precipitous decrease in plasma estrogen levels. Across different stages of life, the presence or absence of endogenous estrogens in women could

affect cardiovascular function, and these effects may be closely related to different stages in the progression of atherosclerosis [1]. Formation of fatty streaks, an early and potentially reversible stage of atherosclerotic lesion, begins during fetal development [2]. The first coronary manifestations of atherosclerosis usually lag behind by ≥ 10 years in premenopausal women (Pre-MW) as compared to age-matched men [3]. Advanced coronary atherosclerotic lesions susceptible to rupture are present in 25% of men and 13% of women aged 30 to 34 years in the United States [4]. Even in women 60-64 years of age, the incidence of CVD is still considerably lower in women than in men, suggesting that previous and prolonged exposure to natural estrogen may be protective for many years beyond menopause [5]. However, as endogenous estrogen levels subside after menopause and following surgical ovariectomy (OVX), or in conditions of impaired ovarian function, these sex-based differences continue to disappear, and 95% of women who develop CVD do so after menopause [3]. These observations suggest that endogenous estrogens attenuate the progression of CVD, and that menopause may be a risk factor for the development of atherosclerosis and CVD [3].

Experimental studies in animal models and vascular cells as well as early epidemiological data in Post-MW who are users and nonusers of menopausal hormone therapy (MHT) have suggested protective cardiovascular effects of estradiol (E2). Observational studies published in the late 1980s and early 1990s almost shared the same opinion that using MHT in Post-MW reduced the risk of morbidity and mortality of coronary heart disease (CHD) for both primary and secondary cardiovascular prevention [6-10] (Fig. 1). In the late 1990s, the hypothesized protective role of MHT against postmenopausal CVD began to be questioned by the results of the first randomized clinical trial (RCT) of secondary prevention in women with known CHD, the Heart and Estrogen/progestin Replacement Study (HERS) [11]. The hypothesis of a vascular protective role of MHT was totally crushed from the year 2000 to 2003 by the publication of four more RCTs focusing on secondary prevention of ischemic heart disease or cerebrovascular disease, and on the progression of coronary-artery atherosclerosis in subjects with angiographically verified coronary disease [12-15]. Over the past decade, controversies regarding the benefits versus the risks of using MHT in CVD have flared when emerging evidence from RCTs appeared to be at odds with previous epidemiological and observational data [15]. Data from prospective RCTs for primary [16] and secondary prevention of CVD [11] by MHT have shown evidence of increased risk for cancer and adverse effects on the cardiovascular system [17, 18]. After few years of confusion about MHT and CVD, reevaluation of the results of previous RCTs such as HERS and the Women's Health Initiative (WHI) has led to reconsideration of the traditional MHT approaches, implementation of new MHT strategies, and the initiation of new clinical trials such as Kronos Early Estrogen Protection Study (KEEPS) and Early Versus Late Intervention Trial with Estradiol (ELITE) (Fig. 1).

The purpose of this review is to use published reports from the Pubmed database to provide an insight into the reasons why the beneficial vascular effects of estrogen observed in experimental studies did not translate into vascular benefits of MHT in clinical trials. We will first discuss the relationship between menopause and CVD and the evidence for protective effects of endogenous estrogen. We will describe the vascular ERs, the post-ER downstream signaling mechanisms and the effects of estrogen on the endothelium, vascular smooth muscle (VSM), and extracellular matrix (ECM) as well as on the lipid profile and atherosclerosis. We will then highlight the discrepant effects of estrogen observed in RCTs, and the potential causes of MHT failure in CVD. The role of the dose, type and route of administration of estrogen and related estrogenic compounds, and their interactions with other sex hormones will also be described. Finally, we will discuss the recent RCTs, and future directions to enhance the benefits of MHT on vascular function and CVD.

Menopause and Cardiovascular Risk

CVD such as myocardial infarction (MI), stroke and venous thromboembolism (VTE) are a major cause of death in men and women. While CVD develops 10 to 15 years later in women than in men, it is the major cause of death in women older than 65 years of age [19]. Deficiency of endogenous estrogen may play a role in the progression of atherosclerosis and the increased CVD in Post-MW. In normally cycling adult women, the ovarian follicle secretes 70 to 500 μg E2 per day, causing changes in plasma estrogen levels from 210 pmol/L in the early follicular phase and 720 pmol/L in the late follicular phase, to 490 pmol/L in the late luteal phase. The half-life of E2 is ~ 3 hours, and much of it is converted into estrone (E1) and estriol (E3) [20]. In the perimenopausal period, plasma estrogen levels decline to about 20% of the levels in the fertile period. For each year delay in the onset of menopause, the cardiovascular mortality risk decreases by 2%. Also, data from the Framingham Heart Study and the Nurses' Health Study (NHS) have shown an increased risk of CVD in young women after bilateral oophorectomy, a risk that was not observed in women using MHT after surgery [21, 22].

Age and estrogen deficiency have been linked to morbidity in women [23]. The cessation of the ovarian function and the reduction of sex steroid hormone levels could have significant metabolic and pathological consequences on the cardiovascular system. Menopause is associated with modifications in lipid metabolism and blood pressure. Post-MW have higher total cholesterol, low-density lipoprotein cholesterol (LDL-c), triglycerides, and lipoprotein(a) [Lp(a)] levels, and lower high-density lipoprotein cholesterol (HDL-c) levels than Pre-MW [24, 25]. The transition to postmenopause is associated with a 16% increase in the levels of triglycerides [26]. While triglyceride levels increase with age in both men and women, a higher prevalence of increased LDL-c is observed in Post-MW [27]. Also, a strong correlation has been demonstrated between weight gain throughout the menopausal transition and the increase in CVD risk factors [28]. Even in the absence of weight gain, the body fat distribution changes from a peripheral to a more central distribution during the menopause transitional period [26]. Additionally, menopause is an independent risk factor for the metabolic syndrome and its various components such as high blood pressure, abdominal adiposity, insulin resistance, and dyslipidemia [29]. Recent cross-sectional study of 1,002 women, 618 Pre-MW and 384 Post-MW, revealed that the risk for metabolic syndrome increases with age to reach peak levels up to 14 years since menopause, then decreases. For the individual components of metabolic syndrome, Post-MW with less than 5 years since menopause had an increased risk of abdominal obesity and high glucose, while those with 5 to 9 years since menopause had the highest risk of high blood pressure, and those with 10 to 14 years since menopause had increased risk of high triglycerides [28]. With regard to insulin resistance, studies have also shown an increase in fasting insulin [26, 29] and glucose levels in Post-MW compared with Pre-MW [30].

In Pre-MW enrolled in the Women's Ischemia Syndrome Evaluation (WISE) Study and undergoing coronary angiography for suspected myocardial ischemia, during an annual follow-up for a median of 5.9 years, diabetes mellitus was associated with hypothalamic hypoenestrogenemia. Importantly, the presence of both diabetes mellitus and hypoenestrogenemia predicted a higher percentage of angiographic coronary artery disease [31]. Also, in the Study of Women's Health Across the Nation (SWAN), the transition to menopause and the decreased estrogen levels were associated with changes in the common carotid intima-media thickness (IMT) and adventitial thickness, which are indicative of the increased risk of CVD with menopause [32]. Thus, deficiency of endogenous estrogen may have significant role in the progression of atherosclerosis and increase CVD in Post-MW.

Protective Effects of Endogenous Estrogen

Estrogen may have protective effects in the cardiovascular system including modification of the composition of circulating lipoproteins, e.g. decreased LDL-c and Lp(a) [25, 33], increased HDL-c, decreased lipid peroxidation [34], decreased insulin resistance [35], changes in blood coagulation, inhibition of intravascular accumulation of collagen, decreased VSM cell growth and proliferation, and direct vasodilation of blood vessels [36]. Many of the vascular effects of estrogen are mediated via vascular estrogen receptors (ERs).

Vascular Estrogen Receptors

In the early 1960's, an estrogen binding protein was discovered, and since then two estrogen receptor (ER) subtypes, ER α and ER β , as well as several variants have been identified. Most of the mammalian tissues and cells express ER α and ER β , and the expression of both ERs may be similar in some organs but different in other tissues where one subtype predominates [37]. ERs have been localized in human aorta, coronary, carotid, uterine and internal mammary artery, as well as umbilical vein [38]. In experimental animals, ERs have been identified in ovine uterine artery, rat aorta, mesenteric and carotid arteries, and mouse aorta [39, 40]. ERs have also been identified in veins of different species, but their responsiveness to estrogen has not been fully examined. A recent study has demonstrated that E2/ER induces venous relaxation and decreases contraction in inferior vena cava (IVC) of female rat [41]. In both arteries and veins, ERs are localized in the endothelium and VSM. At the subcellular level ERs have been localized to the cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus, and mitochondria. Also, in the plasma membrane of ECs, ERs localize to discrete structures known as caveolae [38, 42].

ER α and ER β share common structural domains, A-F. At the N terminal of ER, the A/B domain contains the transcriptional activation function-1 (AF-1). Human ER α and ER β share less than 20% amino acid identity in the AF-1 region, suggesting that this region may contribute to ER subtype-specific actions on target genes. The DNA binding domain (C domain) has 95% homology between the ER subtypes. The D domain is a hinge region that connects the DNA binding domain with the ligand binding domain. D domain also functions in promoting the association of the ER with heat shock protein 90 (HSP90) and in the nuclear localization of ER [38]. D domain has only 30% homology between ER α and ER β . The ligand binding domain (E domain) contains the hormone dependent transcriptional activation function-2 (AF-2), the ligand binding cavity, and a receptor dimerization function. E Domain has 55% homology between the ER subtypes. Domain F has <20% amino acid homology between the two ERs, and the function of this domain is unclear. Full transcriptional activation by ERs is mediated by synergism between AF-1 and AF-2 [20, 38].

Human ER α and ER β are encoded by distinct genes located on chromosomes 6 (q24-q27) and 14 (q21-q22), respectively [38]. Genetic polymorphism in ERs and truncated forms of ER α and ER β have been described in many tissues, including the vascular endothelium. In addition to the classic ERs, a G protein-coupled 7-transmembrane receptor, GPR30 (also known as GPER), was cloned in 1998. GPER is structurally unrelated to ER α and ER β , but binds E2 with high affinity and may be involved in estrogen signaling [43]. The human GPR30 gene is located on chromosome 7 (p22) [44].

