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Authors
Miller, Virginia M
Naftolin, Fredrick
Asthana, Sanjay
et al.

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The Kronos Early Estrogen Prevention Study (KEEPS): what have we learned?

Running title: Lessons from KEEPS

Virginia M Miller, PhD[1], Fredrick Naftolin, MD[2], Sanjay Asthana, MD[3], Dennis M Black, PhD[4], Eliot A Brinton, MD[5], Matthew J Budoff, MD[6], Marcelle I Cedars, MD[7], N Maritza Dowling, PhD[8], Carey E Gleason, PhD[9], Howard N Hodis, MD[10], Muthuvel Jayachandran, PhD[11], Kejal Kantarci, MD[12], Rogerio A Lobo, MD[13], JoAnn E Manson, MD[14], Lubna Pal, MD[15], Nanette F Santoro, MD[16], Hugh S Taylor, MD[17], S Mitchell Harman, MD, PhD[18]

Affiliations:

[1]Virginia M Miller: Departments of Surgery and Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN 55905 USA

[2]Fredrick Naftolin: Department of Obstetrics and Gynecology, New York University School of Medicine, New York, NY 10014 USA

[3]Sanjay Asthana: University of Wisconsin School of Medicine and Public Health and the Geriatric Research, Education and Clinical Center, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin 53705

[4]Dennis M Black: School of Medicine, University of California, San Francisco, CA 94143 USA

[5]Eliot A Brinton: Utah Lipid Center, Salt Lake City, UT 84108 USA

[6]Matthew J Budoff: Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-University of California Los Angeles, Torrance, CA 90520 USA

[7]Marcelle I Cedars: University of California, San Francisco, Department of Obstetrics, Gynecology and Reproductive Sciences, San Francisco, California 94143 USA

[8]N Maritza Dowling: Department of Acute and Chronic Care, Department of Epidemiology and Biostatistics. George Washington University School of Nursing and Milken Institute School of Public Health, Washington, DC 20006 USA

[9]Carey E Gleason: Division of Geriatrics, Department of Medicine, University of Wisconsin School of Medicine and Public Health and the William S. Middleton Memorial VA, Geriatric Research, Education and Clinical Center, Madison, WI 53705 USA

[10]Howard N Hodis: Atherosclerosis Research Unit, University of Southern California, Los Angeles, CA 90033 USA

[11]Muthuvel Jayachandran: Department of Physiology and Biomedical Engineering, Division of Nephrology and Hypertension, Division of Hematology Research, Mayo Clinic Rochester, MN USA

[12]Kejal Kantarci: Department of Radiology, Mayo Clinic, Rochester, MN 55905 USA

[13]Rogerio A Lobo: Department of Obstetrics and Gynecology, Columbia University, New York, NY 10032 USA

[14]JoAnn E Manson: Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215 USA

[15]Lubna Pal: Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT 06520 USA

[16]Nanette F Santoro: Department of Obstetrics & Gynecology, University of Colorado School of Medicine, Aurora, CO 80045 USA

[17]Hugh S Taylor: Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT 06520 USA

[18]S Mitchell Harman: Phoenix Veterans Administration Health Care system, Phoenix, AZ 85012 USA

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Corresponding author: Virginia M Miller, PhD
Professor, Surgery and Physiology
Medical Science 4-20
Mayo Clinic
200 First St SW
Rochester, MN 55905
Phone: 507-284-2290
Fax: 507-266-2233
Email: miller.virginia@mayo.edu

Abstract:

Objective: The Kronos Early Estrogen Prevention Study (KEEPS) was designed in the aftermath of premature halting of the Women's Health Initiative to address gaps in understanding of the timely effects of menopausal hormone treatments (HT) on cardiovascular health and other effects of menopause. Method: The original KEEPS was a randomized, double blinded, placebo controlled trial to test the hypothesis that use of HT in the early post-menopause would slow progression of atherosclerosis as measured by changes in carotid artery intima-media thickness (CIMT). Recruitment was conducted from 2005-2008 with total enrollment of 727 women. The intervention phase of the trial concluded in 2012. Results: In addition to the primary outcome of CIMT and the secondary outcome of changes in coronary artery calcification, several ancillary studies were conducted to examine the effects of HT on cognition and mood, brain structure, menopausal symptoms including sleep and sexual function, bone health, metabolism, intravascular biomarkers for disease processes, breast pain, and pharmacogenomics of HT. The KEEPS continues to study these issues in follow-up. Conclusion: This review summarizes the current main findings from the KEEPS and its ancillary studies, and provides a perspective for future research to optimize HT for menopausal women.

Key Words: cardiovascular disease, cognition, hormone therapy, menopause, menopausal symptoms, osteoporosis

Introduction

Early termination of the estrogen plus progestin (E+P) portion of the Women's Health Initiative's (WHI) trial in 2002, for net harm, and of the WHI estrogen-only trial (E-only in 2004 for futility) left scientists, physicians, and their patients with many unanswered questions about benefits and risks of menopausal hormone treatments (HT). Unfortunately, despite the large scale and high cost of the WHI, many previous questions remained unanswered, and some new questions arose. Nevertheless, it seemed unlikely that the National Institutes of Health would fund another study to address these remaining and emerging questions, the most critical being whether the WHI findings were affected by the fact that most women in the hormone trials were five or more years beyond their menopause on entry into the study and many had existing cardiovascular disease[1]. In this aftermath, the Kronos Longevity Research Institute, a privately funded non-profit located in from Phoenix, AZ, brought together basic and clinical scientists, some of whom had been investigators in the WHI, from nine academic centers across the country to design a new study named the Kronos Early Estrogen Prevention Study (KEEPS). The primary goal of the KEEPS was to determine if HT begun within three years of a woman's last menstrual period (two years after the World Health Organization definition of menopause) would slow progression of subclinical atherosclerosis as indicated by changes in intima-media thickness of the carotid arteries (carotid intima-artery media thickness, CIMT). Secondary goals included evaluation of risk factors for cardiovascular disease. Other goals included aspects of post-menopausal health and evidence for adverse side effects. This review summarizes what has been learned from the KEEPS, including multiple ancillary studies that have provided new insights into cognitive function, mood/affective outcomes, brain structure,

menopausal symptoms including sleep quality and sexual function, bone health, metabolism, biomarkers for disease processes, breast pain, and pharmacogenomics of HT. This review will discuss strengths and short-comings of the KEEPS and its ancillary studies to provide information to women considering HT and to guide to future research.

