EFFECT OF HORMONE THERAPY AND CALCITRIOL ON SERUM LIPID PROFILE IN POSTMENOPAUSAL ELDERLY WOMEN: ASSOCIATION WITH ESTROGEN RECEPTOR ALPHA GENOTYPES

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Abstract

Objective—Examine the effect of conjugated equine estrogens alone (ET), conjugated equine estrogens plus medroxyprogesterone (EPT), calcitriol alone, calcitriol plus EPT/ET or placebo on serum lipid profile and analyze the interaction with estrogen receptor alpha gene single nucleotide polymorphisms (ESR-α SNPs) on response to therapy.

Methods—489 postmenopausal women > 65 years age enrolled in 3-year double blind placebo-controlled clinical trial

Results—In both intent to treat and complier (>80% adherent) analysis, there was significant increase in serum high-density lipoproteins (HDL), significant decrease in serum low-density lipoproteins (LDL) and LDL:HDL ratio in all hormone treatment groups compared to placebo (p<0.05). However, serum triglycerides and very low-density lipoproteins (VLDL) increased in EPT and ET+ calcitriol groups' versus placebo (p <0.05). ESR-α SNPs PvuII and XbaI appeared to have a significant effect on response to treatment. Genotypes containing p allele showed significantly greater decrease in serum cholesterol and VLDL than those having P allele in the ET plus calcitriol group (p<0.05) and those with x allele had significantly greater decrease in serum cholesterol in HT plus calcitriol group at the end of 3 years versus X allele and a greater decrease in serum LDL in alleles x versus X in ET plus calcitriol group (p<0.05).

Conclusions—ET with or without progesterone had a favorable effect on lipid profile in postmenopausal elderly women and this was dependent on estrogen receptor SNP's – PvuII and XbaI. However, this interaction with ESR-α SNPs need to be confirmed in larger studies.

Keywords

hormone therapy; lipids; conjugated equine estrogen; medroxyprogesterone acetate; postmenopausal women; estrogen receptor SNPs

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in both men and women in the United States and other developed countries. However, the incidence of the disease is delayed in women by about a decade, probably as a consequence of a protective effect of estrogen before menopause (1–3). The risk of CVD in women increases markedly after menopause (4,5). Several lines of evidence indicated that the unfavorable lipid-lipoprotein changes seen after menopause were related to lack of estrogen and had led to the suggestion that hormone therapy (HT) might reduce the risk of CVD.

A number of observational studies reported a lower risk of CVD in women taking estrogen. A meta-analysis of more than 30 observational studies showed a 56% reduction in risk of a major coronary event in healthy current estrogen users compared with women who have never used estrogen (6,7). The cardio-protective effect of ET (Estrogen Therapy) has been attributed to a number of potential mechanisms of which favorable changes in lipid-lipoprotein profile is estimated to contribute to at least one third of cardio-protective effect (8). Observational studies and clinical trials that evaluated the effect of exogenous estrogen on cardiovascular risk have demonstrated favorable changes in serum lipid and lipoprotein levels including a decrease in serum levels of total cholesterol and LDL and increased serum HDL and triglyceride levels (8–13). However other factors including route, type and dose of estrogen administration and the co-administration of a progestin affects the response in terms of serum lipids (11–19). Medroxyprogesterone acetate (MPA) is the most commonly used progestogen in HT regimens in United States, for women with intact uterus to reduce the increased incidence of endometrial cancer that accompanies unopposed estrogen use. MPA was reported to attenuate the beneficial effects of estrogen on cardiovascular system in postmenopausal estrogen progestin intervention trial (11,20) and also by other investigators (21). Debate is continuing on whether there is an impact of the added progestins on the beneficial effects of estradiol on the cardiovascular system (22,23). Also studies in the cynomolgus macaque had previously indicated an attenuation of ET effects' by MPA on the development of atherosclerotic plaque, as well as coronary artery reactivity (24–27). However, observational studies of HT report no differences in risk for clinical cardiovascular events between users of estrogen alone and users of estrogen plus progestins, including women using predominantly MPA (18,19,28). In two large randomized clinical trials, HERS (Heart and Estrogen/progestin Replacement Study) and ERA (Estrogen Replacement and Atherosclerosis Study) which examined the use of HT in postmenopausal women with established CHD, no beneficial effect of HT in reducing the CHD risk was found (29,30). Then came the results from the Women's Health Initiative (WHI), a large, randomized trial that reported that women on estrogen in combination with progestin had increased incidence of coronary heart disease compared to placebo (31).

The biological rationale for the beneficial effect of ET is that estrogen increases the hepatic synthesis of LDL receptor (apo B-100), resulting in an increased LDL uptake and therefore decreased circulating LDL levels (32). It also increases the activity of the enzyme lipoprotein lipase, thus raising HDL levels (33).

Though the positive role of ET treatment on serum lipids is well known, the influence of HT on lipid parameters is still controversial.

Most of the biological actions of estrogen are exerted through estrogen receptors (ER) that belong to the family of ligand gated nuclear transcription factors (34,35). There are two types of estrogen receptors – alpha (α) and beta (β). ER-α is considered to be the main receptor for estrogen. There are various single nucleotide polymorphisms (SNPs) present in ER-α - PvuII and XbaI. These are present in intron 1 of the ER-α gene (Figure 1). PvuII is

present −397 base pairs from exon 2 and produces C to T transition (36). XbaI is present −351 base pairs from exon 2 and produces A to G transition (37). These SNPs have previously been shown to be associated with bone markers (38), bone mineral density and fractures (39,40) and cardiovascular disease including myocardial infarction (41,42) and stroke (43). Few studies have examined the association of change in serum lipids in response to HT with estrogen receptor alpha gene SNPs (37, 44,45).

In a randomized, double blinded clinical trial; we examined the effect of conjugated equine estrogen alone (ET, in women who underwent hysterectomy) or in combination with medroxyprogesterone (EPT, in women with intact uterus) on lipid profile in elderly postmenopausal women and further analyzed the impact of estrogen receptor α gene single nucleotide polymorphisms (SNPs) on the response to therapy.

METHODS

Four hundred and eighty nine elderly women aged 65–77 years were recruited to participate in a 3-year double blind placebo-controlled clinical trial, STOP IT (Sites Testing Osteoporosis Prevention or Intervention Treatment). The study was originally designed to test the effects of estrogen in women without a uterus (ET) plus medroxyprogesterone acetate in women with a uterus (EPT), calcitriol, and the combination of ET/EPT and calcitriol on bone density, biochemical markers of bone remodeling, falls and fractures, compared to placebo. The women were recruited into the study through advertisements in local newspapers or by mass mailing of letters inviting them to participate in a three-year study. Women were excluded if they had severe chronic illness, had primary hyperparathyroidism or active renal stone disease, and were on medications, such as bisphosphonates, anticonvulsants, estrogen, fluoride, or thiazide diuretics in the previous 6 months. Additional inclusion criteria were normal liver and kidney function. All participants were free living, in good health and ambulatory. Of the 489 women, 470 were white, 13 were black, 4 were Hispanic, 1 Asian and one of mixed race. Creighton University Institutional Review Board approved the study and all participants signed an informed consent to participate in the study. .

