Menopause: The Journal of The North American Menopause Society
Vol. 20, No. 3, pp. 342-353
DOI: 10.1097/gme.0b013e3182843aad

Invited Review

Timing hypothesis for postmenopausal hormone therapy: its origin, current status, and future

Thomas B. Clarkson, DVM, Giselle C. Meléndez, MD, and Susan E. Appt, DVM

Abstract

Objective: This work aims to review preclinical/clinical cardiovascular studies that led to randomized trials of the risks and benefits of postmenopausal hormone therapy (HT), the pathobiological basis for the timing hypothesis, and subset analyses of randomized trials that tend to support the timing hypothesis; to elaborate experimental data that might inform the results of recent trials; and to summarize evidence regarding how early is early enough for the initiation of HT.

Methods: This work used interpretive literature review.

Results: Preclinical and large observational studies provided what was considered at the time to be convincing evidence that HT provided protection against progressing coronary artery atherosclerosis. Those findings prompted three randomized, placebo-controlled, prospective trials to determine the risks and benefits of HT. None provided any evidence that HT had any beneficial effects on preexisting coronary artery atherosclerosis. Monkey studies provided clear evidence that HT was effective in slowing the progression of coronary artery atherosclerosis only when administered soon after surgical menopause and that benefit was lost if estrogen therapy was delayed until the plaques had become complicated. The phenomenon was referred to as the "timing hypothesis," and evidence for its translation into postmenopausal women was sought in subset analyses of data from the Women's Health Initiative and from newly planned prospective trials.

Conclusions: Current data are both supportive and not supportive of the timing hypothesis. However, evidence indicating that estrogens administered in the perimenopausal transition or early in menopause are not harmful to the cardiovascular system and, when given for a few years for the treatment of menopausal symptoms, may slow the progression of atherosclerosis and reduce the postmenopausal cardiovascular disease burden seems convincing.

Key Words: Timing hypothesis – Cardiovascular disease – Hormone therapy.

It seems timely and important to review the timing hypothesis as it relates to the possible cardiovascular benefits of hormone therapy (HT) for postmenopausal women. The timing hypothesis has two main components. The first component is that HT initiated during the perimenopausal transition or early menopause (a time when atherosclerosis is usually at the fatty streak or uncomplicated plaque stage) will slow the progression of lesions into larger, more complicated plaques. The second component is that the beneficial effects of HT will be lost several years after menopause, with atherosclerosis having progressed to the complicated plaque stage; furthermore, at that stage, exposure to HT may induce plaque instability and thus an increased risk for clinical events. Clearly, HT should not be used for the sole purpose of its potential early cardiovascular benefits. However, the timing hypothesis is timely because there is currently increased interest in the use of nonhormonal drugs for the treatment of menopausal symptoms during the perimenopausal transition and early menopause. The use of estrogens for the treatment of menopausal symptoms is not only efficacious for this purpose, as indirect epidemiologic evidence and preclinical investigations (primarily those conducted in nonhuman primates) indicate that it probably slows the progression of early atherosclerosis and reduces the subsequent cardiovascular burden in later postmenopausal years.

In this essay, we review the events that led to randomized trials evaluating the risks and benefits of HT and the pathobiological basis for the timing hypothesis. We also review recent trials and subset analyses of those trials that tend to support the timing hypothesis. The numbers of publications relating to this subject are large, and we made no effort to be comprehensive in this review. The publications cited are those that seem to bear directly on the validity of the timing

Received October 30, 2012; revised and accepted December 20, 2012.

From the Center for Comparative Medicine and Research, Wake Forest University School of Medicine, Winston-Salem, NC.

Financial disclosure/conflicts of interest: T.B.C. serves in the advisory committee of Pfizer and has current grants from Pfizer and Merck. S.E.A. participates in the grant from Merck. G.C.M. is a Wake-Merck Cardiovascular Research Fellow supported by a grant from Merck.

Address correspondence to: Thomas B. Clarkson, DVM, Department of Pathology/Comparative Medicine, Center for Comparative Medicine and Research, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1040. E-mail: tclarkso@wakehealth.edu

hypothesis. Finally, we speculate about future needs for research.

Events leading to randomized trials of the risks and benefits of HT

During the 1980s and 1990s, numerous observational studies seemed to report convincing evidence that both postmenopausal estrogen therapy (ET) and postmenopausal estrogen + progestin therapy (EPT) provided protection from cardiovascular disease (CVD), particularly coronary heart disease (CHD), even among those who have already developed clinical evidence of CHD. Notably, investigators studying women who were part of the Lipid Research Clinics Program Follow-up Study were among the first to report (1987) that postmenopausal estrogen use was associated with reduced risks for CHD.[1] That observation prompted a number of additional observational studies that similarly indicated an association between HT and protection from CHD.[2] Generally, those observational studies concluded that there were large reductions in CHD events associated with HT use. By far, the largest of the observational studies was the Nurses' Health Study, which began in 1976 when 121,700 female nurses aged 30 to 55 years completed a mailed questionnaire about their postmenopausal hormone use and medical history.[3] Based on a 20-year follow-up of 70,533 postmenopausal women, the current use of postmenopausal estrogens was associated with a relative risk (RR) for a major coronary event of 0.61 (95% CI, 0.52-0.71) when adjusted for age and common cardiovascular risk factors.[4] Those findings encouraged the widespread use of ET and EPT for the prevention of postmenopausal CVD.

Bolstered by the results of the aforementioned observational studies and data from the work of Sullivan et al[5] indicating that the more severe is a postmenopausal woman's coronary artery disease, the more benefit she derives from ET, three randomized prospective trials were designed. The first trial was the Heart and Estrogen/progestin Replacement Study (HERS). HERS involved women who were generally older than 65 years and had definitive evidence of CHD. The EPT used in that study comprised conjugated equine estrogens (CEE) 0.625 mg/day and medroxyprogesterone acetate (MPA) 2.5 mg/day. No reductions in coronary events were observed after approximately 4.1 years of EPT. In fact, during the first year of the trial, there was an increase in events (RR, 1.52; 95% CI, 1.0-2.29), an outcome observed primarily in the first 4 months.

In the second trial, Estrogen Replacement Atherosclerosis (ERA), Herrington et al[6] investigated the effect of ET (CEE 0.625 mg/d) and EPT (CEE 0.625 mg/d + MPA 2.5 mg/d) on the progression of preexisting coronary artery atherosclerotic plaques, as assessed by angiography. The mean age of the women in ERA was 65 years, with half of them having experienced a previous myocardial infarction. After 3.5 years of treatment, there were no angiographically detectable differences in coronary artery atherosclerosis progression in either the ET cohort or the EPT cohort. The third trial, Women's Estrogen-progestin Lipid-Lowering Hormone Atherosclerosis Regression Trial (WELL-HART), studied postmenopausal women (mean age, 63.5 y) who had at least one coronary artery atherosclerotic plaque demonstrated angiographically.[7] Women received oral micronized estradiol (E_2) therapy (ET) or oral micronized E_2 + MPA (EPT). Results from this trial provided additional evidence for the lack of effect of either ET or EPT on plaque progression in older women with preexisting coronary artery atherosclerosis.

The negative findings from these secondary intervention trials resulted in negative reactions both from the lay press and from many investigators, while engendering considerable confusion among healthcare providers and their patients. Mendelsohn and Karas[8] were prompted to write an editorial titled "The time has come to stop letting the HERS tale wag the dogma." It was also at about that point in time (2001) that the US International Foundation for Studies in Reproduction dedicated its meeting to Trudy Bush and invited one of us (T.B.C.) to present a lecture in her honor, "The new conundrum: do estrogens have any cardiovascular benefits?", which represented the first presentation of what has become known as the "timing hypothesis." The lecture and its subsequent publication[9] emphasized the pathobiological basis for the interpretations of secondary intervention trials, how vascular biologists view "primary prevention," and how that differed from the popular definition of primary prevention. To vascular biologists, primary prevention is the inhibition of the progression of fatty streaks into atherosclerotic plaques. To epidemiologists, primary prevention is the reduction in CHD events regardless of the extensiveness or complexity of preexisting coronary artery atherosclerotic plaques.