Design and study population

Design: KEEPS (NCT00154180) was a multi-center, 4-year randomized, double-blind, placebo controlled national trial.[2] The age of KEEPS participants (42-58 years within 6-months to 3 years of natural menopause) was representative of the majority of women who would likely seek HT for clinical purposes, as were women included in many of the previous observational studies and the 50-54 year old groups in the WHI.[3,4] This age range for KEEPS intentionally contrasted with those applied in the WHI, which was a trial of older women on average 12 years past menopause when randomized to HT, and differed from the Early vs Late Intervention Trial with Estrogen (ELITE) that included two strata of women (< 6y years since menopause and >10 years since menopause when randomized), who were not screened for sub-clinical atherosclerosis prior to randomization, and who may have undergone hysterectomy or bilateral oophorectomy. [5,6] Other exclusion criteria for KEEPS that differentiates this cohort from those of other studies included body mass index (BMI) >35 kg/m^2, untreated hypertension, dyslipidemia (including use of statins), diabetes, history of cardiovascular disease, smoking more than 10 cigarettes a day, and a history of cancer or other major chronic diseases.[7] To exclude women with sub-clinical cardiovascular disease in KEEPS, a coronary artery calcium (CAC) score had to be <50 Agatston Units (AU) at the screening visit.

The choice of HT used in KEEPS was dictated, in part, by the prescription guidelines and recommendations developed by professional societies following the WHI, specifically, to “use the lowest dose for the shortest possible time”.[8] Therefore, the dose of oral conjugated equine estrogens (o-CEE) was selected at 0.45 mg/day rather than the higher 0.625 mg/day used in the WHI. A second treatment group received transdermal 17β-estradiol (t-E2; 50µg/day), making KEEPS the first randomized, placebo-controlled trial to include two different formulations of HT in the same study of cardiovascular disease.[2] Both treatment groups received micronized progesterone (200mg/day) was used for the first 12 days each month instead of the continuous, synthetic medroxyprogesterone acetate (MPA) that was used in women with a uterus in the WHI. Women in the placebo group were administered matching placebo pill, patch, and 12 days of a placebo capsule. It was understood that the use in KEEPS of these formulations and the dose of o-CEE and of progestin, and the cyclical, instead of continuous use of the latter, differed from that in WHI and other contemporary trials, making direct comparisons of outcomes from KEEPS with those of other trials would be limited.

Study cohort: A full CONSORT diagram for recruitment and inclusion is published elsewhere.[9] In brief, of the 4,533 phone enquiries for the study, 3,455 being disqualified including those who did not complete the screening process. Of the 1,078 who consented for the study, 290 were excluded based on screening medical history or laboratory tests. The majority of KEEPS participants were non-Hispanic white (80%) and held a bachelor’s degree or higher (74%).

In contrast to all other reported studies, the KEEPS participants were free of sub-clinical cardiovascular disease at the beginning of the study. They were younger than women enrolled in the E+P trial of the WHI, and they also tended to

have lower body mass index, systolic blood pressure, total cholesterol, triglycerides, and fasting blood glucose, but higher high density lipoprotein-cholesterol (HDL-C) levels (Table 1).[7]

Primary and secondary cardiovascular outcomes

Primary outcome - changes in CIMT: CIMT was similar between women meeting inclusion criterion at screening vs those who did not [0.726±0.90 mm (n=718) vs0.725±0.084 mm (n=113), respectively].[7] At baseline, CIMT correlated with age but not with menopausal symptoms or serum levels of estradiol.[10] Of the 727 women enrolled in KEEPS, 89.3% had at least one follow-up CIMT during the treatment phase of the trial and 79.8% had a CIMT measurement at the conclusion (48 months) of the study. The average annual increases in CIMT(0.007 mm/year) were similar across all 3 treatment groups (Table 2). KEEPS estimated an effect size of -0.0034 mm/year, with a group size of 145 to provide a 99% probability of detecting a difference significant at the 0.05 level.

The absence of a treatment effect on CIMT differs from those from the ELITE trial in which women within 6 years of menopause who were randomized to estradiol 1 mg daily showed a lower mean annual rate of CIMT progression relative to women randomized to placebo. In the early postmenopausal stratum of ELITE (women within women within 6 years of their last menstrual period), CIMT averaged 0.75 mm prior to randomization and increased by 0.0078 mm per year in the placebo group, a rate comparable to that in KEEPS, but was significantly less (0.0044 mm per year) in the estradiol group (oral estradiol 1 mg/d plus 45 mg cyclical vaginal progesterone).[11]

There are several major differences in design between KEEPS and ELITE. ELITE included women with natural and surgically induced menopause who in the

early postmenopausal strata averaged about 3 years older (average age 55 years) and 3 years from their last menstrual period compared to an average of 52 years of age and 1.5 years from the last menstrual period for women in KEEPS all of whom underwent natural menopause. The type of HT in ELITE was oral (1 mg per day) 17β-estradiol with vaginal progesterone, whereas, in KEEPS the HT was transdermal 17β-estradiol or low dose oral CEE (0.45 mg per day) with oral progesterone. HT in both KEEPS and ELITE were cyclic (the progesterone was not taken every day) while the WHI used daily a synthetic progestin for women with a uterus. Thus, differences in age of the women, their ovarian status, formulations, doses and mode of delivery of the HT may all be relevant to the observed differences in the primary outcomes of these two clinical trials.[12] The oral 17β-estradiol used in ELITE had greater metabolic effects on lipid metabolism than the transdermal product used in KEEPS. The mixed estrogens and androgens in CEE would have both agonist and perhaps some antagonistic effects on some signaling pathways due to differences in affinity of the various estrogen metabolites for the estrogen receptors in the liver and on cells in the blood and vascular wall.[13,14] Other differences in formulations of estrogen and cardiovascular outcomes from other clinical studies have been reviewed elsewhere.[15] In addition, in KEEPS women with CAC scores >50 AU were excluded from the trial, resulting in a lower average cardiovascular risk of the KEEPS cohort than the ELITE cohort that randomized women regardless of CAC status.

