SUPPORTIVE STUDIES

**ESTRADIOL LEVELS AND SERUM LIPIDS**


- Pellets produce more reproducible estrogen levels faster and remain steady longer than patches.
  Estradiol levels with patches were less than half of pellet patient levels and fluctuated widely.

**SIGNIFICANT INCREASE IN HDL**

Maturitas 1984: 5: 177-84, Linda Cardozo

- HDL increased at 12 weeks with pellets (sub-cutaneous hormone implants: bio-identical estradiol and testosterone).
- 24 weeks with patches
- Hot flashes were resolved in 100% of patients.
- Depression resolved in 99%
- Loss of libido resolved in 92%

**76% REDUCTION IN HOT FLASHES**


- Using CEE and Bazedoxifene demonstrated 76% success in hot flash reduction
  *This is but one example of the inferiority of synthetic HRT vs Bio-identical hormone pellets.*

**DECREASED TOTAL CHOLESTEROL, SERUM TRIGLYCERIDES, AND INCREASED HDL.**

Susan Davis, et al Menopause Volume 7, No. 6, pp.395-401

- Using subcutaneous bio-identical hormone pellets
  *This again is superior to oral HRT where triglycerides are increased and there is a null effect on HDL.*
**BONE DENSITY**


- Testosterone and estradiol pellets increased BMD 8.3% per year vs 1-2% for oral HRT.

Morris Notelovitz et al Obstetrics & Gynecology, Volume 70, No. 5, Nov 1987 Metabolic & Hormonal Effects of 17-B-Estradiol Implants

- Two year study: **marked increase in bone density with no adverse** effects were noted in the coagulation inhibition and fibrinolysis assays in the pellet patients
- Multiple others showing similar results
  
  Testosterone that stimulates the osteoblast working with the estradiol inhibiting the osteoclast.

**THE BRAIN**


- HRT and particularly ERT plays an efficacious role in **preventing neurodegenerative conditions**.
- 17B Estradiol reduced risk for Alzheimer’s disease.
- Minimizes cognitive decline in otherwise healthy women
- Estradiol (E2) protects against B-amyloid induced degeneration.  
  *Progestins may actually dampen this affect.*
- Compared to E2 users vs Non-users E2:  
  For avg. 15 years had increased cerebral blood flow

**CEREBRAL METABOLIC ACTIVITY**

Psychoneuroendocrinology 2010; 36:502-513, Silverman et al looked at

- 17B Estradiol vs CEE vs CEE plus progestin on 17-B Estradiol performed the best on verbal memory (an early warning of Alzheimer’s disease) by more than 3 standard deviations.


- Both Estrogen and Testosterone have neuro-protective roles. Women with lower E2 levels have an even greater risk of AD.
- There is overwhelming evidence that E and T help decrease apoptosis. Protective effect of both hormones decreases beta amyloid deposition.
THE HEART


- Multiple benefits including men given aromatize-able testosterone
- Increase blood flow to the coronary arteries (even in patients with C.A.D.)
- Decrease plaque in the coronary arteries
- Decrease inflammation in the coronary arteries

Coupled with the lipid data above is impressive and safe way to reduce the number one killer of men and women in America.

THE BREAST


- Protected by the use of estradiol and testosterone
- Clinical and non-human primate studies suggest androgens inhibit mammary epithelial proliferation and breast growth. Estrogen, particularly in oral form, stimulates SHBG and reduces free testosterone. Testosterone is being used worldwide as a treatment for breast cancer.

Rebecca Glaser M.D., a renowned breast cancer surgeon: Testosterone pellets reduce significantly tumor volume in an active breast cancer patient. Patient achieved physiologic testosterone blood levels.

GARY DONOVITZ, MD

- Over the past 6 years followed nearly 10,000 patients on sub-cutaneous hormone pellet therapy
- A total of 9 breast cancers - zero mortality
- 30 breast cancers per year in W.H.I. in the placebo arm following nearly the same number of patients

THE CONSENSUS:

Testosterone in bio-identical form is certainly protective to the breast. There have been NO CONTRADICTORY STUDIES in the world literature.
ESTRADIOL LEVELS AND SERUM LIPIDS


- **Pellets** produce **more reproducible estrogen levels faster and remain steady longer than patches**. Estradiol levels with patches were less than half of pellet patient levels and fluctuated widely.
A randomized comparison of nonoral estradiol delivery in postmenopausal women

Frank Z. Stanczyk, PhD, Donna Shoupe, MD, Victoria Nunez, LVN, Priscilla Macias-Gonzales, Marcela A. Vijod, BS, Rogerio A. Lobo, MD
Los Angeles, California

We compared the transdermal and subdermal routes of estrogen administration with respect to the constancy of estrogen delivery and metabolic effects. Twenty postmenopausal women were randomized to receive either two 25 mg estradiol pellets subdermally \( (n = 10) \) or a 0.1 mg estradiol transdermal patch twice weekly \( (n = 10) \). Blood was sampled at 0, 2, 4, 6, 8, 12, 24, and 72 hours and 1, 2, 4, 8, 12, 16, 20, and 24 weeks (fasting samples at 0, 12, and 24 weeks), and a fasting urine was obtained after diuresis at 0, 12, and 24 weeks. In a 72-hour profile, serum estradiol levels (mean \( \pm \) SE) were highest at 24 hours \( (179 \pm 20 \text{ pg/ml}) \) and fell to \( 139 \pm 16 \text{ pg/ml} \) at 72 hours in the pellet group. In the patch group, estradiol levels rose rapidly to \( 152 \pm 33 \text{ pg/ml} \) at 4 hours, remained relatively constant over 8 hours, and fell to \( 46 \pm 10 \text{ pg/ml} \) at 72 hours. At 1 week, estradiol levels in the pellet group were \( 113 \pm 12 \text{ pg/ml} \) and remained relatively constant for 24 weeks. In contrast, estradiol levels in the patch group were \( 52 \pm 11 \text{ pg/ml} \) at 1 week and then varied widely until 24 weeks, when the levels were \( 89 \pm 26 \text{ pg/ml} \). The mean estradiol/estrone ratio ranged between 1 and 2.5 in both groups but fluctuated widely in the patch group. Follicle-stimulating hormone was suppressed in both groups; however, the decrement in the pellet group was greater \( (p < 0.002) \). There was a significant increase in high-density lipoprotein cholesterol and a decrease in total cholesterol/high-density lipoprotein cholesterol at 12 weeks with the pellet but only at 24 weeks with the patch. The urinary calcium/creatinine ratio was reduced more consistently with the pellet than with the patch. Hot flushes were eliminated in all subjects. (Am J Obstet Gynecol 1988;159:1540-6.)

Key words: Estradiol, postmenopausal women, estrogen replacement therapy

Although the oral route is the most common form of estrogen replacement therapy in postmenopausal women, parenteral estrogen replacement therapy offers several theoretic advantages. Perhaps the most important advantage of parenteral over oral estrogen replacement therapy is that the former route avoids the first-pass effect. There is evidence that the hepatic effects of oral estrogen replacement therapy may contribute to complications sometimes associated with oral estrogen replacement therapy, such as hypertension, intravascular clotting, and gallbladder disease.1-3 Parenteral routes of estrogen replacement therapy may avoid these exaggerated hepatic actions and in addition may provide postmenopausal women with an alternative to oral estrogen replacement therapy.

Although several parenteral routes of estrogen replacement therapy are currently available, there is a paucity of data on estrogen levels achieved by these delivery systems and their effects on gonadotropins and metabolic parameters. The objective of this study was to compare the transdermal and subdermal routes of estrogen administration. These methods are considered the most available and practical routes of estrogen...
Table I. Percentage of subjects in the pellet and patch groups with serum $E_2$ concentrations $<20$ and $40$ pg/ml

<table>
<thead>
<tr>
<th>Time of sample</th>
<th>$&lt;20$ pg/ml, % of subjects</th>
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delivery and are generally preferred over intramuscular and vaginal routes. Specifically, we studied the constancy of estrogen delivery and the estrogen effect on follicle-stimulating hormone (FSH), lipids, and the calcium calcium/creatinine ratio.

**Methods**

The study group consisted of 20 women between 40 and 65 years of age. All the women had undergone total abdominal hysterectomy, and 12 had also undergone bilateral salpingo-oophorectomy. All the subjects were considered postmenopausal on the basis of serum estradiol ($E_2$) levels $<20$ pg/ml and serum FSH levels $>40$ mIU/ml. The mean ($\pm$ SD) weights of the subjects in the pellet and patch groups were 148 $\pm$ 13 and 136 $\pm$ 13 pounds, respectively. Each subject was within 20% of ideal body weight; however, the subjects’ diets were not controlled. All subjects were nonsmokers.

The study subjects were randomized according to a table of random numbers. They received either a transdermal $E_2$ system (Estraderm, CIBA-GEIGY Pharmaceutical Co., Summit, N.J.), which was 4 cm in diameter, contained 8 mg of $E_2$, and delivered 0.1 mg of $E_2$/day,

\[ \text{or a subdermal } E_2 \text{ system (Progynon Associates, Rosemont, Ill.) consisting of two 25 mg crystalline } E_2 \text{ pellets. These systems will be subsequently referred to as patch and pellet, respectively. Ten subjects were in each study group. Pellets were inserted subdermally with a trocar through a small skin incision in the lower abdominal area. The patch was attached to the anterior lower abdominal wall and was changed twice weekly at 3- and 4-day intervals according to the manufacturer’s directions. Each subject in the patch group was instructed to return the used patch before receipt of a new one. Ninety-five percent of the patches were returned.} \]

Both blood and urine samples were obtained from the subjects. Blood was sampled before treatment and

\[ \text{2, 4, 6, 8, 12, 24, and 72 hours and 1, 2, 4, 8, 12, 16, 20, and 24 weeks after treatment was initiated. After the first 72 hours, blood sampling occurred between 8:00 and 10:00 AM in all patients. In patients receiving the patch, blood was obtained before the patch was changed on specified days. Pretreatment and 12- and 24-week specimens were taken after a 12-hour fast. The blood was allowed to clot at 4°C for 1 hour and after centrifugation, the separated serum was stored at } -20°C \text{ until analyzed. Urine samples were obtained before treatment and 12 and 24 weeks after treatment started after a 12-hour fast. The subjects were required first to void and then to drink 250 ml of distilled water 2 hours before urine collection.} \]

Estradiol, estrone ($E_1$), FSH, and lipids were measured in the serum samples, and the calcium/creatinine ratio was measured in each urine specimen. $E_2$ was quantitated, which followed extraction with ethyl acetate:hexane (3:2), by means of an iodinated $E_2$ radioimmunoassay kit from Pantex (Santa Monica, Calif.). The lowest $E_2$ concentration that could be measured reliably was 8 pg/ml. Accuracy of the $E_2$ assay was assessed in a recovery experiment in which a known amount of $E_2$, which ranged from 25 to 200 pg, was added to individual 1 ml aliquots of serum in duplicate. Linear regression analysis of the $E_2$ concentration measured ($y$) versus the $E_2$ concentration added ($x$) yielded the following equation and correlation coefficient:

\[ y = 1.15x + 16.2, r = 0.998. \]
Table II. Percentage of subjects in the pellet and patch groups with serum $E_2$ concentrations $>150$ and $200$ pg/ml

<table>
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terminated at pooled serum $E_2$ concentrations of 52 pg/ml (low pool) and 119 pg/ml (high pool). The intra-assay coefficients of variation (n = 6) were 9.2% and 10.9%, and the interassay coefficients of variation (n = 28) were 17.5% and 9.1% for the low and high pools, respectively. The $E_2$ antiserum cross-reacted 0.36% with $E_1$. $E_1$ was measured by radioimmunoassay after diethyl ether extraction and celite column chromatography as described previously. FSH was assayed by means of a double-antibody $^{125}$I-FSH radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, Calif.) in which the calibrators contained 0 to 100 mIU/ml of FSH in World Health Organization second international reference preparation—human menopausal gonadotropin. The lipid profiles and calcium/creatinine ratios were determined at SmithKline Bio-Science Laboratories (Van Nuys, Calif.). Each lipid profile consisted of total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and total cholesterol/high-density lipoprotein cholesterol ratio. Total cholesterol and triglycerides were measured on an Olympus high-speed clinical analyzer with the use of enzymatic and spectrophotometric methods. High-density lipoprotein cholesterol was quantitated spectrophotometrically on an Abbott VP analyzer after phosphotungstic–magnesium chloride precipitation and centrifugation. Low-density lipoprotein cholesterol was calculated as described by Friedwald et al. Urine calcium and creatinine were measured by arsenazo dye and modified Jaffe reaction methods, respectively, and the calcium/creatinine ratio was then calculated.

Although not the primary focus of this study, subjects recorded symptoms weekly. From these diary cards, changes in vasomotor symptoms and reports of adverse effects were monitored.

Longitudinal data were analyzed by the nonparametric one-way analysis of variance (Friedman) for significance among the time variates. To determine the significance of difference between the baseline value and the values at different time intervals, the Wilcoxon matched-pairs sign ranks statistic was used. Differences between the two treatment groups were analyzed by the Mann-Whitney U test.

Results

A 72-hour profile of serum $E_2$ was obtained and is shown in Fig. 1. In the pellet group, $E_2$ increased steadily during the first 24 hours after treatment and reached a mean $E_2$ concentration of 179 pg/ml at 24 hours. Peak levels were reached between 24 and 72
hours, and the mean $E_2$ concentration at 72 hours was 139 pg/ml. In the patch group $E_2$ levels increased rapidly and reached a mean concentration of 152 pg/ml 4 hours after treatment. This level remained relatively constant during the next 8 hours but fell to a mean level of 46 pg/ml at 72 hours. In one subject in the patch group, the serum $E_2$ concentration was measured 48 hours after treatment (data not shown). The $E_2$ value at this time was approximately 40% higher than the corresponding 72-hour $E_2$ value.

The mean serum $E_2$ levels at different intervals from 1 to 24 weeks in the pellet and patch groups are depicted in Fig. 2. One week after treatment began, the $E_2$ concentration in the pellet group averaged 113 pg/ml. This level did not vary significantly during the rest of the study period. Furthermore, in two of the subjects in the pellet group, serum $E_2$ concentrations were measured weekly beyond 24 weeks (data not shown). In each subject the $E_2$ value at 32 weeks was not significantly different from the corresponding value at 24 weeks. In contrast, mean $E_2$ concentrations in the patch group varied widely during the study. The $E_2$ level at 1 week was 46 pg/ml, and this value was approximately doubled at 24 weeks. During this interval, the $E_2$ levels fell below or near 40 pg/ml at three different times (2, 8, and 16 weeks).

Variation in $E_2$ levels between the subjects in each treatment group was examined by expressing the mean $E_2$ levels at the different time intervals from 1 to 24 weeks as a percentage change from the mean $E_2$ concentration at 72 hours in each group (Fig. 3). In the patch group the mean percentage change ranged from approximately 25% to 225%. In comparison, the percentage change of mean $E_2$ levels in the pellet group was small and remained relatively constant throughout the study.