ER subtypes have distinct tissue distribution, with ER β having a wider tissue distribution than ER α , and being the predominant ER subtype in VSM [36]. One study has shown that ER β is expressed in higher amounts in VSM of human coronary artery, iliac artery, aorta, and saphenous vein specimens from women, while in specimens from men ER α and ER β are detected in equal amounts, and 72 h exposure of VSM to E2 does not alter the ER α and

ER β ratio irrespective of whether the specimens were taken from women or men [45]. Studies have also demonstrated the predominance of ER β in both endothelium and media of coronary arteries from Pre-MW and Post-MW [46]. In contrast, immunohistochemical staining in small peripheral subcutaneous arteries from healthy Post-MW revealed similar expression of ER α and ER β in the media, while ER α was dominant in the endothelium [47]. ERs have also been localized in the atherosclerotic plaque suggesting a role in the regulation of the atherosclerotic process. One study examined the expression of ER α and ER β in coronary artery of Pre-MW and Post-MW as a function of plaque area, calcium area, calcium-to-plaque ratio, and estrogen status. The study demonstrated that ER β is the predominant ER in human coronary arteries and suggested that increased ER β expression may be linked to advanced atherosclerosis and calcification independent of age or hormone status [46]. In coronary arteries from men, ER β expression in the intima, but not in the media, positively correlated with plaque area, supporting the importance of the intima's ER β in atherosclerosis, which could be mediated by altered endothelial function [48]. A recent study has also shown a 10-fold greater expression of ER β versus ER α or GPR30 in internal mammary arteries isolated from male and female patients with atherosclerosis [43]. It has been suggested that epigenetic changes in ER β may contribute to the development of atherosclerosis and vascular ageing in human arteries [49]. In contrast, a study showed that expression of HSP-27, a potential biomarker of atherosclerosis and an ER β associated protein, was absent in human coronary arteries with complex atherosclerotic lesions [50]. Also, another study showed that the relative abundance of both ER α and ER β in the media of human aorta was not correlated with the degree of atherosclerosis [51].

Experimental studies on ER α knock-out (KO), ER β KO, and double ER α and ER β KO mice revealed that life is possible without either or both ERs, although the reproductive functions are severely impaired [52]. ER α and ER β have been localized in ECs and VSM [53]. In rats, a prominent nuclear expression of ER β is observed in the aorta, uterine and tail artery, while ER α is predominantly expressed in uterine vessels, suggesting that ER α and β may have different roles in vascular function [54].

Polymorphisms in ER genes may account for differences in ER function and may modify the outcome of MHT. Two common polymorphisms of ER α at positions c.454–397 T>C (PvuII) and c.454–351 A>G (XbaI) may be associated with the severity of CAD in Post-MW undergoing angiography for suspected CAD [55]. Post-MW with the ER α IVS1-397 polymorphism C/C genotype (recessive) have greater levels of HDL and apolipoprotein A-1 as well as a greater forearm blood flow, brachial artery diameter, and endothelium-dependent dilation compared to those with the CT/TT genotype [56]. In Post-MW with established CAD and being treated with estrogen alone or estrogen+progesterone, a two-times greater increase in HDL levels was seen in women with the ER α IVS1-401 polymorphism C/C genotype as compared to those without the polymorphism [57].

Methylation of the *ER α* gene in the cardiovascular system is linked to aging and atherogenesis. In some women, increased methylation of *ER α* gene in certain vascular sites may partially negate the benefit of MHT in these women. One study demonstrated an age-related increase in *ER α* gene methylation in the right atrium, and significant levels of *ER α* methylation in both arteries and veins [58]. Further studies are needed to determine the predictors of *ER α* gene methylation in humans, and the impact of its prevention or reversal using demethylating agents. *ER α* gene methylation is potentially reversible pharmacologically using inhibitors of the DNA-methyltransferase enzyme. ER gene demethylation could be relevant for the control VSMC proliferation in pathological processes such as plaque formation and restenosis post-angioplasty [54]. Thus ERs may exhibit regional differences in their expression and activity in different blood vessels and

their vascular function may be influenced by genetic polymorphism and age-related epigenetic modifications such as gene methylation.

Genomic and Non-Genomic Effects of Estrogen

E2 induces both genomic and non-genomic effects in the vasculature (Fig. 2). In the classic genomic pathway, nuclear ERs act as transcription factors that modulate gene expression by directly binding to specific DNA sequences known as estrogen response element (ERE). In the absence of estrogen, ER exists as an inactivated monomer bound to HSP90. Upon binding to estrogen, ER undergoes conformational changes that result in dissociation of HSP90 and formation of a homo- or heterodimer with high affinity for estrogen and DNA [20]. ER then binds to EREs residing in estrogen responsive genes, and recruits co-regulatory proteins to initiate changes in the expression of genes involved in cell growth, proliferation and differentiation [59]. Estrogen increases the gene expression of vasodilatory enzymes such as nitric oxide synthase (NOS) and prostacyclin synthase. Also, estrogen/ER α may mediate downregulation of mRNAs for nuclear-encoded subunits of major complexes of the mitochondrial respiratory chain [60]. ER α and ER β may have differential and opposing effects at the same promoter sites [61]. For example, where both ERs are co-expressed, ER β may exhibit inhibitory action over ER α -mediated gene expression [62]. Also, studies on the aorta of wild-type and ER knockout mice have suggested that ER β mediates estrogen-induced increase in gene expression, while ER α may be responsible for estrogen-mediated decrease in gene expression. ER transcriptional activity may also be regulated by intracellular signaling pathways even in the absence of ER ligands [38].

Non-genomic effects are rapid responses that occur too quickly to be mediated by gene transcription, are independent of protein synthesis, and typically involve modulation of membrane bound and cytoplasmic regulatory proteins. E2-induced vasodilation occurs within seconds or minutes, and involves activation of kinases and phosphatases and changes in ion fluxes across membranes [20].

Estrogen and Endothelium-Dependent Vascular Function

In endothelial cells (ECs), estrogen modifies the production, release, and bioactivity of endothelium-derived relaxing factors such as NO, prostacyclin (PGI₂), and hyperpolarizing factors (EDHF), as well as contracting factors such as endothelin (ET-1). Estrogen affects the intracellular Ca²⁺ kinetics in coronary ECs [63]. In EC caveolae, E2/ER cause an increase in intracellular Ca²⁺, which binds calmodulin, and together displace endothelial NOS (eNOS) from its binding with caveolin-1 at the plasma membrane caveolae into the cytosol, thus allowing eNOS to associate with HSP90. HSP90, a highly conserved stress protein, is essential for Akt-mediated activation of eNOS and prevention of proteasomal degradation of PI₃K (Fig. 2) [64]. E2/ER interact with and activate the p85 regulatory subunit of PI₃K, increasing intracellular production of PIP₃, which activates Akt [65]. E2 binding to membrane ER also activates mitogen-activated protein kinase (MAPK). Activation of eNOS by HSP90, Akt, and MAPK leads to the translocation of eNOS back to the plasma membrane where it undergoes myristoylation and palmitoylation to become fully activated, transforms L-arginine to L-citrulline, and releases NO [36].

In support of beneficial effects of estrogen on the endothelium, studies on small subcutaneous arteries isolated from healthy Post-MW not receiving MHT have shown that the morphology and function of the endothelium are impaired, and these impairments are improved upon treating the isolated vessels with E2 [66]. Also, endothelium-dependent vascular relaxation is greater in female than male spontaneous hypertensive rat (SHR) [67]. Selective ER α agonists such as Cpd1471 also improve EC dysfunction in blood vessels of ovariectomized (OVX) female SHR [68]. Also, E2 administration in OVX female mice

causes rapid non-genomic arterial dilation of elastic and muscular arteries, as a result of ER-mediated NO production. Studies in KO mice demonstrated that ER- α , but not ER- β , mediates the beneficial effect of E2 on NO basal production [69]. These results indicate that E2 modulation of arterial tone through plasma membrane ER and rapid signaling could underlie some of the observed actions of estrogen *in vivo* [70].

E2 has antioxidant properties that reduce reactive oxygen species and thereby increase NO bioactivity [38]. In OVX female rats, increased blood pressure is associated with increased plasma lipoperoxides and vascular free radicals, and E2 replacement prevents these effects. Also, superoxide ($O_2^{\bullet-}$) production is greater in blood vessels of male than female rats. Furthermore, E2 inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression and the generation of $O_2^{\bullet-}$ and peroxynitrite, thereby enhancing NO bioactivity [71]. Studies on isolated porcine coronary artery rings with intact endothelium have shown that xanthine/xanthine oxidase, which generate $O_2^{\bullet-}$, causes slow contraction, and that the phytoestrogens genistein and resveratrol inhibit xanthine/xanthine oxidase-induced contraction even to a greater extent than E2 [72]. Isoflavones may protect against CVD by activating intracellular signaling pathways, leading to increased NO bioavailability and upregulation of antioxidant gene expression via the transcription factors nuclear factor κ B (NF κ B) and NF-E2-related factor 2 (Nrf2). These signaling cascades may represent a mechanism by which dietary phytoestrogens maintain the reduction/oxidation reaction homeostasis in the vasculature [64].

PGI₂ is an endothelial derived relaxing factor produced from arachidonic acid through the catalytic activity of two cyclooxygenases, COX-1 and COX-2. E2 may stimulate endothelium-dependent vascular relaxation by increasing COX-1 expression/activity and PGI₂ production. Sex differences in indomethacin-sensitive porcine coronary artery relaxation have been related to differences in COX products [73]. Estrogen increases urinary excretion of COX-2-derived PGI₂ metabolites in ER β but not ER α knockout mice, suggesting a role of ER α in PGI₂ metabolism [74].

E2/ER could also increase the production of EDHF, which activates K⁺ channels, causes hyperpolarization, and in turn inhibits Ca²⁺ influx and leads to VSM relaxation (Fig. 2) [75]. E2 may also inhibit endothelium derived contracting factors such as ET-1. E2 administration is associated with rapid vasodilation, increased blood flow that is regulated by NO and, and reduction of ET-1 [76]. Also, prolonged treatment of cultured ECs of female porcine coronary artery with E2 and its metabolites 2-hydroxy-E2 and 2-methoxy-E2 inhibit the expression and release of ET-1 in response to serum, tumor necrosis factor- α , and angiotensin II [77].

Estrogen and VSM

E2 inhibits VSM growth and proliferation, likely through inhibition of MAPK transactivation and the nuclear transcription and expression of growth factors. E2 also causes rapid relaxation of endothelium-denuded vascular segments, suggesting direct effects on VSM contraction mechanisms [20]. VSM contraction is triggered by increases in $[Ca^{2+}]_i$ due to Ca²⁺ release from the sarcoplasmic reticulum and Ca²⁺ entry from the extracellular space. Activation of myosin light chain (MLC) kinase, protein kinase C (PKC) and Rho kinase as well as inhibition of MLC phosphatase contribute to VSM contraction [78]. Our laboratory has shown that the contraction to the α -adrenergic agonist phenylephrine (Phe) is greater in aortic segments from intact male compared to female Sprague-Dawley rats. Phe-induced contraction is greater in OVX compared with intact female rats, suggesting a role of estrogen in the reduced vascular response in females. Also, contraction in response to membrane depolarization by high KCl solution, which stimulates Ca²⁺ influx through voltage-gated Ca²⁺ channels, is reduced in the aorta of intact female compared with male

rats and OVX female rats. The reduced vasoconstriction in intact female rats is likely due to long-term effects of estrogen on the expression and permeability of voltage-gated Ca^{2+} channels [79].