Women were randomly assigned to either conjugated equine estrogens (Premarin) 0.625 mg daily plus Medroxyprogesterone acetate (Provera) 2.5 mg daily (EPT), calcitriol (Rocaltrol) 0.25 mcg b.i.d., the combination of Premarin plus Provera + calcitriol, or matching placebos; hysterectomized women (n=290) assigned to estrogen were not given the progestin (ET). The detailed study design has been depicted in Fig 2. Details about the safety assessment and study compliance have been published elsewhere (46).

Dietary intake at baseline and at the end of the study (36 months) was assessed using 7-day food diaries. A dietitian asked the women to complete a 7-day food dairy and nutrient supplement record. Plastic food models (NASCO, Fort Artinson, WI) were used to help participants to better estimate the quantities consumed. The average daily caffeine and calcium intakes were calculated using the FOOD PROCESSOR II PLUS nutrition and diet analysis system (version 5.1; Esha Research, Salem, OR).

On entry into the study, women underwent a physical examination and a detailed medical history was obtained from a questionnaire. At baseline recruitment and at 36 months, participants were also provided with a questionnaire to report their smoking and alcohol history, reproductive history, and present use of medications, vitamins and mineral supplements. Current smokers were considered as smokers while past and women who never smoked were classified as nonsmokers. Alcohol use was stratified into drinkers and nondrinkers.

At baseline and at the end of study, a fasting blood sample was collected from each participant between 0700 h and 0900 h for measurement of serum total cholesterol, triglycerides, HDL cholesterol, and LDL cholesterol levels. Serum total cholesterol, triglycerides and HDL cholesterol were estimated using an auto-analyzer (Vitros 750 and Vitros 950 chemistry analyzer, Ortho-Clinical Diagnostics, NY). The method used for total cholesterol measurement is based on an enzymatic method proposed by Allain et al (47). Triglyceride analysis is based on an enzymatic method described by Spayd et al (48). HDL cholesterol is estimated after pretreatment of the sample to precipitate LDL and VLDL. VLDL cholesterol level is calculated as triglyceride/5. LDL cholesterol is calculated as, [total cholesterol-(HDL+VLDL)].

Estrogen Receptor Genotype Analysis

Genomic DNA was isolated from peripheral white blood cells using the Puregene kit (Gentra systems). The samples were genotyped for PvuII (rs2234693) and XbaI (rs9340799) restriction fragment length polymorphisms (RFLPs) as has been published by us before (38). Briefly, the oligonucleotide primers used included forward, 5'-CTGCCACCCTATCTGTATCTTT-3' and reverse, 5'-ACCCTGGCGTCGATTATCTGA-3'. Conventional polymerase chain reaction (PCR) was performed for 30 cycles under the following conditions: denaturation for 60 seconds at 94°C, annealing at 50°C for 60 seconds and extension at 72°C for 90 seconds. The PCR products (~1.3 kbp long fragments) were digested with restriction endonuclease enzymes – PvuII and XbaI. The resulting fragments were separated by gel electrophoresis on 2% agarose gel stained with ethidium bromide and visualized on a transilluminator under UV light and photographed. These were coded as P/p (PvuII) and X/x (XbaI) with upper case letters signifying the absence and lower case signifying the presence of restriction sites respectively.

Statistical analysis

Data were analyzed using SAS package 8.0. The following procedures were performed for both the intent to treat group and adherent to treatment group. In each case, the baseline characteristics between the six treatment groups were compared using One-Way ANOVA and the averages and standard errors were computed. The percentage of smokers and alcohol drinkers by treatment group, and their standard errors, were also found by Chi-Square test. The unadjusted (crude means) baseline lipid variables and the unadjusted percent change over baseline lipid parameters of the six treatment groups were compared again using One-Way ANOVA. When the treatment effect was significant at 0.05 level in the ANOVA models, multiple comparisons of the least squared means by treatment were performed using Tukey's method. In addition to these "crude" means, in an ANCOVA (analysis of covariance) model, the treatment effects on (a) the baseline values and (b) the percent change from the baseline in variables of interest were assessed. The effects of the covariates, age weight, height, BMI, dietary calorie intake, fiber intake, calcium intake, caffeine intake, alcohol status, smoking status and age at menopause were adjusted for in these analyses. When the percent change was the outcome variable, the corresponding baseline value was also used for the adjustment. The least–squares means (i.e. the adjusted means) were computed by the MIXED procedure of SAS (v. 8.2). When the treatment effects were significant at 0.05 level in the ANCOVA models, multiple comparisons of the least squared means by treatment were performed using Tukey's method. The impact of RFLP on the treatment response in terms of serum lipids was assessed by ANCOVA models after adjusting for the above mentioned confounders plus statin users and the baseline serum lipid for each of the corresponding values, for example in the analysis of final serum cholesterol at 36 months, baseline serum cholesterol was used a co-variate in the analysis in addition to the above mentioned covariates. When the treatment effects were significant at 0.05 level in

the ANCOVA models, multiple comparisons of the least squared means by treatment were performed using Bonferroni adjustment.

RESULTS

Of the 489 women enrolled into the study, 416 women came for the final visit (36 month), of which 337 women were adherent to treatment (> 80 % compliant with study medication). The data of the 416 women were included for the intent to treat analysis. Of these, one woman with suspected Paget's disease was excluded from the analyses and 3 women had no lipid profile data available. So the final intent to treat analyses was performed on the data of 412 women. The data of the 337 women who were adherent to the treatment regimen were used for complier analyses (> 80% compliance with study medication). The initial randomization of women into different treatment groups has been depicted in Fig 2.

In both intent to treat and complier analyses, there were no significant differences between the treatment groups with regard to their age, height, weight, BMI, dietary calcium and caffeine intake (Table 1). The distribution of smokers and alcohol drinkers was also not different between the six treatment groups (Table 1).

Intent to Treat Cross- sectional Analysis

At baseline, there were no significant differences in means of serum cholesterol and HDL cholesterol between any of the treatment groups (Table 2). Unadjusted mean serum triglycerides and serum VLDL were significantly lower in women assigned to EPT +calcitriol treatment regimen compared to women assigned to ET treatment (123.4 ± 7.7 vs 180.7 ± 15.0; $p < 0.01$ and 24.7 ± 1.5 vs 34.8 ± 2.7; $p < 0.05$ respectively -Table 2), however, after adjusting for differences in clinical characteristics and dietary factors, the changes became non significant (adjusted means for serum triglycerides were 124.6 ± 11.5 mg/dl in EPT+calcitriol group vs 174.2 ± 13.3 on ET, $p > 0.05$ and for serum VLDL were 24.4 ± 2.1 vs 33.0 ± 2.5, $p > 0.05$ respectively). Adjusted mean serum LDL cholesterol was significantly higher in women receiving the EPT treatment at baseline compared to women receiving the calcitriol (145.8 ± 4.6 in EPT group vs 128.5 ± 4.0 in calcitriol group; $p < 0.05$). The unadjusted ratio of mean LDL/ HDL cholesterol in the EPT group was significantly greater compared to women in the calcitriol group (3.15 ± 0.15 vs 2.65 ± 0.08; $p < 0.05$) and was similar in adjusted analysis (3.05 ± 0.13 vs 2.49 ± 0.11; $p < 0.01$ respectively). The unadjusted LDL/HDL cholesterol was also significantly higher on ET compared to women in the calcitriol group (3.20 ± 0.19 vs 2.65 ± 0.08; $p < 0.05$ respectively: see Table 2). Also, the mean LDL/HDL ratio in women on EPT+calcitriol was significantly different from the group on EPT (both adjusted and unadjusted) (Table 2).