Table 1 shows the percentage of study subjects with $E_2$ values <20 and 40 pg/ml. In the pellet group no subjects had $E_2$ levels <40 pg/ml. However, in the patch group 10% to 30% of the subjects had values <20 pg/ml at five different time intervals, and 20% to 70% had $E_2$ concentrations <40 pg/ml at all time intervals.

We also assessed the percentage of study subjects with $E_2$ values >150 and 200 pg/ml (Table 1). Only a small number of subjects in the patch group had values >150 pg/ml, whereas 10% to 40% of the subjects in the pellet group exceeded this concentration at every time interval except one. However, only one subject in each treatment group exceeded $E_2$ concentrations >200 pg/ml.

Serum $E_2$ concentrations in the pellet and patch groups were compared over a 16-week period (data not depicted). In the pellet group, $E_4$ levels averaged 83 pg/ml after 1 week of treatment, and this level was maintained with little fluctuation. In the patch group the mean $E_2$ level at 1 week of the study was 32 pg/ml, and this value increased gradually during the study until the sixteenth week, at which time the level had doubled. The mean $E_2/E_1$ ratio was calculated for the different time intervals from 1 to 16 weeks in each treatment group and ranged between 1 and 2.5 (Fig. 4). The ratio was relatively constant in the pellet group when compared with that of the patch group.

Mean serum FSH levels measured at different time intervals in the two treatment groups are shown in Fig. 5. In the pellet group FSH was significantly ($p < 0.05$)
suppressed from baseline values at every time interval, whereas in the patch group there was a significant decrease in FSH at 4, 8, 12, 20, and 24 weeks. The greatest suppression of FSH in both groups was at 12 weeks. Comparison of the percentage change from baseline between the two groups at 12 weeks showed a significantly greater ($p < 0.002$) change in the pellet group.

Total cholesterol and triglycerides (data not depicted) did not change significantly in either treatment group. In the pellet group, high-density lipoprotein cholesterol increased significantly ($p < 0.05$) from baseline at 12 and 24 weeks of the study, whereas in the patch group, high-density lipoprotein cholesterol also increased but was significantly greater ($p < 0.03$) from baseline only at 24 weeks (Fig. 6). There was no significant difference from baseline in low-density lipoprotein cholesterol levels in either treatment group (Fig. 6). Comparison of the total cholesterol/high-density lipoprotein cholesterol ratios (Fig. 7) showed a significant ($p < 0.01$) decrease from baseline at 12 and 24 weeks in the pellet group. In the patch group the ratios decreased from baseline at both time intervals but the difference was significant ($p < 0.03$) only at the 24-week interval.

A reduction in urinary calcium/creatinine ratios (Fig. 8) from baseline occurred at both 12 and 24 weeks in the pellet and patch groups. However, these reductions were significant ($p < 0.01$) only in the pellet group.

All subjects in our study had elimination of hot flushes; however, most subjects complained about local reactions caused by the $E_2$ delivery systems. In the pellet group seven subjects complained of breast tenderness, especially around the areolar area. Two of the subjects had moderate to severe reactions and the rest had mild reactions. In the patch group, one subject complained about moderate to severe breast tenderness, and four other subjects had mild reactions. The duration of the complaints ranged from 8 to 24 weeks. None of the subjects in the pellet group complained of problems at the incision site. In the patch group, nine patients had a transient local skin reaction, and this effect persisted for a longer time in one subject, who also experienced some skin discoloration.

**Comment**

In this study we compared subdermal and transdermal routes of estrogen administration. It has been previously shown that the subdermal $E_2$ pellet offers many practical and theoretic advantages over other forms of estrogen replacement therapy.\(^6\)\(^7\) We reported that a single 25 mg $E_2$ subdermal pellet gives serum $E_2$ concentrations in the range of 40 to 70 pg/ml over a 6-month period. To achieve higher serum $E_2$ levels in the present study, we administered two 25 mg pellets subdermally in each subject in the pellet group. The mean $E_2$ levels attained in these subjects ranged between 95 and 126 pg/ml (Fig. 2). The patch group
used the 0.1 mg E₂ transdermal system. Although patches are available commercially in two doses, 0.05 and 0.1 mg of E₂, the former dose results in E₂ levels that are closer to the single 25 mg pellet.

Our data clearly show that the pellet provides a more reproducible estrogen delivery system than the patch. The serum E₂ levels of each subject in the pellet group were relatively constant throughout the study, whereas the E₂ values for each subject in the patch group varied widely. The fluctuation in E₂ levels in the patch group may be related to inherent properties of the patch or compliance issues. One inherent property of the patch is that its release of E₂ gives rise to a peak and nadir of E₂ levels over a 72-hour period (Fig. 1), which is similar to that observed during a 24-hour period after oral ingestion of an E₂ preparation. Furthermore, lack of patient compliance in following the manufacturer’s directions in using the patch can result in altered profiles of E₂ levels such as the ones observed in this study (Figs. 2 and 3). Although the subjects in the present study were encouraged to comply with the experimental protocol, our data suggest that there could have been a lack of compliance by subjects in the patch group.

Suppression of serum FSH levels was observed in both the pellet and patch groups. However, the FSH decrement was greater in the pellet group, presumably because of the higher E₂ concentrations and less fluctuations of these levels in this group. The FSH values in the pellet group were lower than the values obtained in our previous study in which a single E₂ pellet was implanted subdermally in women who underwent hysterectomy and bilateral salpingo-oophorectomy. The lower FSH levels were expected since higher serum E₂ concentrations were attained in the present study. With respect to the FSH levels in the patch group, our results are in agreement with those of other investigators, albeit the degree of suppression varied with the E₂ dose of the patch.

Although total cholesterol and triglycerides did not change significantly in either treatment group, there was a significant increase in high-density lipoprotein cholesterol and a decrease in the total cholesterol/high-density lipoprotein cholesterol ratio by 12 weeks of the study in the pellet group but only at 24 weeks in the patch group. Very few studies have found changes in high-density lipoprotein cholesterol with parenteral forms of estrogen. The estrogen effect on lipoproteins may occur only after sufficient estrogen is delivered over a prolonged time, such as 6 months. Although patients wearing a patch had fluctuating levels of E₂, which were extremely low at times, the finding of an increase in high-density lipoprotein cholesterol at 6 months is novel and important. Nevertheless, larger numbers of subjects and more sophisticated lipoprotein determinations are needed to confirm these data.

Although there was a reduction in the fasting urinary calcium/creatinine ratio at 12 and 24 weeks in the patch group, our data show a more consistent reduction of this ratio in the pellet group. However, the calcium/creatinine ratio is a relatively crude marker of bone turnover and merely provides a guide for the effects of estrogen on bone.

All subjects in our study had elimination of hot flushes. Although differences in the frequency and severity of the flushes were not analyzed, the benefits of the pellet and patch with respect to suppression of hot flushes appear to be comparable.

Our data show that over 24 weeks, the pellet offers a more reproducible estrogen delivery and greater es-
trogen effect. These advantages of the pellet over the patch may be related to inherent properties of the patch or issues of patient compliance. In our study group dermatologic complaints were fairly common. However, there was no difference in serum E2 levels between patients having and not having such complaints.

REFERENCES

**SIGNIFICANT INCREASE IN HDL**

Maturitas 1984: 5: 177-84, Linda Cardozo

- HDL increased at 12 weeks with pellets *(sub-cutaneous hormone implants: bio-identical estradiol and testosterone).*
- 24 weeks with patches
- Hot flashes were resolved in 100% of patients.
- Depression resolved in 99%
- Loss of libido resolved in 92%
The effects of subcutaneous hormone implants during the climacteric

Linda Cardozo 1,*, Donald M.F. Gibb 1, Susan M. Tuck 1, Margaret H. Thom 1, John W.W. Studd 1 and Derek J. Cooper 2

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(Received 25 April 1983; accepted after revision 31 August 1983)

Climacteric symptoms in 120 women were treated with a total of 469 hormone implants (oestradiol 50 mg and testosterone 100 mg) over a period of four years. All patients with a uterus were given an oral progestogen to prevent endometrial hyperplasia. There was a marked response to treatment, hot flushes being improved in all patients, depression in 99% and loss of libido in 92%. Patient acceptability of this type of treatment was good and there were few side effects or complications.

After therapy, the serum oestradiol exceeded the serum oestrone but remained within normal limits. When climacteric symptoms returned and re-implantation occurred the serum levels of oestrone, oestradiol, luteinising hormone (LH), follicle stimulating hormone (FSH) and testosterone were within the normal range for the reproductive age. This indicates that the return of symptoms is due to a change in the hormone levels rather than absolute hypo-oestrogenism.

(Key words: Climacteric, Hormone implants, Clinical effects, Oestradiol-serum levels)

Introduction

Oestrogen replacement therapy is now accepted as the appropriate treatment for the climacteric syndrome. It is usually administered orally, but this requires daily patient compliance, may produce side effects of nausea and vomiting and does not allow for the use of testosterone, for psychosexual problems, which is hepatotoxic when given orally as methyltestosterone [1].

The use of implants, which release their active components over a period of several months, was first described by Bishop [2] and their use as an alternative treatment for the climacteric syndrome was introduced in 1949 [3]. Despite some early interest [4], hormone implant therapy was not widely used for over twenty years, and although its use was later advocated, especially for castrated women [5], it has not gained the same popularity as oral oestrogen therapy. This is unfortunate because the technique is simple. Hormone pellets can be implanted under local

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anaesthetic as an out-patient procedure [6], and the inconvenience of daily tablet taking is avoided. They also produce a physiological serum oestradiol to serum oestrone ratio greater than unity [7], in contrast to the abnormally high serum oestosterone levels produced by most orally administered oestrone or oestradiol [8].

It has been stated that hormone implants should never be used for patients with a uterus [9] but it is our policy to use implants in such patients as first line therapy, particularly in women who complain of co-existent lethargy, depression or loss of libido.

The diagnosis of the menopause is retrospective and some patients may present with symptoms whilst still menstruating whereas others attend up to several years later. The climacteric symptoms of hot flushes, night sweats and vaginal dryness are well defined, but others such as insomnia, depression and psychological problems may be due to other social and family factors operative at this age. In this study, we have divided patients into pre-menopausal and post-menopausal groups to assess any differences in symptom pattern, response to treatment and hormone blood levels following treatment.

**Patients and methods**

One hundred and twenty women attending the Dulwich Hospital Menopause Clinic for their climacteric symptoms, all of whom had already received at least one hormone implant, were admitted to the study. These patients were consecutive and unselected. They were divided into two groups according to their last pre-treatment menstrual period. Those who had not stopped menstruating or who had had a period within the last 12 mth before treatment was commenced were classified as ‘pre-menopausal’, and those who had not menstruated for 12 mth before the onset of implant therapy were classed as ‘post-menopausal’. Of the 53 women in the pre-menopausal group (A), 7 had undergone a hysterectomy with conservation of the ovaries, within the previous year, whilst still menstruating. There were 67 post-menopausal women (B) of whom 18 had undergone a hysterectomy. The mean age for Group A patients was 44.5 yr (range 35–54 yr) and for Group B patients was 53.3 yr (range 34–71 yr). Oral hormone replacement therapy had previously been prescribed for 66% of Group A patients and 71% of Group B patients. This had proved unsatisfactory in 43 and 51%, respectively. Psychoactive drugs had been used for 51% of Group A patients and 42% of Group B patients, with 41 and 64%, respectively, finding these drugs helpful.

Hormone implants were prescribed for a variety of indications, including unsatisfactory oral therapy, the predominance of psychosexual symptoms (because of the availability of testosterone), convenience following a hysterectomy (because progestogens were no longer required) and patient request. No patient had any contraindication to hormone replacement therapy and all had received between one and six consecutive implants of oestradiol 50 mg and testosterone 100 mg at 4–12 monthly intervals over a period of up to 4 yr.
All patients with a uterus were given norethisterone 5 mg daily for the first 7 days of each month. This regime was only altered if heavy or irregular bleeding was noticed or if endometrial hyperplasia was discovered.

A full history was taken and clinical examination including breasts, abdomen and pelvis was performed prior to hormone administration and on entry into the study. The patient’s weight and blood pressure were recorded during each visit and a Vabra curettage was performed (when appropriate) before hormones were prescribed and annually thereafter.

On admission to the study a questionnaire was completed comparing the patient’s symptoms before and after implant therapy together with any side effects of treatment. In the majority of patients, symptoms started to recur at approximately 6 mth; at this time, blood was taken for serum hormone estimations (FSH, LH, oestrone and testosterone). All patients chose to continue implant therapy when their symptoms returned.

Results

A total of 469 implants were given, 183 to Group A and 286 to Group B. The duration of symptom relief was 6 mth in 85% of Group A and 69% of Group B (range 3–12 mth for both groups).

The effects of implants on symptoms is shown in Table II. The $\chi^2$ test was used to compare the two groups of patients. A significantly greater proportion of patients in Group B suffered from flushes ($P < 0.03$). There was no significant difference in any of the other symptoms. Log linear models were used to test for differences in the effect of treatment on each symptom; no significant differences were noted.

**Table I**

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Present (% of patients)</th>
<th>Complete relief (% of patients with symptom)</th>
<th>No relief (% of patients with symptom)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
<td>Group A</td>
</tr>
<tr>
<td>Hot flushes/sweats</td>
<td>71.7</td>
<td>89.6</td>
<td>97.4</td>
</tr>
<tr>
<td>Headaches</td>
<td>83.0</td>
<td>73.1</td>
<td>65.9</td>
</tr>
<tr>
<td>Insomnia</td>
<td>71.7</td>
<td>74.6</td>
<td>63.2</td>
</tr>
<tr>
<td>Palpitations</td>
<td>50.9</td>
<td>40.3</td>
<td>55.6</td>
</tr>
<tr>
<td>Bone pains</td>
<td>54.7</td>
<td>64.2</td>
<td>55.2</td>
</tr>
<tr>
<td>Dyspareunia</td>
<td>45.3</td>
<td>53.7</td>
<td>62.5</td>
</tr>
<tr>
<td>Loss of libido</td>
<td>84.9</td>
<td>82.1</td>
<td>66.7</td>
</tr>
<tr>
<td>Irritability</td>
<td>90.6</td>
<td>79.1</td>
<td>68.8</td>
</tr>
<tr>
<td>Poor memory/concentration</td>
<td>79.2</td>
<td>62.7</td>
<td>59.5</td>
</tr>
<tr>
<td>Depression</td>
<td>81.1</td>
<td>77.6</td>
<td>79.1</td>
</tr>
<tr>
<td>Lethargy</td>
<td>79.2</td>
<td>73.1</td>
<td>61.9</td>
</tr>
<tr>
<td>Urethral syndrome</td>
<td>13.2</td>
<td>26.9</td>
<td>28.6</td>
</tr>
</tbody>
</table>
TABLE II
SIDE EFFECTS OF HORMONE IMPLANTS.