Secondary outcome - coronary artery calcification: Of recruited women 14% were excluded from KEEPS due to a CAC scores > 50 AU, an exclusion criterion for the study. Women excluded based on CAC score had similar measures of CIMT to women with CAC scores <50 AU. This observation of apparent discordance in the

degree of atherosclerosis defined by CAC and CIMT suggests that different cellular process and risk factors may contribute to progression of atherosclerosis at various anatomical locations.[7]

Because of the CAC exclusion criterion, 87% of the women enrolled in KEEPS did not have any detectable levels of calcium in their coronary arteries prior to randomization (CAC scores of 0 AU at baseline.) About six percent of participants had CAC scores between 1-5 AU and six percent had scores > 5-50 AU (Table 3). Of the KEEPS participants who had CAC scores assessed both at baseline and after 48 months of treatment, there were no statistically significant differences in changes in CAC scores among the three treatment groups with an average increase of about 19% over the 4-year course of the KEEPS trial. However, the percentage of women who had CAC scores >5 trended to be less in the o-CEE group than in the placebo (Table 3). Although this trend of less accumulation of calcium in the coronary arteries in the HT groups did not reach statistical significance, it is consistent with the finding of lower CAC scores after o- CEE (albiet at a somewhat higher dose than in KEEPS) in the E-only group of the WHI.[16] KEEPS had excluded women with more significant CAC at screening and was underpowered to detect statistical significance for these small changes in CAC among groups. Whether HT doses used in KEEPS or higher doses used in WHI or ELITE would have slowed CAC had the study continued for more than four years remains a matter of speculation.

Other cardiovascular outcomes

Blood pressure did not change significantly across any of the treatment groups during the study (Table 1). Regarding adverse events, there were no venous thrombotic events and the single myocardial infarction was reported in a woman assigned to t-E2 but it occurred before the onset of treatment.[9]

Several ancillary studies (Table 4) to KEEPS were conducted that provide an integrated picture of the cardiovascular health of the KEEPS cohort, and insight into potential mechanisms by which HT might affect progression of cardiovascular disease. Although the number of participants is relatively small, these results highlight technical details and preliminary data from which other hypotheses can be developed and tested.

Endothelial function: One of the earliest changes in vascular function associated with development of atherosclerosis is loss of endothelium-derived nitric oxide. Nitric oxide causes vasodilatation, reduces platelet activation, and reduces adhesion of leukocytes and platelets to the uninjured vascular wall.[17] The endothelial production of nitric oxide is modulated by estrogen.[18] To investigate the potential impact of HT on endothelial function, a non-invasive test, digital peripheral tonometry (EndoPat®, model 200, Itamar Medical, Ltd., Caesarea, Israel), was used to measures changes in digital pulse volume following occlusion of the brachial arterial, a measure of shear-stress induced vasodilatation caused by release of nitric oxide,(reactive hyperemia).[19] In a subset of KEEPS participants (n=102), the reactive hyperemic index (RHI) prior to randomization (baseline) ranged from 1.22 to 5.44. An RHI <1.35 has a sensitivity of 80% and a specificity of 85% to identify persons with coronary endothelial dysfunction defined by the degree of vasodilatation invoked by an intra-coronary arterial infusion of acetylcholine.[20] At baseline, RHI did not associate with CIMT or CAC scores. The conventional cardiovascular risk factors of BMI (ρ = -0.21, P = 0.031) and waist circumference (ρ = -0.19, P = 0.05) negatively associated with RHI, an association that was not observed in never smokers (n=73; P = 0.375).[21]

By the completion of KEEPS, 83 women had RHI measurements at both baseline and at their exit visit at 48 months (during the non-progesterone phase of treatment). At 48 months, RHI showed again wide variation among women (range from 1.0-4.26), but did not show a significant change from the baseline measurement in any of the treatment groups. However, considering all groups combined, there was a significant inverse association between the change in RHI and the change in CIMT (P = 0.012) but not CAC, suggesting that a decrease in endothelial function is associated with an increase in progression of sub-clinical atherosclerosis in carotid but not coronary arteries.[22]

These results are consistent with the general hypothesis that decreased endothelial vasodilatory function coincides with progression of atherosclerosis. However, the absence of an effect of long-term estrogen on reactive hyperemia in KEEPS is inconsistent with observations from other studies.[23,24] The small sample size, choice of digital tonometry rather than a more sensitive measure of reactive hyperemia by ultrasound of the brachial artery, as well as the low doses of HT utilized in KEEPS may account, in part, for this discrepancy. [25]

Blood elements and cell-derived microvesicles: Activation of platelets and migration of monocytes/macrophages into the vascular wall at regions of endothelial dysfunction or damage are initiating steps in the development of atherosclerotic lesions.[26-28] In a subset of KEEPS participants at their baseline visit, *in vitro* measures of platelet aggregation and secretion showed statistical association with individual components of the metabolic syndrome (waist circumference, systolic blood pressure, fasting glucose, high density lipoprotein cholesterol and triglycerides). At the baseline visit, the number of platelets in the blood was associated with increasing waist circumference, whereas platelet

secretion of adenosine triphosphate and expression of P-selectin association decreased with increasing blood glucose and blood pressure.[29]. Thus, associations of “metabolic syndrome” an index or summary measure based on recommended cut-off scores may mask associations of its individual components. Identifying the individual components associated with platelet activation will help to target preventive strategies on manageable or treatable cardiovascular risk factors.