Compliers analysis

At baseline, there were no significant differences in unadjusted and adjusted means between the six treatment groups with regard to serum cholesterol, triglycerides, HDL cholesterol and VLDL cholesterol (Table 2) but There was a significant difference in baseline adjusted serum LDL cholesterol between the women assigned to EPT and calcitriol treatment regimens (154.9 ± 5.4 vs 132.9 ± 4.3; $p < 0.01$), between the women assigned to EPT +calcitriol and EPT treatment regimens (132.0 ± 5.4 vs 154.9 ± 5.4; $p < 0.05$) and between the women of ET+calcitriol and EPT treatment groups (128.5 ± 6.3 vs 154.9 ± 5.4; $p < 0.05$). The LDL:HDL cholesterol ratio (both unadjusted and adjusted) was significantly higher in women receiving EPT treatment compared to that of women assigned to calcitriol alone (3.30 ± 0.17 vs 2.70 ± 0.09 respectively $p < 0.01$ in unadjusted analysis and 3.22 ± 0.15 vs 2.55 ± 0.12; $p < 0.01$ in adjusted analysis respectively), EPT+calcitriol treatment (2.66 ± 0.13 in EPT+calcitriol vs 3.30 ± 0.17 in EPT; $p < 0.05$ for unadjusted and $p < 0.01$ for adjusted) and

ET+calcitriol treatment (2.54 ± 0.18 in ET+calcitriol vs 3.22 ± 0.15 in EPT; adjusted, p<0.05).

Longitudinal Analysis

Intent to Treat—Serum total cholesterol - The results of unadjusted analysis are presented in Table 3. The decrease in total cholesterol in women receiving EPT and ET alone was significantly greater than placebo (−12.1 ± 4.4 in EPT and −14.4 ± 4.8 in ET vs 3.5 ± 2.4 mg/dl in placebo; p <0.05: see Table 3), however, it became non-significant after adjustment for relevant confounders (Fig 3).

Serum triglycerides - The increase in serum triglycerides was significantly higher in women receiving ET+calcitriol compared to placebo (41.6 ± 10.7 vs 10.5 ± 6.0 mg/dl; p<0.05 - Table 3); the adjusted increase was significantly higher in EPT and ET+calcitriol groups compared to placebo and EPT+calcitriol (only EPT group) groups (35.3 ± 5.6 % in EPT vs 12.7 ± 4.6 % in placebo; p<0.01; 37.7 ± 7.1 % in ET+calcitriol vs 12.7 ± 4.6 % in placebo; p<0.05 and 14 ± 5.7 % in EPT+calcitriol vs 35.3 ± 5.6 % in EPT alone; p<0.05 – see Fig 3). No significant differences existed between other treatment groups of intent to treat analyses.

Serum HDL - There was a significantly greater increase in serum HDL in all of the hormone treatment groups as compared to placebo and calcitriol groups; both unadjusted and after adjustment for confounders (Unadjusted change was 6.4 ± 1.3, 7.3 ± 1.5, 6.2 ± 1.3, 8.4 ± 1.6 mg/dl in EPT, ET, EPT+calcitriol and ET+calcitriol groups vs 1.3 ± 0.7 and −1.2 ± 0.8 in placebo and calcitriol respectively, p<0.05 – see Table 3). For adjusted analysis, serum HDL increased by 13.9 ± 2.5 % in EPT, 13.1 ± 2.9 % in ET, 12.7 ± 2.5 % in EPT+calcitriol and 15.6 ± 3.2 % in ET+calcitriol vs 2.66 ± 2.1 % in placebo (p<0.05) and decreased in calcitriol only group (−0.1 ± 2.2 %; p <0.05 – see Figure 4).

Serum LDL - There was a significantly greater decrease in serum LDL in all of the hormone treatment groups as compared to placebo and calcitriol groups; both unadjusted and after adjustment for confounders (Unadjusted change was −25 ± 4.2, −26.3 ± 4.7, −11.5 ± 3.5, −19.9 ± 5.5 mg/dl in EPT, ET, EPT+calcitriol and ET+calcitriol groups vs −1.3 ± 2.1 and −0.84 ± 2.3 in placebo and calcitriol respectively, p<0.05 – see Table 3). For adjusted analysis, serum LDL decreased in EPT (−13 ± 2.6 %), ET (−15.5 ± 3 %), EPT+calcitriol (−7.2 ± 2.5 %) and ET+calcitriol (−14.2 ± 3.2 %) vs 0.3 ± 2.1 % increase in placebo (p<0.05) and 1.1 ± 2.2 % increase in calcitriol only group (p <0.05 – see Figure 4). The exception was the adjusted and unadjusted percent change in LDL- C in EPT+calcitriol group, which was not significantly different.

Serum LDL/HDL ratio - The adjusted and unadjusted serum LDL/HDL ratio was also significantly lowered in all the 4 treatment groups receiving the hormones compared to that of placebo and calcitriol groups (Unadjusted change was −0.84 ± 0.12, −0.82 ± 0.11, −0.41 ± 0.09, −0.68 ± 0.13 in EPT, ET, EPT+calcitriol and ET+calcitriol groups vs −0.05 ± 0.06 and 0.05 ± 0.05 in placebo and calcitriol respectively, p<0.05 − see Table 3). For adjusted analysis, the results were similar: −21.1 ± 3.3 % in EPT, −22.3 ± 3.9 % in ET, −14 ± 3.3 % in EPT+calcitriol and −22.5 ± 4.1 % in ET+calcitriol groups vs 0.35 ± 2.7 % and 4.7 ± 2.8 % in placebo and calcitriol respectively, p<0.05 – see Figure 4).

Serum VLDL - The unadjusted serum VLDL of the women receiving ET+calcitriol treatment increased significantly more than placebo (8.3 ± 2.1 mg/dl increase in ET +calcitriol vs 2.7 ± 1.0 in placebo, p <0.05; see Table 3). However, adjusted percent change in VLDL levels was significantly higher in ET+calcitriol group compared to that of women belonging to placebo, calcitriol and EPT+calcitriol treatment groups (40 ± 6.6 % in ET

+calcitriol vs 13.7 ± 4.4 %, 15.8 ± 4.6 %, 13.6 ± 5.3 % in placebo, calcitriol and EPT +calcitriol respectively, p< 0.05; see Fig 4).