<table>
<thead>
<tr>
<th>Side effect</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
</tr>
<tr>
<td>Breast discomfort</td>
<td>28.3</td>
</tr>
<tr>
<td>Increased facial hair</td>
<td>22.6</td>
</tr>
<tr>
<td>Acne</td>
<td>5.7</td>
</tr>
<tr>
<td>Abnormal bleeding</td>
<td>16.3</td>
</tr>
</tbody>
</table>

Changes in systolic and diastolic blood pressure were not significant, nor was weight change in either of the groups. Side effects are shown in Table II. The $\chi^2$ test showed that there was no significant difference between the two groups of patients in the occurrence of side effects due to hormone implants. No patient wanted to stop implant therapy because of the severity of side effects. Abnormal bleeding (in the presence of a normal Vabra curettage) was treated by increasing the duration of norethisterone to 5 mg daily for the first 10 or 13 days of each month. There were no complaints of nausea or vomiting.

No side effects were attributed to the 7 days of norethisterone in 52.2% of Group A and 57.1% of Group B. However, 39.1% of Group A and 34.7% of Group B complained of pre-menstrual tension during progestogen therapy and 17.4% of Group A and 12.2% of Group B suffered from dysmenorrhoea. A log mean model was used to test for differences in the side effects of progestogens; no significant difference was found. The incidence of pre-menstrual tension and dysmenorrhoea were reduced by changing the norethisterone to a 5-day course (if the Vabra curettage was normal) or by halving the dose.

Prior to the index implant two of the pre-menopausal and five of the post-menopausal patients had developed cystic glandular hyperplasia during implant therapy. In all cases this had been corrected by norethisterone 5 mg twice daily for 21 out of 28 days for two cycles [10]. The complications encountered during the study were few. 5.7% of Group A and 3.0% of Group B developed benign breast pathology. Vabra curettage revealed a 5.7- and 6.0%-incidence of cystic endometrial hyperplasia in Groups A and B, respectively. There was one case of deep venous thrombosis in a Group B patient. Fisher’s Exact Test showed no difference in the presence of complications in the two groups.

The endometrial histology (from Vabra curettage) on admission to the study is shown in Table III. All patients who developed cystic glandular hyperplasia were also treated with two courses of norethisterone as described above. In all cases the abnormality was corrected and the patient’s routine duration of norethisterone increased to 10 or 13 days each cycle. No patient required a hysterectomy for endometrial pathology or heavy uterine bleeding.

Blood hormone levels at six months after varying numbers of implants are shown in Tables IV and V. The serum FSH and LH fell consistently after successive implants, although the levels or oestrone and oestadiol showed little evidence of
### TABLE III
**ENDOMETRIAL HISTOLOGY.**

<table>
<thead>
<tr>
<th>Endometrium</th>
<th>Group A</th>
<th></th>
<th>Group B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Non applicable</td>
<td>7</td>
<td>13.2</td>
<td>18</td>
<td>26.9</td>
</tr>
<tr>
<td>Unsatisfactory</td>
<td>4</td>
<td>7.5</td>
<td>9</td>
<td>13.4</td>
</tr>
<tr>
<td>Proliferative</td>
<td>31</td>
<td>58.5</td>
<td>28</td>
<td>41.8</td>
</tr>
<tr>
<td>Secretory</td>
<td>8</td>
<td>15.1</td>
<td>8</td>
<td>11.9</td>
</tr>
<tr>
<td>Cystic glandular hyperplasia</td>
<td>3</td>
<td>5.7</td>
<td>4</td>
<td>6.0</td>
</tr>
</tbody>
</table>

### TABLE IV
**GROUP A: HORMONE LEVELS AT 6 MONTHS.**

<table>
<thead>
<tr>
<th>Implant number</th>
<th>1 (n = 10)</th>
<th>2 (n = 8)</th>
<th>3 (n = 10)</th>
<th>4 (n = 8)</th>
<th>5 (n = 10)</th>
<th>6 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (µ/l)</td>
<td>8.1</td>
<td>7.0</td>
<td>4.5</td>
<td>4.8</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>LH (µ/l)</td>
<td>15.4</td>
<td>5.5</td>
<td>6.3</td>
<td>4.6</td>
<td>7.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Oestrone (pmol/l)</td>
<td>278</td>
<td>622</td>
<td>401</td>
<td>413</td>
<td>491</td>
<td>722</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>484</td>
<td>748</td>
<td>854</td>
<td>582</td>
<td>828</td>
<td>951</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>2.7</td>
<td>2.2</td>
<td>2.7</td>
<td>2.5</td>
<td>3.8</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*Normal pre-menopausal values: FSH 1–13 µ/l, LH 1–100 µ/l, oestrone 150–1000 pmol/l, oestradiol 90–1050 pmol/l, testosterone 0.6–2.28 nmol/l.*

### TABLE V
**GROUP B: HORMONE LEVELS AT 6 MONTHS**

<table>
<thead>
<tr>
<th>Implant number</th>
<th>1 (n = 4)</th>
<th>2 (n = 9)</th>
<th>3 (n = 12)</th>
<th>4 (n = 13)</th>
<th>5 (n = 8)</th>
<th>6 (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (µ/l)</td>
<td>16.3</td>
<td>17.4</td>
<td>9.3</td>
<td>3.8</td>
<td>5.9</td>
<td>3.1</td>
</tr>
<tr>
<td>LH (µ/l)</td>
<td>14.2</td>
<td>14.9</td>
<td>7.9</td>
<td>4.3</td>
<td>4.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Oestrone (pmol/l)</td>
<td>353</td>
<td>543</td>
<td>390</td>
<td>470</td>
<td>340</td>
<td>416</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>539</td>
<td>684</td>
<td>860</td>
<td>691</td>
<td>608</td>
<td>704</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>2.3</td>
<td>3.1</td>
<td>2.4</td>
<td>3.3</td>
<td>2.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>


Accumulation and did not exceed the normal pre-menopausal range. The oestradiol to oestrone ratio remained greater than unity throughout. Plasma testosterone levels at the time of return of symptoms and re-implantation remained in the upper normal range.

A significant difference ($P < 0.05$) was found in the FSH level between the groups after taking the number of implants into account using covariate analysis. There was no significant difference for the other hormones.
Discussion

Women complain of climacteric symptoms before or for several years after their menopause. Pre-menopausal patients have already been shown to complain of emotional problems more frequently than their post-menopausal counterparts [11]. We would confirm these findings, the proportion of post-menopausal patients complaining of hot flushes being significantly greater than the pre-menopausal group and the number of pre-menopausal women complaining of psychological symptoms being greater than the post-menopausal group, although these did not quite reach significance. We also note that 59.3% of pre-menopausal patients but only 35.7% of post-menopausal patients had found psycho-active drugs helpful for their symptoms.

The FSH level in the pre-menopausal patients with alleged climacteric symptoms was lower than the level found in the post-menopausal patients even after 6 and 12 mth of therapy. This confirms the findings of Chakravarti et al. [12] using oral conjugated equine oestrogens. In spite of this difference the response to oestradiol and testosterone implant therapy was the same.

Loss of libido was a presenting symptom in over 80% of patients in both groups and hormone implant therapy produced a complete cure in two-thirds of the patients. This is an important finding. Studd et al. [11] have shown that psychosexual problems occurring after the menopause respond to testosterone, and as it cannot be administered orally, 6 monthly subcutaneous implantation provides an ideal alternative.

Side effects from hormone implant therapy were few. Although mild breast discomfort was noted by some patients, early in treatment, this always resolved spontaneously and did not prevent any patient from continuing treatment. A 20% incidence of hirsutism may appear alarming but this was no more than a slight increase in downy facial hair elicited on direct questioning and was always improved when the dose of testosterone was halved or discontinued in subsequent implants. Heavy or irregular bleeding was easily controlled by modifying the progestogen regime.

We find that the main problem with implant therapy is the side effects caused by the progestogens. There is a high risk of endometrial hyperplasia in unopposed oestrogen therapy [13] but this can be completely avoided by prescribing progestogens for 10 or 13 days each cycle [10]. We have found that the prescription of progestogens for 7 days/mth is an acceptable compromise. Seven or our 95 patients with a uterus (7.4%) developed cystic glandular hyperplasia which, in all cases, was reversed by long courses of progestogens and did not recur when the duration of the regular monthly course was subsequently increased. It is clear that the protection of the endometrium is related to the duration of progestogen and the troublesome symptomatic side effects a result of an excess daily dose of progestogen.

The only major complication which we felt required cessation of hormone replacement therapy was one case of deep venous thrombosis which occurred in a post-menopausal patient. Because of the increased incidence of deep venous thrombosis amongst oral contraceptive pill users there is anxiety regarding changes
in clotting factors in peri-menopausal patients taking hormone replacement therapy. However, there is no good evidence that healthy post-menopausal women taking oestrogen replacement therapy are at greater risk of developing arteriovenous thrombosis [14], and the results of a large epidemiological study suggest that the real incidence of coronary thrombosis is less than half that of the controls [15]. It is probable that oestradiol does not cause any increase in thromboembolic phenomena, and we found no increase in blood pressure.

It has been inferred that oestradiol is poorly absorbed from the gastro-intestinal tract [16], although more recently it has been shown that an oral preparation containing oestradiol with oestrone and estriol given in high dosage does increase the serum oestradiol level for up to 48 h after ingestion [17]. With subcutaneous oestradiol implants however the serum oestradiol exceeds the serum oestrone from the start of treatment, and as this is the normal ratio in pre-menopausal women it would seem to be preferable to the reversed ratio caused by most oral preparations. In spite of the increasing suppression of FSH and LH with successive implants, there was no evidence of cumulative levels of oestradiol, oestrone or testosterone. But the fact that the serum levels of these hormones were above normal at the time of return of convincing symptoms and re-implantation poses many questions regarding basic endocrinology and the relationship of symptoms to hormone levels. It would seem that these typical climacteric symptoms are due to a change of serum oestrogen and testosterone levels from moderately high levels to normal, rather than a result of low post-menopausal values of oestradiol and testosterone. From this study, we conclude that subcutaneous hormone implants are an effective, acceptable treatment for climacteric symptoms in both pre- and post-menopausal women with few side effects or complications. The presence of a uterus is not a contra-indication as endometrial pathology and irregular bleeding can be prevented or corrected by cyclical oral progestogens for 5-13 days each month.

References

1 Martindale. The extra pharmacopoeia, 28th ed. 1982; 1420.


76% REDUCTION IN HOT FLASHES


- Using CEE and Bazedoxifene demonstrated 76% success in hot flash reduction

This is but one example of the inferiority of synthetic HRT vs Bio-identical hormone pellets.
Evaluation of bazedoxifene/conjugated estrogens for the treatment of menopausal symptoms and effects on metabolic parameters and overall safety profile

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a Columbia University Medical Center, New York, New York; b University of Virginia Health System, Charlottesville, Virginia; c University of Cincinnati College of Medicine, Cincinnati, Ohio; d University of New Mexico Medical School, Albuquerque, New Mexico; and e Wyeth Research, Collegeville, Pennsylvania

Objective: To evaluate the effects of a tissue-selective estrogen complex (TSEC) composed of bazedoxifene/conjugated estrogens (BZA/CE) on menopausal symptoms, metabolic parameters, and overall safety.

Design: Multicenter, double-blind, placebo- and active-controlled phase 3 trial (Selective estrogens, Menopause, And Response to Therapy [SMART]-1).

Setting: Outpatient clinical.

Patient(s): Healthy, postmenopausal women (n = 3,397) age 40 to 75 with an intact uterus.

Intervention(s): Single tablets of BZA (10, 20, or 40 mg), each with CE (0.625 or 0.45 mg); raloxifene 60 mg; or placebo taken daily for 2 years.

Main Outcome Measure(s): Hot flushes, breast pain, vaginal atrophy, metabolic parameters, and adverse events.

Result(s): BZA (20 mg)/CE (0.625 or 0.45 mg) significantly reduced the frequency and severity of hot flushes and improved measures of vaginal atrophy compared with placebo. At week 12, the daily number of hot flushes decreased by 51.7% to 85.7% with all BZA/CE doses vs. 17.1% for placebo. BZA/CE improved lipid parameters and homocysteine levels, did not significantly change carbohydrate metabolism, and had only minor effects on some coagulation parameters. The incidences of breast pain and adverse events were similar between BZA/CE and placebo.

Conclusion: The TSEC composed of BZA (20 mg)/CE (0.625 or 0.45 mg) is an effective and safe treatment for menopausal symptoms. (Fertil Steril® 2009;92:1025–38. ©2009 by American Society for Reproductive Medicine.)

Key Words: Bazedoxifene, breast pain, coagulation, conjugated estrogens, hot flushes, metabolism, vaginal atrophy

An increasing number of women in the United States are postmenopausal. According to the U.S. Census Bureau, an estimated 60 million women will be over 45 years of age by the year 2010 (1). Because of the decreasing levels of estrogen associated with menopause, many women might experience bothersome symptoms, with the hallmark feature being vasomotor symptoms or hot flushes. Recent analyses of the risks and benefits of hormone therapy concluded that it is an appropriate option for such symptomatic women (2). Another significant concern for postmenopausal women is the risk of developing osteoporosis, with an estimated 8 million women affected and another 34 million women at risk (3).

Selective estrogen receptor modulators (SERMs) have been developed to treat postmenopausal osteoporosis. As a class, SERMs generally provide estrogen agonistic effects on bone and the liver, but may be agonistic or antagonistic for the uterus and vagina, and are usually antagonistic for the breast and brain, thus potentially inducing or worsening hot flushes in some women. An indole-based SERM, bazedoxifene (BZA), has been shown to be effective for the prevention and treatment of osteoporosis (4, 5) and to provide antagonistic effects on the breast and uterus (6–9).

In light of continued efforts to provide women with additional therapeutic options for the treatment of menopausal symptoms, consideration has been given to the pairing of BZA with estrogens in the form of a tissue-selective estrogen complex (TSEC). The partnering of BZA with conjugated estrogens might offer advantages over the use of progestins in women with an intact uterus receiving hormone therapy, including an improved safety and tolerability profile. The BZA/conjugated estrogens (CEs) combination has the potential to not only reduce vasomotor symptoms and prevent osteoporosis, but also to minimize or antagonize stimulatory effects on the breast and uterus, which may reduce breast tenderness (10) and decrease the occurrence of vaginal bleeding. The results of a preliminary phase 2 study (11) evaluating a TSEC at varying doses of BZA...
(5–20 mg) with CEs (0.3 or 0.625 mg) indicated that doses of CE higher than 0.3 mg were necessary to consistently reduce the occurrence of hot flushes when combined with BZA at doses that prevented estrogen-induced endometrial proliferation.