WOMEN'S HEALTH INITIATIVE

The Women's Health Initiative (WHI) was a large, randomized, placebo-controlled trial designed to assess the long-term risk-to-benefit ratio of ET and EPT. The prespecified primary beneficial outcome was CHD, and the primary adverse outcome was invasive breast cancer. ET comprised CEE (0.625 mg/d), and EPT comprised CEE (0.625 mg/d) + MPA (2.5 mg/d). The CEE + MPA arm of the trial was terminated early after 5.2 years of a planned 8.5 years of follow-up because of an excess in breast cancer cases among the CEE + MPA–treated women. Relevant to this review was the finding of increased CHD events in those women (seven more CHD events and eight more strokes per 10,000 person-years).[10] That finding prompted many to conclude that EPT does not have any beneficial effects on the prevention of postmenopausal CVD. It now seems probable, based on subset analyses of WHI data and other studies, that such a conclusion was premature and failed to take into account that the effects of ET and EPT on atherosclerosis initiation and progression are complex and influenced strongly by the extent and stage of subclinical atherosclerosis at the time of initiation of treatment. These subset analyses are discussed in detail later in the review.

ORIGINS OF THE "TIMING HYPOTHESIS"

After the WHI trial report in 2002, there were even more confusion, controversy, and uncertainty about the ET, EPT, and their effects on the cardiovascular health of postmenopausal women, particularly for those in the late stages of the perimenopausal transition or in early postmenopause. Fortunately, soon after, a unifying hypothesis (soon to be known as the timing hypothesis), based to a large extent on previous monkey studies, began to emerge.[11]

Observations from cynomolgus monkey studies

An important part of the experimental basis for the timing hypothesis came from a study that compared the robust inhibition of coronary artery atherosclerosis resulting from the administration of CEE to cynomolgus monkeys immediately after ovariectomy with the absence of an effect of CEE on monkeys with preexisting atherosclerosis (Fig. 1).[9,12] In that study, cynomolgus monkeys were made surgically postmenopausal, and the initiation of ET and EPT was delayed for 2 years (comparable to six postmenopausal years for women). Delayed initiation of ET and EPT allowed coronary artery plaques to progress to a stage of atheronecrosis with inflammatory complications comparable to those found in women aged 60 to 65 years. In contrast to the observed large reduction in plaque size when hormone treatments are initiated early, no inhibition of coronary artery atherosclerosis was seen in monkeys in which ET or EPT was initiated 2 years after the induction of surgical menopause, a time comparable to six postmenopausal years for women.

The absence of a beneficial effect of ET and EPT on preexisting complicated plaques was useful in the interpretation of findings from HERS, ERA, and WHI; that is, estrogens exert a beneficial effect on atherogenesis if started early, but no benefit is observed if ET is initiated after a prolonged period with diminished estrogen exposure—the beginnings of the timing hypothesis.

The timing hypothesis is usually interpreted as age modulating the effect of estrogen on arteries; however, experimental evidence seems clear that it is not age, but rather the stage of progression of subclinical atherosclerosis, that determines if ET will be atheroprotective.[13] The relationship between atherosclerosis stage and the effectiveness of ET in inhibiting the progression of atherosclerosis was investigated directly using surgically postmenopausal cynomolgus monkeys[14] (Fig. 2). At the time the monkeys were ovariectomized, a section of the common iliac artery was removed and atherosclerosis extent was quantified. The animals were divided into tertiles based on atherosclerosis extent, and the effect of 3-year ET on the contralateral iliac artery was evaluated. ET was highly effective in inhibiting atherosclerosis progression among those monkeys in the lowest tertile when treatment was begun (P = 0.0001). The plaques among those animals were comparable to those in women at the end of the perimenopausal transition and early postmenopausal years. On the other hand, monkeys in the tertile with the largest plaques, comparable to plaques of women about 55 to 60 years old, did not experience any inhibition in atherosclerosis progression because of ET (P = 0.71).

Natural history of coronary artery atherosclerosis and effect of the stage of atherosclerosis progression on estrogen's effect on arteries

Understanding the timing hypothesis requires a review of the stages in the progression of atherosclerosis across the life span of the average American woman. Coronary artery atherosclerosis has its origin in childhood and progresses with each reproductive stage (Fig. 3). Substantial data now describe the progression of subclinical coronary artery atherosclerosis in the average American woman. Data on the premenopausal precursors (fatty streaks) of postmenopausal coronary artery plaques (raised lesions) come primarily for the multicenter study Pathological Determinants of Atherosclerosis in Youth.[15]

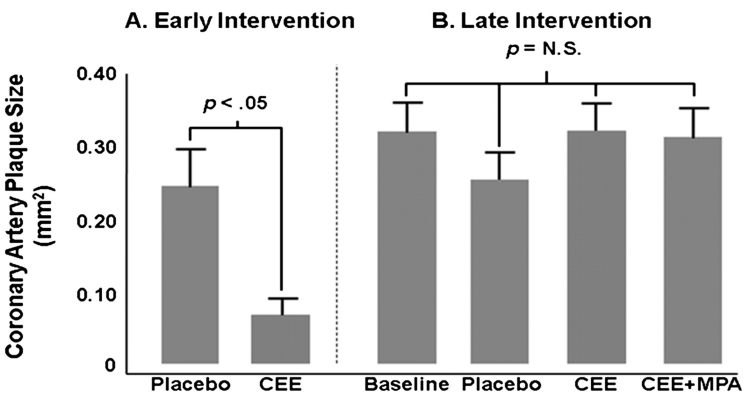

FIG. 1. Effect of timing the initiation of hormone therapy on coronary artery atherosclerosis extent in surgically postmenopausal monkeys. **A:** When estrogen therapy is begun at the onset of surgical menopause, coronary artery atherosclerosis of cynomolgus monkeys is reduced by about 70%, based on at least five studies. Treatment was carried out for 34 months (comparable to about 9 y in women). **B:** When either ET (CEE) or EPT (CEE + MPA) was delayed for 2 years (comparable to 6 y in women), coronary artery atherosclerosis in postmenopausal cynomolgus monkeys was not reduced. The treatment period was 2 years (again, comparable to 6 y in women). CEE, conjugated equine estrogens; MPA, medroxyprogesterone acetate. Reproduced with permission from Karas and Clarkson.[11] (Copyright 2011 by the American Society for Reproductive Medicine. All rights reserved. No part of this presentation may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or by any information storage and retrieval system without permission in writing from the American Society for Reproductive Medicine, 1209 Montgomery Highway, Birmingham, AL 35216.)

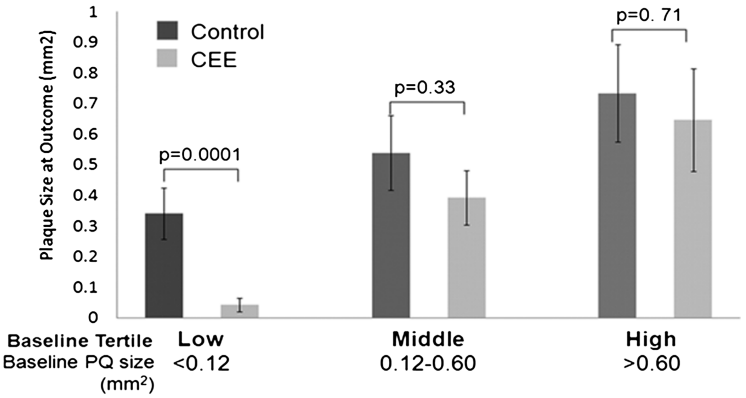

FIG. 2. The extent of preexisting atherosclerosis modulates the effectiveness or the lack of effectiveness of preventing the progression of atherosclerosis after conjugated equine estrogens (CEE) treatment of surgically postmenopausal cynomolgus monkeys. Only the low tertile with plaque extents comparable to women at the end of the perimenopausal transition and early postmenopausal years derived a significant atheroinhibitory benefit of CEE treatment (P = 0.0001). PQ, plaque. Data from Anthony and Clarkson.[14]

The numbers shown in the lumen of premenopausal women refer to the surface of coronary arteries with fatty streaks. Notably, by 35 years of age, approximately 70% of women have fatty streaks, although not large, covering about 15% of the surface of coronary arteries.

Data on adult and postmenopausal women come largely from autopsies of women who died of accidental deaths. During the perimenopausal transition and early years of menopause (35-45 y), plaques become larger, and fibrous caps begin to develop. By the age of 55 years, plaques continue to increase in size, fibrous caps become more distinct, and atheronecrosis and calcification are often present.[16] Arterial imaging also reveals the presence of plaques in the premenopausal phase and the perimenopausal transition.[17,18] Among women older than 65 years, plaques become increasingly complicated, the amount of atheronecrosis increases, and inflammatory processes are often apparent within plaques, particularly within the shoulder regions of fibrous caps. In vivo studies, using electron bean tomography to evaluate coronary artery calcification, tend to support pathological studies. Coronary artery calcification, which is always associated with necrosis, begins at approximately the age of 55 years and becomes distinct (60% of women reaching the 90th percentile of coronary calcification) by the age of 60 to 64 years.[19] There is general agreement that calcification, necrosis, and activation of metalloproteinases, often referred to as "negative remodeling," are associated with plaque instability.