Complier Analysis—There were no major differences from the intent to treat analysis in terms of serum total cholesterol, serum HDL, serum LDL, LDL/HDL ratio and serum VLDL (Table 3, Figures 5 and 6). Serum triglycerides increased more in EPT and ET +calcitriol groups compared to placebo and calcitriol groups (Unadjusted analysis: Change in serum triglycerides was 69.5 ± 22.3, 45.8 ± 12.0 mg/dl in EPT and ET+calcitriol vs 9.3 ± 6.6 and 16.5 ± 6.0 mg/dl in placebo and calcitriol respectively, p <0.05; see Table 3). For adjusted analysis, change in serum triglycerides was 46.5 ± 6.8 % in EPT, 42.7 ± 7.8 % in ET+calcitriol vs 12.8 ± 5.1 % in placebo and 16.4 ± 5.4 % in calcitriol respectively (p < 0.05, see Figure 5).

Effect of Estrogen Genotypes

Estrogen receptor alpha gene RFLPs were done in 324 women for both PvuII and XbaI. These were in apparent linkage disequilibrium and the distribution did not differ from that expected under Hardy-Weinberg equilibrium. There were no significant differences in baseline serum lipids according to genotypes.

There was a significant association between PvuII RFLP and the response to treatment in terms of serum cholesterol and VLDL. Genotypes containing the p allele had a greater decrease in serum cholesterol (−19 mg/dl in pp vs 7 mg/dl in Pp, p = 0.06) and increase in serum VLDL (13.3 mg/dl in pp vs −5 mg/dl in PP, p = 0.0006) compared to those having the P allele in the ET plus calcitriol group (Table 4). In terms of serum triglycerides and serum LDL, there was a trend (overall p value < 0.05) for greater decrease in the genotypes containing p allele in the ET plus calcitriol group (−66.8 mg/dl in pp vs −25.8 mg/dl in PP for serum triglycerides and −40.4 mg/dl in pp vs −19.5 mg/dl in PP for serum LDL) (Table 4).

XbaI RFLP also showed a significant association in the response to treatment in terms of serum cholesterol and serum LDL. Genotypes including the x allele had a greater decrease in serum cholesterol in the EPT plus calcitriol group at the end of 3 years compared to X allele alone (−8.9 mg/dl in Xx vs 36.8 mg/dl in XX, p = 0.03) and a greater decrease in serum LDL in ET plus calcitriol group (−40.4 mg/dl in xx vs 9.2 mg/dl in Xx, p = 0.01) (Table 4). In terms of serum VLDL, there was a trend (overall p value < 0.05) for greater increase in xx vs XX (13.3 mg/dl vs −2.5 mg/dl respectively). There were no significant differences in other serum lipids in terms of response to treatment according to genotypes.

Discussion

The results of this study show that hormone therapy (estrogen with or without medroxyprogesterone acetate) favorably lowers serum LDL and increases serum HDL but also increases serum triglycerides in healthy elderly post-menopausal women, and further indicate that the effect of treatment (HT and/or calcitriol) is modified by estrogen receptor single nucleotide polymorphisms (SNPs). This is the first study of its kind looking at the interaction between estrogen receptor SNPs, hormone therapy, calcitriol and treatment response in terms of serum lipids in a longitudinal follow up.

The positive effects of estrogen therapy on serum lipids have been reported in previous studies. In a randomized, double blind trial in younger postmenopausal women, treatment with ET resulted in a significant dose-dependent reduction in LDL-cholesterol and increase in serum HDL over the dose range 0.3–0.625 mg/day. At all three doses of ET studied (0.3, 0.45, and 0.65 mg/day), LDL-cholesterol was significantly reduced and HDL was increased

relative to both baseline and placebo treatment (49). In addition, there were favorable effects on hemostatic factors including fibrinogen and plasminogen activator inhibitor −1. Similar findings were reported in another randomized double blind cross-over trial (50).

There has been interest in the cardiovascular effect profile of HT in postmenopausal women. At the center of debate are two large studies, one observational – Nurses Health Study (NHS) and the other a randomized controlled trial – Women's Health Initiative (WHI) (31,51).

NHS was a large observational study involving 121,700 female nurses 30 to 55 years of age at the time of enrollment in 1976 (18, 52). In the latest analysis involving 70,533 postmenopausal women with no previous cardiovascular disease followed for up to 20 years, there was a significant reduction in major CHD (nonfatal myocardial infarction and coronary death) after adjustment for age, body mass index, history of diabetes, hypertension, high cholesterol level, age at menopause, smoking status and parental history of premature heart disease (Relative Risk, RR = 0.61, 95% Confidence Limits (CL) = 0.52–0.71 for current users and RR = 0.82, CL = 0.72–0.94 for past users of hormone therapy). There was a small difference in risk reduction based on the type of hormone therapy (RR = 0.55, CL = 0.45–0.68 for women taking oral conjugated estrogen alone and RR = 0.64, CL = 0.49–0.85 for women taking estrogen plus progestin) (52).

However, the initial results of WHI trial showed that estrogen plus progestin therapy increased the risk of coronary heart disease (CHD) (nonfatal myocardial infarction [MI] and CHD death), stroke and venous thromboembolism (31). These results were later modified based on more data and after central adjudication – estrogen plus progestin therapy still significantly increased the risk of CHD (HR = 1.24, nominal 95% confidence interval (nCI) = 1.00–1.54, adjusted confidence interval (aCI) = 0.97–1.60) and stroke (HR = 1.31, nCI = 1.02–1.68, aCI = 0.93–1.84) (53). The absolute risk was still very low – the findings suggest that over one year, one per 1600 users women taking EPT compared with placebo developed 6 more CHD events. There was no increase in the CHD death or overall mortality, congestive heart failure and a composite of CHD, revascularization or angina. Therefore, although the risk for MI increased the risk for angina was not increased. But there were other adverse events- invasive breast cancers, strokes, VTEs, PEs and benefits- less colorectal cancers, hip fractures and total fractures that increased the risk benefit of HT. In the ET arm of the WHI trial, there was no significant effect on CHD although risk of stroke and VTE was elevated however; none of the cardiovascular events were significant after adjustment (51). There was no increase in breast cancer on ET.

In a subgroup analysis to determine specific populations at increased risk of CHD, no significant interaction between age or years since menopause and treatment was observed (*P* = 0.36 for the age analysis and *P* = 0.33 for years-since-menopause analysis), although there was a numerical trend towards a higher risk with EPT more years after menopause (53). Although the tests for interaction were non-significant, women not using statins or aspirin (≥80 mg/d) were at increased risk of CHD. In addition, women with baseline serum LDL cholesterol levels (> 155 mg/dl) had increased risk of CHD when started on EPT compared to those with lower LDL-C. Age was also a determinant of CHD risk. The average age of women started on HT was 63 years. BMI was also a possible risk because the average BMI was 28.5 kg/m^2 (53). In the Nurses Health study the risk of CHD increased with increasing BMI; the risk was increased 356% in women with a BMI of ≥ 29 kg/m^2 compared to women with a BMI of < 21 kg/m^2 (54).