This article reports findings from the Selective estrogens, Menopause, And Response to Therapy-1 (SMART-1) trial, a 2-year, randomized, double-blind, placebo- and active-controlled clinical trial evaluating the efficacy and safety of varying doses of BZA/CE in postmenopausal women. This report describes the effects of BZA/CE on menopausal symptoms (hot flushes, vaginal atrophy), metabolic parameters, as well as its overall safety profile. The effects of BZA/CE on endometrium, bone mineral density (BMD), and uterine bleeding are reported separately in this journal issue (12–14).

MATERIALS AND METHODS

Study Design

Healthy women (40–75 years of age) who were postmenopausal for at least 1 year were eligible for participation in this 2-year, double-blind, randomized, multicenter, placebo- and active–controlled phase 3 trial conducted at 94 study sites worldwide. All subjects were required to have an intact uterus and acceptable endometrial biopsy results at screening.

A subset of women was enrolled in the Osteoporosis Prevention I Substudy or the Osteoporosis Prevention II and Metabolic Substudy. The two osteoporosis substudies were designed to assess BMD changes in later and earlier postmenopausal women. Subjects in the Osteoporosis Prevention I Substudy were >5 years postmenopausal, with a BMD T-score between 1 and 2.5 at the lumbar spine or total hip and at least one additional risk factor for osteoporosis (family history of osteoporosis, early menopause, current history of smoking, past history of excessive alcohol use, diet low in calcium, inactive lifestyle, thin and/or small frame, Caucasian or Asian). Subjects in the Osteoporosis Prevention II Substudy and Metabolic Substudy were ≥1 year but ≤5 years postmenopausal with at least 1 risk factor for osteoporosis.

Treatments

Subjects were randomly assigned through a computerized randomization/enrollment interactive voice recognition system to one of eight treatment regimens, including six BZA/CE doses (BZA [0.45 or 0.625 mg], raloxifene 60 mg, or placebo). Subjects were required to take one capsule orally at approximately the same time each day and maintain a consistent daily intake of dietary and supplemental calcium and vitamin D (total daily calcium intake, 1,000–1,600 mg; vitamin D, 200–400 IU).

Use of the following concomitant medications was permitted: acetaminophen; inhaled steroids (maximum daily intake, 1,000 μg); dermal steroids; intra-articular injections (maximum of three injections during the treatment period); oral corticoids at standard therapeutic doses for periods of up to 10 days; up to two antihypertensive medications; and vitamin/mineral supplements if they had been taken continuously for ≥12 weeks before the study. Prohibited therapy included estrogen–, progestin–, androgen–, or SERM-containing medications other than the study drug. Also prohibited was the continued use of medications that could affect bone metabolism (osteoporosis substudies) or prescription lipid–lowering agents and anticoagulants other than aspirin (metabolic substudy).

Assessments

Subjects were instructed to record in daily diaries information on hot flushes, sexual activity/dyspareunia, and breast pain. Vaginal atrophy was measured by vaginal smears at months 6, 12, 18, and 24, which quantified the degree of maturation of the vaginal epithelium by the proportion of parabasal, intermediate, and superficial cells obtained in the sample.

Safety assessments included adverse event (AE) reporting and clinical laboratory evaluations (e.g., hematology, blood chemistry), which were performed at the screening visit and at months 3, 6, 12, 18, and 24. Reports of AEs were summarized using terms from the Medical Dictionary for Regulatory Activities. In the metabolic substudy, fasting serum samples were collected at randomization and at months 6, 12, and 18 to evaluate insulin and glucose levels. Coagulation factors (prothrombin time, partial thromboplastin time, fibrinogen, antithrombin III activity, protein C activity, protein S activity, plasminogen activity, plasminogen activator inhibitor-1 [PAI-1] activity, PAI-1 antigen, and D–dimer) and lipid parameters (total cholesterol, high-density lipoprotein [HDL] cholesterol, low–density lipoprotein [LDL] cholesterol, very-low-density lipoprotein [VLDL] cholesterol, triglycerides, VLDL triglycerides, HDL2 cholesterol, HDL3 cholesterol, apolipoprotein A1, apolipoprotein B, and lipoprotein [a]) were assessed in the metabolic substudy. Safety and metabolic data for month 24 (study end) are presented here.

Statistical Analyses

Analyses of any data of interest included only those subjects who received at least one dose of the study drug, in addition to criteria specific for the parameter of interest. Hot flush data were analyzed for the efficacy evaluable population or those subjects who reported at least seven moderate or severe hot flushes per day or 50 per week during screening (n = 216). Complete data were summarized for the baseline week, for each week during weeks 1–12 (using a last observation carried forward approach), and for every four-week period thereafter. The mean daily number of hot flushes was calculated using only moderate and severe hot flushes, whereas the mean daily severity of hot flushes was calculated using all three intensities (1 = mild; 2 = moderate; and 3 = severe). Pairwise comparisons vs. placebo were made using an analysis of covariance (ANCOVA).

Vaginal epithelial maturation data were analyzed for the vulvar/vaginal atrophy population, or for those subjects with no more than 5% superficial cells at screening and those who had a baseline and at least one on-therapy assessment (n = 1,867). Vaginal atrophy was measured by the change from baseline in the percentage of superficial, intermediate, and parabasal cells at each time point using an ANCOVA, with treatment and center as factors and baseline value as covariate. The proportion of each cell type was analyzed using a non-parametric one-way Kruskal-Wallis test for between-group comparisons (vs. placebo) and a signed-rank test for within-group comparisons.

The incidence of sexual activity and dyspareunia was summarized for seven consecutive baseline days before treatment initiation and for sequential 28-day periods during the study. Breast pain data were analyzed for subjects with diary data for at least 5 of the 7 days during screening and at least 20 days for the relevant 4-week interval. Among-group differences in the incidence of breast pain during each time period (weeks 1–4, 5–8, and 9–12) were evaluated using Fisher’s exact test. The mean change from baseline in the percentage of days with breast pain in a given 4-week interval was evaluated using ANCOVA, and pairwise comparisons versus placebo were made using the t test.

Data from the metabolic substudy were analyzed in those subjects who had baseline and on–therapy values for the parameter of interest. For lipid parameters, differences in the mean percent change from baseline at each time point between each BZA/CE treatment group and placebo were evaluated using an analysis of variance (ANOVA) model. For all other parameters (e.g., coagulation, carbohydrate), the mean absolute change from baseline was calculated using all three intensities (1 = mild; 2 = moderate; and 3 = severe). Among-group differences in the incidence of AEs were evaluated using χ2 analysis (overall P value), and Fisher’s exact test was used to compare the incidence of AEs between each BZA/CE group and the placebo group. For each laboratory parameter, the adjusted mean change from baseline as well as the number and percentage of subjects with potentially clinically important (PCI) values were summarized. Within- and among-group differences in the mean change from baseline for all laboratory tests were evaluated using ANCOVA. Pairwise comparisons for the incidence of PCI values were made using Fisher’s exact test.

RESULTS Subjects

A total of 3,397 subjects were randomly assigned to a treatment group and received at least 1 dose of the study drug (Fig. 1). Subject demographics and baseline characteristics were similar across all treatment groups (Table 1). The rates of study discontinuation were not significantly different among treatment groups (range,
29.8–35.7%; Fig. 1). The most frequent reason for discontinuation was AEs, followed by subject request unrelated to the study. A significantly higher percentage of subjects in the placebo group withdrew from the study because of unsatisfactory response compared with those in any other treatment group (P = 0.002).

**Hot Flushes**

Examination of the mean daily number of moderate and severe hot flushes demonstrated that all doses of BZA/CE provided significantly better relief of hot flushes than placebo at most time points (P < 0.01; Fig. 2A). At week 12, the adjusted mean change from baseline in the average daily number of hot flushes for the BZA/CE treatment groups ranged from −5.53 to −8.98 (−51.7% to −85.7%) compared with −2.45 (−17.1%) and −5.29 (−44.1%) for the placebo and raloxifene treatment groups, respectively. Treatment with BZA (20 mg)/CE (0.625 or 0.45 mg) was significantly more effective than placebo at every weekly time point from weeks 6 to 12. Improvements in the frequency and severity of hot flushes observed with BZA (10 or 20 mg)/CE (0.625 or 0.45 mg) were sustained through the second year of therapy (data not shown). Although the daily number of hot flushes reported with BZA (40 mg)/CE (0.625 mg) or BZA (40 mg)/CE (0.45 mg) was also significantly reduced compared with placebo at week 12, this decrease was not as great as that noted with BZA (10 or 20 mg)/CE (0.625 or 0.45 mg) at most time points (Fig. 2). Bazedoxifene/CE groups demonstrated significant decreases in hot flush number and severity compared with raloxifene; significant differences in number and/or severity were seen as early as week 2 for BZA (10 mg)/CE (0.625 or 0.45 mg) and at week 6 for BZA (20 mg)/CE (0.625 or 0.45 mg), and continued through week 12.

**Vaginal Atrophy**

Treatment with BZA (10 mg)/CE (0.625 mg or 0.45 mg) and BZA (20 mg)/CE (0.625 or 0.45 mg) was significantly more effective than placebo in increasing the mean proportion of superficial cells from baseline to most time points (P < 0.001; Fig. 3A). Furthermore, all four BZA/CE doses containing BZA (10 or 20 mg)/CE (0.625 or 0.45 mg) were significantly more effective than placebo in increasing the mean proportion of intermediate cells and decreasing the proportion of parabasal cells from baseline to all time points (P < 0.001; Fig. 3B, C). There was a dose–related attenuation of the beneficial estrogenic effect on vaginal atrophy with increasing doses of BZA, which was most noted with BZA (40 mg)/CE (0.625 or 0.45 mg). However, with BZA doses of 10 and 20 mg, the effects on vaginal endpoints were substantially improved compared with raloxifene or placebo.

**Sexual Activity, Dyspareunia, and Breast Pain**

At baseline, sexual activity was reported by 34–43% of the participants and dyspareunia was reported by 16–26%. There were no significant among-group differences in the incidence of sexual activity throughout the study. Compared with subjects who received placebo or raloxifene, subjects treated with BZA (10 mg)/CE (0.625 mg) had a lower incidence of dyspareunia at weeks 5–8 (P < 0.05). With BZA (10 mg)/CE (0.625 mg) and BZA (20 mg)/CE (0.625 or 0.45 mg) there was a significantly lower incidence of dyspareunia during weeks 9–12 (P < 0.05).

For most of the weeks analyzed, BZA (10 and 20 mg)/CE (0.625 or 0.45 mg) had significantly less dyspareunia than with raloxifene (P < 0.05). Breast pain occurred with similar frequency for subjects in the BZA/CE, raloxifene, and placebo groups, and there were no significant differences in the incidence of breast pain among the groups for any 28-day interval.

**Metabolic Parameters**

The adjusted mean percent changes from baseline in LDL and HDL cholesterol are presented in Figure 4. Reductions in LDL cholesterol for all BZA/CE doses (range, −5.7% to −10.9%) were significantly higher than placebo.
greater compared with placebo (range, –0.1 to 2.2%) at all time points (\(P < 0.01\)). Increases in HDL cholesterol for all BZA/CE doses (range, 7.0–13.5%) were significantly greater compared with placebo (range, 1.3% to 5.4%) at all time points (\(P < 0.05\)), and significantly greater compared with raloxifene (range, 3.1–6.6%) at most time points (\(P < 0.05\)). There was no apparent dose-related attenuation of HDL cholesterol levels with BZA, and the observed increases were sustained throughout 2 years of therapy.

Changes in other lipid parameters are provided in Table 2. Total cholesterol decreased from baseline for all BZA/CE treatment groups at all time points (range, –0.8 to –3.7%). At month 24, the increase from baseline in triglycerides was higher for all BZA/CE treatment groups at months 12 (\(P < 0.01\)), and significantly greater compared with placebo (range, 1.3% to 5.4%) at all time points (\(P < 0.05\)). With the exception of BZA (10 mg)/CE (0.625 mg) at months 18 and 24. Decreases in plasma homocysteine with BZA (40 mg)/CE (0.625 mg) and BZA (10 mg)/CE (0.45 mg) were significantly greater than that observed with placebo at all time points (\(P < 0.05\)).

There was no significant change from baseline in protein C activity for any BZA/CE treatment groups (range, –0.3 to –0.5 mg/day), which were significantly different from those observed in the placebo group (\(P < 0.001\)). There was no significant change with raloxifene. (Note: Overall for these coagulation parameters, changes in the raloxifene group were similar to those seen with BZA/CE with no statistically significant difference between raloxifene 60 mg and either BZA/CE dose).

There was no significant change from baseline in protein C activity for any BZA/CE doses relative to placebo. However, some increases in protein C activity observed with BZA/CE were significantly different from the decreases observed with raloxifene at some time points (\(P < 0.05\)). With the exception of BZA (20 mg)/CE (0.45 mg) at month 6, all doses of BZA/CE were associated with small but significantly greater changes in protein S activity (range, –0.1 to 0.1 mg/day) compared with placebo at all time points (\(P < 0.05\)). At month 24, minor increases in protein S activity

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<th>Characteristic</th>
<th>BZA (10 mg)</th>
<th>BZA (20 mg)</th>
<th>BZA (40 mg)</th>
<th>BZA (10 mg)</th>
<th>BZA (20 mg)</th>
<th>BZA (40 mg)</th>
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<td>417</td>
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<td>433</td>
<td>423</td>
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<td>56.29 (5.98)</td>
<td>56.68 (5.81)</td>
<td>56.84 (5.73)</td>
<td>56.22 (5.80)</td>
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<td>5 (1.20)</td>
<td>5 (1.20)</td>
<td>12 (2.79)</td>
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<td>BMI, kg/m²</td>
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<td>Years since</td>
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<td>8.29 (5.71)</td>
<td>7.94 (5.60)</td>
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<td>417</td>
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</table>

Note: BMI = body mass index.

Mean changes from baseline in (A) the daily number and (B) the severity of hot flushes in each treatment group. (A) Mean change in the daily number of moderate or severe hot flushes for weeks 1–12. \( P < 0.05 \) for all BZA/CE doses compared with placebo for weeks 5–12. \( P < 0.05 \) for BZA (10 mg)/CE (0.625 mg) at all time points and for BZA (10 mg)/CE (0.45 mg) at all time points except Week 1. (B) Adjusted mean change in total hot flush severity (mild, moderate, and severe) from baseline at weeks 4 and 12. Note: BZA = bazedoxifene; CE = conjugated estrogens. \(^a\) \( P < 0.001 \) vs. placebo.
were noted in all BZA/CE treatment groups, whereas decreases were observed at earlier time points. Changes in antithrombin III activity (range, 0.1 to 0.3 mg/day) were also small but significantly greater compared with placebo for all BZA/CE doses (P < 0.05), with the exception of BZA (20 mg)/CE (0.625 mg) at month 6. Changes from baseline in antithrombin III activity were similar for subjects who received raloxifene or BZA/CE. There were no appreciable dose-related effects of BZA/CE on anticoagulation factors.