Figure 3 indicates the stages in the progression of atherosclerosis where there are probable benefits of endogenous and exogenous estrogens, primary benefits of ET/EPT, no benefits of ET/EPT, and maybe deleterious effects of ET/EPT. Those designations are based on monkey studies, basic vascular biological studies, clinical observations, and clinical trial outcomes. Taken together, they constitute the basis for the timing hypothesis.

CELLULAR AND MOLECULAR MECHANISMS SUPPORTING EARLY BENEFIT AND LATE HARM

Estrogen exerts its effects on arteries primarily by binding to estrogen receptors (ERs). The two most common subtypes in arteries are ER-α and ER-β. Decreasing arterial ERs are a characteristic of progressing atherosclerosis and vascular aging.[20,21] Even during the premenopausal years, ER-α expression in circulating vascular endothelial cells varies across the menstrual cycle, as it is reduced by 30% in the early follicular phase to the late follicular phase.[22] During the perimenopausal period, fluctuations in menstrual cycle hormones induce arterial changes in the ratio of ER-α expression to

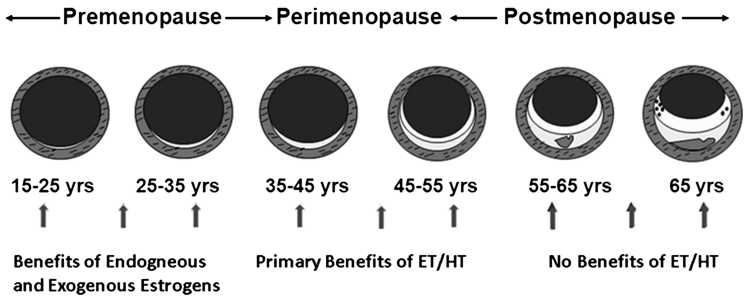

FIG. 3. The natural history of coronary artery atherosclerosis among women in the United States is depicted schematically. The benefits or the lack of benefits of ET/EPT on atherosclerosis is based on the body of evidence from preclinical and clinical studies. ET, estrogen therapy; EPT, estrogen + progestin therapy; HT, hormone therapy. Reprinted from Mikkola TS, Clarkson TB, Notelovitz M. Postmenopausal hormone therapy before and after the Women's Health Initiative study: what consequences? *Ann Med* 2004;36:402-413, with permission from Taylor and Francis Ltd. (http://www.tandf.co.uk/journals).

ER-β expression, which in turn alters tissue responsiveness to estrogen.[23] Notably, after menopause, women express 33% fewer ER-α receptors on endothelial cells than do their premenopausal counterparts evaluated during the late follicular phase.[22,24] It now seems clear that differences in ER expression levels are influenced by estrogen and reproductive stage. The marked reduction of estrogen after menopause contributes to the adverse effects of aging on the cardiovascular system by increasing the atherogenicity of circulating lipoproteins into a more atherogenic profile.[25] Furthermore, studies on human arteries and cultured vascular smooth cells suggest that methylation of the ER gene during menopause results in increased progression of atherosclerosis.[26,27] Because the onset of menopause coincides with the early stages of atherogenesis, the relative importance of reduced ER function relative to other aging changes is difficult to determine.

The mechanisms by which ET of women with complicated atherosclerotic plaques results in an increased occurrence of coronary events remain unclear. Basic to understanding those potential mechanisms, however, is understanding that E_2 has both anti-inflammatory and proinflammatory properties that vary with estrogen concentrations.[23] It is possible that the increase in coronary events in trials involving secondary prevention was due, in part, to the up-regulation of proinflammatory cytokines and adhesion molecules that subsequently triggered secretion, activation, or secretion and activation of matrix metalloproteinases (MMPs).[28] MMPs are key matrix-degrading enzymes that maintain the structural integrity of preexisting coronary artery plaques, especially the shoulder region of the plaques' fibrous caps.[29] Studies suggest that increases in MMP-2 and MMP-9 plasma concentrations in women receiving HT may play a role in weakening the plaques and increasing their vulnerability to rupture.[30]

OBSERVATIONS NOT SUPPORTIVE OF THE TIMING HYPOTHESIS

Although the timing hypothesis was embraced by most as a reasonable explanation for the differences seen in the early observational trials of ET and EPT effects on CHD and the outcomes of the WHI trial, not all experts in the field were convinced.[31] In 2007, Barrett-Connor[32] published a thoughtful commentary in the *American Journal of Epidemiology* elaborating her view that "the hypothesis is stronger than the evidence." Although she acknowledged that it was plausible, based largely on monkey studies, that the effects of estrogens on arteries could differ with the stage in the natural history of the disease and the severity of subclinical atherosclerosis, a subgroup analysis of WHI data did not provide convincing support of the hypothesis. A major point of her commentary was that the best opportunity to test the timing hypothesis was lost because only women in the WHI younger than 60 years underwent coronary artery calcium (CAC) scans to estimate plaque severity; women 60 years or older were not similarly examined. She pointed out that if the timing hypothesis were valid, there would have been the possibility of establishing that the younger WHI women had less CAC in the CEE arm than the placebo group of younger women and little or no difference with the older women.

Manson and Bassuk[33] provided an invited commentary in response to the publication of Barrett-Connor. In a comprehensive review of the evidence, they did not concur with Barrett-Connor's concerns and concluded that the data from a subgroup analysis of WHI women and other studies "suggest that hormone therapy may have a beneficial effect on the heart if started in early menopause, when a woman's arteries are still likely to be relatively healthy, but a harmful effect if started in late menopause, when advanced atherosclerosis may be present."

Prentice et al[34] conducted further analyses on the results of WHI randomized trials (1993-2004) regarding the health benefits versus risks of women who initiated either ET (CEE alone) or EPT (CEE + MPA) soon (≤5 y) after menopause. Data from the WHI observational study (1993-2004) were also included in some of their analyses. They considered incident CHD, stroke, venous thromboembolism, breast cancer, colorectal cancer, endometrial cancer, hip fracture, and death from other causes. For the purposes of this review, we shall consider only their observations about CHD. The understanding of the rationale for the approach used by Prentice et al and its implications for the potential validity of the timing hypothesis was enhanced by a thoughtful commentary by Banks and Canfell.[35]

A difficult challenge to using the WHI data in understanding the cardiovascular benefits or lack of benefits of early-reproductive-stage hormone treatment is that 48% of women in the ET arm and 26% of women in the EPT arm of the trial were prior hormone users.[36] Consequently, it seemed important for these investigators to use a reclassification that took into account prior hormone use by the women both in the trial and in the observational studies. Banks and Canfell[35] pointed out that this was a key methodological difference between this study and previous analyses. They referred to it as the "gap time" between menopause and the first use of hormone treatment. In summary, they found no significant gap time interaction (<5 y vs >5 y postmenopausal) for either ET (P = 0.40) or EPT (P = 0.42) and thus no support for the timing hypothesis.

CLINICAL OBSERVATIONS AND TRIALS SUPPORTIVE OF THE TIMING HYPOTHESIS

One of the earliest (2001) randomized trials to examine the effect of postmenopausal estrogen administration on the progression of atherosclerosis in somewhat younger women was the Estrogen in the Prevention of Atherosclerosis Trial (EPAT).[37] Healthy (no clinical evidence of CVD) postmenopausal women were randomized to unopposed oral micronized E_2 versus placebo. After 2 years, atherosclerosis progression, measured as carotid artery intima-media thickness (CAIMT), was quantified, and atherosclerosis of E_2-treated women was found to be slower (Fig. 4).

In 2004, Lobo[39] reported on the almost total lack of occurrence of CHD among 4,065 young (53-54 y, on average)

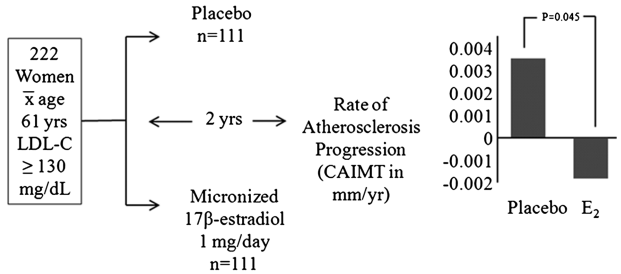

FIG. 4. Effect of estrogen therapy on "preclinical atherosclerosis" determined by imaging carotid artery intima-media thickness (CAIMT). LDL-C, low-density lipoprotein cholesterol; E_2, estradiol. Reprinted from Clarkson TB, Kaplan JR. Stage of reproductive life, atherosclerosis progression and estrogen effects of coronary artery atherosclerosis. In: Lobo RA, ed. *Treatment of the Postmenopausal Woman: Basic and Clinical Aspects*, 3rd ed. San Diego, CA: Elsevier, 2007, with permission from Elsevier. Reproduced with permission from Munir et al.[38]

postmenopausal women who participated in two large randomized clinical trials conducted between 1989 and 2001. No cases of CHD occurred among the more than 4,000 women given EPT, although the expected rate would have been about 2 cases per 1,000 women at that age. Although entirely observational and lacking adequate controls, the results of the study did tend to support the likelihood of early estrogen benefit.