In a randomized double blind trial involving 222 post-menopausal women ≥ 45 years old without pre-existing cardiovascular disease and serum LDL ≥ 130 mg/dl, the average rate of

progression of subclinical atherosclerosis measured by change in intimal-media thickness of the right distal common carotid artery was slower in women taking ET with 17beta-estradiol than in women taking placebo (55). However, the results of other studies are less convincing (30).

These apparent differences in observational and intervention studies could be partly explained by findings of various animal studies carried out by Clarkson et al and others (56,57). Specifically, these studies demonstrate that the extent of subclinical atherosclerosis at baseline appears to be a significant modulator of the atheroprotective effects of HT and that estrogen inhibits the initiation of fatty streaks in vasculature but does not inhibit the progression of established lesions. This "timing hypothesis" might play an important role in determining the effects of estrogen therapy in various populations. In a meta-analysis of 30 trials of hormone therapy involving 26708 women with mean follow of 4.5 years, hormone replacement reduced mortality in the younger age group < 60 years, (OR 0.61; CI, 0.39 to 0.95), but not in the older age group > 60 years, (OR 1.03; CI, 0.90 to 1.18) (58).

Estrogen receptor SNPs appear to affect the response of serum lipids to HT treatment. The genotypes that included the x and p alleles showing significantly greater response in terms of serum cholesterol, serum LDL and serum VLDL compared to X and P alleles respectively. We have shown previously from the same dataset that PvuII and XbaI SNPs interact with treatment response to hormone therapy in terms of bone loss measured by changes in bone mineral density and bone markers (38).

Only a few studies have looked at the interaction between ERα genotypes and the response in serum lipids to hormone therapy. In fact, the first study done by Herrington et al (37) found a significant association between PvuII SNPs and treatment response to hormone therapy in terms of serum HDL with greater increase in serum HDL in women with PP genotype as compared to Pp or pp. Other small studies have found similar results (44,45).

The mechanism by which ERα-SNPs lead to changes in serum lipids is not clear. Initial reports had suggested an alteration in protein expression secondary to changes in mRNA splicing of PvuII gene (59). As these are present in the non-coding region (activation domain) of gene, the structure of receptor protein should not be altered. Instead there is growing evidence that these SNPs affect the binding of transcription factors like myb to ERα gene leading to decreased expression of ERα receptor. Herrington el al (60) found that the presence of P allele resulted in > 10-fold increase in a downstream reporter activity as compared to only 2.5-fold increase in activity with p allele; both sets of gene stimulated by transcription factor myb. However, whether these SNPs affect the quality or quantity of estrogen receptor alpha gene mRNA transcripts or protein expression remains to be established. Schuit et al. (61) demonstrated that these SNPs (p and x alleles) are associated with lower serum estradiol levels in postmenopausal women. They hypothesized that these SNPs affected the expression of one of the subtypes of 17β-hydroxysteroid dehydrogenase enzyme that catalyzes the transformation of estrone into estradiol. It might also be possible that these SNPs are in linkage disequilibrium with another SNP in ERα gene that affects the expression of ERα.

In a prospective study involving 1739 men and women from Framingham cohort followed for 27 years, the investigators found a significant association between ERα SNPs and MI risk with 3 times greater risk of MI (95% CI = 1.7–5.2) in men with PP genotype as compared to Pp or pp (41). Similar results were found for stroke in 2709 males of the Second Northwick Park Heart study with increased risk in PP genotype independent of established cardiovascular risk factors including diabetes (43). In an autopsy study from Finland, the investigators found that men aged 53 years or over with P/p and P/P genotype

had two to five-fold larger complicated lesions and odds ratio of 6.2 and 10.6 respectively for coronary thrombosis than men with the p/p genotype after adjusting for age and body mass index (62). As noted, all these studies found worse cardiovascular outcomes in men associated with P allele. In contrast, the results of Rotterdam study in 3791 postmenopausal women followed for 8–10 years suggested that p and x alleles were associated with increased MI risk (42). These apparent opposing results in men and women can be explained by the fact that there is no cessation of hypothalamic-pituitary axis in men leading to sufficiently high serum estradiol levels leading to protective effects of p allele in men (42). However in women, the low estradiol levels after menopause coupled with the presence of p allele reducing the expression of ERα receptor gene, leads to worse clinical consequences in terms of cardiovascular endpoints.

There is also the potential effect of calcitriol on the expression of estrogen receptor alpha gene expression. Many studies in human cells have shown that calcitriol down regulates the expression of estrogen receptor alpha gene with major impact on gene transcription (63, 64). These studies also demonstrate a decrease in the bio-responses to estrogen with calcitriol. Although these findings were predominantly found in breast cancer models, it could occur in other tissues also. The results of our study support these findings by showing a more favorable response in serum lipids to hormone therapy and calcitriol in women with p allele as compared to P allele. In women with p allele, ERα receptor gene is already under expressed and calcitriol has little effect on its expression and treatment with hormone therapy infact leads to better response in serum lipids as is found in men (42).

There are some limitations in our study. This is a relatively small sample size for comparing the effects of estrogen receptor SNPs on serum lipids although it is still one of the largest studies reporting the effect of interaction between estrogen receptor alpha SNPs and hormone therapy on serum lipids. Moreover, the genotypes were done by restriction endonuclease digestion and not by Taqman probes that are more accurate.

Although these findings are in older postmenopausal women, if applicable to younger postmenopausal women could partially explain the cardio-protective effect of estrogen when started early after menopause. The results of this study especially the genotype analysis are preliminary in nature and further large trials need to be carried out to confirm our findings in both younger and older postmenopausal women and also the overall impact on event rate in terms of MI and other outcomes.

Acknowledgments

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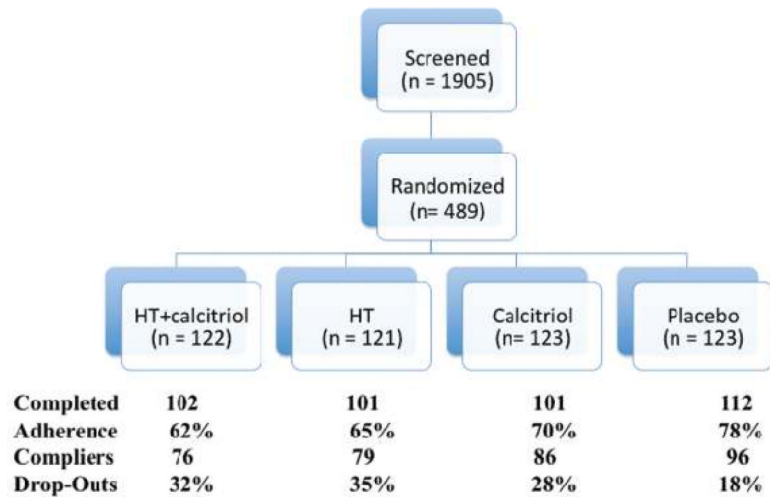
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Estrogen receptor alpha

FIG. 1.
Schematic diagram–estrogen receptor-α gene: it consists of eight exons encompassing six domains. *Pvu*II and *Xba*I restriction fragment length polymorphisms produce C/T and A/G variations, respectively.