Circulating platelets do not contain a nucleus, but their megakaryocyte precursors do, and, thus, represent the target for genomic effects of the sex steroids on the subsequent characteristics of the circulating platelet pool.[30] Although, there were no statistically significant differences in the number, aggregatory reactivity or secretory capacity of platelets among KEEPS groups over the course of the treatment period[31,32], the content of vasoactive and mitogenic factors derived from the platelet lysate decreased within the HT groups compared to the placebo group.[31] In addition, the platelet content of 5-hydroxytryptamine and vasoactive prostanoids (prostacyclin and thromboxane) differed between the o-CEE and t-E2 groups.[33,34] Collectively, differences in the amount and types of vasoactive and mitogenic factors derived from the activated platelets would influence vascular remodeling, such as increases in CIMT, within the vicinity of platelet activation.

Activated platelets, leukocytes and endothelial cells release membrane bound vesicles less than 1 micron in size called microvesicles (MV). These MV carry bioactive molecules (DNAs, RNAs, proteins and metabolites) capable of stimulating other cells.[35] At the baseline KEEPS visit, numbers of platelet-derived and procoagulant MV were directly associated with CIMT but not CAC or RHI.[29] Whereas, monocyte- and endothelial cell-derived MV were associated with systolic blood pressure, which also was associated with CIMT. These observations open new

insight into how activation of cells within the vascular compartment may contribute to progression of vascular lesions. [32,36]

In general, the number of circulating MV varies with serum levels of estradiol.[37] However, following 48 months of treatment, the number of circulating MV did not differ across HT groups in KEEPS, which might reflect the rather narrow range in serum estradiol levels among groups despite the different treatments.[9] The average change in CIMT correlated with the average number of MV derived from leukocytes and vascular endothelium, and those expressing cell adhesion molecules, an observation consistent with that prior to treatment and with the general hypothesis that intravascular cellular activation occurs during vascular remodeling of the carotid arteries. These findings also imply selective influences regulating the number of both types of MV, rather than a simple numerical relationship. Although development of MV as biomarkers for active disease processes may not herald increases in CIMT, the diagnostic potential of specific populations of MV to detect elevation in CAC, particularly those MV with thrombin generating capacity may be most useful. MV derived from stem cells and adipocytes need to be explored in more detail in larger cohorts.[38,39]

General effects of HT on menopausal women

Cognitive function and mood: Prior to the publication of results from the Women's Health Initiative's ancillary Memory Study (WHIMS), there was conflicting but generally favorable evidence regarding effects of HT on cognitive function and risk of Alzheimer's disease.[40] The initial report from WHIMS found that HT increased the risk for dementia with the caveat that the observations were restricted to women who initiated therapy older than 65 years of age, and was most pronounced for therapies using medroxyprogesterone acetate (MPA).[41] It was these observations

that influenced the design of the KEEPS cognitive and affective sub-study (KEEPS Cog)[42], which was based on the underlying hypothesis of a "critical period" in which initiation of HT might benefit or protect brain health. Furthermore, questions remained regarding influence of the type of estrogen and the use of synthetic progestogens on cognition. Therefore, the basic design of KEEPS that enrolled women within three years of menopause, with the inclusion of both o-CEE and t-E2, with the use of natural and cyclic progesterone, provided an ideal background to examine cognition. KEEPS Cog was funded by National Institutes of Health and was open to all KEEPS participants. A set of 19 tests (many of which were included in WHIMS) were selected to examine intelligence, verbal learning and memory, language and mental flexibility, attention and executive function, working memory, and mood. The tests were administered at four time points: baseline and 18, 36, and 48 months after randomization. The 36 month visit was to be conducted within days six-twelve while women were administered cyclic progesterone.[42]

Of the 727 initial enrollees in KEEPS, 693 enrolled in the KEEPS Cog study[43]; 220 were randomized to o-CEE, 211 were randomized to t-E_2, and 262 were randomized to placebo. The demographics of women participating in the KEEPS Cog were the same as those for the parent KEEPS study.[43] Baseline cognitive data collected prior to randomization were assessed relative to latent class membership defined by cardiovascular risk profiles (blood pressure, blood lipids, insulin resistance and Framingham risk score) and relevant sample characteristics [education apolipoprotein E ε4 (APOEε4) status, ethnicity and age]. The latent profile analysis identified two distinct phenotypical classes of cardiovascular risk: low-risk, representing 62% of enrollees and high risk, representing 38% of enrollees. Performance of low-risk women on language and mental flexibility tasks and global

cognition was better than that of high-risk women. Education level was associated with risk classification, and older age and Hispanic ethnicity increased the probability of being in the high-risk group. The presence of the *APOE*ε4 was more prevalent in the high-risk group.[44]

Associations for specific cardiovascular risk factors that might affect cognitive performance were investigated in 571 women for whom a complete data set and *APOE*ε4 genotypes were available. After controlling for age, education and *APOE*ε4 status, systolic blood pressure showed a negative association with performance on auditory attention and working memory, a relationship that was not related to endogenous hormone levels at baseline.[45] These results are consistent with the growing body of evidence of the relationship of systolic blood pressure with structural changes in the brain and negative impact on cognition.[46]

662 women provided sufficient neuropsychological test data to be included in the longitudinal analytic sample.[43] Effects of HT on changes in cognition and mood were analyzed using linear mixed-effects models. Longitudinal outcome measures included: Modified Mini-Mental State examination; four cognitive latent factors (verbal learning/memory, auditory attention/working memory, visual attention/executive function and speeded language/mental flexibility), and mood measured by the Profiles of Mood States (POMS). There were no treatment-related effects found for any of the cognitive outcomes. For mood, compared to placebo, women treated with o-CEE but not t-E2 reported less depression and anxiety symptoms.[43] It should be emphasized that these results are limited to the four years of treatment in women with low cardiovascular risk. At present, long-term follow-up of women enrolled in KEEPS is underway to confirm these effects in women following cessation of treatment. Subsequent reports from WHIMS

examining cognitive function in women who initiated HT (o-CEE at 0.625 mg/day with or without medroxyprogesterone) between the ages of 50-55 years of age found no sustained benefit or risk to cognitive function when these women were examined seven years after cessation of therapy.[41]