Much has been written about the seemingly divergent observations concerning EPT and CHD based on observational studies such as the Nurses' Health Study and randomized trials such as the WHI. Grodstein et al[31] described probable explanations for the discordant findings, suggesting methodological differences in addition to biological differences such as time since menopause and stage of atherosclerosis progression. Given the strength of the evidence that the discordant data might concern the timing hypothesis, Grodstein et al[40] reexamined the Nurses' Health Study data for a relationship among ET and EPT use, CHD, and time since menopause when hormone treatment was initiated. They concluded that the reanalysis supported the possibility that the timing of HT initiation in relation to menopause onset or age might influence coronary risk. Women beginning ET or EPT near menopause (RR, 0.66; 95% CI, 0.54-0.80) and those beginning EPT near menopause (ET: RR, 0.72; 95% CI, 0.56-0.92; EPT: RR, 0.72; 95% CI, 0.56-0.92) had a significantly reduced risk of CHD. The authors point out that although their data support the timing hypothesis, they could not draw firm conclusions because a large majority of the nurses initiated hormone use soon after menopause and only a small proportion of participants initiated hormones long after menopause.

The results of a meta-analysis by Salpeter et al[41] provided additional support for the timing hypothesis. The analysis involved pooled data from 23 trials with 39,049 participants followed for 191,340 person-years. HT (ET and EPT not analyzed separately) significantly reduced CHD events in younger women (time from menopause <10 y or younger than 60 y; odds ratio, 0.68; 95% CI, 0.48-0.96), but not in older women (>10 y postmenopausal or older than 60 y; odds ratio, 1.03; 95% CI, 0.91-1.16). Later, Salpeter et al[42] reported a similar finding for mortality (total deaths and deaths due to CVD, cancer, or other causes).

The results of the analyses of the effects of ET (CEE only) on CHD outcomes in the WHI trial also provide evidence suggestive, albeit not definitive, of support for the timing hypothesis.[43] The ET arm of the WHI involved randomization of 10,739 postmenopausal women who have had hysterectomy to receive CEE (0.625 mg/d) or placebo. This report considered not only their primary outcome (nonfatal myocardial infarction or coronary death) but also their secondary outcomes, such as coronary revascularization. The differences in cumulative hazard ratios for CHD (myocardial infarction or coronary death) by age decade are shown schematically in Fig. 5. In the youngest of the three age groups (50-59 y at baseline), there was a trend (barely missing conventional statistical significance) for a lower risk of CHD in the CEE group compared with the placebo group (hazard ratio, 0.63; nominal 95% CI, 0.36-1.08), in contrast with the oldest age group (70-79 y at baseline; hazard ratio, 1.10; 95% CI, 0.69-1.73). When secondary outcomes were added to the analysis, the youngest age group administered CEE was found to have had coronary revascularization significantly

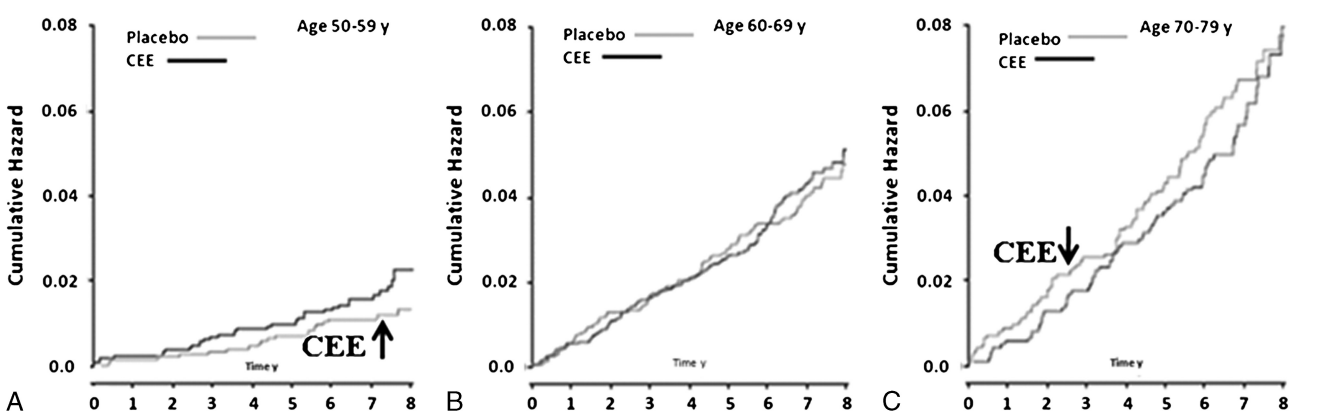

FIG. 5. Estimates of cumulative hazard ratios for coronary heart disease (myocardial infarction or coronary death) by age decade. Note the trend towards beneficial effect in the conjugated equine estrogens (CEE)–treated age 50-59 year group (**A**) and excess events in the CEE treated age 60-69 (**B**) and 70-79 year group (**C**). Adapted with permission from Hsia et al.[43]

less frequently than the placebo group (hazard ratio, 0.55; nominal 95% CI, 0.35-0.86).

Additional evidence supportive of that portion of the timing hypothesis proposing that women administered ET early in menopause will have delayed progression of coronary artery atherosclerosis in subsequent postmenopausal years was found in the results of the WHI-Coronary Artery Calcium Study (WHI-CACS).[44] The investigators took advantage of the well-established relationship between the amount of coronary artery plaque calcium and the extent of plaque complications and future risk of events. Using computer tomography to quantify CAC, they examined the hearts of 1,064 women aged 50 to 59 years at the time of randomization to CEE only in the WHI trial. The examinations occurred after a mean treatment of 7.4 years and 1.3 years after the trial was completed. The women in the youngest of the three age groups (50-59 y) administered CEE had lower prevalence and quantity of CAC than those administered placebo. The odds ratios for having high amounts of CAC were about 30% to 40% lower in intention-to-treat analyses and 60% lower among women who were at least 80% adherent to CEE treatment for at least 5 years.

In 2007, Rossouw et al[36] published the results of a comprehensive subgroup analysis of WHI outcomes describing their investigation of whether HT's effect on cardiovascular risk varied by age or years since menopause. Primary emphasis was placed on CHD and stroke. The important findings from that subgroup analysis were summarized clearly by Manson and Bassuk[33] (Fig. 6), and those data support the timing hypothesis. CHD, expressed as estimated absolute excess risks per 10,000 person-years, was significantly less for those aged 50 to 59 years or for those less than 10 years since menopause ($P = 0.03$).

Recently, Hodis et al[45] reviewed evidence in support of the timing hypothesis for CHD's beneficial effects resulting from postmenopausal HT. They introduced a new suggestion, that

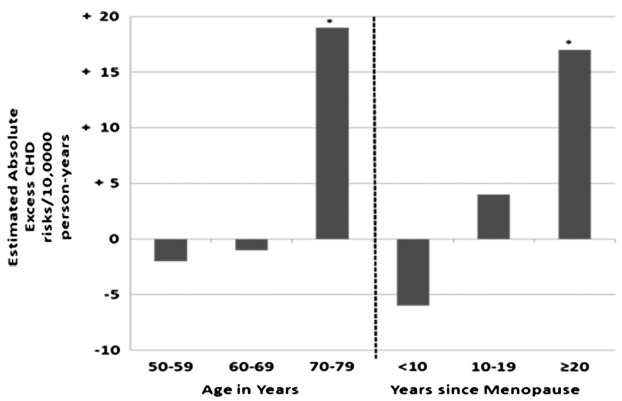

FIG. 6. Coronary heart disease (CHD) risks in the combined trials of menopausal hormone therapy among women in the Women's Health Initiative followed from 1993 to 2002 (EPT trial) and from 1993 to 2004 (ET trial). Estimated absolute excess risks for CHD per 10,000 person-years: (annualized percentage in placebo group) × (hazard ratio in placebo group − 1) × 1,000. Modified from Table 3 in Manson and Bassuk[33] based on data from Rossouw et al.[36] *$P = 0.03$ versus age of 50 to 59 years or less than 10 years since menopause.

is, the implications of the timing hypothesis might extend to selective ER modulators (SERMs) and isoflavones. In a subgroup analysis of the Raloxifene Use for The Heart (RUTH) trial, CHD was significantly reduced by 41% relative to placebo in women younger than 60 years (no CHD benefit across all ages) and by 52% for women less than 10 years postmenopausal.[46] Evidence that the timing hypothesis might extend to isoflavones was noted in data from the Women's Isoflavone Soy Health (WISH) trial, which was designed to test whether soy isoflavones, which bind primarily to ER-β, inhibited the progression of subclinical atherosclerosis (CAIMT).[47] Women randomized to soy isoflavones within 5 years of menopause had a significant reduction in the progression of subclinical atherosclerosis, whereas no such benefit was found for those given soy isoflavones 5 years beyond menopause. These observations provide additional support for the timing hypothesis; that is, the stage of atherosclerosis progression at the time of treatment may influence ER expression, ER function, or both and may extend to SERMs and isoflavones.