**Adverse Events**

Overall, the incidence of AEs and serious AEs was similar among treatment groups (Table 3). There were no significant differences in the incidence of treatment-emergent AEs among groups (range, 90–94%; Table 3). The majority of treatment-emergent AEs, which were generally mild or moderate in severity, were not considered related to the study drug. There were no significant among-group differences in the incidence of these AEs. There were 6 deaths in the study, which were not thought to be study related. These deaths were caused by bronchoaspiration, intracerebral hemorrhage secondary to metastatic lung cancer, chronic obstructive airway disease, unknown causes, and accidental injury (two subjects).

**Venous Thromboembolic Events**

Overall, the incidence of venous thromboembolic events (VTEs) was similar for subjects treated with BZA/CE or placebo (0.76 vs. 1.56 per 1,000 women-years, respectively; relative risk, 0.48; 95% confidence interval [CI], 0.05–4.66). Two subjects in the BZA/CE treatment groups and one subject in the placebo group reported deep vein thrombosis. Pulmonary embolism was reported in one subject who received BZA (40 mg)/CE (0.625 mg). There were no reports of retinal vein thrombosis. Similarly, the incidence of superficial thromboses or phlebitis was low across all treatment groups (<1%), with no statistically significant among-group differences; importantly, none of these cases were classified as VTEs.
The percentage of subjects with PCI increases in cholesterol levels at
Clinical Laboratory Evaluations incidence of cardiovascular AEs was low (0.16–10.34), or an incidence of 2.02 vs. 1.56 per 1,000 women–years. For coronary artery disease and coronary artery insufficiency, a myocardial infarction with BZA/CE was 0.48 (95% CI, 0.05–0.81) across all treatment groups, with no significant differences among groups. Compared with subjects who received placebo, the relative risk of experiencing PCI increases in cholesterol levels was 8.1% in the placebo group and 5.7% in the raloxifene group, and it did not exceed 6.5% in any BZA treatment group. However, significantly higher percentages of subjects in the BZA/CE treatment groups experienced PCI increases in triglyceride levels compared with the placebo group (P < 0.05). A total of five subjects treated with BZA/CE had triglyceride values that were considered clinically important. Analysis of other laboratory safety data for blood chemistry and hematology parameters demonstrated no trends of concern. Results of liver function tests also indicated no values of clinical importance.

**Cardiovascular AEs**
The cardiovascular AEs of interest included myocardial infarction, coronary artery disease, and coronary artery insufficiency. The incidence of cardiovascular AEs was low (<1%) across all treatment groups, with no significant differences among groups. Compared with subjects who received placebo, the relative risk of experiencing a myocardial infarction with BZA/CE was 0.48 (95% CI, 0.05–4.66), or an incidence of 0.76 vs. 1.56 per 1,000 woman-years. For coronary artery disease and coronary artery insufficiency, the relative risk with BZA/CE vs. placebo was 1.29 (95% CI, 0.16–10.34), or an incidence of 2.02 vs. 1.56 per 1,000 women–years.

**Clinical Laboratory Evaluations**
The percentage of subjects with PCI increases in cholesterol levels at any time point was similar across all treatment groups. The incidence of PCI increases in cholesterol levels was 8.1% in the placebo group and 5.7% in the raloxifene group, and it did not exceed 6.5% in any BZA treatment group. However, significantly higher percentages of subjects in the BZA/CE treatment groups experienced PCI increases in triglyceride levels compared with the placebo group (P < 0.05). A total of five subjects treated with BZA/CE had triglyceride values that were considered clinically important. Analysis of other laboratory safety data for blood chemistry and hematology parameters demonstrated no trends of concern. Results of liver function tests also indicated no values of clinical importance.

**Discussion**
The TSEC was designed to provide tissue-selective activities of a SERM with the proven benefits of estrogen therapy (ET). The SMART-1 trial evaluated the efficacy and safety of the first TSEC composed of BZA/CE in postmenopausal women over a 2-year period. Findings from this study demonstrated favorable effects of BZA/CE on the relief of menopausal symptoms. Specifically, BZA (20 mg)/CE (0.625 or 0.45 mg) were significantly and clinically more effective than placebo in reducing the incidence of hot flushes, and BZA (20 mg)/CE (0.625 or 0.45 mg) also significantly reduced the severity of hot flushes compared with placebo. The BZA/CE groups decreased the daily number of hot flushes by 51.7–85.7% compared with only 17.1% with placebo. Whereas raloxifene has been shown to increase hot flushes in this population, in our study, raloxifene was associated with a numeric reduction in flush frequency, but not severity; however, BZA (10 and 20 mg)/CE groups BZA/CE groups reduced both hot flush frequency and severity significantly better than raloxifene. Furthermore, BZA (20 mg)/CE (0.625 or 0.45 mg) was significantly more effective than placebo in improving vaginal atrophy with significant increases in superficial and intermediate cells and reductions in parabasal cells. Accordingly, BZA (20 mg)/CE (0.625 or 0.45 mg) significantly reduced the incidence of dyspareunia relative to placebo. Breast pain is known to be increased in women taking estrogen/progestin therapy (EPT) (10). In this study, none of the BZA/CE doses increased the incidence of breast pain compared with placebo or raloxifene.

The menopausal transition is known to confer unfavorable changes in lipid and carbohydrate metabolism (15, 16), which may be associated with an increased risk of cardiovascular disease in women (17, 18). In this study, treatment with all BZA/CE regimens was associated with decreases in total cholesterol. Importantly, increases in LDL and decreases in HDL cholesterol are known risk factors for cardiovascular disease in women (19, 20). All BZA/CE doses were associated with marked decreases in LDL and increases in HDL cholesterol throughout the 2-year study period. Greater improvements in HDL cholesterol, HDL2 cholesterol, and apolipoprotein A1 were observed with administration of BZA/CE compared with raloxifene. Such favorable effects on lipid parameters have previously been observed in studies that randomized women to receive ET or EPT for up to 3 years (21–23).

A minor attenuating effect on HDL cholesterol levels was noted with increasing dose of BZA. However, the reductions in LDL cholesterol and apolipoprotein B appeared to be even greater with increasing dose of BZA, particularly when paired with CE (0.45 mg). Decreases in PAI-1 activity and PAI-1 antigen levels were also observed with BZA/CE treatment. Improvements in lipoprotein (a) observed with BZA/CE treatment in this study are consistent with those observed with EPT in the Women’s Health, Osteoporosis, Progestin, Estrogen (HOPE) trial (23) and the Heart and Estrogen/progesterin Replacement Study.
### TABLE 2

Adjusted mean changes from baseline in selected lipid and coagulation parameters at month 24 (metabolic substudy).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CE (0.625 mg)</th>
<th>CE (0.45 mg)</th>
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<tbody>
<tr>
<td></td>
<td>BZA (10 mg)</td>
<td>BZA (20 mg)</td>
</tr>
<tr>
<td>Mean (SE) percent change</td>
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<td></td>
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<tr>
<td>Lipid parameters</td>
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<tr>
<td>Total cholesterol</td>
<td>−2.3 (1.4)</td>
<td>−2.6 (1.4)</td>
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<tr>
<td>Triglycerides</td>
<td>18.8 (4.9)</td>
<td>25.1 (5.1)</td>
</tr>
<tr>
<td>HDL₂-C</td>
<td>33.4 (4.9)</td>
<td>28.4 (5.1)</td>
</tr>
<tr>
<td>Apo A1</td>
<td>11.2 (1.3)</td>
<td>11.1 (1.4)</td>
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<tr>
<td>Apo B</td>
<td>1.4 (1.8)</td>
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<tr>
<td>Lp(a)</td>
<td>−21.4 (3.0)</td>
<td>−21.2 (3.1)</td>
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<td>Mean (SE) change, mg/day</td>
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<td>Fibrinogen</td>
<td>−0.38 (0.07)</td>
<td>−0.50 (0.07)</td>
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<tr>
<td>Protein C activity</td>
<td>0.06 (0.02)</td>
<td>0.04 (0.02)</td>
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<tr>
<td>Protein S activity</td>
<td>0.02 (0.02)</td>
<td>0.07 (0.02)</td>
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<tr>
<td>Antithrombin III activity</td>
<td>−0.29 (0.02)</td>
<td>−0.28 (0.02)</td>
</tr>
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</table>

^Note: Apo = apolipoprotein; HDL₂-C = high-density lipoprotein 2 cholesterol; Lp(a) = lipoprotein (a).

^aP < 0.05 vs. placebo.

^bP < 0.01 vs. placebo.

^cP < 0.01 vs. raloxifene.

^dP < 0.05 vs. raloxifene.

^eP < 0.001 vs. placebo.
AEs; however, it is important to note that a longer period of treatment with BZA/CE doses was not associated with an increased risk of VTEs or cardiovascular events. Treatment with BZA/CE doses was generally well tolerated and demonstrated a safety profile similar to that of placebo. The incidence of AEs, serious AEs, and study discontinuations owing to AEs was similar across all treatment groups. Treatment with BZA/CE doses was associated with beneficial effects on lipoprotein (a) observed with BZA/CE in the present study was greater than the response (HERS) (24). This beneficial effect on lipoprotein (a) observed with BZA/CE in the present study was greater than the response observed with ET and EPT in the HOPE trial (23). Also consistent with findings of the HOPE trial were the changes in protein S activity and antithrombin III activity noted with BZA/CE vs. placebo. No significant changes in carbohydrate metabolism or serum concentrations of D-dimer infestations were observed with any BZA/CE regimens throughout the study period. Because CE alone is known to reduce levels of fasting insulin, future studies will help determine whether BZA might affect this beneficial estrogen response. It is also important to note that any beneficial effects of BZA/CE on surrogate markers might not predict clinical events. Overall, BZA/CE was generally well tolerated and demonstrated a safety profile similar to that of placebo. The incidence of AEs, serious AEs, and study discontinuations owing to AEs was similar across all treatment groups. Treatment with BZA/CE doses was associated with beneficial estrogenic effects on metabolism. Among the parameters assessed in this study, the higher BZA dose (40 mg) was found to decrease, somewhat, the beneficial effect of CE on hot flushes and vaginal atrophy. Apart from a relatively minor attenuation noted for HDL2 cholesterol, no other attenuating effects of BZA on clinical laboratory determinations were observed. For instance, triglycerides were unaffected by BZA dose, and some estrogenic effects were enhanced with increasing BZA dose, including decreases in LDL cholesterol. One possible limitation of this study in evaluating the relief of vasomotor symptoms and vaginal atrophy is the wide range in ages of the subject population (45–70 years of age), because the occurrence of menopausal symptoms is typically highest in the early observation in a larger population of subjects will be able to provide definitive risk information regarding possible adverse effects of therapy, as this study was not powered to detect small differences in these cardiovascular safety endpoints. In this study, analysis of most clinical laboratory determinations (e.g., hematology, blood chemistry, liver function) revealed no clinically important differences among treatment groups and no trends of concern. In that the pairing of BZA and CE alleviates the need for a progestin, it is of interest to determine whether BZA attenuates any of the beneficial estrogenic effects of CE on metabolism. Among the parameters assessed in this study, the higher BZA dose (40 mg) was found to decrease, somewhat, the beneficial effect of CE on hot flushes and vaginal atrophy. Apart from a relatively minor attenuation noted for HDL2 cholesterol, no other attenuating effects of BZA on clinical laboratory determinations were observed. For instance, triglycerides were unaffected by BZA dose, and some estrogenic effects were enhanced with increasing BZA dose, including decreases in LDL cholesterol. One possible limitation of this study in evaluating the relief of vasomotor symptoms and vaginal atrophy is the wide range in ages of the subject population (45–70 years of age), because the occurrence of menopausal symptoms is typically highest in the early...
years of menopause. However, an important objective of the SMART-1 trial was to assess the efficacy of BZA/CE for the prevention of postmenopausal osteoporosis, requiring a postmenopausal population at sufficient risk for osteoporosis and enrolling women of increasing age. Nevertheless, BZA/CE was associated with effective relief of menopausal symptoms in postmenopausal women of varying ages.

In conclusion, the SMART-1 trial showed that the administration of a TSEC that partners BZA and CE was effective in treating symptoms associated with menopause, particularly vasomotor symptoms and vaginal atrophy, without increasing the incidence of breast pain or overall AEs, including VTEs and cardiovascular AEs. Favorable effects on the lipid profile and minimal changes in coagulation and carbohydrate parameters were consistent with well known estrogen effects. Based on these findings, BZA/CE demonstrated a favorable benefit-risk profile and represents a promising new menopausal therapy with improved tolerability.

Acknowledgment: The authors acknowledge the contributions of the other investigators of the study (see Appendix).

REFERENCES

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DECREASED TOTAL CHOLESTEROL, SERUM TRIGLYCERIDES, AND INCREASED HDL.

Susan Davis, et al Menopause Volume 7, No. 6, pp.395-401

- Using subcutaneous bio-identical hormone pellets

This again is superior to oral HRT where triglycerides are increased and there is a null effect on HDL.
Effects of estradiol with and without testosterone on body composition and relationships with lipids in postmenopausal women

Susan R. Davis, MD, PhD, Karen Z. Walker, PhD, and Boyd J. G. Strauss, MD

ABSTRACT

Objective: The cardioprotective effects of postmenopausal estrogen replacement therapy are mediated by several mechanisms, including favorable effects on lipids and lipoproteins. The extent to which the latter reflects modification of body fat distribution by sex steroids is not known. Hence, we investigated the relationships between changes in lipids and measures of body composition in postmenopausal women who were administered estrogen therapy with and without testosterone.

Design: We randomized 33 postmenopausal women to treatment with either estradiol 50 mg (E) alone or estradiol 50 mg plus testosterone 50 mg implants (E&T) administered every 3 months for 2 years in conjunction with cyclic oral progestins for women with an intact uterus.

Results: Both therapies were associated with sustained reductions in total cholesterol and low-density lipoprotein (LDL) cholesterol. In women who received E but not E&T, hip (p < 0.001) and abdominal circumferences (p < 0.05) and fat mass:fat-free mass (FM:FFM) ratio over the abdomen (p < 0.05) declined. E&T but not E resulted in increased FFM (p < 0.001) and a reduced FM:FFM ratio (p < 0.05). For E but not E&T, the decrease in LDL cholesterol was significantly related to changes in total and compartmental body fat and to change in the FM:FFM ratio (p < 0.05).

Conclusion: Estrogen replacement has effects on body fat distribution in postmenopausal women that are associated with improved lipid parameters. Addition of parenteral testosterone does not negate the favorable effects of estrogen on LDL cholesterol levels but may attenuate the reduction in centralized body fat achieved with E implants.

Key Words: Testosterone – Estradiol – Menopause – Body composition – Blood lipids.
versely influenced by the use of parenteral testosterone. There are, however, few prospective data pertaining to the effects of ERT on body composition and fat distribution or to the relationships between such changes and the effects of ERT on plasma lipids and lipoproteins and the effects of concomitant testosterone therapy on these relationships.