Brain structure: An ancillary brain magnetic resonance imaging (MRI) study was conducted on the KEEPS Mayo Clinic cohort (n=118) to investigate the effects of o-CEE and t-E2 on changes in brain structure over the four years of KEEPS. Of these women, five participants were excluded due to neurological disorders or MRI contraindications. As well, twelve women declined participation. Of the eligible women who underwent a MRI scan prior to treatment (n=101), MRI data were analyzed for 95 participants with at least one follow-up MRI examination during the course of the study at month 18 (n=92), 36 (n=87), or 48 (n=79). The major finding of this study was that the rates of ventricular volume changes were greater in women who received o-CEE compared to those receiving placebo over four years. Although the rates of changes in structural MRI measures did not differ between the o-CEE and t-E2 groups, the rates of increase in ventricular volume did not reach statistical significance in the t-E2 group, compared to placebo, probably due to our limited sample size and short follow-up. Furthermore, increases in ventricular volume over 18 months of treatment were greater in the oral CEE group if hormone treatment was initiated later in menopause. Changes in structural MRI measures were not accompanied by significant differences in global cognition among groups.[47] The volume of white matter hyperintense (WMH) lesions increased during the treatment phase of the trial and was not associated with treatment assignment. However, the WMH lesion volume correlated with the platelet-derived, thrombogenic microvesicles at baseline, suggesting that *in vivo* platelet activation

may contribute to a cascade of events leading to development of WMH lesions in recently menopausal women.[48]

Menopausal symptoms: Menopausal symptoms are linked to overall health and disease risks, as well as to impaired quality of life (QOL), in most[49-52], but not all[53] studies. Women in KEEPS (N=727) completed brief symptom questionnaires at 6, 12, 24, 36 and 48months.[54] KEEPS, self-reported symptoms of hot flashes, vaginal dryness/dyspareunia, mood swings, sleep, and palpitations were monitored, and linked to baseline health status and improvements over time. The use of both o-CEE and t-E2 also permitted direct comparisons between these two widely-used forms of hormone therapy.

At baseline, menopausal symptoms were not associated with markers of subclinical cardiovascular disease (CIMT and CAC).[10] However, most likely due to the size of the study groups, associations of depressive symptoms and self-reported palpitations did not achieve statistical significance with CAC, but may be worthy of further study in larger cohorts. Symptoms of hot flashes/night sweats, irritability, and insomnia at baseline were more likely among black women, with a very large odds ratio for irritability (19.23, 95% CI 11.72-31.57), a striking finding that requires confirmation and further exploration in larger cohorts.

Moderate-to-severe symptoms of hot flashes and night sweats were similarly reduced by both o-CEE and t-E2 compared to placebo.[54] There was no differentiation of either hormone treatment versus placebo on relief of symptoms of irritability or mood swings. An overall trend for symptom improvement was seen over time in the placebo group. Neither BMI nor race/ethnicity modified these results.[54] These findings confirm a natural time course for regression of common

menopausal symptoms of hot flashes, night sweats, irritability, and mood swings over time for most, but not all women.[55,56]

In addition to examining the role of hormone therapy in addressing symptoms, KEEPS measured estradiol (E2; the principal circulating hormone resulting from t-E2) in a subset of participants (N=426) to link hormone levels to symptom relief. A well validated, immunoradiometric assay was used to determine E1 and E2 levels.[9] This assay is highly sensitive and compares favorably with mass spectrometry.[57] At baseline, there was no relationship between circulating E2 and symptoms. Among women reporting complete relief of hot flashes, those randomized to t-E2 had a mean E2 level of 44 pg/ml (95% CI 39-50) compared to those reporting moderate or severe persistence of hot flashes, who had circulating E2 levels of 9-11 pg/ml (95% CI 5-23). This latter finding is clinically relevant, in that it helps define the therapeutic window of E2 when using t-E2.

Sleep quality: Sleep quality was examined both at baseline and in response to hormones.[58] The Pittsburgh Sleep Quality Index (PSQI) was used for assessments at baseline, and 6, 18, 36 and 48 months.[59] A global PSQI score of >8, consistent with disturbed sleep, was reported by 24% of women at baseline. Sleep quality improved with both o-CEE and t-E2. Individual sleep domain scores were improved similarly by o-CEE and t-E2 for sleep satisfaction and latency; however, the sleep disturbances domain was improved by t-E2, but not o-CEE, suggesting a possible superiority of t-E2 for this aspect of sleep. Taken together, these findings provide new and useful information regarding the effects of HT on menopausal symptoms and quality of life. These studies establish therapeutic equivalence of 0.45mg of CEE to 50 micrograms of t- E2—with some distinction between the two. [56]

Sexual function: Estrogen deficiency impacts both physical and psychological domains that are critical to sexual experience. Estrogen insufficiency is associated with anatomical changes that contribute to sexual dysfunction, including thinning of the skin of external genitalia, loss of subcutaneous fat, parallel involution of the corpora cavernosa, as well as thinning of the epithelium lining the vagina.[60-63] Together these changes can lead to reduced lubrication, pain or discomfort during intercourse. As estrogen is a modulator of serotonergic function, it affects regions of the brain known to regulate mood and desire, including the amygdala, hippocampus, and the hypothalamus.[64] Estrogen loss is associated with alterations in mood, sleep, and cognitive function, all directly or indirectly influencing sexual function.[60]

Improvements in domains of sexual function have been previously noted with use of HT, however, the effects of o-CEE vs t-E2 have not previously been directly compared. Five hundred and seventy five of the 727 KEEPS enrollees agreed to participate in the ancillary study of sexual function.[65] Participants completed the Female Sexual Function Inventory (FSFI), a well validated 19-item questionnaire for assessing the key dimensions of sexual function along six domains of sexual function, including desire, arousal, lubrication, orgasm, satisfaction, and pain.[66,67] Treatment with t-E2 , but not o-CEE, was associated with a significant improvement in the FSFI overall score across all time points compared with placebo. In the individual domains of sexual function, t-E2 and o-CEE both differed from placebo. Symptoms related directly to tissue effects of estrogens on the reproductive track, such as lubrication and pain on penetration, demonstrated a progressive exacerbation with time, yet were reduced with the use of either t-E2 or o-CEE. In contrast, the more subjective domains of desire, arousal, orgasm, and sexual

satisfaction demonstrated a relatively steady state overtime, yet the superiority of t-E2 over o-CEE was apparent in the greater efficacy of t-E2 in improving these non-physical sexual function aspects.