RECENTLY COMPLETED AND ONGOING TRIALS OF THE TIMING HYPOTHESIS

Kronos Early Estrogen Prevention Study

The conceptual framework for the Kronos Early Estrogen Prevention Study (KEEPS) was outlined in a review by Harman and Schierbeck et al,[48] who also composed the investigative team for KEEPS. The review cited evidence for the timing hypothesis regarding the cardiovascular effects of HT administered near the time of menopause and the need to not only consider timing but also compare oral versus transdermal administration of estrogens.

KEEPS was a 4-year, randomized, double-blind, placebo-controlled trial of oral EPT (CEE 0.45 mg/d, with cyclically administered [for 12 d each month] oral micronized progesterone 200 mg), a transdermal patch (E_2 50 μg/d, with the same cyclically administered monthly oral micronized progesterone), and a placebo group. The participants of the study were 727 women aged 42 to 58 years (mean age, 52 y), all of whom were within 3 years of menopause at the time of randomization. The primary endpoints of the trial were increases in CAIMT and CAC during the 4 years of treatment versus placebo. The details of the results have not yet been published; however, they were presented in detail at the 23rd Scientific Meeting of The North American Menopause Society.[49] The effect of the treatments on cardiovascular risk factors was small. Neither type of EPT significantly affected systolic blood pressure or diastolic blood pressure. Treatment with oral CEE resulted in small increases in high-density lipoprotein cholesterol, small decreases in low-density lipoprotein cholesterol, and increases in plasma triglyceride and C-reactive protein concentrations. Transdermal E_2 resulted in improved blood glucose concentrations and insulin sensitivity but had neutral effects on other biomarkers.

Although both forms of HT had beneficial effects on menopausal symptoms, bone density, and several psychological outcomes, for the purposes of this review, we shall discuss

only atherosclerosis outcomes. Somewhat unexpectedly by most, after 4 years of treatment, neither type of EPT administered to recently (≤3 y) postmenopausal women had either beneficial or deleterious effects on atherosclerosis progression, as determined by yearly measures of CAIMT, nor any beneficial effects on CAC progression comparing baseline with end of treatment. There were some trends indicating favorable effects on the progression of CAC, but none reached statistical significance.

KEEPS was a well-designed, well-executed trial that aimed "to learn whether menopausal hormone therapy given to healthy women early in menopause would have an effect on progression of atherosclerosis as indicated by changes over time in arterial imaging." There were high expectations that HT given to these recently postmenopausal women would have slowed the progression of CAIMT and CAC. For some time to come, there will be speculations about the reasons for the neutral finding. Because KEEPS had a number of characteristics in common with our studies of the timing hypothesis in the monkey model, we have considered some aspects of our previous studies that might have some bearing on the neutral atherosclerosis outcome in KEEPS.

Role of progestogen

Interestingly, there are limited definitive experimental data on whether progesterone attenuates the early benefits of ET on the progression of either coronary artery atherosclerosis or common carotid atherosclerosis. About two decades ago, we conducted a study to address that question using surgically postmenopausal monkeys.[50] In that study, we compared continuously administered E_2 (via subcutaneous Silastic implants) with continuously administered E_2 and cyclically administered progesterone (again by subcutaneous Silastic implants). Two findings are relevant to the interpretation of KEEPS data. First, the hormone treatments had a robust effect on inhibiting the progression of coronary artery atherosclerosis; both E_2 alone and E_2 + progesterone reduced the extent by about 50%, suggesting no attenuation of benefit by progesterone. Second, both E_2 alone and E_2 + progesterone, although exerting a major atheroprotective effect on coronary arteries, did not significantly reduce atherosclerosis extent in the common carotid arteries. That finding suggests to us that the common carotid arteries may not be as sensitive as coronary arteries to the atheroprotective effects of estrogens. Furthermore, the neutral effect of hormone treatments in KEEPS, based on observations of the common carotid arteries, may have underestimated a potentially positive effect on coronary arteries.

Time since menopause

The monkey study that first stimulated interest in the timing hypothesis found no beneficial effects of CEE on postmenopausal monkeys when the treatment was delayed for a period comparable to six postmenopausal years for women (note Fig. 2). Largely on that basis, KEEPS chose to study women who were 3 years or less postmenopausal. What we do not know from the monkey study is whether the beneficial effects of CEE might have been lost much earlier than those comparable to 6 years postmenopause.

Dose of CEE

We have reexamined our monkey data to see if there is any evidence to support that inhibition of the progression of atherosclerosis in the common carotid arteries might require a higher dose of CEE than atheroprotective effects on coronary arteries. Whether the dose of CEE for monkeys was women's equivalent of 0.30, 0.45, or 0.625 mg/day, the beneficial effect on coronary artery atherosclerosis progression was comparable (Fig. 7); however, there seems to be a dose-related effect on inhibiting common carotid artery atherosclerosis. The 0.45-mg/day dose (as used in KEEPS) was considerably less effective for the common carotid arteries than was 0.625 mg/day. That experimental observation raises the possibility that a higher dose of CEE (0.625 mg/d) might have had an atheroprotective effect on the common carotid arteries in KEEPS.

Danish Osteoporosis Prevention Study

Although they originally planned to evaluate hormone effects on osteoporosis prevention, Schierbeck et al[48] and the investigative team also considered the long-term effects of HT on cardiovascular outcomes in recently postmenopausal women. The study involved 1,006 perimenopausal or early postmenopausal white women, as well as women who have had hysterectomy when they were aged between of 48 and 52 years. The inclusion criteria were strict on reproductive stage: aged between 48 and 52 years, had the last menstrual bleeding 3 to 24 months before study entry, had perimenopausal symptoms (including irregular menstruation), and had follicle-stimulating hormone (FSH) concentrations more than 2 SDs above that for premenopausal women. For those women who have had hysterectomy, they had to fall within

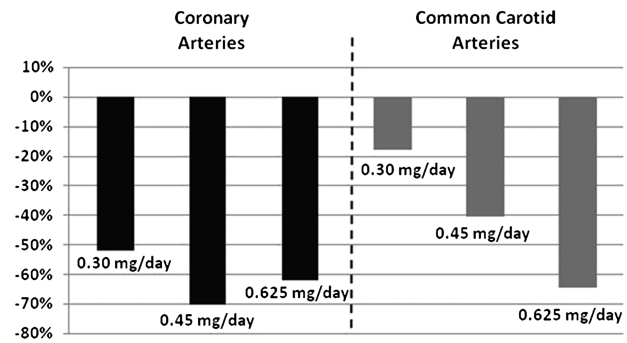

FIG. 7. The results of three trials with the cynomolgus macaque postmenopausal model fed a moderately atherogenic diet and administered CEE at a woman's equivalent of either 0.30, 0.45, or 0.625 mg/day. Data concerning 0.30 mg/day are taken from Appt et al,[51] data concerning coronary artery effects at 0.45 mg/day are taken from Clarkson,[52] and carotid artery data are as yet unpublished. The data for the monkeys treated with the woman's equivalent of 0.625 mg/day are taken from a trial in which CEE alone was compared with control.[53] The atheroprotective effect of CEE on coronary arteries is generally comparable at all three doses. Unlike the coronary arteries, there is a clear CEE dose relationship in inhibiting atherosclerosis in the common carotid arteries, with a minimal effect at 0.30 mg/day, a moderate effect at 0.45 mg/day, and a robust effect at 0.625 mg/day.

the young age range and to have elevated FSH concentrations. Treatment for naturally perimenopausal and postmenopausal women comprised 2 mg of E_2 for 12 days, 2 mg of E_2 + 1 mg of norethisterone acetate for 10 days, followed by 1 mg of E_2 for 6 days. For hysterectomized women, the treatment comprised 2 mg of E_2 each day. The primary endpoint was a composite of death, admission to the hospital for heart failure, and myocardial infarction. After a mean duration of 10.1 years of randomized treatment, the women were advised to discontinue their hormone treatments based on the reasons for terminating the WHI prematurely. However, after the termination of randomization, the investigators continued to follow the women for an additional 5.7 years for a total follow-up time of 15.8 years. Interestingly, 75% of the women adhered to the treatment arm to which they were randomized originally for 80% or more of the remaining 5.7 years of follow-up. Consequently, data have been reported for both 10 years of randomized treatment and 16 years of follow-up.