PKC is a ubiquitous protein kinase that comprises a family of Ca^{2+} -dependent and Ca^{2+} -independent isoforms expressed in VSM of various vascular beds. During cell stimulation, PKC may activate other protein kinases that ultimately interact with the contractile myofilaments and enhance VSM contraction [80]. Sex-related decrease in VSM contraction in female compared with male Wistar-Kyoto (WKY) rats is associated with reduction in the expression and activity of vascular α -, δ -, and ζ -PKC. Treatment of OVX females with subcutaneous E2 implants causes reduction in Phe contraction and PKC activity that is greater in SHR than WKY rats. These findings suggest sex differences in VSM contraction and PKC activity that are possibly mediated by estrogen and are enhanced in the SHR model of genetic HTN [81].

Rho-kinase is known to inhibit MLC phosphatase and to enhance the VSM myofilament sensitivity to $[\text{Ca}^{2+}]_i$. Rho-kinase is upregulated in CVD and may play a role in the pathogenesis of coronary arteriosclerosis and vasospasm [82]. Estrogen inhibits Rho-kinase expression/activity. For instance, Rho-kinase expression may involve a PKC/NF- κ B pathway that is inhibited by estrogen [83]. Also, the vasodilator response to the Rho-kinase inhibitor Y-27632 is similar in OVX female and male rats, and E2 treatment of OVX rats normalizes the vasodilator effects of Y-27632 to those observed in intact females [84]. Furthermore, in cultured human coronary VSM cells, treatment with E2 causes a decrease in Rho-kinase mRNA expression [82]. Collectively, E2/ERs appear to mediate a vasodilator response through both genomic and nongenomic activation of endothelium-derived relaxing factors and inhibition of VSM contraction pathways.

Estrogen, the cytoskeleton and Extracellular Matrix (ECM)

The actin cytoskeleton forms the backbone of the cell, and its spatial organization is crucial for cell movement and migration. Modification of the form and location of actin fibers in relation to membrane anchoring structures such as integrins and focal adhesion complexes allow cell movement in the extracellular environment [85]. $\text{ER}\alpha$ interacts with the G protein $\text{G}\alpha_{13}$ to induce activation of RhoA/Rho-kinase and phosphorylation of the actin-regulatory protein moesin, leading to remodeling of the actin cytoskeleton and EC migration [86]. The ECM provides the architectural framework of the vascular wall, and regulates the behavior of the vascular cells, and their ability to migrate, proliferate and survive injury [87]. Vascular remodeling occurs during all stages of atherosclerotic progression, and matrix metalloproteinases (MMPs), a family of zinc-binding proteolytic enzymes, are involved in these processes [88]. MMP-induced ECM degradation within the atherosclerotic plaque may cause plaque instability and cardiovascular events. It has been shown that increased plasma levels of MMP-2, -9 and -10 in women receiving MHT are associated with elevated risk of plaque instability and cardiovascular events [89].

Estrogen and the Atherosclerotic Process

In women at different stages of life the presence or absence of endogenous estrogens affects cardiovascular function, and these effects may be closely related to different stages in the progression of atherosclerosis. Atherosclerosis is an inflammatory process involving EC dysfunction and excess deposition of oxidized lipids. Atherosclerotic lesions evolve from an initial accumulation of foam cells in the endothelium leading to a fatty streak. This is followed by the accumulation of fatty deposits including cholesterol, creating a true atheroma (Fig. 3). Once an atheroma is formed, collagen in the fibrous cap stabilizes the plaque and prevents it from rupturing. MMPs, which are produced by the inflammatory cells

in the atheroma, degrade collagen, leading to disruption of the fibrous cap, rupture of the plaque, and release of a thrombus that may occlude the artery [90]. E2 reduces the development of early atherosclerotic lesions by affecting lipid metabolism and reducing lipid deposits in the endothelium. However, once an atheroma is established, estrogen increases MMP expression, causing disruption of the fibrous cap and rupture of the plaque. If the capsule ruptures, E2 could become thrombogenic, leading to clot formation and occlusion of the arterial lumen [91]. Thus, estrogen, through different mechanisms, inhibits early development of atherosclerosis, but may increase the risk of cardiovascular events once atherosclerosis has been established (Fig. 3).

Earlier clinical studies have shown that intravenous administration of E2 may cause direct vasodilatation in healthy women as well as in women with atherosclerotic disease [92]. However, data from the Cardiovascular Health Study, a longitudinal study of cardiovascular risk factors in 1,636 women older than 65 years of age, showed an association between MHT and flow-mediated vasodilation only in healthy Post-MW. MHT had no effect on brachial artery blood flow in older women (> 80 years), in women with documented CVD, or in women with a combination of cardiovascular risk factors [93]. A recent study also demonstrated that E2 administration improves flow-mediated vasodilatation to a greater extent in women within 5 years since menopause than in women more than 5 years after menopause [94]. These data demonstrate sensitivity to estrogen in younger women with healthy endothelium, but not in older women where the vascular endothelium could be damaged by atherosclerotic lesions. Experimental studies in monkeys also demonstrated that estrogen inhibited the development of atherosclerotic plaque formation when given directly after ovariectomy; however, when estrogen treatment was started 2 years after ovariectomy, there was no inhibition of coronary atherosclerosis [95]. Similarly, in OVX female rats with carotid intima damage, estrogen inhibits intima hyperplasia and VSM cell proliferation when given on the day of injury, 15 minutes before, and continuing 3 days after injury, but does not have any beneficial effects when administered 7 days after injury [96]. Also, in pigs, a single dose of E2 delivered locally after balloon injury of coronary artery improves reendothelialization and enhances endothelial function at the injured site within one month after percutaneous coronary angioplasty [97]. Thus, both clinical and experimental animal studies support that estrogen has beneficial effects in the early stages of atherosclerotic disease.

Results from MHT Randomized Clinical Trials

Earlier observational studies of MHT in Post-MW have shown reduction in the risk of a cardiovascular event in women using unopposed oral estrogen [6]. Also, a meta-analysis of these studies has shown that MHT is associated with a one-third reduction in fatal CVD [98]. One observational study demonstrated that MHT was associated with favorable outcomes after coronary angioplasty and coronary artery bypass graft surgery [99]. Also, a study in 337 women undergoing elective percutaneous transluminal coronary angioplasty has shown that MHT users have fewer cardiovascular events (12% vs. 35%) and better survival (93% vs. 75%) as compared to nonusers [100]. Another observational study demonstrated that the mortality after MI was reduced in Post-MW receiving MHT [99]. Similarly, some small RCTs have shown beneficial vascular effects of MHT in healthy Post-MW. A study in early Post-MW randomized to E2 showed less progression in carotid artery intima-media thickness as measured by ultrasound than Post-MW randomized to placebo [101]. Similarly, in women randomized to either MHT or placebo for 2 to 3 years around the time of menopause and subsequently followed without treatment for up to 15 years, those initially assigned to MHT had less subsequent aortic calcification and cardiovascular deaths than those assigned to placebo [102]. However, concerns have been raised with respect to the findings of CVD benefits of MHT in the observational studies partly because some

studies were not well-randomized, and women who chose to take MHT might have been healthier than those who did not (“healthy user” bias). Also, MHT mostly started in the direct perimenopausal or postmenopausal period for the relief of menopausal symptoms. Furthermore, it was difficult to compare the findings of these studies because different MHT preparations were used for different periods of time [103].

Despite the benefit of MHT on cardiovascular events in most observational studies and some small clinical trials, some observational studies have shown that short-term use of MHT may be associated with increased risk of CVD in women. The Nurses’ Health Study, a prospective observational cohort study of secondary prevention of CHD which included 2,489 women with a previous MI or documented atherosclerosis showed that the risk for recurrent major coronary events increased among short-term MHT users but decreased among long-term users [104]. Also, most RCTs failed to show tangible cardiovascular benefits of MHT. In a placebo controlled RCT of 1,017 women age 50-69 years who had experienced a first MI, unopposed E2 was neither harmful nor beneficial in terms of frequency of re-infarction or cardiac death after 2 years of treatment. However, in the active treatment group noncompliance was high (> 50%) after 1 year, which weakened the power of this study [105]. Other major RCTs such as HERS and WHI did not support benefits of MHT in CVD [11]. HERS comprised 2763 Post-MW (average age 67 years) with established CVD, and administered daily CEE (0.625 mg) and medroxyprogesterone acetate (MPA) (2.5 mg) or placebo. After approximately 4.1 years of follow-up, HERS found no difference in secondary CVD outcome (nonfatal MI and CVD death), even though MHT reduced LDL-c and increased HDL-c [11]. Importantly, a marked increase (52%) in adverse CVD events was found in the MHT group during the first year of treatment, and no protective effects were observed after an additional 2.7 years of follow-up [106]. WHI was a double-blind RCT conducted in healthy Post-MW between 50 and 79 years of age to evaluate whether MHT was effective in primary prevention. WHI had two arms; one arm studying the impact of CEE (0.626 mg/day) plus MPA (2.5 mg/day) or placebo in 16,608 women with a uterus, and the second arm studying the impact of CEE (0.625 mg/day) alone or placebo in 10,739 women without a uterus. The outcome of WHI was that neither estrogen nor estrogen plus progestin decreased CVD. WHI was prematurely stopped in July 2002, after a mean of 5.2 years of follow-up because the Data and Safety Monitoring Board (DSMB) concluded that the evidence of breast cancer harm, along with some increase in CHD, stroke, and pulmonary emboli, outweighed the evidence of benefit for bone fractures and possible benefit for colon cancer [16] (Fig. 1).

Evidence from observational studies and clinical trials also raised concerns regarding potential relationship between MHT and venous thromboembolism (VTE). VTE is uncommon before menopause, but its incidence increases with age after menopause and reaches 1 per 100 over age 75 years [112]. Observational studies have suggested that MHT use is associated with increased risk of VTE [116, 117]. Also, RCTs have shown a 2-fold increased risk of VTE with MHT use [11,16,110]. Recent large cohort case-control study of 23,505 Post-MW with VTE matched with 231,562 controls revealed that the risk of VTE increased with current use of oral estrogen (relative risk (RR) 1.49; 95% confidence interval (CI) 1.37–1.63) and oral estrogen–progestogen combinations (RR 1.54; 95% CI 1.44–1.65). The increased VTE risk with oral estrogen was highest in the first year after initiation of treatment and decreased in subsequent years. On the other hand, there was no significant increase in VTE risk with the use of transdermal estrogen, even in patients with pre-existing thrombophilia [118]. Other studies have shown a higher risk of VTE with higher doses of estrogen [119]. The WHI study demonstrated a significant overall doubling of VTE events with MHT in the estrogen/progestogen arm [120], whilst the overall effect of MHT on VTE events was not significant in the estrogen-alone arm (Table 2).

Another concern with MHT use is the risk of stroke. The Nurses' Health Study reported that MHT increased the risk of stroke by 35% [10]. WHI reported a 30% to 40% increased risk of stroke for women given estrogen combined with progestin or estrogen alone [121]. The Women's Estrogen for Stroke Trial (WEST) examined the relation between MHT and ischemic stroke and transient ischemic attacks (TIA) among 664 Post-MW randomized to either E2 or placebo [12]. Women were followed for up to 4 years (mean 2.8 years), the primary endpoint was stroke and death, and the secondary endpoints included TIA and nonfatal MI. The results demonstrated no net benefit for the primary endpoint. In a secondary analysis, there was an increased risk of fatal stroke or death within 30 days of a stroke. During the first 6 months, there were 3 fatal strokes and 18 nonfatal strokes in the group randomized to E2, compared with 1 fatal stroke and 8 nonfatal strokes in the placebo group (RR 2.3; 95% CI 1.1-5.0).