Sex hormone binding globulin was greater in the o-CEE compared to the t-E2 group.[9] Given that hepatic sex hormone binding globulin controls levels of free testosterone [68], it may not be surprising that these formulations of HT might influence sexual function differently. Free testosterone may be critically important to desire, arousal, orgasm, and sexual satisfaction. Overall, the proportion of women with low sexual function was significantly decreased after treatment with t-E2, but not o-CEE compared with placebo. Thus, KEEPS findings suggest that menopausal women with sexual concerns are better served by treatment with t-E2 than with o-CEE.

Skin health: Skin wrinkling is another consequence of aging that is believed to be related to menopause and possibly to estrogen deprivation.[69] Estrogens influence skin appearance through regulation of dermal cell functions such as collagen production and hydration. In retrospective trials, long-term HT users were shown to have more elastic skin and less severe wrinkling than women who never used HT.[70] KEEPS evaluated facial wrinkling [71] at 11 locations on the face and neck and skin rigidity (using a durometer) at the forehead and cheek in an ancillary study conducted at a subset of KEEPS sites at baseline and yearly for 4 years.[70,72] Among the 106 women assessed at baseline, skin wrinkling was noted to be lowest among black women. Skin rigidity was not associated with race/ethnicity. Increasing weight was associated with less skin wrinkling, and, among white women, age was associated with wrinkling, and time since menopause was associated with increased skin rigidity. Waist circumference was also associated with increased skin rigidity.

Wrinkling was associated with a past history of smoking, but not current smoking. These relationships were not modified by sun exposure, but overall numbers in this study were small.[72]

Longitudinal evaluation of skin wrinkling and rigidity was completed in 116 women.[73] Neither total wrinkle score nor total rigidity score was significantly different at the end of the 48 months among women randomized to o-CEE, t-E2, or placebo. However, black women had the lowest wrinkle scores compared with white women across all 4 years. Skin rigidity decreased in all groups over time, but the decrease in total facial rigidity was significantly less in black women compared with white women after 4 years. There was no apparent cosmetic benefit to hormone use in this trial. Discrepancies between this randomized trial and retrospective studies could be due to selection bias inherent in retrospective studies or because the timing of skin changes are slow and may take longer than would be detectable in a four-year trial. Differences from other studies may also relate to proportion of women who were current smokers or other lifestyle choices including sun exposure.

Bone health: Efficacy of the HT to prevent loss of bone mineral density was assessed in a subgroup of KEEPS participants (n = 76) by quantitative computed tomography (from the CAC scans) to assess bone geometry and volumetric bone mineral density at the thoracic and lumbar spine, by dual-energy x-ray absorptiometry at the femoral neck, and by high-resolution peripheral quantitative computed tomography of trabecular and cortical bone at the distal radius. In addition to efficacy of the lower doses of HT used in KEEPS to reduce hot flashes and improve sleep, as expected based on previous studies, both treatments maintained density of trabecular and cortical bone of the spine and hip compared to

placebo.[74] these results are consistent with the WHI that showed that HT protects against femoral neck fracture.[75] On the contrary, (cortical) bone fragility in non-estrogenic anti-osteopenia regimens has become an important focus in the debate over which agents should be considered for primary prevention against osteoporosis.

Breast health: Breast pain is common in menopausal women. In the WHI, there was a higher reported incidence of breast pain in women randomized to 0.625 mg/day o-CEE either with or without MPA compared to those randomized to placebo.[76] Using a modification of the McGill Pain Questionnaire, there were no increases in reported severity of cyclic or non-cyclic breast pain in KEEPS participants at Mayo Clinic randomized to the lower dose of o-CEE or t-E2 (n=113).[77] It is likely that the difference between studies reflects the dose of o-CEE and transdermal product, but selection bias may have also contributed as KEEPS participants were required to have a normal mammogram within a year of enrollment into the study. The number of breast cancers in the main KEEPS study was small and the incidence did not differ among groups.[9]

Metabolism: Given the rigorous inclusion and exclusion criteria, KEEPS participants had low metabolic cardiovascular risk profiles based on BMI, serum lipids, triglycerides and homeostasis model assessment of insulin resistance (HOMA-IR), and inflammatory markers of IL-6 and CRP, which did not differ significantly among the randomized groups (Table 1).[7,9] After 4 years of treatment, in the o-CEE group, low-density lipoproteins decreased, whereas total and high density lipoprotein cholesterol and triglycerides increased compared to the placebo and t-E2 groups (Table 1). These changes in lipoprotein cholesterol are consistent with direct hepatic effects expected with oral treatments as was reported with CEE in other

studies.[78,79] Both low density and high density lipoproteins decreased in the t-E2 group with triglycerides remaining unchanged and similar to the placebo group. Insulin resistance showed a decreasing trend in both hormone groups, an interesting finding that requires additional analysis relative to other cardiovascular outcomes (Table 1).

An ancillary study of a sub-set of KEEPS participants (n = 74) assessed orexin-A levels, a neuropeptide implicated in regulation of food intake and energy expenditure.[80,81] At baseline, plasma levels of orexin-A were similar among the treatment groups but showed wide variation with the 25th and 75th percentiles ranging from 1.78 to 6.11 ng/mL. This variability persisted over the 48 months of treatment with levels in the o-CEE group significantly higher than changes observed in either the t-E2 or placebo groups. Although BMI increased significantly more in the placebo group compared to the hormone-treated groups, there was no correlation for BMI with orexin-A in any of the groups.