After 10 years of randomized treatment, hormone treatment (ET and EPT data combined) significantly reduced the primary composite endpoint (mortality, heart failure, or myocardial infarction; hazard ratio, 0.48; 95% CI, 0.26-0.87; P = 0.015). As a single component of the composite endpoint, reduction in mortality did not reach statistical significance (hazard ratio, 0.57; 95% CI, 0.30-1.08; P = 0.084). Similarly, as individual components of the composite endpoint, reductions in heart failure and myocardial infarction did not reach statistical significance (heart failure, P = 0.07; myocardial infarction, P = 0.21).

After 16 years of total follow-up, the beneficial effects of hormone treatment on the composite primary trial endpoint remained statistically significant (hazard ratio, 0.61; 95% CI, 0.39-0.94; P = 0.02). However, none of the components of the composite primary endpoint were improved significantly (mortality, P = 0.10; heart failure, P = 0.15; myocardial infarction, P = 0.14). Unfortunately, this trial does not specifically support the timing hypothesis because it focused on demonstrating cardiovascular benefits when treatment is begun during the perimenopausal transition or early menopause. However, as stated by the authors, it does provide evidence "that hormone therapy started in recently menopausal women and continued for a prolonged duration does not increase or provoke adverse cardiovascular events."

Early versus Late Intervention Trial with Estradiol

The Early versus Late Intervention Trial with Estradiol (ELITE) was designed specifically to test the timing hypothesis.[45] It is a double-blind, placebo-controlled, single-center trial involving 643 postmenopausal women, with the participants assigned to treatment or placebo based on time since menopause (<6 y or >10 y since menopause). The women were randomized to oral micronized E_2 (1 mg/d), placebo with vaginal progesterone gel, or placebo for 10 days each month. The primary endpoint is the progression of atherosclerosis, as determined by CAIMT measured every 6 months. The trial, supported by the National Institutes of Health, has been granted a 3-year extension, which will make possible the extension of randomized treatments for an average of 5 years, and the addition of measures for CAC and plaque visualization. ELITE is expected to be completed in 2014 and to provide important information concerning the timing hypothesis.

The studies of women clearly have important strengths but also some weaknesses. The primary strength is that there is no perfect animal model, so that any observations from studies of women are directly relevant. The weaknesses relate to the low frequency of CHD events among younger postmenopausal women, resulting in challenges to adequate statistical power and the need (in the recent trial) to rely on imaging a surrogate artery, most often the common carotid artery. CAIMT provides information about the progression of plaque size but does not allow for the assessment of plaque characteristics, such as the conversion of uncomplicated plaques into complicated plaques.

WHEN IS EARLY EARLY ENOUGH?

Studies of both monkeys and women seem to agree that for ET to be effective in inhibiting the progression of atherosclerosis, it is necessary that arteries have retained a functional endothelium and adequate ERs. Consequently, recent studies of changes in artery function during the menopausal transition may suggest that this is the optimal time to consider ET. Currently, however, there is a lack of critical information about the menopausal transition and the initiation/progression of subclinical atherosclerosis.

Although there is reasonably good information on the age and stage of progression of average women in the United States (Fig. 3), the relationship between reproductive stage (premenopausal, perimenopausal, and postmenopausal) and atherosclerosis progression has been unclear. To better understand the association between reproductive stage and progression of atherosclerosis, we have studied a subgroup of women from the Study of Women Across the Nation (SWAN). The study of the natural history of subclinical atherosclerosis during the menopausal transition (SWAN Heart) took place at two of the SWAN sites (Pittsburgh and Chicago) and involved women aged 45 to 58 years. Women were categorized into premenopause, early menopausal transition (EMT), late menopausal transition (LMT), and postmenopause. In an initial cross-sectional analysis (n = 483) of CAIMT and carotid adventitial diameter (artery size), no differences in CAIMT were observed among the reproductive stages.[54] However, women in LMT had larger carotid arteries than women in premenopause/EMT (6.96 ± 0.75 vs 6.65 ± 0.54, P < 0.001). That finding, in the absence of increasing CAIMT, suggests that arterial remodeling in response to atherosclerosis progression may be occurring in LMT.

Women at the Pittsburgh site (n = 249) were followed longitudinally for 9 years to determine whether the rate of progression of CAIMT and artery size varied across stages of the menopausal transition in SWAN Heart.[55] The annual rate of change in CAIMT was greater in the late perimenopausal stage (0.017 mm/y) than in either the premenopausal phase

(0.007 mm/y) or the early perimenopausal phase (0.005 mm/y). Reproductive hormones were significantly associated with the progression of CAIMT and artery size. Increases in artery size were negatively associated with plasma E_2 concentrations and positively associated with plasma concentrations of FSH. CAIMT was negatively associated with plasma sex hormone–binding globulin (SHBG) concentrations.[18] Taken together, these two SWAN Heart studies suggest that late perimenopause may be a time of rapid changes in the artery wall and that these changes may be associated with reproductive hormone fluctuations. A third investigation of this group of women has provided evidence that the associations between endogenous hormones and coronary and aortic calcification may be modified by obesity.[56] In that study, a lower SHBG level was associated with increased coronary artery calcification in obese women, but the relationship was reversed in nonobese women.

Currently, there are some inconsistencies in reports concerning atherosclerosis progression across the menopausal transition. A separate investigation reported that women who were premenopausal, transitioning, or postmenopausal (n = 203, aged 45-60 y) had similar rates of CAIMT progression during a 3-year period.[57] Among the transitioning women, however, those undergoing the most rapid transition from premenopause to postmenopause had the highest rate of CAIMT progression. In addition, new data from a prospective, cross-sectional, observational study measuring CAC, CAIMT, and aortic intima-media thickness (ATHENA-CT, n = 123, aged 45-64 y) confirmed that coronary, aortic, and carotid arterial plaques are present in asymptomatic premenopausal and perimenopausal women.[38] Although the overall mean number of coronary plaques did not differ among premenopausal, perimenopausal, and postmenopausal women, plaque severity (number of calcified plaques) was greater in late perimenopausal and postmenopausal women. Similar to other studies, there was an association between atherosclerosis and reproductive hormones. Specifically, the number of coronary plaques was inversely associated with SHBG and positively associated with FSH and free testosterone.

Two other important predictors of CVD risk in women are endothelial dysfunction (reduced endothelial-dependent vasodilation) and measures of arterial stiffness (arterial compliance and pulse wave velocity).[58-61] These changes in arterial function probably occur before, and contribute to, the progression of atherosclerosis. However, little is known about changes in endothelial function and arterial stiffness across menopausal stages. Recently, brachial artery flow-mediated dilation was measured in premenopausal (22-43 y), early and late perimenopausal (43-56 y), and early and late postmenopausal (49-70 y) women.[62] After adjusting for numerous traditional CVD risk factors, flow-mediated dilation was observed to decrease significantly across all stages of the menopausal transition. Furthermore, impairment in endothelial function was greater during the late perimenopausal stage than during the early perimenopausal stage (35% vs 17%). Age and menopausal stage were highly correlated ($r = 0.88$); therefore, the analysis was not adjusted for age. Similar to the previously mentioned atherosclerosis studies, higher FSH ($r = -0.57$, $P < 0.001$) and lower E_2 ($r = 0.46$, $P < 0.001$) were associated with decreased endothelial function in that study. Further supporting an association between reproductive hormones and endothelial function is the finding that surgically postmenopausal women (hysterectomy with and without oophorectomy) have greater arterial stiffness than naturally postmenopausal women, and that lower SHBG levels in those women were associated with decreased arterial compliance.[63]