STOP IT - Randomization

	HT+calcitriol (n = 122)	HT (n = 121)	Calcitriol (n= 123)	Placebo (n = 123)
Completed	102	101	101	112
Adherence	62%	65%	70%	78%
Compliers	76	79	86	96
Drop-Outs	32%	35%	28%	18%

FIG. 2.
STOP IT randomization. STOP IT, Sites Testing Osteoporosis Prevention or Intervention Treatment; HT, hormone therapy.
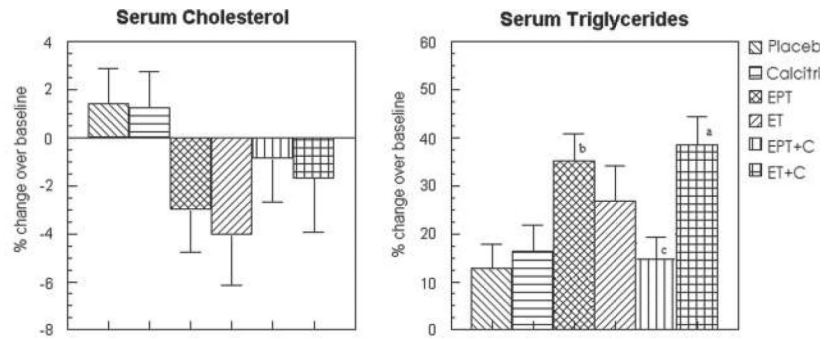
FIG. 3.
Percent change in serum cholesterol and serum triglycerides in the various treatment groups after adjustment for confounders (intent-to-treat analysis). EPT, estrogen + medroxyprogesterone therapy; ET, estrogen therapy; C, calcitriol. [a]$P < 0.05$ as compared with placebo. [b]$P < 0.01$ as compared with placebo. [c]$P < 0.05$ as compared with EPT.
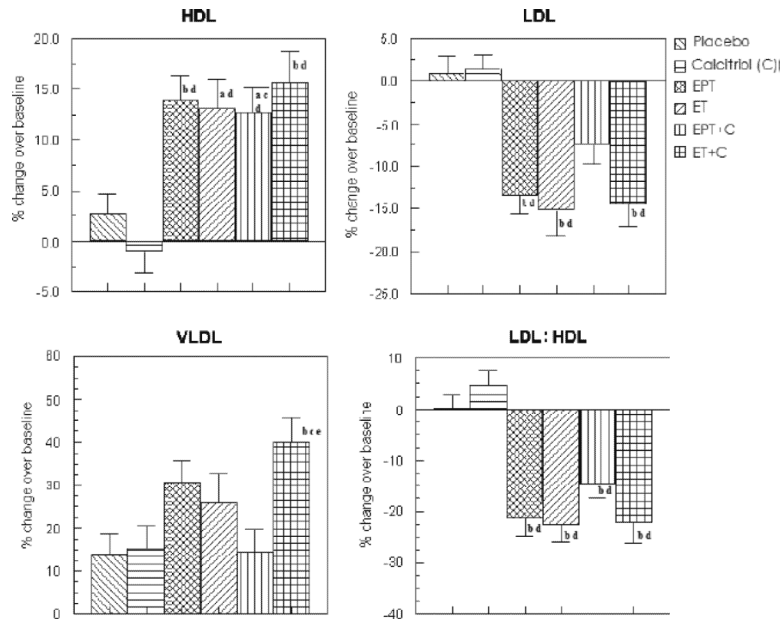
FIG. 4.
Percent change in serum HDL, LDL, LDL/HDL ratio, and VLDL in the various treatment groups after adjustment for confounders (intent-to-treat analysis). EPT, estrogen + medroxyprogesterone therapy; ET, estrogen therapy; C, calcitriol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein. [a]$P < 0.05$ as compared with placebo. [b]$P < 0.01$ as compared with placebo. [c]$P < 0.05$ as compared with C. [d]$P < 0.01$ as compared with C. [e]$P < 0.01$ as compared with EPT + C.
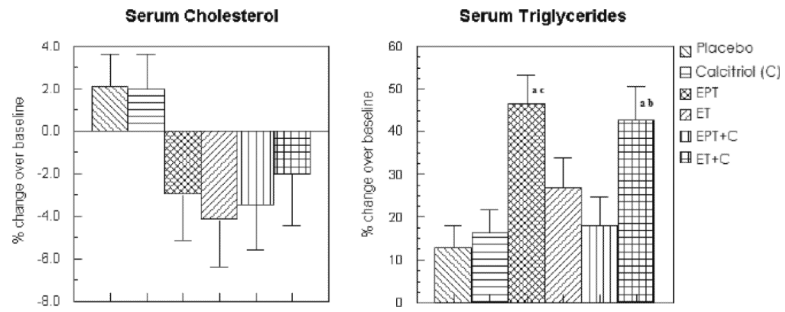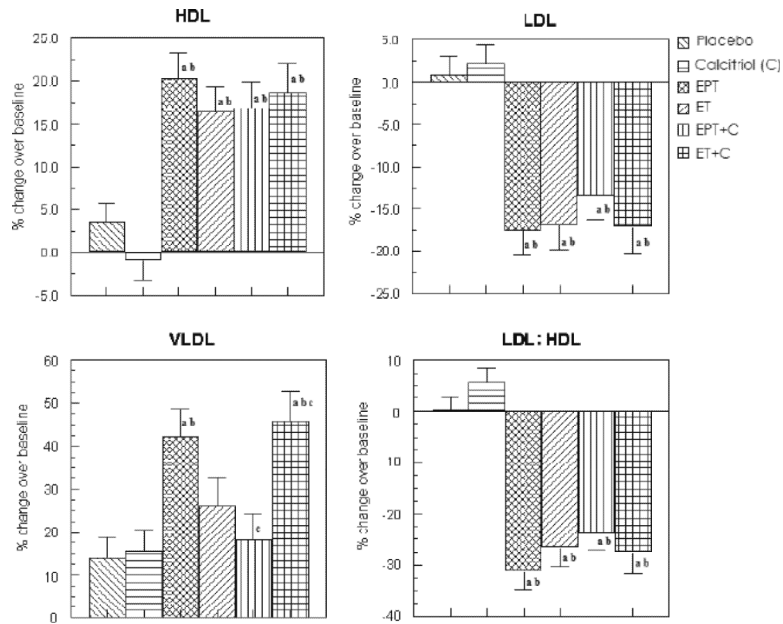
FIG. 5.
Percent change in serum cholesterol and serum triglycerides in the various treatment groups after adjustment for confounders (complier analysis). EPT, estrogen + medroxyprogesterone therapy; ET, estrogen therapy; C, calcitriol. $^a P < 0.01$ as compared with placebo. $^b P < 0.05$ as compared with calcitriol. $^c P < 0.01$ as compared with calcitriol.