There is conflicting evidence regarding the contribution of pericardial and epicardial fat to incidence of coronary artery disease and CAC.[82-84] Amounts of epicardial, pericardial and hepatic fat were measured from women being screened for KEEPS who had sufficient images of the liver and spleen on the CAC computed tomographic scans to calculate hepatic fat compared to BMI (n = 652). Blood levels of adipokines (leptin, soluble leptin receptor and high molecular weight adiponectin) were measured by enzyme-linked immunosorbent assay and were compared relative to BMI of 25Kg/m^2 and none, ≤1 component or ≥2 components of the metabolic syndrome (waist circumference, blood pressure, fasting glucose, low levels of high density lipoproteins and triglycerides).[85] Epicardial and pericardial fat increased while hepatic fat decreased with increasing BMI and components of the

metabolic syndrome. Leptin increased whereas, soluble leptin receptor and adiponectin decreased with increasing BMI and metabolic syndrome characteristics. These results support a continuum of risk with increasing BMI, waist circumference, blood pressure, fasting glucose and triglycerides.[85] Furthermore, they suggest that assessment of individual components rather than the collective heterogeneous grouping to define "metabolic syndrome" may provide information for more targeted preventive strategies.

A cross-sectional analysis was performed on a sub-group of KEEPS participants who had CAC measurements available prior to and following 48 months of randomized treatment (n = 474). In a preliminary analysis presented at the 2018 meeting of the North American Menopause Society[86], epicardial fat depots increased significantly in the placebo group but not in the hormone groups after 48 months of treatment. Preliminary analysis suggests that greater pericardial fat was associated (P=0.02) with greater risk for progression of CAC but only in the t-E2 group. However, further analysis is needed to determine relationships among these fat depots, conventional cardiovascular risk factors, and coronary calcification in menopausal women.

Genetic variance and treatment effect

The KEEPS studied whether a limited set of genetic variants influenced the effect of HT on specific outcomes, i.e. CIMT, CAC, menopausal symptoms, etc. There are multiple genetic variants potentially affecting response to estrogen, including variants in genes regulating estrogen metabolism and receptors, as well as, variants of genes containing estrogen response elements in the promoter regions that have the potential to influence the effect of treatment, specifically HT, on all of the outcomes in KEEPS. A targeted candidate genetic analysis was used to gain insight

into processes contributing to the main outcomes of KEEPS. This analysis included genetic analysis of 610 participants of KEEPS and consisted of a set of 13,229 single nucleotide polymorphisms (SNPs) within 764 genes from anticoagulant, procoagulant, fibrinolytic, and innate immunity pathways, and variants of estrogen receptors α and β.[87]

Genetic variance and CIMT: After adjusting for age and pulse pressure, variables that associated positively with CIMT at baseline, SNPS of the mitogen-activated protein kinase 4 (MAP4K4 on chromosome 2) and of the interleukin 5 (IL5 on chromosome 5) genes correlated positively with CIMT (β = 0.03697, P = 2.36E-06 and β 0.05122, P = 5.02E-05, respectively). However, two variants for chemokine ligand 5 (CCL5 or RANTES on chromosome 17) gene correlated negatively with CIMT (β = -0.0427, P = 3.59E-05). All of these genetic variants are related to regulation of innate immunity.[87]

In 606 women who completed KEEPS on protocol (Placebo = 194, t-E2 = 161, o-CEE = 157), none of the top SNPs within the innate immunity pathway that associated with CIMT prior to treatment were among those that associated with CIMT after treatment. However, there was a significant interaction between genetic variants in genes of the innate immunity pathway and treatment on the changes in CIMT over the four years of the trial. Although individual SNPs did not reach statistical significance with changes in CIMT during the treatment period, collectively considering all of the SNPs evaluated and the frequency of the allele in the population, these pharmacogenomic effects could account for the increase in the overall variance observed in CIMT and reduce the ability to observe a specific effect by treatment.[88] The contribution of genetic variants within the innate immunity pathway to progression of CIMT is consistent with the overall hypothesis

of an inflammatory component of the disease demonstrated by changes in the biomarkers (activated cells, platelets and microvesicles) measured in KEEPS and by observations from other genetic studies.[89]

Genetic variance and CAC: After adjusting for waist circumference, interleukin-1 receptor-associated kinase-2 (IRAK2 on chromosome 3) and serpin family A member 1 (SERPINA 1 on chromosome 14) associated positively with CAC; ABO on chromosome 9 associated negatively with CAC. These genes code for enzymes involved in the breaking down of elastase and posttranslational modification of proteins including those related to thrombosis.[87] There were no associations of SNPs by treatment effect on increases in CAC>5 AU.[88] The inability to identify any of these candidate genes with CAC most likely reflects the complexity of processes involved with cellular differentiation and mineralization, and perhaps the interaction of metabolic and immunological factors on the calcification process.[90-92]

Genetic variance and menopausal symptoms: To better understand factors accounting for individual variation in response to HT, two genes associated with estrogen metabolism were evaluated in a subset of KEEPS participants. The gene *SULT1A1* encodes the enzyme sulfotransferase family 1A member 1 (SULT1A1) that sulfates estrone and 17β-estradiol. This enzyme is ubiquitous affecting the circulating ratios of these estrogens to their sulfated conjugates. *SULT1A1* is polymorphic, that is, there are multiple variations in copy number and SNPs. The number of SNPs and gene copies associate with enzyme activity, thus, making it a reasonable candidate gene to study variation in responses to HT. In a subset of 155 women screened for KEEPS, 8 women had a single copy, 101 women had 2 copies, 37 women had 3 copies, and 9 women had 4 copies of the gene. Of the SNPs evaluated, the number of G alleles at rs9282861, associated with younger age at

menopause, and women with 4 G alleles had less severe insomnia and lower frequency of night sweats[93], which may reflect effects on melatonin metabolism.[94]

After treatment, as described above, menopausal symptoms decreased in the HT groups. The *SULT1A1* did not associate with total number of symptoms nor did the rs9282861variant associate with estrogen levels in any of the HT groups. However, in women randomized to o-CEE, the ratio of unconjugated to conjugated estrogen was lower with increasing number of variants. These differences in effects of the genetic variants on circulating ratios of the sulfonated estrogen may reflect, in part, that the o-CEE formulations contains significant amounts of these estrogen conjugates.