Changes in plasma E_2 concentrations during the menopausal transition may be modulating endothelial dysfunction. The menopausal transition is divided into EMT and LMT based on menstrual cycle characteristics.[64] EMT generally begins approximately 4 years before the final menstrual period, and its initiation is characterized by a missed cycle, a variation in intermenstrual intervals of more than 7 days, and intermittent elevations in follicular-phase FSH. LMT generally begins approximately 1 to 3 years before the final menstrual period and is defined by 60 days or more of amenorrhea, more sustained increases in follicular-phase FSH, and declining follicular-phase E_2.[64,65] Changes in E_2 across the menopausal transition are variable and may not be uniform across women of differing body mass index and race/ethnicity. For example, the mean area-under-the-curve E1G (urinary estrogen metabolite) has been reported in some women to increase during EMT and to decline during LMT.[66] In addition, in a recent menopausal transition study, women were subdivided by follicular-phase plasma E_2 trajectories into four subgroups: slow decline (~27%), flat (~27%), rise/slow decline (~13%), and rise/steep decline (~31%).[67] These trajectories were strongly related to body mass index and race/ethnicity, with obese women generally experiencing a flat trajectory. Normal-weight white and African-American women tended to be in the rise/steep decline group, and Asian women experienced a slow overall decline. Interestingly, a large group (~45%) of primarily nonobese women experienced an elevation in E_2 before the final menstrual period. Declining E_2 levels or E_2 fluctuations across the menopausal transition probably have adverse effects on endothelial cells and subsequently predispose arteries to accelerated atherosclerosis progression.[68,69]

Whether arterial pathobiological changes that have occurred by the time of the final menstrual period determine postmenopausal CVD progression remains to be determined. However, evidence from nonhuman primate trials suggests that hormonal interventions before menopause can affect the trajectory of CVD postmenopausally.[70]

Relative to the issue of "When is early early enough?", the late menopausal transition seems to be a time of accelerated atherosclerosis progression and endothelial dysfunction, independent of traditional plasma CVD risk factors. These arterial changes occur simultaneously with increasing FSH and declining E_2 and SHBG, thus implicating reproductive hormonal changes as a potential mediator in the process. However, these relationships may be modified by obesity. Whether

intervention during this phase can decrease the trajectory of CVD postmenopausally remains to be determined.

FUTURE RESEARCH

It is improbable that the timing hypothesis will be subjected to a future double-blind, randomized, prospective trial to test the safety and efficacy of HT administered to perimenopausal and early postmenopausal women with coronary events as the primary outcome. As pointed out by Hsia et al,[43] given the low coronary even rate in that age/reproductive stage group, the sample size needed to attain 80% power would be 17,231 women, assuming equal numbers of women in the active and placebo groups and complete adherence to study medication. Consequently, it is more probable that understanding the validity or lack of validity of the timing hypothesis will depend on studies using surrogate endpoints and on more definitive studies of estrogen's effects on arteries with increasing reproductive age and atherosclerosis stage.

Although there is considerable evidence that "early" initiation may inhibit the progression of atherosclerosis, it is entirely unclear how early is early enough. There is an important need to focus future research on better understanding endothelial dysfunction during the perimenopausal transition and on the stage in the progression of atherosclerosis when substantial numbers of ERs are lost. The effects of dose, type of estrogen, and route of administration on the pathogenesis of atherosclerosis are also issues that must receive more research attention. The question of whether progestogens attenuate estrogen's beneficial effects on arteries seems unending. Particularly important seems to be the issue of whether progesterone differs in that regard from some of the synthetic progestins. Issues regarding the progestin component of HT may become less urgent if they are used less frequently and tissue-specific SERMs are used increasingly to protect the breast and the uterus during ET.

CONCLUSIONS

Studies using the monkey model have clearly shown that both ET and EPT administered soon after the induction of surgical menopause robustly inhibit the initiation and progression of diet-induced coronary artery atherosclerosis and that this inhibition of atherosclerosis progression by HT is lost after a period equivalent to six postmenopausal years for women. There are no experimental data, however, concerning whether the atheroprotective effects of estrogens might have been lost much earlier. A lack of information about "When is early early enough?" is a constraint for future studies of the timing hypothesis.

Clearly, there are data that are both supportive and nonsupportive of the timing hypothesis. Taken in balance, however, the evidence indicating that estrogens administered in the perimenopausal transition or early in menopause are not harmful to the cardiovascular system and, when given for a few years for the treatment of menopausal symptoms, may slow the progression of atherosclerosis and reduce the future burden of postmenopausal CVD in later years seems convincing.

REFERENCES

1. Bush TL, Barrett-Connor E, Cowan LD, et al. Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. *Circulation* 1987;75:1102-1109.
2. Grady D, Rubin SM, Petitti DB, et al. Hormone therapy to prevent disease and prolong life in postmenopausal women. *Ann Intern Med* 1992;117:1016-1037.
3. Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH. A prospective study of postmenopausal estrogen therapy and coronary heart disease. *N Engl J Med* 1985;313:1044-1049.
4. Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 2000;133:933-941.
5. Sullivan JM, Vander ZR, Hughes JP, et al. Estrogen replacement and coronary artery disease. Effect on survival in postmenopausal women. *Arch Intern Med* 1990;150:2557-2562.
6. Herrington DM, Reboussin DM, Brosnihan KB, et al. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med* 2000;343:522-529.
7. Hodis HN, Mack WJ, Azen SP, et al. Hormone therapy and the progression of coronary-artery atherosclerosis in postmenopausal women. *N Engl J Med* 2003;349:535-545.
8. Mendelsohn ME, Karas RH. The time has come to stop letting the HERS tale wag the dogma. *Circulation* 2001;104:2256-2259.
9. Clarkson TB. The new conundrum: do estrogens have any cardiovascular benefits? *Int J Fertil Womens Med* 2002;47:61-68.
10. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 2002;288:321-333.
11. Karas RH, Clarkson TB. Considerations in interpreting the cardiovascular effects of hormone replacement therapy observed in the WHI: timing is everything. *Menopausal Med* 2003;10:8-12.
12. Williams JK, Anthony MS, Honore EK, et al. Regression of atherosclerosis in female monkeys. *Arterioscler Thromb Vasc Biol* 1995;15:827-836.
13. Clarkson TB. Estrogen effects on arteries vary with stage of reproductive life and extent of subclinical atherosclerosis progression. *Menopause* 2007;14:373-384.
14. Anthony MS, Clarkson TB. Does extent of pretreatment atherosclerosis influence the effects of conjugated equine estrogens on atherosclerosis pregression? *J Am Coll Cardiol* 2002;39:248A.
15. McGill HC Jr, McMahan CA, Zieske AW, Malcom GT, Tracy RE, Strong JP. Effects of nonlipid risk factors on atherosclerosis in youth with a favorable lipoprotein profile. *Circulation* 2001;103:1546-1550.
16. McGill HC Jr, Stern MP. Sex and atherosclerosis. In: Paoletti R, Gotto AM Jr (Editors). *Atherosclerosis Reviews*, vol 4. New York, NY: Raven Press, 1979, 157-242.
17. Sutton-Tyrrell K, Lassila HC, Meilahn E, Bunker C, Matthews KA, Kuller LH. Carotid atherosclerosis in premenopausal and postmenopausal women and its association with risk factors measured after menopause. *Stroke* 1998;29:1116-1121.
18. El Khoudary SR, Wildman RP, Matthews K, Thurston RC, Bromberger JT, Sutton-Tyrrell K. Endogenous sex hormones impact the progression of subclinical atherosclerosis in women during the menopausal transition. *Atherosclerosis* 2012;225:180-186.
19. Raggi P, Callister TQ, Cooil B, et al. Identification of patients at increased risk of first unheralded acute myocardial infarction by electron-beam computed tomography. *Circulation* 2000;101:850-855.
20. Losordo DW, Kearney M, Kim EA, Jekanowski J, Isner JM. Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women. *Circulation* 1994;89:1501-1510.
21. Wynne FL, Payne JA, Cain AE, Reckelhoff JF, Khalil RA. Age-related reduction in estrogen receptor–mediated mechanisms of vascular relaxation in female spontaneously hypertensive rats. *Hypertension* 2004;43:405-412.
22. Gavin KM, Seals DR, Silver AE, Moreau KL. Vascular endothelial estrogen receptor α is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. *J Clin Endocrinol Metab* 2009;94:3513-3520.