FIG. 6.
Percent change in serum HDL, LDL, LDL/HDL ratio, and VLDL in the various treatment groups after adjustment for confounders (complier analysis). EPT, estrogen + medroxyprogesterone therapy; ET, estrogen therapy; C, calcitriol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein. [a]$P < 0.01$ as compared with placebo. [b]$P < 0.01$ as compared with C. [c]$P < 0.05$ as compared with EPT.

Table 1

Baseline Characteristics

	Placebo	Calcitriol	EPT	ET	EPT+Calcitriol	ET+Calcitriol	P value
N	112	101	57	42	62	38	
Age (y)	71.0 ± 0.4	71.4 ± 0.3	71.4 ± 0.5	71.3 ± 0.5	71.1 ± 0.4	71.9 ± 0.6	0.83
Height (cm)	160.0 ± 0.6	159.7 ± 0.6	158.9 ± 0.7	159.7 ± 1.6	159.0 ± 0.8	158.2 ± 0.8	0.67
Weight (Kg)	69.3 ± 1.3	67.7 ± 1.3	67.4 ± 1.4	73.3 ± 2.2	66.7 ± 1.5	69.4 ± 2.1	0.14
BMI	27.2 ± 0.5	26.5 ± 0.5	26.8 ± 0.6	28.7 ± 0.8	26.4 ± 0.5	27.7 ± 0.8	0.14
Calcium intake (mg/d)	782.2 ± 28.6	762.1 ± 25.6	806.3 ± 41.1	691.2 ± 39.8	767.0 ± 38.0	654.3 ± 34.5	0.07
Caffeine intake (mg/d)	267.0 ± 22.5	218.8 ± 16.1	234.5 ± 22.6	261.2 ± 22.7	252.4 ± 19.3	330.5 ± 51.7	0.09
Smokers (%)[a]	11.6 ± 3.0	8.9 ± 2.8	17.5 ± 5.1	21.4 ± 6.4	11.3 ± 4.1	13.2 ± 5.6	0.35
Alcohol use (%)[a]	32.1 ± 4.4	40.6 ± 4.9	45.6 ± 6.7	21.4 ± 6.4	27.4 ± 5.7	31.6 ± 7.6	0.08
Statin Use N (%)[a]	8 (7.14)	5 (5)	2 (3.5)	2 (4.8)	1 (1.6)	5 (13.2)	0.243

Abbreviation: EPT, estrogen plus medroxyprogesterone therapy; ET, estrogen therapy

The values represented in the table are means ± SE.

Comparisons were done using the One-Way Analysis of Variance or Kruskal-Wallis test, as appropriate.

[a] The distribution was tested using the Chi-square statistic.

Table 2

Baseline lipid profile in treatment groups of Intent to treat and complier analyses[a]

	Placebo	Calcitriol	EPT	ET	EPT+Calcitriol	ET+Calcitriol
INTENT TO TREAT (n)	112	101	57	42	62	38
Serum cholesterol	224.4 ± 2.8	218.2 ± 3.6	231.6 ± 5.1	232.8 ± 6.2	218.6 ± 4.7	220.0 ± 5.6
Serum triglycerides	148.2 ± 7.8	144.9 ± 8.0	155.9 ± 10.4	180.7 ± 15.0	123.4 ± 7.7[f]	154.3 ± 13.8
Serum HDL	50.5 ± 1.1	53.3 ± 1.2	50.9 ± 1.7	48.9 ± 1.9	54.2 ± 1.2	51.8 ± 2.4
Serum VLDL	28.5 ± 1.4	28.5 ± 1.5	30.2 ± 1.9	34.8 ± 2.7	24.7 ± 1.5[e]	30.9 ± 2.8
Serum LDL	143.2 ± 2.7	135.1 ± 3.3	150.7 ± 4.7	146.1 ± 5.6	139.7 ± 4.3	136.2 ± 5.3
LDL : HDL cholesterol	2.96 ± 0.08	2.65 ± 0.08	3.15 ± 0.15[b]	3.20 ± 0.19[b]	2.67 ± 0.10[be]	2.78 ± 0.13
COMPLIERS (n)	96	86	41	38	43	33
Serum cholesterol	223.0 ± 3.0	221.1 ± 4.0	235.7 ± 6.0	231.8 ± 6.6	217.1 ± 5.9	218.7 ± 5.9
Serum triglycerides	148.9 ± 8.8	145.1 ± 9.1	156.5 ± 12.1	177.5 ± 16.2	130.3 ± 9.8	155.4 ± 14.7
Serum HDL	50.5 ± 1.1	53.3 ± 1.3	49.9 ± 1.9	49.2 ± 2.0	53.6 ± 1.4	51.3 ± 2.7
Serum VLDL	28.5 ± 1.6	28.4 ± 1.7	29.9 ± 2.1	34.0 ± 2.9	26.1 ± 1.9	31.1 ± 2.9
Serum LDL	143.9 ± 2.9	137.9 ± 3.7	156.3 ± 5.1[b]	145.2 ± 6.0	137.4 ± 5.3	134.9 ± 5.7
LDL : HDL cholesterol	2.97 ± 0.09	2.70 ± 0.09	3.30 ± 0.17[c]	3.16 ± 0.20	2.66 ± 0.13[d]	2.78 ± 0.14

Abbreviation: EPT, estrogen plus medroxyprogesterone therapy; ET, estrogen therapy

[a] The values are means ± SE in mg/dl. Comparison between the treatment groups was done using either ANOVA (for crude means) or ANCOVA (for adjusted means; adjusted for age, weight, BMI dietary factors). Post-hoc significance was computed by Tukey's multiple comparison method.