Factors Responsible for the Divergent Outcomes of MHT on CVD

Despite the initially reported favorable effects of MHT on endothelial function, lipid metabolism and plasma lipoproteins, MHT appears to be associated with disturbances in other cardiovascular mechanisms that increase the risk of CVD. The lack of vascular benefit of MHT in RCTs may be explained by several factors including the age of Post-MW, timing of MHT, type, dose and route of administration of MHT, and interaction with other sex hormones/metabolites.

MHT and Age

The discrepancy between the results of the observational studies and the RCTs may be explained by various confounding factors or selection biases, but the age of participating women may have played a major role. The menopausal and cardiovascular changes in aging women are partly related to the substantial decrease in plasma estrogen levels to <100 pmol/L. Age-related changes in ER amount, distribution or affinity may also contribute to menopausal and cardiovascular symptoms [38]. One study demonstrated that ER β expression exceeded ER α expression in all layers of human coronary arteries; the ER β expression correlated with advanced atherosclerosis and tended to be greater in non-MHT than in MHT users [46]. DNA methylation has been proposed to play a role in age-related disease, and the decrease in ER α expression could be attributed to age-related methylation of the ER α gene, particularly in human coronary atherosclerotic plaques [127]. Methylation of a cytosine and guanine rich area in the promoter region of the ER α gene, called CpG island, is a major mechanism for downregulation of gene expression. Methylation-induced inactivation of ER α gene in vascular tissue may play a role in atherogenesis and aging of the vascular system [58]. This is supported by reports that human coronary atherosclerotic tissues show higher methylation levels (28.7%) than normal arterial (6.7-10.1%) and venous (18.2%) tissues [49].

The vascular effects of estrogen could also be modified by other vascular changes associated with aging such as endothelial dysfunction, changes in the mechanisms of VSM contraction, and increased production of inflammatory cytokines. Age-related vascular remodeling involves endothelial dysfunction, enhanced growth of VSMCs, and increased vascular plaques. Vascular aging also involves changes in the mechanical and structural properties of the vascular wall. Arteries become stiffer and less compliant due to quantitatively less elastin and more collagen, and impaired endothelial-mediated vasodilation [38]. There are also changes in wall thickness, media to lumen ratio and EC-derived vascular mediators. With age, NO production decreases and ET-1 production increases. This favors a procoagulant state and promotes VSM growth. Also, atherosclerosis progresses through lifetime and is enhanced by smoking, dyslipidemia, diabetes, and HTN. Atherosclerosis leads to thick and stiff arterial wall, calcification and plaque formation, and thereby changes

the mechanical properties of the arteries and increases the risk of cardiovascular events [128]. Arteries of Post-MW have some degree of atherosclerosis that could mechanically impede the vasodilator effects of estrogen.

Early loss of endogenous E2 is associated with CVD. Most autopsy studies demonstrate increased CVD in young oophorectomized women [6]. The Framingham Study shows that women entering menopause early naturally or surgically have a greater likelihood of developing CVD compared to age-matched Pre-MW. The Nurses' Health Study has shown an increased risk of CVD in women with bilateral oophorectomy not receiving MHT [21]. In Pre-MW, endogenous E2 may counteract the age-related manifestations of vascular remodeling including endothelial dysfunction, enhanced growth of VSMCs, and increased prevalence of vascular plaques. Although it is difficult to separate vascular changes due to aging from those due to menopause, comparisons between men versus age-matched Post-MW and between Pre-MW versus Post-MW suggest that endogenous E2 delays CVD. In support of this concept, E2 inhibits many processes involved in age-associated vascular remodeling, including endothelial dysfunction and VSMC proliferation, and lowers cholesterol and improves vascular tone [3].

Time since menopause, rather than type of menopause (natural or surgical), is a major factor in subclinical atherosclerosis [129]. In women 35 years of age, only fatty streaks and minimal atherosclerotic plaques occur in coronary arteries. The incidence of plaques is only evident in women around perimenopause (40–50 years age), and a steep increase in the incidence of plaque is seen after 50 years of age (around menopause). Active progression of atherosclerotic lesions takes place at 45–55 years of age when menopause occurs, and these lesions start to develop complications when women are ≥ 65 years of age [3]. Menopause is also associated with marked endothelial dysfunction, decreased endothelium-dependent relaxation and flow-mediated dilation, and intimal thickening [3]. A significant increase in the prevalence of plaques and intima-media thickening (IMT) occurs 5–8 years after menopause [130]. Also, compared to Pre-MW, Post-MW have greater levels of total cholesterol, LDL cholesterol, and apolipoprotein B, which represent primary cardiovascular risk factors during menopause [131]. The progression rate of vascular disease may also depend on age at menopause and status of subclinical atherosclerosis. In women who underwent bilateral oophorectomy, intimal thickening increases with years since menopause and reaches significance 15 years after menopause [129]. Age-dependent progression of carotid intimal thickening in Post-MW is reduced by MHT [132]. These findings suggest that estrogen plays a role in regulating vascular intimal thickening.

HTN may contribute to acceleration of atherosclerosis following menopause. Post-MW ≥ 60 years old make up the majority of hypertensives and are more likely to develop CVD [133]. In Post-MW the activity and synthesis of pro-hypertensive factors are increased, whereas the synthesis of blood pressure-lowering factors is decreased [134]. Thus age-related changes in plasma estrogen levels and vascular ERs are major factors in the progression of atherosclerosis and vascular dysfunction, and therefore could affect the cardiovascular effects of MHT in aging women.

Role of Timing of MHT

The discrepancy between the results of the observational studies and the RCT may also be related to the timing of initiation of MHT. It has been hypothesized that the onset of atherosclerosis may be delayed by estrogen, while the more advanced atherosclerotic lesions may not be influenced by MHT, or may even become more unstable [135]. Data from the Nurses' Health Study suggest that the time when MHT is initiated influences cardiovascular benefit. Women who participated in this study were between 30 to 55 years of age, and approximately 80% of participants initiated MHT within 2 years of the onset of menopause

[10]. In contrast with observational studies such as the Nurses' Health Study, RCTs enrolled older women who initiated MHT 10 years after the beginning of menopause. Women in HERS were on average 67 years of age and had been postmenopausal for many years at the time of enrollment. In HERS, the time from menopause to randomization was 23 years compared with 13 years in the Estrogen in the Prevention of Atherosclerosis Trial (EPAT) [15]. Although WHI was a primary prevention trial, similar to HERS the participants in WHI were older (50–79 years) with only 10% of the participants between 50 and 54 years and 20% between 54 and 59 years. Time since menopause may also be associated with other factors that affect the cardiovascular health of women. In women assigned MHT in WHI, 36% had HTN, 13% were being treated for hypercholesterolemia, 4.4% were being treated for diabetes, and 10.5% were current smokers. Although WHI was supposed to be a study of healthy women, more than two-thirds of women studied were overweight or obese, more than one-third were HTN, and 8% reported previous CVD [17].

Experimental studies have measured atheromatous plaque size in ovariectomized cynomolgus monkeys fed a moderately atherogenic diet, and then treated with CEE or placebo. Treatment with CEE resulted in approximately 70% reduction in coronary artery atheromatous plaque size relative to that in the placebo group [136]. In OVX monkeys, a 70% protection was observed when hormone therapy was initiated simultaneously with the atherosclerotic diet. A marginal delay in initiation of hormone therapy, i.e. after moderate atherosclerosis, resulted in only a 50% protection. When hormone therapy was begun 2 years into the atherosclerotic diet, no protection was observed [95]. Similarly, in rats, administration of E2 prior to and during, but not 7 days after balloon injury resulted in inhibition of neointima formation [96]. Thus the timing of initiation of MHT with respect to the onset of menopause may have important ramifications on the potential benefits in preventing or delaying the progression of atherosclerosis and CVD. Around the time of menopause, women still have relatively healthy arteries, allowing a 'window of opportunity' for MHT to produce cardiovascular benefit. However, arterial disease increases as a function of time after menopause, and the diseased arteries become less responsive to the beneficial effects of estrogen.

Role of Type of MHT

The outcome of MHT in Post-MW could be affected by the type of estrogen used. Natural estrogens are structurally similar to E2 produced in the ovaries, and are readily metabolized and excreted. CEE is a common form of MHT derived from urine of pregnant mares and is available in formulas like Premarin, Prempro, Premphase and Prempac. CEE contains at least 10 estrogens in sulfate ester form including the saturated estrogens E1, 17 β -E2 and 17 α -E2, and the unsaturated estrogens equilin, 17 β -dihydroequilin, 17 α -dihydroequilin, equilinenin, 17 β -dihydroequilenin, 17 α -dihydroequilenin, and Δ^8 -E1 [137]. CEEs are metabolized in the body. Unconjugated estrogens (e.g. equilin) are absorbed more rapidly than conjugated estrogens (e.g. equilin sulfate), but they are soon conjugated in the liver (first pass effect) and circulate together with E1-sulfate as a hormonally inert estrogen reservoir. Tissue enzymes such as sulfatases and 17 β -hydroxysteroid dehydrogenases secreted by the bowel bacterial flora and the intestinal mucosa activate these inert estrogens to E1 and E2 [138]. These estrogens are excreted in the urine, along with glucuronide and sulfate conjugates. CEE in both oral and transdermal forms increase flow-mediated vasodilation in healthy Post-MW [38]. In the Postmenopausal Estrogen/Progestin Interventions (PEPI) study, treatment of Post-MW with CEE caused a 6-9% decline in intracellular adhesion molecules and 26-33% decline in MMP-9 levels, but increased C-reactive protein (CRP) levels by 121-150% after one year of treatment [139]. Also, in hysterectomized Post-MW treated with unopposed CEE there was increased risk of adverse arterial events including carotid arterial events, lower extremity arterial events, and

abdominal aortic aneurysm [140]. The WHI estrogen alone arm showed an increased risk of stroke in women receiving 0.625mg of CEE per day [121]. Also, *in vitro* studies of human aortic VSM cells have demonstrated that the major components of CEE, i.e. estrone, estriol, and estrone sulfate, are less potent in inhibiting mitogen-induced VSM cell growth and MAPK activity as compared to E2 [3].

Synthetic estrogens such as diethylstilbestrol, esterified estrogen, ethinyl estradiol, estradiol benzoate, cypionate, and valerate may provide cardiovascular benefits. Post-MW treated with esterified estrogen alone have lower risk of ischemic stroke and myocardial infarction, and no increase in venous thrombosis risk compared to Post-MW treated with CEE [117, 141]. Also, experimental studies demonstrated that treatment of ovariectomized hypertensive rats with estradiol valerate causes an increase in serum vascular endothelial growth factor (VEGF), which accelerates endothelial cell growth both *in vitro* and *in vivo* [142].