The administration of androgen replacement therapy, usually in the form of testosterone, is used for the restoration of libido in symptomatic women and for the prevention of bone loss after menopause. With the increasing availability of testosterone and other androgen supplements for women, the inclusion of androgen therapy in postmenopausal hormone regimens is becoming more widespread. However, the effects of adding testosterone to estrogen therapy on body composition have not been reported. We report the relationships between changes in body composition and fat distribution and lipids and lipoproteins after long-term administration of estradiol alone or estradiol plus testosterone.

MATERIALS AND METHODS

Subjects

As reported previously, 34 postmenopausal women (>12 months of amenorrhea and had serum follicle-stimulating hormone levels > 15 IU/L) who attended the menopause clinic of Monash Medical Centre, Melbourne, Australia, volunteered for this study, which was approved by the Human Research and Ethics Advisory Committee of Monash Medical Centre, Melbourne. All subjects gave their written informed consent. None of these women had previously been treated with androgens or had received hormone implants, although some had received oral ERT.

Methods

Women were randomized independently to single blind treatment with either estradiol 50-mg implants alone (E) or estradiol 50-mg plus testosterone 50-mg implants (E&T), both donated for the study by Organon Australia Ltd. Implants were administered every 3 months for a period of 2 years. E or T implants were not inserted if a preceding blood test indicated that serum estradiol or testosterone levels exceeded 500 pmol/L or 4 nmol/L, respectively. During the study, 13 estradiol implants were withheld from seven women who received E&T treatment, and 7 estradiol implants were withheld from four women who received E alone. Thirteen testosterone implants were similarly withheld. Women with an intact uterus were treated with either cyclical medroxyprogesterone acetate (Provera, Upjohn Pty. Ltd, Rydalmere, NSW, Australia) 5–10 mg or norethisterone (Primolut N, Schering Pty. Ltd., Alexandria, NSW, Australia) 2.5 mg orally for 12 days per month. All investigations were performed at entry into the study and then at six monthly intervals for 2 years. All investigators and research assistants involved in body composition measurements and data analysis were blinded as to each patients therapy.

Women were weighed without shoes and while wearing light clothing or underwear. Weight was measured to the nearest 0.1 kg on a digital scale, and body height was measured using a wall-mounted stadiometer. Body mass index was calculated as weight (kg) divided by height (m) squared. Measurements of body circumferences and skinfold thicknesses were undertaken by a single skilled individual using standard procedures. The World Health Organization abdominal circumference was taken as the greatest circumference between the lowest rib and the top of the pelvis. Skinfold thicknesses were measured at the triceps, biceps, and subscapular and suprailiac sites with Harpenden calipers (Holtain Ltd., Crymych, UK). Body composition was measured by dual-energy X-ray absorptiometry (DXA) after a whole-body scan taken on a DPX-L scanner (DPX-L; Lunar Corporation, Madison, WI), which was standardized daily against a calibration block. Total body fat mass (FM), the sum of fatty elements in all fat tissue, was derived according to computer algorithms supplied by the manufacturer (DPX-L software version 3.4, Lunar Corporation), and free-fat mass (FFM) was taken as total body tissue minus FM. In addition, an abdominal region of interest was defined manually by delineating a superior border at the level of the top of the L2 vertebra, an inferior border at the bottom of the L4 vertebra, and vertical borders drawn through the intersection of the superior border with the left and right costal margins. The ratio of abdominal FM to FFM was then determined after compositional analysis of this region of interest.

Accuracy of total body fat by DXA has been assessed by comparison to underwater hydrodensitometry in 12 healthy adult volunteers. The correlation was $r = 0.895$ \((p < 0.0001)\) with a between-method bias of $+4.8\%$ (range = 2–9%). Precision of the DXA was assessed by 10 repeated measures on one healthy volunteer. The coefficient of variability (CV) was 1% for percentage of fat, 0.6% for FM, and 2.1% for FM. Precision of the sum of the thicknesses of four skinfolds was assessed by eight repeated measures in three healthy volunteers by two trained technicians. The CV varied from 5.9% to 6.3%, depending on the technician.
For accuracy of the sum of the four skinfold thicknesses, the correlation with DXA was \( r = 0.921 \) (\( p < 0.00001 \)).

Serum estradiol and testosterone were measured by radioimmunoassay. Total cholesterol, TG, and HDL cholesterol were measured by automated standard methods, and LDL cholesterol was calculated according to Friedwald.

**Statistical analyses**

The data comprised repeated measurements on each individual at baseline and then every 6 months for 2 years. Results are expressed as the mean ± standard deviation (SD). The baseline data were tested for treatment differences by two sample \( t \) tests. For 6-, 12-, 18-, and 24-month data, parameters were analyzed by multivariate analysis of covariance (MANCOVA). Data were also compared at baseline and after 2 years in each group by Student’s paired \( t \) test. Relations between variables were established by Pearson’s correlation coefficient. Analyses were performed using Microsoft Excel 5.0 for the Macintosh. The level of significance was taken as 0.05.

**RESULTS**

Thirty-two women completed the study: 17 in the E group and 15 in the E&T group. One woman discontinued for personal reasons early after commencement, and the other discontinued after 12 months because of weight gain. At baseline, after randomization, the E group did not differ from the E&T group in smoking or alcohol habits, hysterectomy or ovariectomy status, estrogen or testosterone levels, or levels of blood lipids. Body mass index also did not differ between the two groups (24.6 ± 3.1 and 24.6 ± 3.3 kg/m\(^2\) for E and E&T therapy, respectively). The mean age of the 17 women in the E group, however, was significantly lower than that of the 15 women in the E&T group (51.3 ± 5.7 years and 57.0 ± 5.2 years, respectively, \( p < 0.01 \)). All body composition variables were analyzed using age as a covariate; no significant effect of age was demonstrated.

Changes in the hormonal status of women before and after the study intervention are shown in Fig. 1. At baseline, the women who were receiving E and the women who were receiving E&T did not differ significantly in their levels of estradiol or testosterone. After 2 years of therapy, serum estradiol levels were significantly higher than at baseline in both treatment groups (\( p < 0.001 \)), whereas, as expected, serum testosterone remained unchanged in the E group but increased with E&T treatment (\( p < 0.001 \)).

The effects of therapy on bone mineral density have been previously reported. Here we report the detailed analysis of change in body composition and fat distribution and the relationships of these changes and change in lipoprotein lipids.

Over 2 years, mean body weight decreased slightly in the E group (from 65.1 ± 9.2 kg to 64.1 ± 8.9 kg) and increased slightly in the E&T group (from 63.4 ± 7.8 kg to 64.6 ± 9.7 kg), but these changes were not statistically significant. Similarly, total body FM as determined by DXA did not change significantly throughout the study (Fig. 2). Although there was a modest decline in FM with E&T therapy over 2 years (37.4 ± 7.1 kg to 35.9 ± 7.7 kg), this change did not achieve statistical
significance (Table 1). In the E&T group but not in the E group, levels of FFM increased significantly over the 2-year period (24.8 ± 5.9 kg to 27.9 ± 5.9 kg, \( p < 0.01 \)), whereas the FM:FFM ratio declined (\( p < 0.05 \)). The MANCOVA of body variables gave a significant treatment effect (\( F = 17.26, 9 \text{ df}, p < 0.05 \)).

Change in the deposition of abdominal fat was also examined. In the group that was given estradiol implants alone, there was a significant decline in the FM:FFM ratio in a region drawn directly over the abdomen (\( p < 0.05 \)). This reflected both a decrease in fat over the abdomen from 1.51 ± 0.57 to 1.43 ± 0.59 kg and a concurrent increase in FFM in this area, from 3.12 ± 0.55 to 3.30 ± 0.52 kg, although these changes in themselves did not reach statistical significance.

Changes in anthropometric measures of girth over the 2 years of the study are given in Table 2. Women who received E implants alone showed significant decreases in hip and abdominal circumferences (\( p < 0.01 \) and \( p < 0.05 \), respectively). There was also a trend toward a decrease in the umbilical circumference (\( p < 0.08 \)). These changes were not observed in women who were given E&T.

Over 2 years, both total cholesterol and LDL cholesterol declined in both treatment groups (Fig. 3). LDL cholesterol fell from 4.0 ± 0.89 mmol/L to 3.3 ± 0.94 mmol/L (\( p < 0.01 \)) in women who were given E implants and from 4.1 ± 0.77 mmol/L to 3.4 ± 0.91 mmol/L (\( p < 0.01 \)) in women who were given E&T. The decline in the ratio of LDL cholesterol to HDL cholesterol, however, was only significant in women who were given E alone (\( p < 0.01 \)).

Relationships between changes in LDL cholesterol and change in body fat were also examined. With E alone, there was a positive and significant relationship between the change in LDL cholesterol over 2 years and the change in total body fat (\( r = 0.494, p < 0.05 \)) (Table 3). Change in LDL cholesterol was also significantly related to change in the FM:FFM ratio over the abdomen (\( r = 0.642, p < 0.01 \)), to change in the total body FM:FFM ratio (\( r = 0.519, p < 0.05 \)), and to the change in hip circumference (\( r = 0.670, p < 0.01 \)). Similarly, the change in the ratio of LDL cholesterol to HDL cholesterol over 2 years was significantly related to change in total body FM, to change in the total body FM:FFM ratio, to change in the FM:FFM ratio over the abdomen, and to the change in hip circumference (all \( p < 0.05 \)) (Table 3). Analysis of comparable data in women who received treatment with E&T indicated that none of the relationships was of statistical significance.

**DISCUSSION**

Women who are commencing ERT are often anxious that their treatment will exacerbate weight gain. This study confirms that estradiol implants, which result in relatively high circulating levels of estradiol, do not significantly increase body weight or total body fat in postmenopausal women over a 2-year period. Moreover, the addition of testosterone did not adversely affect body weight or total body fat.

Our results with E alone are in agreement with many previous studies. Although weight gain with ERT has been reported, most studies indicate that ERT either decreases or has no influence on body weight. Similarly, despite one report that ERT increased the percentage of body fat, the present study is consistent with other findings that total body FM by DXA is unaffected by ERT and with a recent twin study that indicated that in monozygotic twins discordant for ERT use, the twin that received the hormone therapy had the lower FM.

After menopause, women typically experience a loss of FFM, a decline that is not alleviated by ERT. In this study, FFM remained stable over 2 years of ERT alone, but treatment with E&T significantly increased total FFM (\( p < 0.01 \)) while decreasing the FM:FFM ratio (\( p < 0.05 \)). This change has the potential to influence the lipid profile; a study of 426 women in the Virgilio Menopausal Health Project indicated that levels of both total cholesterol and LDL cholesterol are inversely related to FFM.
Although E and E&T treatment seem similar in their effects on body weight and total body fat, they differed in their effect on tissue composition in the abdomen. Although the women who were treated with E implants alone exhibited a mean decrease in the FM:FFM ratio in a region drawn over the abdomen (p < 0.05), the FM:FFM ratio in this region remained unchanged in women who received E&T. There were, however, only 15 women in the E&T group; this caused a lack of statistical power. Nevertheless, our sample size calculations indicate that when observed differences in body composition failed to reach significance in the E&T

**TABLE 1.** Change in body composition in postmenopausal women who received hormonal replacement therapy with estradiol (n = 17) or with estradiol plus testosterone (n = 15) implants for a period of 2 years

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estradiol Baseline value</th>
<th>Estradiol After 2 y</th>
<th>Estradiol plus testosterone Baseline value</th>
<th>Estradiol plus testosterone After 2 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body fat mass (kg)</td>
<td>35.7 ± 6.6</td>
<td>35.1 ± 6.6</td>
<td>37.4 ± 7.1</td>
<td>35.9 ± 8.0</td>
</tr>
<tr>
<td>Total body FFM (kg)</td>
<td>28.0 ± 6.5</td>
<td>28.2 ± 5.8</td>
<td>24.8 ± 5.9</td>
<td>27.9 ± 5.9</td>
</tr>
<tr>
<td>FM:FFM ratio</td>
<td>1.34 ± 0.41</td>
<td>1.31 ± 0.42</td>
<td>1.62 ± 0.58</td>
<td>1.38 ± 0.52</td>
</tr>
<tr>
<td>Fat mass over abdomen (kg)</td>
<td>1.51 ± 0.57</td>
<td>1.43 ± 0.59</td>
<td>1.60 ± 0.55</td>
<td>1.64 ± 0.65</td>
</tr>
<tr>
<td>FFM over abdomen (kg)</td>
<td>3.12 ± 0.55</td>
<td>3.30 ± 0.52</td>
<td>3.17 ± 0.45</td>
<td>3.31 ± 0.38</td>
</tr>
<tr>
<td>FM:FFM over the abdomen</td>
<td>0.48 ± 0.16</td>
<td>0.42 ± 0.14</td>
<td>0.51 ± 0.18</td>
<td>0.51 ± 0.21</td>
</tr>
</tbody>
</table>

Data are the mean ± SD.  
*Significant change over 2 y, p < 0.01.  
*Significant change over 2 y, p < 0.05.

**TABLE 2.** Change in anthropometric measures of girth in postmenopausal women who received hormonal replacement therapy with estradiol (n = 17) or with estradiol plus testosterone (n = 15) implants for a period of 2 years

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment: estrogen Baseline value</th>
<th>Treatment: estrogen Baseline value</th>
<th>Treatment: estrogen After 2 y</th>
<th>Treatment: estrogen After 2 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO abdominal circumference (cm)</td>
<td>92.8 ± 10.9</td>
<td>93.1 ± 11.5</td>
<td>88.4 ± 11.8c</td>
<td>89.9 ± 9.3</td>
</tr>
<tr>
<td>Umbilical circumference (cm)</td>
<td>89.7 ± 11.1</td>
<td>89.6 ± 11.1</td>
<td>86.7 ± 10.8b</td>
<td>83.3 ± 9.4</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>100.4 ± 8.3</td>
<td>101.2 ± 6.0</td>
<td>95.5 ± 9.4d</td>
<td>97.9 ± 6.2</td>
</tr>
</tbody>
</table>

Data are the mean ± SD.  
*Significant change over 2 y, p < 0.05.  
Trend towards a change over 2 y, p < 0.08.  
*Significant change over 2 y, p < 0.01.

**TABLE 3.** Relationships between change in LDL-cholesterol or in change in the ratio of LDL-cholesterol to HDL-cholesterol with change in body fat or hip circumference in postmenopausal women treated with estradiol (n = 17) or with estradiol plus testosterone (n = 15) implants for a period of 2 y

<table>
<thead>
<tr>
<th>Change in LDL-cholesterol</th>
<th>Change in ratio of LDL: HDL-cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>E &amp; T</td>
</tr>
<tr>
<td>E</td>
<td>E &amp; T</td>
</tr>
</tbody>
</table>

| Change in total body fat    | 0.49c                                  | 0.49c                                 |
| Change in the total body FM:FFM ratio | 0.52c                          | 0.49c                                 |
| Change in FM:FFM over the abdomen | 0.64c                          | 0.52c                                 |
| Change in hip circumference | 0.67c                                  | 0.56c                                 |

Data given are r values.  
*Significant relationship, p < 0.05.  
*Significant relationship, p < 0.01.
group, this was due to a lack of biologically relevant difference because the sample size that was required to show significance was unrealistically high.