23. Novella S, Heras M, Hermenegildo C, Dantas AP. Effects of estrogen on vascular inflammation: a matter of timing. *Arterioscler Thromb Vasc Biol* 2012;32:2035-2042.
24. Murphy E. Estrogen signaling and cardiovascular disease. *Circ Res* 2011;109:687-696.
25. Hermenegildo C, Garcia-Martinez MC, Tarin JJ, Cano A. Inhibition of low-density lipoprotein oxidation by the pure antiestrogens ICI 182780 and EM-652 (SCH 57068). *Menopause* 2002;9:430-435.
26. Post WS, Goldschmidt-Clermont PJ, Wilhide CC, et al. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res* 1999;43:985-991.
27. Kim J, Kim JY, Song KS, et al. Epigenetic changes in estrogen receptor β gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence. *Biochim Biophys Acta* 2007;1772:72-80.
28. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. *Arterioscler Thromb Vasc Biol* 2010;30: 1282-1292.
29. Johnson JL, Jackson CL, Angelini GD, George SJ. Activation of matrix-degrading metalloproteinases by mast cell proteases in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 1998;18:1707-1715.
30. Heo SH, Cho CH, Kim HO, et al. Plaque rupture is a determinant of vascular events in carotid artery atherosclerotic disease: involvement of matrix metalloproteinases 2 and 9. *J Clin Neurol* 2011;7:69-76.
31. Grodstein F, Clarkson TB, Manson JE. Understanding the divergent data on postmenopausal hormone therapy. *N Engl J Med* 2003;348:645-650.
32. Barrett-Connor E. Hormones and heart disease in women: the timing hypothesis. *Am J Epidemiol* 2007;166:506-510.
33. Manson JE, Bassuk SS. Invited commentary: Hormone therapy and risk of coronary heart disease why renew the focus on the early years of menopause? *Am J Epidemiol* 2007;166:511-517.
34. Prentice RL, Manson JE, Langer RD, et al. Benefits and risks of postmenopausal hormone therapy when it is initiated soon after menopause. *Am J Epidemiol* 2009;170:12-23.
35. Banks E, Canfell K. Invited commentary: Hormone therapy risks and benefits—the Women's Health Initiative findings and the postmenopausal estrogen timing hypothesis. *Am J Epidemiol* 2009;170:24-28.
36. Rossouw JE, Prentice RL, Manson JE, et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA* 2007;297:1465-1477.
37. Hodis HN, Mack WJ, Lobo RA, et al. Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 2001;135:939-953.
38. Munir JA, Wu H, Bauer K, et al. The perimenopausal atherosclerosis transition: relationships between calcified and noncalcified coronary, aortic, and carotid atherosclerosis and risk factors and hormone levels. *Menopause* 2012;19:10-15.
39. Lobo RA. Evaluation of cardiovascular event rates with hormone therapy in healthy, early postmenopausal women: results from 2 large clinical trials. *Arch Intern Med* 2004;164:482-484.
40. Grodstein F, Manson JE, Stampfer MJ. Hormone therapy and coronary heart disease: the role of time since menopause and age at hormone initiation. *J Womens Health* 2006;15:35-44.
41. Salpeter SR, Walsh JM, Greyber E, Salpeter EE. Brief report: Coronary heart disease events associated with hormone therapy in younger and older women. A meta-analysis. *J Gen Intern Med* 2006;21:363-366.
42. Salpeter SR, Walsh JM, Greyber E, Ormiston TM, Salpeter EE. Mortality associated with hormone replacement therapy in younger and older women: a meta-analysis. *J Gen Intern Med* 2004;19:791-804.
43. Hsia J, Langer RD, Manson JE, et al. Conjugated equine estrogens and coronary heart disease: the Women's Health Initiative. *Arch Intern Med* 2006;166:357-365.
44. Manson JE, Allison MA, Rossouw JE, et al. Estrogen therapy and coronary-artery calcification. *N Engl J Med* 2007;356:2591-2602.
45. Hodis HN, Collins P, Mack WJ, Schierbeck LL. The timing hypothesis for coronary heart disease prevention with hormone therapy: past, present and future in perspective. *Climacteric* 2012;15:217-228.
46. Collins P, Mosca L, Geiger MJ, et al. Effects of the selective estrogen receptor modulator raloxifene on coronary outcomes in the Raloxifene Use for The Heart trial: results of subgroup analyses by age and other factors. *Circulation* 2009;119:922-930.
47. Hodis HN, Mack WJ, Kono N, et al. Isoflavone soy protein supplementation and atherosclerosis progression in healthy postmenopausal women: a randomized controlled trial. *Stroke* 2011;42:3168-3175.
48. Schierbeck LL, Rejnmark L, Tofteng CL, et al. Effect of hormone replacement therapy on cardiovascular events in recently postmenopausal women: randomised trial. *BMJ* 2012;345:e6409.
49. Harman SM. New findings from the Kronos Early Prevention Study (KEEPS) randomized trial. The North American Menopause Society 23rd Annual Meeting, October 6, 2012.
50. Adams MR, Kaplan JR, Manuck SB, et al. Inhibition of coronary artery atherosclerosis by 17-β estradiol in ovariectomized monkeys. Lack of an effect of added progesterone. *Arteriosclerosis* 1990;10:1051-1057.
51. Appt SE, Clarkson TB, Lees CJ, Anthony MS. Low dose estrogens inhibit coronary artery atherosclerosis in postmenopausal monkeys. *Maturitas* 2006;55:187-194.
52. Clarkson TB. Effects of bazedoxifene alone and with conjugated equine estrogens on coronary and peripheral artery atherosclerosis of postmenopausal monkeys. *Menopause* 2013;20:274-281.
53. Clarkson TB, Anthony MS, Wagner JD. A comparison of tibolone and conjugated equine estrogens effects on coronary artery atherosclerosis and bone density of postmenopausal monkeys. *J Clin Endocrinol Metab* 2001;86:5396-5404.
54. Wildman RP, Colvin AB, Powell LH, et al. Associations of endogenous sex hormones with the vasculature in menopausal women: the Study of Women's Health Across the Nation (SWAN). *Menopause* 2008;15: 414-421.
55. El Khoudary SR, Wildman RP, Matthews K, Thurston RC, Bromberger JT, Sutton-Tyrrell K. Progression rates of carotid intima-media thickness and adventitial diameter during the menopausal transition. *Menopause* 2012;1:8-14.
56. El Khoudary SR, Wildman RP, Matthews K, et al. Effect modification of obesity on associations between endogenous steroid sex hormones and arterial calcification in women at midlife. *Menopause* 2011;18:906-914.
57. Johnson BD, Dwyer KM, Stanczyk FZ, et al. The relationship of menopausal status and rapid menopausal transition with carotid intima-media thickness progression in women: a report from the Los Angeles Atherosclerosis Study. *J Clin Endocrinol Metab* 2010;95:4432-4440.
58. Mattace-Raso FU, van der Cammen TJ, Hofman A, et al. Arterial stiffness and risk of coronary heart disease and stroke: the Rotterdam Study. *Circulation* 2006;113:657-663.
59. Kazmierski M, Michalewska-Wludarczyk A, Krzych LJ. Intima-media thickness and flow-mediated dilatation in the diagnosis of coronary artery disease in perimenopausal women. *Pol Arch Med Wewn* 2010;120: 181-188.
60. Willum-Hansen T, Staessen JA, Torp-Pedersen C, et al. Prognostic value of aortic pulse wave velocity as index of arterial stiffness in the general population. *Circulation* 2006;113:664-670.
61. Rossi R, Nuzzo A, Origliani G, Modena MG. Prognostic role of flow-mediated dilation and cardiac risk factors in post-menopausal women. *J Am Coll Cardiol* 2008;51:997-1002.
62. Moreau KL, Hildreth KL, Meditz AL, Deane KD, Kohrt WM. Endothelial function is impaired across the stages of the menopause transition in healthy women. *J Clin Endocrinol Metab* 2012;12:4692-4700.
63. Gavin KM, Jankowski C, Kohrt WM, Stauffer BL, Seals DR, Moreau KL. Hysterectomy is associated with large artery stiffening in estrogen-deficient postmenopausal women. *Menopause* 2012;19:1000-1007.
64. Harlow SD, Gass M, Hall JE, et al. Executive summary of the Stages of Reproductive Aging Workshop + 10: addressing the unfinished agenda of staging reproductive aging. *J Clin Endocrinol Metab* 2012;97:1159-1168.
65. Santoro N, Randolph JF Jr. Reproductive hormones and the menopause transition. *Obstet Gynecol Clin North Am* 2011;38:455-466.
66. O'Connor KA, Ferrell RJ, Brindle E, et al. Total and unopposed estrogen exposure across stages of the transition to menopause. *Cancer Epidemiol Biomarkers Prev* 2009;18:828-836.
67. Tepper PG, Randolph JF Jr, McConnell DS, et al. Trajectory clustering of estradiol and follicle-stimulating hormone during the menopausal transition among women in the Study of Women's Health Across the Nation (SWAN). *J Clin Endocrinol Metab* 2012;97:2872-2880.
68. Ihionkhan CE, Chambliss KL, Gibson LL, Hahner LD, Mendelsohn ME, Shaul PW. Estrogen causes dynamic alterations in endothelial estrogen receptor expression. *Circ Res* 2002;91:814-820.
69. Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. *Am J Cardiol* 2002;89:12E-17E.
70. Kaplan JR, Manuck SB, Anthony MS, Clarkson TB. Premenopausal social status and hormone exposure predict postmenopausal atherosclerosis in female monkeys. *Obstet Gynecol* 2002;99:381-388.