The realization that MHT may not be as safe as previously thought has increased the interest in phytoestrogens. Phytoestrogens are a large group of plant-derived compounds containing a phenolic ring that allows them to bind ERs and produce estrogenic effects. Phytoestrogens are found in peas, beans, alfalfa and other plants in relatively high concentrations [128]. Phytoestrogens are also found in a variety of foods particularly soybeans, red clover, and wheat grains [143]. Interestingly, food containing phytoestrogens is consumed in significant quantities in cultures with lower rate of menopausal symptoms, osteoporosis, cancer and CVD [144]. Phytoestrogens are classified into groups according to their chemical structure. The greatest estrogenic activity is found in flavones, isoflavones, flavonols, flavanones, and lignans. The most common of these are the isoflavones and lignans, which are found mainly in vegetables and whole grains. These compounds have a steric structure similar to that of steroidal estrogens allowing them to bind to ER and exert various estrogenic or anti-estrogenic effects. Phytoestrogens, notably isoflavones, bind with a greater affinity to ER β than to ER α [145]. Phytoestrogens' affinity for and activation of ER is weak, with a binding affinity between 10^{-4} and 10^{-2} of that of E2; however phytoestrogens can be present in the blood at levels up to 10,000 times that of steroidal estrogens [145]. Isoflavones have a limited distribution in nature and amongst commonly consumed foods are found in physiologically relevant amounts only in soybeans and foods derived from soy. Greater reliance on vegetables and particularly legumes such as soy for dietary protein is observed in communities such as Japan, China, South East Asia and Central America, and those communities typically have substantially higher dietary isoflavone levels than those found in Western countries [145]. Soy beans contain three primary isoflavones in their glycoside form – genistin, daidzin and glycitin. Digestion leads to the cleavage of the sugar moiety resulting in the formation of the respective aglycones, genistein, daidzein and glycitein [145]. Isoflavones activate eNOS, induce vasodilatation and may have anti-atherogenic and anti-thrombotic effects. Also, genistein and daidzein decrease monocytes chemoattraction protein 1 (MCP-1) and collagen-induced platelet aggregation [145]. A meta-analysis of randomized placebo controlled trials to evaluate the effects of oral isoflavone supplementation on endothelial function in Post-MW as measured by flow mediated dilatation (FMD) showed that isoflavone supplementation improved flow in women with low baseline FMD, but not in women with a high baseline FMD [146]. Another meta-analysis of 38 controlled human studies of soy consumption provided evidence of a positive effect on lipid profiles including reduction in the levels of LDL-c and triglycerides and increases in HDL-c [147]. However, a 12 month double-blind RCT that compared the effects of soy protein containing 99 mg isoflavones/day (aglycone weights) with those of milk protein (placebo) on blood pressure and endothelial function in 202 post-MW aged 60–75 years did not find any effect on blood pressure, body weight or endothelial function [148].

In search for alternative MHT, research has focused on the potential benefits of selective estrogen receptor modulators (SERMs). SERM is a non-steroidal molecule that binds with high affinity to ERs, but has tissue-specific effects distinct from estrogen [149]. An ideal SERM will act as an ER agonist on the cardiovascular system, bone, vagina and bladder, while acting as an ER antagonist on the breast and endometrium. Based on the positive effects of SERMs recorded on intermediate markers (blood lipids and markers of inflammation), it has been postulated that the use of SERMs in Post-MW may reduce coronary artery disease (CAD). All SERMs reduce the levels of total cholesterol, LDL-c, triglycerides, fibrinogen and proinflammatory cytokines such as interleukin-6 and tumor necrosis factor- α [150]. An analysis of the large scale clinical trial “Raloxifene Use for the Heart” (RUTH), evaluating long-term efficacy and safety of this SERM in the prevention of CHD and breast cancer in Post-MW at risk of coronary events, demonstrated no cardiovascular benefits of raloxifene over a 5.6-year follow-up period and cautioned against increased risks of VTE and stroke [113]. Also, the Multiple Outcomes of Raloxifene Evaluation (MORE) study showed that raloxifene therapy for 4 years did not significantly affect the risk of cardiovascular events in the overall cohort (RR 0.86; 95% CI 0.64–1.15), but reduced the risk of cardiovascular events (RR 0.60; 95% CI 0.38–0.95) in a subset of women with increased cardiovascular risk and multiple risk factors. The risk of stroke was unchanged (RR 0.68; 95% CI 0.43–1.07) and the risk of VTE was increased over the first 4 years of MORE (RR 2.1; 95% CI 1.2–3.8), but significantly increased (RR 1.7; 95% CI 0.9–3.1) over 8 years (including 4 years’ extension) [151].

27-hydroxycholesterol (27HC), an abundant cholesterol metabolite that is elevated with hypercholesterolemia and found in atherosclerotic lesions, is a competitive antagonist for vascular ERs and may serve as endogenous SERM. Although the binding affinity of 27HC to ER α and ER β is similar, its inhibitory effect of E2-induced transcription is stronger for ER β than ER α [152]. Under normal conditions in Pre-MW, the amount of 27HC generated from cholesterol is lower than the level of estrogen, thus the ER function would be preserved leading to vascular protection. In contrast, when the level of 27HC is higher relative to that of estrogen, such as during the postmenopausal period or as a consequence of hypercholesterolemia, the vascular function of ER would be inhibited, resulting in loss of vascular protection. This may help explain why women are better protected than men from cardiovascular disease until they reach the age of menopause [152]. The ER antagonistic properties of 27HC may also partly explain why the MHT clinical trials failed to show a cardioprotective benefit in Post-MW [152].

Ex vivo studies on rat renal and pulmonary artery rings and porcine coronary arterial rings have shown that raloxifene inhibits VSM contraction through inhibition of Ca²⁺ influx *via* voltage-gated Ca²⁺ channels [153]. Raloxifene and E2 have shown different effects in human umbilical venous endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs). Treatment with E2 or raloxifene increased both IGF-I and COX-2 mRNA expression in HUVECs, but reduced serum-induced expression of COX-2 in HASMCs. Treatment with E2 and raloxifene induced recruitment of co-activator complexes and histone acetylation at both the IGF-I and COX-2 gene promoter in HUVECs, but decreased these parameters in HASMCs [154]. These findings indicate that SERMs have different effects on multiple processes in different tissues. Although currently available SERMs show limited cardiovascular benefits, as new SERMs are developed and more studies are done on existing SERMs, they may be used as MHT to decrease the risk of CVD in Post-MW.

Significant progress has been made to develop compounds that act selectively on ER subtypes. Selective ER agonists such as propylpyrazole triol (PPT) and diarylpropionitrile (DPN) have been developed. Members of the triarylpyrazole class such as propylpyrazole trisphenol (PPT) are 400-fold more potent on ER α than ER β [155]. Studies with selective

ER agonists suggest that ER α mediates most of the vascular actions of estrogen [156]. PPT increases flow-mediated relaxation in small mesenteric arteries from female, but not male mice [157]. Diarylpropionitrile (DPN) is a potent ER β agonist with a 30- to 70-fold selectivity over ER α . DPN induces rapid NO-dependent vasodilation [158]. Specific ER antagonists may be useful in preventing some of the undesirable effects of E2 such as breast and uterine cancer. ER antagonists bind to the ER ligand-binding pocket, thereby occluding agonist access and inducing a conformational change in ER that prevents efficient interaction with transcriptional coactivators [20]. The search for ER antagonist has led to the synthesis of a series of steroidal 7-alkylamide analogs of E2. ICI 164,384, the first estrogen antagonist described, blocks the uterotrophic effects of E2 or tamoxifen in rats. ICI 182,780 (fulvestrant) is a more potent E2 antagonist approved for treatment of advanced breast cancer in Post-MW [20]. Both ICI 164,384 and ICI 182,780 are classical ER antagonists that block the activity of E2 *via* inhibition of both AF-1 and AF-2 action [20]. By introducing basic side chain substituents such as those found in tamoxifen, ER α selective antagonists, such as methyl-piperidino-pyrazole (MPP) have been developed [159]. Further research is needed to develop specific estrogenic compounds with high cardiovascular benefits in Post-MW.

Dose of MHT

The adverse effects of MHT observed in RCTs may be related to the relatively high dose of estrogens used in these studies. Estrogens are highly lipophilic compounds and their circulating levels may not reflect their vascular tissue level. Also, estrogens are extensively bound to plasma proteins, particularly globulin. Furthermore, the hormone pharmacokinetics and volume of distribution may change during menopause particularly in women with liver or kidney disease. Thus, an estrogen dose that may seem normal in Pre-MW could produce superphysiological plasma levels in Pos-MW, and lead to estrogenic side-effects [20]. These observations have suggested that smaller doses of estrogen might be a safer strategy. Data now suggest that the benefits of MHT can be maintained with a dose lower than previously used. Women's Health, Osteoporosis, Progestin, Estrogen (Women's HOPE) trial have demonstrated efficacy and improved benefit/risk ratios of lower-dose combinations of CEE/MPA (0.45/ 1.5 mg and 0.3/ 1.5 mg) for prevention of bone loss, relief of vaginal atrophy and vasomotor symptoms, and reduction in endometrial bleeding [160]. Also, the Clinical study on Hormone dose Optimization In Climacteric symptom Evaluation (CHOICE) trial has shown efficacy of ultra-low-dose of MHT (0.5 mg E2+0.1 mg norethisterone acetate (NETA) or 0.5 mg E2+0.25 mg NETA) in the reduction of moderate to severe hot flushes, with a rapid effect after 3 weeks. Low dose and ultra-low-dose of combined estrogen plus progestin regimens have also been shown to prevent osteoporosis [161]. Also, in a 2-year study of low- and ultra-low-dose combinations of oral E2 alone or with norethisterone, doses as low as 0.25 mg E2 produced marked increases in bone mineral density (BMD) and therefore can prevent osteoporosis [162].

Studies have also shown that the effect of estrogen on lipids and lipoproteins may change with the dose used. In the HOPE trial, the degree of LDL-c reduction and HDL-c increase was less with the lower doses of CEE [163]. However, even at the lower doses of CEE, the beneficial effects were maintained, whilst the impact on hemostatic parameters, e.g. the reduction in protein S activity, was minimized. The increase in triglyceride levels with lower doses of CEE was less pronounced. Also, a study comparing low dose of MHT (CEE 0.3 mg/MPA 2.5mg) versus standard-dose of MHT (CEE 0.625 mg/MPA 2.5mg) demonstrated similar increase in the maximal forearm blood flow (FBF), decrease in serum concentrations of LDL-c and malondialdehyde (MDA)-modified LDL in both treatment groups [164].

The vascular effect of estrogen may in part reflect its effect on MMP activity. At low doses, E2 may inhibit MMPs and thereby attenuate excess collagen deposition, whereas at high

doses E2 may activate MMPs and thereby promote vascular lesion formation or plaque destabilization. Studies in cultured human coronary artery and umbilical artery VSM cells have shown that increasing levels of E2 from physiological to supraphysiological levels were associated with dose-dependent increases in MMP-2 levels in culture media. E2 appeared to be a specific stimulator of MMP-2 release from human vascular cells. The concentration dependence of this effect suggested a basis for the differential effects of low and high estrogen levels on vascular integrity [165]. Thus, the dose of estrogen could influence its vascular effects and the relatively high dose of MHT may explain the adverse effects observed in RCT.