In addition to sulfation of estrogens, their transport into the liver, particularly estrone sulfate, is by the membrane-bound, sodium-independent organic anion transporter protein (OATP1B1) encoded by *SLCO1B1* gene. Variant rs4149057of this gene has been studied in relationship to statin-induced myopathy and breast cancer. In an exploratory study of 100 KEEPS participants, 75 women were homozygous for the TT allele (normal function), 24 were heterozygous for the CT allele and one woman was homozygous for the CC allele (reduced function). Prior to randomization, this genetic variant was not associated with total menopausal symptoms, or disaggregated scores for hot flashes, night sweats or insomnia.[95] After treatment, although the variant did not associate with either total symptom score or scores for hot flashes or insomnia, the reduction in number of serve night sweats was greater in women assigned to HT with the CT genotype compared to those with the TT genotype. Women with the CT genotype had higher levels of sulfonated E2 and E1 than those with the TT genotype especially in those assigned

to t-E2. Women assigned to t-E2 also reported greater alleviation of sleep disturbances which may include those triggered by night sweats.[58]

In spite of the fact that these studies of genetic variants with responses to HT were obtained on small numbers of women, the women were well-characterized allowing for these explorations in the absence of comorbidities. In addition, the direct comparisons of effects of the genetic variants on responses to two different but clinically relevant HT formulations provides the background upon which to develop a genetic screen or algorithm that would help to personalize dosing and formulation of HT for menopausal women in the future.

The future

KEEPS provides a defined cohort in which to study healthy aging and the subsequent cardiovascular and cognitive risks following cessation of HT.[96-100] The National Institutes of Aging has funded a continuation of KEEPS (NCT03718494) that will expand these preliminary studies, allowing all former KEEPS participants to enroll for follow-up assessment of cognition and brain structures, including assessments of brain volumes, white matter hyperintensities, and β-amyloid load. Furthermore, imaging of tau deposition, another marker for Alzheimer's disease-related pathology, will be performed in a subset of these women. Enrollment is ongoing and it is anticipated for the study to be completed in 2023. Results of these follow-up evaluations will provide important confirmation of physiological consequences following cessation of HT in women from KEEPS. Funding for other new modalities against specific aspects of menopause (anti-bone loss, and anti-hot flashes, etc.) are needed so that effectiveness of those modalities can be compared to the general value of the wide systems effects of HT.

Conclusion

The KEEPS and its numerous ancillary studies have taught us many things regarding the effects of two HT methods commonly used in contemporary clinical practice in generally healthy menopausal women (Table 4). Negativity following the WHI led to many women not receiving prescriptions for estrogen even with moderate to severe symptoms, and decreased funding for further studies. Although KEEPS can be criticized, as have other studies, for not being generalizable to other groups, the alternative is also true. That is, KEEPS has provided valuable information regarding a carefully characterized group of women for whom HT would be beneficial to manage symptoms (hot flashes, sleep disturbances, sexual function) and chronic diseases of menopause, specifically to reduce the risk of osteoporosis without increasing the risk for cardiovascular disease and cognitive impairment. Indeed, since KEEPS, other analyses of the WHI and several meta-analyses have shown that younger women, similar to the participants of KEEPS, demonstrate no harm from HT and may benefit in terms of cardiovascular disease risk.[11,101-107] Clinicians should be encouraged by these results to help better characterize the benefits and risk of HT for their patients. Investigators should be encouraged to continue to obtain and evaluate data from the KEEPS cohort. With the additional follow-up of the KEEPS cohort, it is expected that the KEEPS will “keep on giving” important data upon which to base clinical recommendations for menopausal women as they age.

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Brigham and Women's Hospital (Partners): #2004-P-002144 BWH
Mayo Clinic: 2241-04
Columbia: IRB#: AAAA-8062
Yale: 0409027022
University of Utah: 13257
Einstein/Montefiore: 04-08-213
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KEEPs: Investigators and Staff:
Albert Einstein College of Medicine: Genevieve Neal-Perry, Ruth Freeman, Hussein Amin (deceased), Barbara Isaac, Maureen Magnani, Rachel Wildman
Brigham and Women's Hospital/Harvard Medical School: JoAnn Manson (PI), Maria Bueche, Marie Gerhard-Herman, Kate Kalan, Jan Lieson, Kathryn M. Rexrode, Barbara Richmond, Frank Rybicki, Brian Walsh
Columbia College of Physicians and Surgeons: Rogerio Lobo (PI), Luz Sanabria, Maria Soto, Michelle P. Warren, Ralf C. Zimmerman
Kronos Longevity Research Institute: S. Mitchell Harman (PI), Mary Dunn, Panayiotis D. Tsitouras, Viola Zepeda
Mayo Clinic: Virginia M. Mille (PI)r, Muthuvel Jayachandran, Philip A. Araoz, Rebecca Beck, Dalene Bott-Kitslaar, Sharon L. Mulvagh, Lynne T. Shuster, Teresa G. Zais (deceased)
University of California, Los Angeles, CAC Reading Center: Matthew Budoff (PI), Chris Dailing, Yanlin Gao, Angel Solano
University of California, San Francisco Medical Center: Marcelle I. Cedars (PI), Nancy Jancar, Jean Perry, Rebecca S. Wong, Robyn Pearl, Judy Yee, Brett Elicker, Gretchen A.W. Gooding; UCSF Statistical Center: Dennis Black, Eric Vittinghof, Lisa Palermo
University of Southern California, Atherosclerosis Research Unit/Core Imaging and Reading Center: Howard N. Hodis (PI), Yanjie Li, Mingzhu Yan
University of Utah School of Medicine: Eliot A. Brinton (PI), Paul N. Hopkins, M. Nazeem Nanjee, Kirtly Jones, Timothy Beals, Stacey Larrinaga-Shum
VA Puget Sound Health Care System and University of Washington School of Medicine: George R. Merriam (PI,deceased), Pamela Asberry, Sue Ann Brickle, Colleen Carney, Molly Carr, Monica Kletke, Lynna C. Smith
Yale University, School of Medicine: Hugh Taylor (PI), Kathryn Czarkowski, Lubna Pal, Linda McDonald, Mary Jane Minkin, Diane Wall, Erin Wolff.
Others: Frederick Naftolin (Co-PI,New York University), Nanette Santoro (PI, formerly from Albert Einstein College of Medicine, currentlyUniversity of Colorado)

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