Anthropometric measures corroborate our DXA findings. E therapy alone was associated with significant decreases in both hip and abdominal circumferences ($p < 0.01$, $p < 0.05$, respectively). This was not seen for women who were treated with E&T. The anthropometric and DXA data from women who were given E implants are consistent with previous studies that showed by DXA $^4$, $^5$, $^{23}$, $^{26}$ or anthropometry $^6$, $^{18}$, $^{21}$, $^{22}$, $^{56}$, $^{27}$ that ERT either prevents the increase of central body fat after menopause or has a neutral effect, but the difference in the response of the women who were given E&T suggests that the addition of T may attenuate the favorable effect of reducing post-menopausal centralized fat accumulation with E alone. The clinical implication of this observation is that the positive relationships between changes in body fat distribution and LDL cholesterol and the LDL cholesterol: HDL cholesterol ratio seen with E alone were not seen with E&T. Differences in circulating lipoprotein-lipid concentrations have previously been reported to be associated with variations in the regional distribution of body fat. $^{28}$ Over the 2-year period of our study, beneficial lipid changes were observed in women who were treated with E. This outcome is consistent with other studies $^{10}$, $^{11}$, $^{29}$ and supports the hypothesis that the beneficial effects on lipid parameters observed with postmenopausal estrogen replacement are the result of direct effects of estrogen on lipid metabolism combined with favorable effects on central fat deposition. It is of interest that although the addition of testosterone to the estrogen therapy resulted in less pronounced change in central body fat, it was nevertheless associated with an improved lipid profile (Fig. 3). Whether the increase in FFM with testosterone acted as a metabolic counterbalance is not known.

Testosterone levels decline with age, $^{30}$ and bioavailable testosterone may decrease further in postmenopausal women who take oral estrogen. $^{31}$ A case therefore can be argued for concurrent androgen and estrogen replacement after menopause. $^{31}$ In particular, the addition of testosterone can significantly increase bone mineral density, $^{9}$, $^{13}$ and it also markedly improves measures of sexuality. $^{9}$ In addition, as we showed here, the addition of testosterone to ERT reverses the decline of FFM seen after menopause, $^{2}$ $^{3}$ but in considering the use of testosterone therapy, these advantages need to be balanced against possible long-term adverse effects on deposition of centralized body fat.

Endogenous androgen excess in postmenopausal women is clearly associated with increased cardiovascular risk, because of perturbations in lipid and carbohydrate metabolism, and a more android weight distribution. $^{26}$, $^{28}$ In this study, undertaken with women of normal body weight, it was found that although testosterone therapy offset the reduction of centralized body fat evident after estrogen alone, it nevertheless still preserved the favorable effects of estrogen on the lipid profile. Therefore, undesirable effects are extremely unlikely and uncommon with testosterone replacement therapy, with the caveat that circulating androgen levels are maintained close to or within the normal female reproductive range and that patients are closely clinically monitored. $^{9}$ Additional studies are needed to establish whether testosterone therapy is inappropriate for more obese postmenopausal women, in whom an adverse effect on blood lipids might become apparent.

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Twenty-three postmenopausal women with a median of 2 years past menopause (range, 1 to 12 years) and a median age of 52 years (range, 39 to 62 years) were recruited to participate in a longitudinal study designed to investigate the factors that influence the increase in bone density with subcutaneous estradiol and testosterone implants. All women received 75 mg of estradiol with 100 mg testosterone subcutaneously. Bone density was measured at the spine and hip by dual-photon absorptiometry before therapy and after 1 year of subcutaneous hormonal therapy. The mean pretreatment bone density at the lumbar vertebrae and neck of the femur was 0.84 grams of hydroxyapatite per square centimeter (SD, 0.11) and 0.73 grams of hydroxyapatite per square centimeter (SD, 0.10), respectively. The bone density at both sites increased with values of 0.91 grams of hydroxyapatite per square centimeter (SD, 0.11) and 0.75 grams of hydroxyapatite per square centimeter (SD, 0.11), respectively. These values represent an increase of 8.3% (p < 0.0001) at the spine and 2.8% (p < 0.01) at the neck of the femur. The plasma estradiol level increased from a median of 80.5 pmol/L to 453 pmol/L (p < 0.001). The percentage increase of vertebral bone density was not related to age, number of years past the menopause, pretreatment bone density, or serum testosterone levels, but a significant correlation was found between the percentage increase in bone density at the spine and the serum estradiol level (p < 0.02, r = 0.45).

Key words: Osteoporosis, menopause, estradiol

After Albright's1 observation that estrogen therapy can reduce urinary calcium excretion in postmenopausal women a number of prospective studies confirmed the values of estrogen replacement therapy in the prevention of postmenopausal osteoporosis.2-4 Epidemiologic studies5, 6 also showed a reduction in the incidence of osteoporotic fractures with such therapy.

Most studies used oral estrogen therapy, which although effective in the suppression of climacteric symptoms usually results in plasma estradiol and follicle-stimulating hormone (FSH) levels that are still in the postmenopausal range.7 Although it has been claimed that the minimum dose of oral estrogen required to prevent postmenopausal osteoporosis is 0.625 mg conjugated equine estrogen8 the optimal dose and route of estrogen necessary to achieve an increase in bone density is not known.

The percutaneous route of administration avoids the enterohepatic circulation and is associated with physiologic plasma levels of estradiol and estrone. This is in contrast to the low levels of estradiol and high levels of estrone found after oral therapy with both conjugated equine estrogens or estradiol valerate.8, 10 Subcutaneous implants of estradiol and testosterone are effective in the alleviation of climacteric symptoms,11 and a cross-sectional study12 showed an apparent superiority of implant therapy over oral therapy in the therapeutic effect on bone density. The suggestion was made in this study that the greater bone density after implant therapy was a result of the greater plasma estradiol levels achieved with this route when compared with oral estrogen therapy.

We present the results of a prospective study of estradiol and testosterone implants on the bone density and plasma hormone levels in postmenopausal women over 1 year of therapy.

Patients and methods

A total of 23 postmenopausal women with a median age of 52 years (range, 39 to 62) were recruited into
this prospective study. The median number of years past the menopause was 2 (range, 1 to 12). Menopausal status was defined as amenorrhea > 1 year’s duration with a serum FSH level >20 IU/L.

All women received hormone replacement therapy by subcutaneous implants of 75 mg estradiol and 100 mg testosterone. A further implant of the same dose of estradiol and testosterone was given after 6 months and again at 1 year.

Assays of FSH, estradiol, and testosterone were performed before treatment and at 1 year. The bone density was estimated in the spine of L2-4 and in the neck of the right femur with a Novo 22A BMC-LAB with gadolinium 153 as the source of radiation. The absorptiometer was standardized with a solution radiologically equivalent to hydroxyapatite, and the results were expressed as grams of hydroxyapatite per unit projected area of bone in square centimeters (gHA/cm²). The precision of the machine with the use of the phantom was 0.69% and short-term precision for normal subjects was 2.04%. Measurements were made before insertion of the hormone implant and at 12 months after therapy within 1 week of the insertion of the third implant.

Statistical analysis. The bone density values before and after treatment approximated to a normal distribution and the increase was therefore analyzed with the paired Student t test. The hormonal parameters could not be regarded as normally distributed and the changes were analyzed with the Wilcoxon matched-pairs signed-rank test. The Spearman correlation coefficient was calculated to express the relationship between the changes in bone density and the serum estradiol levels, serum testosterone levels, age, number of years past the menopause, and pretreatment bone density.

Results

Table I. Mean values of bone density at lumbar spine and neck of femur, body weight, blood pressure, and plasma hormone values before therapy and after 1 year of estradiol and testosterone implants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pretreatment</th>
<th>1 Year after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean bone density at spine gHA/cm² (SD)</td>
<td>0.84 (0.11)</td>
<td>0.91 (0.11)*</td>
</tr>
<tr>
<td>Mean bone density at femur gHA/cm² (SD)</td>
<td>0.73 (0.10)</td>
<td>0.75 (0.11)†</td>
</tr>
<tr>
<td>FSH (IU/L) median (range)</td>
<td>71 (28-100)</td>
<td>12 (1-62)‡</td>
</tr>
<tr>
<td>Estradiol (pmol/L) median (range)</td>
<td>80.5 (30-580)</td>
<td>453 (204-883)†</td>
</tr>
<tr>
<td>Testosterone (nmol/L) median (range)</td>
<td>0.6 (0.3-1.8)</td>
<td>0.9 (0.4-2.4)§</td>
</tr>
<tr>
<td>Mean weight in kg (SD)</td>
<td>67.3 (7.7)</td>
<td>66.7 (7.0)</td>
</tr>
<tr>
<td>Mean blood pressure (mm Hg) (SD)</td>
<td>120 (37)/73 (12)</td>
<td>125 (21)/75 (13)</td>
</tr>
</tbody>
</table>

*p < 0.0001, Student’s paired t test.
†p < 0.01, Student’s paired t test.
‡p < 0.001, Wilcoxon matched-pair signed-rank test.
§p < 0.01, Wilcoxon matched-pair signed-rank test.

Of the 23 patients studied 22 patients showed an increase in the bone density after 1 year of therapy (Table I). The mean bone density at the lumbar spine before therapy was 0.84 gHA/cm² (SD 0.11), which increased to 0.91 gHA/cm² (SD, 0.11) after 1 year (p < 0.0001). The value for the proximal femur was 0.73 gHA/cm² (SD, 0.10), which increased to 0.75 gHA/cm² (SD, 0.11, p < 0.01). The mean increase in the lumbar spine was 0.07 gHA/cm² (95% confidence interval, 0.06 to 0.08 gHA/cm²). The mean increase at the femur was 0.02 gHA/cm² (95% confidence interval, 0.01 to 0.04 gHA/cm²). These values represent a mean increase in the bone density of 8.3% at the spine and 2.8% at the neck of the femur after 1 year.

The median serum FSH level before therapy was 71 IU/L (range, 28 to 100 IU/L) with a median serum estradiol level of 80.5 pmol/L (range, 30 to 580 pmol/L) and a serum testosterone level of 0.6 nmol/L (range, 0.3 to 1.8). After 1 year of therapy the serum FSH level significantly reduced to 12 IU/L (range, 1 to 62; p < 0.001), the serum estradiol level increased to 453 pmol/L (range, 204 to 883; p < 0.001), and the serum testosterone level increased to 0.9 nmol/L (range, 0.4 to 2.4; p < 0.01).

A significant correlation was found between the percentage increase of vertebral bone density and the
plasma estradiol levels achieved after 1 year of therapy (Fig. 1, \( r = 0.45; p < 0.02 \)). There was no significant correlation between the increase in bone density and the initial bone density (Fig. 2), age (Fig. 3), the number of years past the menopause (Fig. 4), and the serum testosterone level (Fig. 5).

**Comment**

These data show an increase of 8.3% in the bone density at the spine and 2.8% at the hip after 1 year of therapy with subcutaneous estradiol and testosterone implants. Evidence from our cross-sectional study with the use of the same method of bone densitometry reporting the vertebral bone density of 1.02 gHA/cm² and proximal femur values of 0.80 gHA/cm² after 8 years of such therapy strongly suggest that this increase is not transient and will be maintained over the years.

Christiansen et al. reported that the use of a combined preparation of oral estradiol, estriol, and norethindrone within 3 years of menopause resulted in an increase in bone density of 3% after 3 years of therapy. Lindsay et al. (1984) found an increase of 2% to 4% over 5 years when mestranol 25 mg was prescribed within 3 years of menopause but this dose only maintained bone density if it was started more than 3 years after menopause. More recently Munk-Jensen et al. reported an increase of 6.4% in the vertebral bone density of women treated within 2 years of menopause with continuous oral estradiol and norethindrone for 1 year. None of these authors reported the estradiol levels obtained with the therapy. In our study we were able to show an increase in bone mass even 10 years after menopause. This increase was the same as found in the younger postmenopausal woman and was also inde-
The use of a combination of estriol and norethisterone resulted in an improvement of 2% to 4% in bone density with some patients requiring 1% to 2% more. The correlation between the percentage increase in bone density and the serum estradiol levels supports the hypothesis that the greater effectiveness of this mode of therapy may be a result of the higher serum estradiol levels achieved. Oral estrogen therapy is associated with lower estradiol levels and a less good skeletal response. The importance of the testosterone component of the treatment is unknown but there was no correlation in this study between plasma testosterone levels and the increase in bone density. Barlow et al. could not show that the addition of testosterone to estrogen therapy improved bone density. However, there is some evidence that adrenal androgens may have a role in maintaining the bone mass in postmenopausal women in the study of postmenopausal women with treated Addison's disease. It is thus possible that the anabolic effects of testosterone on the skeleton may partly explain these results and this component of the therapy is subject to further investigation.

The increase in bone density was greater in the spine than that at the neck of the femur because the more active trabecular bone predominates in the vertebra. There are several models that show such substantial reversal of bone loss, particularly in the vertebra. Treasure et al. showed in a cross-sectional study that the bone loss of anorexia nervosa is reversed when menstruation returns. Greenspan showed that the osteoporosis of hyperprolactinemic men with hypogonadism recovers when the hyperprolactinemia is treated. Matta et al. (1988) reported that the 5.9% loss of bone density after the use of buserelin for 6 months is reversed when it is discontinued. These conditions are all characterized by hypogonadism and the studies indicate...
that bone can be replaced in both men and women when plasma sex hormones return to normal physiological levels.

The current recommended oral dosage of estrogens will suppress menopausal symptoms and prevent further bone loss but the serum estradiol and FSH levels achieved often remain in the postmenopausal range. The dosage of orally administered estrogen may be limited by gastrointestinal symptoms and the liver impact on estrogen metabolism. It is probable that the safest way to achieve serum estradiol levels adequate to produce significant increases in bone mass is by the percutaneous route with either transdermal patches or subcutaneous implants.

The mechanism by which estrogen deficiency causes loss of bone remains unclear but the original Albright hypothesis of an association with a generalized loss of collagen including the collagenous matrix of the bone and the collagen of the skin is relevant. The substantial increase in vertebral spine density shown in this study can be compared with the 30% increase in skin collagen and a 25% increase in skin thickness that occurs in postmenopausal women who receive percutaneous estradiol therapy. Only serial histomorphometric studies of bone biopsy specimens in these patients will reveal whether the estrogen-promoted changes in skin collagen are reproduced in the collagenous matrix of postmenopausal women.

It is reassuring from this study that estradiol and testosterone implants may not only prevent osteoporosis but will also be valuable in the older woman who might believe that it is too late to commence estrogen therapy. This therapy is also appropriate for the younger woman with premature ovarian failure who has already suffered substantial bone loss.