Route of Administration of MHT

The route of administration could also be a factor for successful MHT. Estrogen can be administered orally, or non-orally as a percutaneous gel, transdermal patch or subcutaneous implant. Studies have examined the benefits and side effects of oral versus transdermal application and found that transdermal applications of MHT may have similar vascular benefits and less adverse side effects than oral preparations [38]. Oral E2 increases CRP levels and induces the expression of MMPs in the vascular wall, which in turn degrade and weaken fibrous caps of vulnerable plaques, and lead to plaque rupture and release of thrombus in the arterial lumen. In healthy post-MW, transdermal E2 increases brachial artery flow-mediated vasodilation similar to oral administration, but does not increase CRP levels [38]. Transdermal E2 also avoids exaggerated peaks in plasma estrogen concentration that are common with oral administration and thus results in a rate of conversion to estrone that is similar to that observed during the menstrual cycle [38]. A study evaluating the routes of administration and the risks of breast cancer in post-MW found that oral E2/progesterone elevated the risk of breast cancer, while oral unopposed estrogen and transdermal E2/progesterone did not [166].

Most of the earlier observational MHT studies relate almost entirely to oral therapy. These observational studies have shown that MHT use is associated with a reduction in CHD of around 40%. Also, analyses from the Nurses' Health Study have shown that MHT use is effective for both primary and secondary prevention of CHD [10]. One epidemiological study reported a decreased risk of CHD with transdermal therapy, whereas there was no overall effect of oral MHT [167]. However, to date, epidemiological data suggest that the route of administration of MHT has no impact on the risk of breast cancer and hip fracture, and the results on the risk of CHD and colorectal cancer are inconsistent. Also, few MHT studies have evaluated the risk of stroke and diabetes to allow meaningful conclusions. There is also a suggestion that transdermal MHT may be less deleterious than oral MHT regarding VTE which needs to be confirmed. Thus, the route of administration of MHT should remain an active area of research to identify treatment modalities that would have the least adverse effects [168].

Estrogen Interaction with Other Sex Hormones

Other sex hormones may modify the vascular actions of estrogen, or have direct effects on the vasculature. Studies have suggested that the relationship between circulating levels of free E2, free testosterone, and sex hormone-binding globulin (SHBG) may be more predictive of changes in carotid intimal thickening than the levels of any of these hormones alone [169]. Also, conversion of testosterone to E2 may contribute to the regulation of the peripheral circulation in men, and administration of an aromatase inhibitor to young men results in a decrease in endothelial vasodilator function. Additionally, the circulating levels of estrogen and androgens may not reflect those at the tissue level, as both aromatase and 5- α -reductase are found in a number of tissues including blood vessels [20]. Furthermore, steroid hormone metabolism is a complex process that allows extensive inter-conversion of

sex hormones precursors and metabolites. With the growing use of steroid metabolism inhibitors, including aromatase inhibitors and 5- α -reductase inhibitors, particularly in women with a history of breast cancer, cardiovascular side effects are predicted [20].

Role of Progestins

Progesterone is a steroid hormone produced by the gonads and adrenal cortex, and by the placenta during pregnancy. Progesterone receptors have been identified in ECs and VSM of humans, mice, rats, rabbits and primates [170]. Similar to E2, progesterone has anti-atherosclerotic effects, decreases LDL-c, and increases HDL-c. Progesterone causes pulmonary vasodilation via endothelium-dependant pathways. It stimulates eNOS expression, and NO-mediated relaxation in rat aorta and ovine uterine artery [171]. It also causes activation of COX and increases vascular PGI₂ production. It inhibits VSM proliferation/migration and facilitates the inhibitory effects of estrogen. Progesterone also causes rapid relaxation of agonist- or KCl-induced contraction in endothelium-denuded porcine coronary artery [172].

However, progesterone produces less vasorelaxation than estrogen, and may even antagonize the vasoprotective effects of estrogen. MPA may attenuate the E2-induced increase of nitrate/nitrite, surrogate markers for NO production, in Post-MW [173], although intermittent addition of MPA for 10 days every 3 months to E2 did not inhibit E2-induced increase in plasma NO levels after 6 months [174]. A study comparing the effect of sequential estrogen/progestin treatment cycle, i.e. two weeks with E2 alone followed by two weeks with an E2/NETA MHT in Post-MW found that the excretion of cGMP was increased with both forms of administration [175]. The same study compared the effect of oral versus transdermal sequential E2/NETA MHT on the PGI₂ to thromboxane ratio and found an increase of PGI₂ metabolite during the E2 phase, and an increase of thromboxane metabolite during combined E2/NETA phases with both oral and transdermal administration, but failed to show significance possibly due to inter-individual variations [175]. Another study demonstrated an increase in thromboxane metabolites with oral E2/NETA but not with transdermal E2/MPA treatment after 12 months [176]. Also, combined estrogens/progestins decreased the production of ET-1 in cultured bovine aortic ECs [177].

Despite the potential beneficial effects of combined estrogens/progestins, progesterone has been shown to counteract E2-induced NO production and vascular relaxation in canine coronary artery. In porcine coronary artery rings, progesterone-induced reduction in NO production is blocked by E2 [178]. Progesterone antagonizes the anti-oxidant effects of E2, and enhances NADPH oxidase expression/activity and the production of reactive oxygen species in OVX mice [179]. Progesterone also promotes upregulation of vascular AT₁R [180, 181].

It has been suggested that estrogenic MHT in Post-W is associated with increased inflammatory response and CRP levels that may trigger acute cardiovascular events. A study of 389 Post-W demonstrated that combined MHT markedly increases CRP but decreases plasma levels of soluble intracellular adhesion molecule-1, vascular cell adhesion molecule-1, soluble E-selectin, IL-6, and s-thrombomodulin, suggesting that the inflammatory pathways may be attenuated by progesterone [182]. Also, the PEPI study has shown that estrogen in combination with micronized progesterone or MPA increased the levels of CRP but decreased the plasma levels of soluble E-selectin, supporting an anti-inflammatory effect of progesterone [183]. However, one study demonstrated that E2 has anti-inflammatory effects and this effects was diminished in the presence of progesterone [184]. *In vitro* experiments indicate that there may be a difference between the various progestogens on the expression of stimulated cell adhesion molecules [185]; however, when combined with estrogens no detrimental effects are observed [173]. Progesterone also

decreases the levels of plasminogen activator inhibitor-1 (PAI-1) in cultured bovine aortic ECs [173]. Neither MPA nor NET seems to exert an antagonistic effect on the E2-induced decrease of PAI-1 synthesis in human coronary aortic ECs [186]. MHT using transdermal E2-gel and MPA or E2/progesterone demonstrated no decrease in PAI-1 levels, whereas oral E2/P, CEE/P, CEE/MPA and E2/NETA reduced serum PAI-1 levels [187]. This effect was also observed for low dose CEE combined with a progestogen [173]. *In vitro* experiments indicate that the addition of MPA or NET to E2 on EC cultures from human female coronary arteries enhanced E2-induced reduction of MMP-1 and therefore affects the atherosclerotic plaque stability [186]. However, CEE alone or combined with MPA increased MMP-9 levels after 4 weeks in Post-MW with established CAD [173]. These complex interactions of estrogen and progesterone on the vasculature highlight the need to determine the benefits vs. risk of combined estrogen/progestins in postmenopausal CVD.

Drospirenone (DRSP), derived from 17 α -spironolactone, binds to the progesterone receptor with the same affinity as progesterone. It has a higher anti-mineralocorticoid potency, and is a more potent anti-androgenic progestin than progesterone [188]. DRSP has been used as MHT in combination with E2 and as an oral contraceptive in combination with ethinylestradiol. The pharmacodynamic properties of DRSP are similar to natural progesterone. It exerts anti-mineralocorticoid effects that may counteract the salt-retaining actions of estrogens. Large multicenter trials comparing ethinylestradiol-DRSP with ethinylestradiol-desogestrel showed good ovulation inhibition, cycle control, and safety and greater weight reduction in the ethinylestradiol-DRSP group [189]. Some clinical trials have demonstrated that DRSP/E2 lowers blood pressure in hypertensive Post-MW. Furthermore, DRSP/E2 has an additive depressor effect on blood pressure when administered in combination with existing antihypertensive therapy such as angiotensin-converting enzyme inhibitors and angiotensin receptor blockers [190]. Angeliq, an MHT composed of DRSP 2 mg and E2 1 mg, reduces carotid intima-media thickness and climacteric complaints including vertigo/dizziness in Post-MW and this may be related to antiandrogenic and anti-mineralocorticoid effects of Angeliq, respectively [191].

Role of Testosterone

In addition to estrogen and progesterone, androgens may play a role in determining the cardiovascular risk in post-MW. In men, androgens control the development and maintenance of masculine secondary sexual characteristics. In women, androgens are important for maintaining bone mass, secondary sex characteristics and libido [20]. Androgens are produced in the testis, adrenal glands and ovaries. Dehydroepiandrosterone (DHEA), a precursor of sex steroids, and its sulfate ester (DHEAS) are abundant in the human circulation [192]. DHEA and androstenedione do not have significant biological activity, but are converted to testosterone. Testosterone is converted to dihydrotestosterone (DHT), which has higher binding affinity to androgen receptor. Testosterone is also aromatized mainly in adipose tissue to E2.

During the normal female reproductive years, approximately 25% of circulating testosterone originates from the ovaries, 25% from the adrenal glands and 50% from conversion of peripheral androstenedione. Plasma testosterone levels range from 1.0 to 1.5 nmol/L in Pre-MW, and decrease to 0.3-0.5 nmol/L in Post-MW [38]. Serum testosterone level is markedly lower in women than in men, but its level after menopause is unclear [193]. Serial measurements of sex hormones in Post-MW for 10 years after cessation of cycling showed age-related increase in serum testosterone and androstenedione and decrease in E2 and dihydrotestosterone [194]. In cross-sectional studies of women in the Rancho Bernardo cohort, serum testosterone decreased immediately after menopause, but then increased with age, reaching Pre-MW levels at 70-79 years of age. Also, in women with surgical menopause serum testosterone levels did not increase with age, but were 40-50% lower than

in women with natural menopause [195]. These data suggest that natural postmenopause is a relatively hyperandrogenic state [193, 196].

The observations that men have higher BP than women, and CVD develops at an earlier age in men than in women have led to the suggestion that androgens promote CVD in men [197]. However, serum testosterone levels are lower in men with chronic CVD than in healthy age-matched men [197, 198]. Although this suggests that androgens may not mediate CVD, the downregulation of androgen synthesis may be a protective compensatory mechanism that occurs in response to CVD [197]. Experimental studies suggest that androgens may mediate CVD in males. Adult male SHR have higher BP than females, and castration of male rats is associated with reduction in BP to the levels found in females. Also, testosterone treatment of OVX female rats increases BP [193]. Sex differences in BP have also been demonstrated in Dahl salt-sensitive and deoxycorticosterone acetate (DOCA)-salt treated rats [193, 199]. One study has demonstrated that increases in the androgen-to-estradiol ratio may promote pro-hypertensive mechanisms [134]. The effects of menopause on HTN and atherosclerosis may vary between subjects and may be enhanced in women with diabetes mellitus (associated with increased testosterone levels) or obesity [134].