[b] $p < 0.05$ compared to calcitriol

[c] $p < 0.01$ compared to calcitriol

[d] $p < 0.05$ compared to EPT

[e] $p < 0.05$ compared to ET

[f] $p < 0.01$ compared to ET

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Table 3

Unadjusted change from baseline (mg/dl) of lipid parameters in treatment groups of Intent to treat and complier analyses

	Placebo	Calcitriol	EPT	ET	EPT+Calcitriol	ET+Calcitriol
INTENT TO TREAT						
N	112	101	57	42	62	38
Serum cholesterol	3.5 ± 2.4	0.95 ± 2.8	-12.1 ± 4.4^a	-14.4 ± 4.8^a	-2.04 ± 3.5	-3.5 ± 5.6
Serum triglycerides	10.5 ± 6.0	17.1 ± 5.3	48.4 ± 19	32.3 ± 9.6	16.1 ± 7.3	41.6 ± 10.7^a
Serum HDL	1.3 ± 0.7	-1.2 ± 0.8	6.4 ± 1.3^{bd}	7.3 ± 1.5^{bd}	6.2 ± 1.3^{ad}	8.4 ± 1.6^{bd}
Serum VLDL	2.7 ± 1.0	3.7 ± 0.9	5.8 ± 2.0	5.02 ± 1.8	3.2 ± 1.4	8.3 ± 2.1^a
Serum LDL	-1.3 ± 2.1	-0.84 ± 2.3	-25 ± 4.2^{bd}	-26.3 ± 4.7^{bd}	-11.5 ± 3.5	-19.9 ± 5.5^{bd}
LDL: HDL cholesterol	-0.05 ± 0.06	0.05 ± 0.05	-0.84 ± 0.12^{bd}	-0.82 ± 0.11^{bd}	-0.41 ± 0.09^{bd}	-0.68 ± 0.13^{bd}
COMPLIERS						
N	96	86	41	38	43	33
Serum cholesterol	4.7 ± 2.7	1.7 ± 3.0	-13.4 ± 5.3^a	-14.4 ± 5.2^a	-6.8 ± 4.4	-4.2 ± 6.2
Serum triglycerides	9.3 ± 6.6	16.5 ± 6.0	69.5 ± 22.3^{bd}	30.9 ± 10.5	18.3 ± 9.9	45.8 ± 12.0^{ad}
Serum HDL	1.4 ± 0.8	-1.4 ± 0.9	9.7 ± 1.5^{bd}	8.6 ± 1.4^{bd}	8.6 ± 1.4^{bd}	9.8 ± 1.6^{bd}
Serum VLDL	2.5 ± 1.1	3.6 ± 1.0	8.9 ± 2.5^{ac}	4.5 ± 2.0	3.7 ± 2.0	9.2 ± 2.4^{ac}
Serum LDL	-0.03 ± 2.3	0.31 ± 2.5	-32.5 ± 4.6^{bd}	-27.2 ± 5.0^{bd}	-19.04 ± 4.3^{bd}	-22.9 ± 5.8^{bd}
LDL : HDL cholesterol	-0.02 ± 0.06	0.09 ± 0.06	-1.1 ± 0.14^{bd}	-0.9 ± 0.1^{bd}	-0.65 ± 0.09^{bd}	-0.82 ± 0.12^{bd}

Abbreviation: EPT, estrogen plus medroxyprogesterone therapy; ET, estrogen therapy

The values are means ± SE. Comparison between the treatment groups was done using ANOVA. Post-hoc significance was computed by Tukey's multiple comparison method.

[a] p<0.05 compared to placebo

[b] p<0.01 compared to placebo

[c] p<0.05 compared to calcitriol

[d] p<0.01 compared to calcitriol

Table 4

Change in serum lipids from baseline to the end of 3 years according to treatment group and estrogen receptor alpha genotypes

Change in mean serum cholesterol; mg/dl

Treatment	PP	Pp	pp	Overall P-value	XX	Xx	xx	Overall P-value
N	67	164	93		45	150	129	
ET+Calcitriol	−21	7[ac]	−19[b]	0.0006	−5.9	3.8	−19.1	0.08
Placebo	−3.8	3.4	12.1	0.13	−6.4	3.8	10.2	0.06
ET	−8.6	−16	−23	0.52	−19.6	−9.6	−20	0.47
Calcitriol	1.7	1.4	−5.4	0.96	17.8	−5.8	−2	0.14
EPT+Calcitriol	10	−11.8	−6.3	0.32	36.8	−8.9[d]	−8.2[e]	0.036
EPT	−17.1	−13.3	−8.5	0.35	−22.7	−8.4	−17.6	0.27

Change in mean serum triglycerides; mg/dl

Treatment	PP	Pp	pp	Overall P-value	XX	Xx	xx	Overall P-value
ET+Calcitriol	−25.8	−40.3	−66.8	0.01	−14.0	37.8	66.8	0.07
Placebo	−11.3	8.4	19	0.43	4.3	8	11.6	0.85
ET	15.4	18.9	42.3	0.99	12.4	14	34.8	0.99
Calcitriol	16.8	21.6	11.3	0.98	35.5	4.8	21	0.42
EPT+Calcitriol	15.6	15.8	13.6	0.93	28.7	9.1	19.9	0.78
EPT	39.9	60.9	60.2	0.94	67.2	75.9	24.8	0.09

Change in mean serum VLDL; mg/dl

Treatment	PP	Pp	pp	Overall P-value	XX	Xx	xx	Overall P-value
ET+Calcitriol	−5[fg]	8	13.3	0.0005	−2.5	13.3		0.006
Placebo	2.9	1.7	1.7	0.34	3.8	1.7	0.6	0.34
ET	3.3	1.3	−1.6	0.94	2.5	1.8		0.87
Calcitriol	−1	4.2	2.2	0.96	2.5	4.1	0.3	0.93
EPT+Calcitriol	3	3.1	2.8	0.91	5.2	4.0		0.75
EPT	−0.7	5.8	12.1	0.21	−2.3	8.1	5.0	0.42

Change in mean serum LDL; mg/dl

	PP	Pp	pp	Overall P-value	XX	Xx	xx	Overall P-value
ET+Calcitriol	−19.5	−9.1	−40.4	0.007	−19	−9.2	−40.4[h]	0.01
Placebo	−7.6	−0.3	2.4	0.28	−12.3	−0.6	4.7	0.05
ET	−24.3	−25.3	−34.5	0.65	−36.1	−17.9	−32.4	0.63
Calcitriol	6.3	2.3	−8.2	0.69	15.1	0.6	−5.2	0.36
EPT+Calcitriol	−5.6	−21.5	−14.2	0.53	18.4	−19.5	−16.7	0.06
EPT	−26.1	−29.5	−31.2	0.75	−37.8	−25.6	−33.8	0.50

Change in mean serum HDL; mg/dl

	PP	Pp	pp	Overall P-value	XX	Xx	xx	Overall P-value
ET+Calcitriol	3.8	10.7	8	0.31	15.5	8.1	8.0	0.54
Placebo	1.4	1.5	2	0.68	1.6	2.6	0.5	0.9
ET	12.3	8	5.2	0.08	14	7.9	6.8	0.046
Calcitriol	−3.2	−2.6	0.65	0.58	−0.8	−2.6	−0.9	0.48
EPT+Calcitriol	12.7	6.6	5.2	0.13	13.2	8.9	4.5	0.13
EPT	4.6	8.7	10.6	0.54	3.7	7.5	11.2	0.28

Abbreviation: EPT, estrogen plus medroxyprogesterone therapy; ET, estrogen therapy

[a] p = 0.001 for PP as compared to Pp

[b] p = 0.057 for PP as compared to pp

[c] p = 0.06 for Pp as compared to pp

[d] p = 0.03 for XX as compared to Xx

[e] p = 0.07 for XX as compared to xx

[f] p = 0.01 for PP as compared to Pp

[g] = 0.0006 for PP as compared to pp

[h] p = 0.01 for Xx as compared to xx