Although testosterone is thought to exert harmful cardiovascular effects, some studies suggest that it may be beneficial. A cross-sectional, population-based study of 6440 perimenopausal women aged 50-59 years demonstrated that testosterone was positively associated with low LDL and high HDL levels in all women, and women with CVD had lower serum androgen levels [200]. In healthy post-MW, plasma levels of testosterone and DHEA-S, a precursor of testosterone, positively correlate with flow-mediated vasodilation in the brachial artery, suggesting a protective effect of testosterone on the endothelium [38]. In surgically Post-MW an oral combination of methyl-testosterone and esterified estrogen reduces apolipoprotein CIII, a major protein in VLDL and triglycerides [201]. Also, low circulating testosterone levels in men are positively correlated with risk factors for CAD [202]. The mechanisms behind testosterone-induced protection are not fully understood, but could comprise effects on traditional risk factors such as lipids and lipoproteins and anti-atherogenic effects via reduction of the atherogenic serum lipid profile. Testosterone also stimulates hepatic lipase and lipoprotein lipase activity and thereby remove serum triglycerides. Interestingly, in men with established CVD, short-term intracoronary administration of testosterone increases coronary blood flow within minutes and is not antagonized by androgen receptor blockers [203]. Experimental studies have shown that testosterone may produce vasodilation independently of estrogen. In porcine coronary artery, testosterone causes relaxation and decreases VSM $[Ca^{2+}]_i$ [172, 204]. Similarly, in porcine coronary artery treatment with dihydrotestosterone, which cannot be aromatized to estrogen, causes vasodilation [203]. These data suggest that testosterone could be protective against CVD in post-MW. Thus, the effects of testosterone on the cardiovascular system and CVD are not clear and warrant further investigation.

Polycystic Ovarian Syndrome (PCOS) is a heterogeneous disorder characterized by polycystic ovaries, menstrual abnormalities (oligomenorrhoea/amenorrhoea), hirsutism, anovulatory infertility, elevated androgens (hyperandrogenemia) and estrogens, as well as obesity, insulin resistance, and lipid abnormalities. Women with PCOS demonstrate low HDL-C (68%), elevated BMI (67%), high blood pressure (45%), hypertriglyceridemia (35%), and high fasting serum glucose (4%), and these abnormalities increase the risk of CVD. A study of 161 PCOS patients has shown that components of the metabolic syndrome are common in PCOS [205]. Also, a retrospective cohort study of 33 women ages 40–59 years with ovarian histopathology typical of PCOS at wedge resection and followed for 22–31 years demonstrated more central obesity and an increased prevalence of diabetes and

HTN in PCOS women compared with age-matched controls [206]. Regarding cardiovascular events, although an association between PCOS and the metabolic syndrome has been suggested, the limited studies conducted in women with PCOS yielded conflicting results. Large prospective cohort studies such as the Framingham or the Nurses Health Study, which focused on hard outcomes such as heart disease or cancer, have failed to identify hyperandrogenemia and anovulation as a separate risk phenotype. A retrospective study from the United Kingdom involving 786 PCOS women diagnosed between 1930 and 1979 failed to establish increased cardiovascular mortality or morbidity [207]. Hence, despite the existence of classical risk factors for CVD such as diabetes, HTN and dyslipidemia in PCOS and the impact of PCOS on surrogate outcomes for CVD, studies to date have failed to establish an effect of PCOS on cardiovascular events.

Lessons Learned and Recent MHT Clinical trials

An important factor that has emerged from the results of experimental studies and what appears to be a critical difference between previous observational studies in humans and the WHI clinical trial is the time of initiation of MHT relative to menopause [15]. The mean age of menopause in the USA is 51 years, which corresponds to the age of women in the Nurses Health Study (NHS) who initiated MHT as part of the usual clinical practice for menopausal symptoms in the perimenopause [115]. This age and timing of MHT initiation contrasts with that of participants in the WHI who were on average 63 years of age at MHT initiation, approximately 12 years past menopause [115]. Because atherosclerosis progresses gradually before being manifested as a clinical event, a significant number of women in the WHI likely had subclinical atherosclerosis at randomization [115]. The presence of subclinical atherosclerotic disease may account for the tendency for increased CVD events early in WHI. Also, HERS showed that in women with preexisting disease, there was an increase in CVD events in the first year or two of the trial. Although continued use of MHT in HERS was associated with a reversal of this trend, the major conclusion from that trial, contrary to conclusions drawn from meta-analyses of the literature, was that women with preexisting CHD were not candidates for preventive therapy with MHT [115].

Two hypotheses emerged from these historical considerations, which require rigorous testing in humans: 1) MHT initiated early after menopause, i.e., prior to the appearance of advanced atherosclerotic lesions, will prevent progression of atherosclerosis, and 2) oral CEE and transdermal E2 may be similarly efficacious in their arterial effects. These hypotheses have prompted two new RCTs: The Kronos Early Estrogen Prevention Study (KEEPS) and Early versus Late Intervention Trial with Estradiol (ELITE) [115].

KEEPS was designed as a randomized placebo-controlled double-blinded prospective trial to evaluate effects of MHT on progression of atherosclerosis as defined by CIMT and coronary arterial calcification (CAC) in women who more closely match the age of initiation of MHT reported by prior observational studies. Women were randomized to daily placebo, oral CEE, or transdermal E2 with placebo or pulsed progesterone for 12 days/month. The women were between 42 and 58 years of age and at least 6 months and no more than 36 months from their last menses with plasma follicle-stimulating hormone (FSH) level ≥ 35 ng/mL and/or E2 levels < 40 pg/mL [115]. Thus, women in KEEPS are younger, healthier, reported more menopausal symptoms, have fewer cardiovascular risk factors than women in the WHI, and more closely match the demographics of women in observational studies. Although observational studies have been criticized for reflecting a “healthy user bias” [208], KEEPS is randomized and blinded such that the outcomes will not be biased by participant group assignment. Also, KEEPS is more representative of the typical clinical experience, i.e. women who are relatively healthy but who are experiencing menopausal symptoms and who seek MHT around the time of their menopausal transition [115]. One limitation of KEEPS is that the majority of participants are Caucasian and results may not be

applicable to diverse ethnic populations. On the other hand, ELITE will randomize 643 women (504 initially proposed), either < 6 years or > 10 years postmenopausal, to receive oral E2 for 2 to 5 years, and as in KEEPS, the primary endpoint is change in CIMT. ELITE will test for differences between early and late initiation of MHT, whereas KEEPS will examine differences between low-dose oral CEE and low-dose transdermal E2. The results of these two studies might help clarify the benefits of MHT timing, and the possible differences in atherosclerosis effects between oral E2 and oral CEE, and between oral and transdermal E2 [20].

Conclusions and Future Directions of MHT in Postmenopausal CVD

The failure of the beneficial effects of estrogen on the vasculature to materialize in RCTs is likely related to age-related changes in ER amount, distribution, integrity, and downstream signaling mechanisms as well as structural changes in the vasculature. These changes may be attributed to age-related ER gene mutations or gene methylation. Further research is needed to further understand the ERs subtype, their distribution, and function in various blood vessels and vascular cells. Differences in ER subtype and postreceptor signaling mechanisms may explain why estrogen promotes EC growth, while inhibiting VSM proliferation.

Subtype-specific ER agonists coupled with specific targeting or drug-delivery techniques (drug-eluting stents, perivascular gel) could be useful in modulating vascular ER activity in a specific blood vessel without affecting other vessels in the systemic circulation. PPT (ER α agonist), DPN (ER β agonist), or G1 (GPER agonist) could be more selective in targeting the vascular ERs while having little undesirable effects on other cell types. Also, SERM could be selective in targeting ERs while having little undesirable effect on breast cancer. Natural hormones that avoid first-pass metabolism in the liver could be more efficient MHT. Also, phytoestrogens may provide a more natural MHT than synthetic compounds. Lower doses of estrogen (0.3 mg) displayed comparable protective effects as those observed with normal doses (0.625 mg), including decreased LDL-c and lipoproteins and marked reductions in coronary atherosclerosis. Similarly, transdermal estrogens are expected to enhance vascular relaxation, and reduce platelet aggregation and vascular inflammation. Further characterization of currently available estrogens and the exploration of novel estrogenic compounds would provide more efficient and specific ER modulators.

Despite the disappointing outcomes of RCTs of estrogen treatment in postmenopausal CVD, there is considerable evidence of beneficial effects of estrogens in the early stages of atherogenesis particularly during the menopausal transition and the early years of postmenopause. The majority of adverse events seen in clinical trials occurred in women who did not begin MHT until 5 years after the onset of menopause, while women who began MHT after surgical menopause demonstrated reductions in coronary atherosclerosis. Therefore, the timing of MHT with respect to the onset of menopause and subject's age should be taken into consideration. The role of other sex hormones such as progesterone and testosterone should also be considered in the design of MHT. Study of diseases associated with abnormal estrogen production or metabolism such as PCOS may provide further insights into the sex hormone environment and ER regulation in Post-MW.

Further research into vascular ERs and alternatives to traditional MHT such as selective ER agonists, SERMs and phytoestrogens, as well as the appropriate route of administration, dose, and timing could provide better approaches to increase the benefits of MHT in CVD.

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List of Abbreviations

CHD	coronary heart disease
CVD	cardiovascular disease
E2	17 β -estradiol
EC	endothelial cell
ECM	extracellular matrix
ELITE	Early versus Late Intervention Trial with Estradiol
eNOS	endothelial nitric oxide synthase
E2	estradiol
EPAT	Estrogen in the Prevention of Atherosclerosis Trial
ER	estrogen receptor
HERS	Heart and Estrogen/progestin Replacement Study
HTN	hypertension
KEEPS	Kronos Early Estrogen Prevention Study
MAPK	mitogen-activated protein kinase
MHT	menopausal hormone therapy
MMP	matrix metalloproteinase
MPA	medroxyprogesterone acetate
NHS	Nurses' Health Study
NO	nitric oxide
PEPI	Postmenopausal Estrogen/Progestin Intervention
Post-MW	postmenopausal women
Pre-MW	premenopausal women
RUTH	Raloxifene Use for The Heart
SHBG	sex hormone-binding globulin
VSM	vascular smooth muscle
VTE	venous thromboembolism
WHI	Women's Health Initiative
WISE	Women's Ischemia Syndrome Evaluation

Fig. 1.

Discrepancies in the vascular effects of estrogen in experimental studies and RCT. Experimental studies largely and consistently demonstrate vascular benefits of estrogen. The excitement generated from the vascular benefits of estrogen observed in initial observational studies, was followed by disappointment from the lack of vascular benefits in RCTs. Recent RCTs such as KEEPS and ELITE may resolve some of the discrepancies regarding the vascular benefits of estrogen and bridge the gap between the findings of experimental studies and RCTs. CVD, cardiovascular disease; ELITE, Early versus Late Intervention Trial with Estradiol; HERS, Heart and Estrogen/progestin Replacement Study; HDL, high density lipoprotein; KEEPS, Kronos Early Estrogen Prevention Study; LDL, low density lipoprotein; MHT, menopausal hormone therapy; NO, Nitric Oxide; VSMC, vascular smooth muscle cell; WHI, Women's Health Initiative

Genomic and Nongenomic Vascular Effects of Estrogen

Fig. 2.

Genomic and nongenomic vascular effects of estrogen. In the genomic pathway in endothelial cells, E2 binds to cytoplasmic ER leading to ER dimerization and localization to the nucleus where the complex interacts with EREs to increase gene transcription and eNOS expression. In the nongenomic pathway, E2 binds to endothelial ER and activates phospholipase C (PLC), generating inositol 1,4,5-triphosphate (IP₃) and DAG. IP₃ causes Ca²⁺ release from the endoplasmic reticulum. Ca²⁺ forms a complex with calmodulin (CAM), which activates eNOS. E2/ER also interacts with Src and activates Modulator of Nongenomic Action of ER (MNAR). They interact with the p85 regulatory subunit of PI₃-kinase (PI₃K), which transforms phosphatidylinositol-4,5-bisphosphate (PIP₂) into phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which activates Akt. ER-mediated activation of Akt or MAPK pathway causes phosphorylation and full activation of eNOS, transformation of L-arginine to L-citrulline and production of NO, which causes VSM relaxation. E2 also binds to membrane GPR30 and activates adenylate cyclase (AC) leading to increased cAMP and activation of protein kinase A (PKA), which activates eNOS and COX1 to produce NO and PGI₂, respectively. E2 also induces production of EDHF. In the genomic pathway in VSM, E2 binds to ER, inhibiting growth factor (GF) receptors, which are known to activate MAPK translocation to the nucleus. E2 binding to ERs also stimulates ER translocation to the nucleus where it may affect gene transcription and VSM growth. In the non-genomic pathway, E2 binds to membrane ERs to inhibit the mechanisms of VSM contraction including [Ca²⁺]_i, Ca²⁺-dependent MLC phosphorylation, protein kinase C (PKC), and Rho-kinase (Rho-K). Endothelial NO and PGI₂ activate guanylate cyclase (GC) and AC, respectively, leading to increased cGMP/cAMP and increased activity of protein kinase G and A (PKG and PKA), respectively. PKG/PKA activate Ca²⁺ extrusion via plasmalemmal Ca²⁺ pump and Ca²⁺ uptake by SR, and inhibit Ca²⁺ entry through membrane Ca²⁺ channels. E2 may also bind plasma membrane ERs and activate K⁺ channels and other EDHF leading to hyperpolarization and inhibition of membrane Ca²⁺ channels. COX1, cyclooxygenase-1; DAG, diacylglycerol; EDHF, Endothelial derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor;

ERE, estrogen response elements; PGI₂, prostacyclin; MAPK, mitogen-activated protein kinase; SR, sarcoplasmic reticulum; VSM, vascular smooth muscle

Fig. 3.

Differential protective effects of estrogenic MHT in early atherogenesis and harmful effects in established atherosclerosis. In early atherogenesis cardiovascular risk factors, hemodynamic forces, and circulating inflammatory factors cause endothelial cell injury resulting in decreased NO production and increased EC permeability. Once injured, the endothelium increases the expression of leukocyte adhesion molecules, which increases the adherence of macrophages and other leukocytes. The increased EC permeability allows entry of leukocytes and lipoproteins into the subendothelial space. Oxidized lipoproteins are taken up by macrophages and SMCs to form foam cells (fatty streak). E2 has beneficial effects on early atherosclerotic lesions by changing the plasma lipid profile, maintaining EC integrity and promoting NO production. In established atherosclerosis foam cells at the central-most position of the developing atheroma become necrotic and form the central lipid core, whereas the shoulder regions contain SMCs, macrophages, and other leukocytes. Platelet-derived growth factor and transforming growth factor- β stimulate SMC migration and collagen formation in the subendothelial space, as well as formation of the fibrous cap. E2 increases MMP expression in established atherosclerosis, causing instability of the fibrous cap and rupture of the plaque. CAM, cell adhesion molecule; EC, endothelial cell; ET-1, endothelin-1; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; NO, nitric oxide; PGI2, prostacyclin; TNF- α , tumor necrosis factor- α ; VSMC, vascular smooth muscle cell

Table 1

Representative MHT clinical trials and their CVD outcome

Clinical Trial	Type	Patient no	Mean follow-up	MHT		Outcome Summary	Ref
				Type	Route		
PEPI	Multicenter, RDBPC	875	3 yr (1991-1994)	CEE alone or CEE +Progestin	Oral	Estrogen alone or in combination with progesterone improves lipoproteins and lowers fibrinogen levels in Post-MW.	[107]
HERS	RDB	2763	4.1yr (1993-1997)	CEE+Progestin	Oral	No overall reduction in CVD events in women with established coronary disease. High risk of CVD in first year	[11]
HERS II	RDBPC	2321	6.8 (2.7 yr added to HERS) (1993-1999)	CEE+Progestin	Oral	MHT did not reduce risk of cardiovascular events in women with CVD	[106]
HERS - UA	RDBPC	2763	4.1 yr (1993-1997)	CEE+Progestin	Oral	The study examined the relation of MHT to serum uric acid (UA) levels and the risk of CVD. Estrogen+progestin lowered serum UA levels slightly, but neither baseline UA nor change in UA affected CHD risk.	[108]
WHI	RDB	16608	5.2 yr (1993-1998)	CEE+Progestin	Oral	Overall health risks exceeded benefits from use of combined estrogen+progestin for an average 5.2-year follow-up among healthy Post-MW	[16]
WEST	RDBPC	664	Mean 2.8 yr (recruited between December 1993 and May 1998)	Estrogen (E2)	Oral	In Post-MW with recent Cerebrovascular accident (CVA) MHT did not reduce risk of recurrence of stroke.	[12]
PHOREA	RDBPC	321	48wk (1995-1996)	E2, E2 + gestodene or placebo	Oral	Women > 55 yr with atherosclerosis (IMT-carotid) No benefit of MHT	[109]
ERA	Three-arm, RDBPC	309	3.2 yr (1996-1999)	CEE alone or CEE +Progestin	Oral	Angiography detected no difference in disease progression despite increased HDL and decreased LDL	[14]
WISDOM	Multicenter, RDBPC	5692	10 yr (started 1997-stopped 2002)	CEE or CEE+ MPA	Oral	MHT increases CV and VTE risk when started many years after menopause. Results are consistent with the findings of WHI study and secondary prevention studies.	[110]

Clinical Trial	Type	Patient no	Mean follow-up	MHT		Outcome Summary	Ref
				Type	Route		
WAVE	RDBPC	423	2.8yr (July 1999-January 2002)	CEE or CEE +MPA and/or Vit. E, C, or placebo	Oral	Women with ≥ 1 stenosis coronary artery 15%-75% (QCA) No benefit MHT & no benefit Vit. C, E	[111]
ESTHER	Multicenter, case-control study	881 271-case 610-control	6 yr (1999-2006)	MHT classified according to the route of estrogen and type of progestogen	Oral vs Transdermal	Oral but not transdermal estrogen increases VTE risk. Norpregnanes may be thrombogenic. Micronized progesterone and pregnanes are safe with respect to thrombotic risk.	[112]
RUTH	Multicenter, RDBPC	10,101	5.6 yr (2000-2005)	Raloxifene daily or placebo	Oral	Raloxifene did not affect risk of CVD. Benefits of raloxifene in reducing risk of invasive breast cancer and vertebral fracture should be weighed against risk of VTE and stroke.	[113]
EPAT	RDBPC	222	2 yr (2001-2003)	E2	Oral	Average rate of progression of subclinical atherosclerosis was slower in healthy Post-MW taking E2	[114]
KEEPS	DB RCT of secondary prevention	720	5yr (2005-2010)	CEE 0.45mg/d +E2 50µg/d/wk +Micronized Progesterone 200mg/d/12d/m	Oral Transdermal Oral	The study will examine whether MHT prevents progression of carotid IMT and accrual of coronary calcium. Outcome in press.	[115]
ELITE	DBRCT	643	Start-2005 End-2012	E2 1mg/day ±progesterone gel 4% or placebo	Oral Vaginal	The study will examine the primary outcome-Rate the change of distal common carotid artery (CCA) Secondary outcome-neurocognitive function & coronary artery Ca+2 and lesions	[20]

CEE, Conjugated equine estrogen; CVD, cardiovascular disease; CHD, coronary heart disease; IMT, intima media thickness; MPA, medroxyprogesterone acetate; QCA, quantitative carotid angiography; RCT, Randomized Clinical Trial; RDBPC, Randomized Double-Blinded Placebo-Controlled

Table 2

Studies Comparing the Risk of VTEs between MHT Users and Nonusers

Study Design	Patient No	Mean Follow-up	MHT		Outcome Summary	Ref
			Type	Route		
Case-control	Cases 66 Control 163	1990 to 1994	MHT containing E2	Oral or Transdermal	The combination of MHT use and thrombophilias (especially if multiple) increases the relative risk of VTE. MHT increases the risk of VTE 4-fold even in women without thrombotic abnormalities	[122]
Case-control	Cases 77 Control 163	1990 to 1994	MHT containing E2	Oral or Transdermal	Investigating whether VTE risk is affected by carriership of hereditary prothrombotic abnormalities (factor V Leiden and prothrombin A) Women who had factor V Leiden and used MHT had a 15-fold increased risk of VTE.	[123]
Case-control	Cases 176 Control 352	1990 to 1996	MHT containing E2	Oral or Transdermal	MHT containing E2 was associated with a 3-fold increased risk of VTE. Increased risk was restricted to the first year of use.	[116]
Case-control	Cases 292 Control 10,000	<6 months 6m to 1yr >1yr (1991-1994)	CEE $\pm$ progestin E2 $\pm$ progestin	Oral Trans-dermal	The risk of VTE among non-users of MHT was 1.3 per 10,000 women/yr. Among MHT users, VTE occurred in 37 per 292 women/yr (13%).	[124]
Case-control	Cases 171 Control 10,000	> 1yr (1991-1995)	E2 alone or CEE +progestin group	Oral or Transdermal	MHT users had 2.3 times higher risk of VTE compared with nonusers (1.3 per 10,000 women/yr). The increased risk was restricted to the first year of treatment	[125]
RCT HERS	2763	4.1 yr (1993-1997)	CEE+Progestin or placebo	Oral	MHT increases risk for VTE in women with CHD.	[126]

CEE, Conjugated equine estrogen; CHD; coronary heart disease; E2, 17 β -estradiol; MHT; Menopausal Hormone Therapy; VTE; venous thromboembolism