We gratefully acknowledge the assistance of Derec Lowe, medical statistician, King's College Hospital, with the statistical analysis.

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Two year study: marked increase in bone density with no adverse effects were noted in the coagulation inhibition and fibrinolysis assays in the pellet patients. Multiple others showing similar results. Testosterone that stimulates the osteoblast working with the estradiol inhibiting the osteoclast.
Metabolic and Hormonal Effects of 25-mg and 50-mg 17\(\beta\)-Estradiol Implants in Surgically Menopausal Women

MORRIS NOTELOVITZ, MD, PhD, MATTHEW JOHNSTON, MD, STEVEN SMITH, MD, AND CRAIG KITCHENS, MD

A prospective study involving 12 surgically menopausal women was undertaken to determine whether 17\(\beta\)-estradiol pellets could maintain bone mineral content without inducing adverse cardiovascular side effects. Surgically menopausal women were randomly selected to have either 25-mg or 50-mg pellets implanted subcutaneously. The bone mineral content of the midshaft of the nondominant radius in the combined group—measured by single photon absorptiometry—increased by 1.8% over the two-year period of observation (\(P < .03\)); the distal bone mineral content of the radius was maintained at 0.8% per annum. No adverse effects were noted in the coagulation profiles or in the coagulation inhibition and fibrinolysis assays of both groups. Serum high-density lipoprotein cholesterol and triglycerides were unaltered, but serum cholesterol values decreased during the six-month period of observation by 14 mg/dL (\(P < .05\)) and 11 mg/dL in the 25- and 50-mg groups, respectively. Carbohydrate and insulin metabolism was unaffected, as was the systolic and diastolic blood pressure. There were no significant intergroup differences in any of the parameters measured. The serum estradiol/estrone ratios of 1.45 and 1.59 reflected a physiologic estrogen milieu at the 25- and 50-mg dosages. Subcutaneous 17\(\beta\)-estradiol pellets can effectively maintain the bone mineral content of surgically menopausal women without inducing adverse cardiovascular side effects. (Obstet Gynecol 70:749, 1987)

Women who experience a premature surgical menopause are at increased risk of developing osteoporosis and atherosclerotic cardiovascular disease. Long-term estrogen replacement therapy has been shown to lessen the risk of menopause-related osteoporosis and, according to some, cardiovascular disease. The beneficial effect on the conservation of bone mass is lost rapidly in surgically menopausal women once estrogen therapy is stopped. In addition, orally administered estrogen is associated with alterations in biologic parameters, such as plasma renin substrate and coagulation factors, that may predispose some women to hypertension and other cardiovascular-related complications. The need for a method to ensure long-term compliance and safety is thus self-evident. Subcutaneous estradiol implants have the potential advantage of achieving patient compliance because they must be administered by a physician, usually at four- to six-month intervals. Irregular hormonal usage or noncompliance can thus be monitored easily. Furthermore, this form of therapy bypasses the enterohpatic circulation, thus avoiding the induction of hepatic factors that may have a negative effect on the cardiovascular system.

Subcutaneous 17\(\beta\)-estradiol pellets have proved effective in the management of the symptomatic menopause. However, there are very few data to document that they are as effective for the preservation of bone mass, the most important indication for long-term estrogen therapy in the menopause. The following study posed four questions: 1) Is parenterally administered 17\(\beta\)-estradiol effective in maintaining the bone mineral content of surgically menopausal women? 2) What effect has this route of administration on cardiovascular-related parameters: blood pressure, lipids and lipoproteins, coagulation and anticoagulation factors, and glucose and insulin metabolism? 3) Do the therapeutic and potential side effects of 25 mg of 17\(\beta\)-estradiol vary significantly from those associated with a 50-mg dosage? 4) Are the hormonal levels obtained by this method compatible with a "physiologic" approach to estrogen replacement therapy?

Materials and Methods

Twelve women who had each had a total hysterectomy and bilateral salpingo-oophorectomy for benign dis-
ease were admitted into the study and observed for two years. They were randomly selected to receive either 25 or 50 mg of 17β-estradiol by subcutaneous implant. Because of the remote possibility of an adverse reaction, we routinely administer a test dose of 10 mg 17β-estradiol by intramuscular injection and monitor the patient's response. No side effects were noted in the study subjects. Pellets were inserted eight or more weeks after this test injection. There were six women in the 25- and 50-mg dosage groups, closely matched for age (38.3 ± 6.9 and 35.7 ± 5.9 years), height (164 ± 6.6 and 160 ± 3.0 cm), weight (64.7 ± 16.8 and 61.2 ± 7.6 kg), and years since surgical menopause (2.5 ± 1.1 and 2.7 ± 2.0 years, respectively). Both groups had previously taken oral contraceptives for similar periods of time, 2.5 ± 1.1 and 2.7 ± 2.0 years, respectively.

We performed tests for the coagulation and anticoagulation profiles and lipid and carbohydrate metabolism at approximately 8:00 AM, after a 12-hour overnight fast. Sampling was performed before treatment (baseline) and repeated three and six months after pellet insertion. Sex steroid levels were measured at monthly intervals for six months. Bone mineral content measurements were taken and blood pressure and weight recorded at six-month intervals for two years.

Glucose tolerance was evaluated by the serum glucose and insulin response to 100 g of orally administered Glucola. Blood samples were taken before and at 30-minute intervals for two hours after the glucose stimulus. Glucose was measured by the hexokinase method, and insulin by the technique of Horowitz et al.8 We also tested an aliquot of the first sample of blood for serum cholesterol, triglycerides, and high-density lipoprotein cholesterol, as described.9 Coagulation (prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen antigen and activity: α1-antitrypsin, α2-macroglobulin, α2-antiplasmin) studies were performed on platelet-free plasma obtained from the first sample of blood and prepared by centrifugation of 9 mL of blood mixed with 1 mL of 3.8% sodium citrate solution.10 Hormones (follicle-stimulating hormone [FSH], luteinizing hormone [LH], estrone, estradiol, and total and free testosterone) were measured in serum from three batched samples obtained at 15-minute intervals, starting with the first blood specimen.10

The bone mineral content of the nondominant radius was measured by single photon absorptiometry (I-125) using the Norland Cameron Model 278 densitometer (Ft. Atkinson, WI). The midshaft and distal portions of the radius were measured at the standard one-third length and 1.5 cm proximal to the tip of the styloid process, respectively. The midshaft represents almost entirely cortical bone, whereas the distal portion contains a mixture of cortical and trabecular bone. Three measurements were recorded at each point.

After the baseline studies, we implanted one or two 25-mg pellets of crystalline 17β-estradiol (Barter Corp.) in the subcutaneous tissues of the lower abdomen, just above the groin. The pellets were inserted after local infiltration of the skin with 5 mL 2% xylacaine. Using an aseptic technique, we made a small stab incision in the skin and inserted the pellets with a Kearns's pellet implanter. Firm pressure for a few minutes was all that was required for hemostasis. The implantation site was covered with a Band-aid.

The data were examined statistically with Student's t test and linear regression analysis.

Results

In the analysis and interpretation of the results, we used subjects as their own controls and evaluated the effect of treatment by changes over a six-month or two-year period. Comparisons between each group were also made.

Both doses of estrogen maintained or increased the bone mineral content of the midshaft and distal radius. The distal bone density (bone mineral content divided by bone width) of the 25-mg group changed slightly from a baseline value of 0.436 ± 0.024 to 0.441 ± 0.023 mg/cm² at 24 months. The respective values for patients given 50 mg were 0.423 ± 0.043 and 0.432 ± 0.045 mg/cm². When these results were pooled, the mean percentage increase in the distal radial density over the two-year period was 0.8%, a change that was not statistically significant. A better response was noted at the midshaft: The first detectable increase occurred after 12–18 months of treatment. The combined improvement of the midshaft bone mineral density over two years was 1.8% (P < .03). The bone density measurements did not differ significantly between the 25- and 50-mg dosages.

Because of the known loss of bone in surgically menopausal women, we considered it inappropriate to include a comparative placebo group. Using single photon absorptiometry in an untreated surgically menopausal population, Lindsay et al noticed a 0.95% loss of bone mineral content per year. Linear regression analysis of the bone mineral content of 56 untreated surgically menopausal women who had attended the Climacteric Center's Osteoporosis Clinic, but who did not participate in the study, revealed an annual bone loss that increased with age and years since menopause from 0.4–0.8% per annum.
Table 1. Effect of 25-mg and 50-mg 17β-Estradiol Implants on Coagulation and Lipids/Lipoproteins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Baseline</th>
<th>3 mo</th>
<th>6 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin time(s) (normal 9.5-12.0)</td>
<td>25 mg</td>
<td>12.2 ± 0.24</td>
<td>12.2 ± 0.26</td>
<td>12.2 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50 mg</td>
<td>12.9 ± 0.22</td>
<td>12.9 ± 0.12</td>
<td>12.9 ± 0.17</td>
</tr>
<tr>
<td>Activated partial thromboplastin time(s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(normal 35)</td>
<td>25 mg</td>
<td>26.0 ± 1.3</td>
<td>26.0 ± 1.5</td>
<td>27.0 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>50 mg</td>
<td>32.0 ± 1.38</td>
<td>32.0 ± 0.96</td>
<td>32.0 ± 1.38</td>
</tr>
<tr>
<td>Antithrombin III activity (normal 80-120%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg</td>
<td>112.0 ± 2.1</td>
<td>108.0 ± 2.9</td>
<td>105.0 ± 2.5</td>
<td>106.0 ± 2.8</td>
</tr>
<tr>
<td>50 mg</td>
<td>108.0 ± 4.1</td>
<td>106.0 ± 1.4</td>
<td>106.0 ± 2.8</td>
<td>106.0 ± 2.8</td>
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<tr>
<td>Plasminogen activity (CTA U/mL) (normal 2.4-3.8)</td>
<td>25 mg</td>
<td>4.2 ± 2.6</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50 mg</td>
<td>3.1 ± 0.3</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>α-antiplasmin activity (normal 80-120%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg</td>
<td>112.0 ± 12.1</td>
<td>107.0 ± 10.6</td>
<td>97.0 ± 7.4</td>
<td>97.0 ± 7.1</td>
</tr>
<tr>
<td>50 mg</td>
<td>92.0 ± 10.7</td>
<td>96.0 ± 5.2</td>
<td>95.0 ± 7.1</td>
<td>95.0 ± 7.1</td>
</tr>
</tbody>
</table>

Lipids/lipoproteins

| Cholesterol (normal 130-250 mg/dL)              |       |                |              |              |
| 25 mg                                          | 180 ± 14 | 167 ± 7       | 166 ± 12*    | 166 ± 12*    |
| 50 mg                                          | 195 ± 15 | 182 ± 13      | 185 ± 16     | 185 ± 16     |
| Triglycerides (normal 40-170 mg/dL)            |       |                |              |              |
| 25 mg                                          | 68 ± 14  | 72 ± 18       | 82 ± 21      | 82 ± 21      |
| 50 mg                                          | 113 ± 26 | 95 ± 25       | 110 ± 31     | 110 ± 31     |
| HDL cholesterol (normal 36-80 mg/dL)           |       |                |              |              |
| 25 mg                                          | 51 ± 4  | 49 ± 3        | 48 ± 2       | 48 ± 2       |
| 50 mg                                          | 46 ± 5  | 41 ± 3        | 48 ± 2       | 48 ± 2       |

CTA = cyano-trimethyl-androstosterone; HDL = high-density lipoprotein.
* P < .05.

The lipid and lipoprotein profile of the two groups responded favorably to both the 25-mg and the 50-mg doses. The serum cholesterol decreased from 180 ± 14 to 166 ± 12 mg/dL (P < .05) and from 195 ± 15 to 185 ± 16 mg/dL in the 25- and 50-mg groups, respectively. The latter difference was not statistically significant. The serum triglyceride and high-density lipoprotein cholesterol values were maintained throughout the study (Table 1).

No differences in prothrombin times, activated partial thromboplastin times, or thrombin times were observed between the 25- and 50-mg groups. The mean fibrinogen activity decreased in the women in the 25-mg group over the six-month period of observation, from 434 ± 58.6 to 390 ± 30.8 to 377 ± 32.9 mg/dL, but this trend was both statistically nonsignificant and well within the range of normal for our laboratory. We noted no changes in factors associated with inhibition of coagulation either between or within the groups. The antithrombin III activity in the 25-mg group subjects decreased slightly from 112% of normal to 105% of normal, but this change was not significant. Fibrinolysis was assessed by plasminogen antigen and activity and by α2-antiplasmin activity. No significant changes occurred.

Glucose tolerance was normal in both groups. One subject in the 25-mg group was an insulin “hypersecretory,” resulting in insulin values in these subjects significantly greater than in the 50-mg group (P < .01). Carbohydrate metabolism was assessed by the glucose tolerance and insulin curves, by the areas under the curve for glucose and insulin, and by the cumulative insulinogenic index. The latter is calculated by dividing the area of insulin under the curve by the area of glucose under the curve. Absolute values at all of the measure points—baseline and 30, 60, 90, and 120 minutes after a glucose load—were within normal limits. The shape of the curve improved in the 25-mg group as treatment progressed, with both the glucose and insulin values approaching fasting values more closely. The insulinogenic index, however, remained the same at 0.6. The 50-mg group maintained normal glucose tolerance more efficiently, with an insulinogenic index of only 0.4. Their insulin pattern also appeared to improve qualitatively with estrogen therapy (eg, the two-hour mean insulin level decreased from the baseline tests of 50 to 34 μU/mL at the six-month test interval). The fasting values were identical at 14 μU/mL (Figure 1).

The serum estradiol values increased significantly in both groups (P < .0005). This resulted in a reversal of the original 25-mg group estradiol/estrone ratio of 0.71 to a six-month value of 1.45. The baseline estradiol/estrone ratio in the 50-mg group (1.19) also improved to 1.59 at six months. Figure 2 reflects these changes and illustrates stable postimplantation blood levels. Serum testosterone values were suppressed by approximately 25% from the baseline. This was also reflected in decreased free testosterone values: a mean decrease of 0.9 ng/mL in the 25-mg group (P < .05) and a lesser and statistically nonsignificant drop of 0.4 ng/mL in the 50-mg group. Dihydrotestosterone values were unchanged. Expected decreases occurred in both
FSH and LH, LH by 56 and 51% in the 25-mg and 50-mg groups, respectively.

The systolic and diastolic blood pressures of the women in both groups were unaffected, but we noted an average increase of 1.35 kg in weight over a two-year period (P < .09).

Discussion

Using single photon absorptiometry, investigators have demonstrated that the bone mineral density of the distal radius decreases by 1.01% and that of the midshaft by 1.04% per year in women between the ages of 50-65 years. This “normal” bone loss pattern is exaggerated in women experiencing a surgical menopause; thus patients who have had an oophorectomy before the menopause have a greater risk of osteoporosis. The exogenous use of estrogens maintains the bone mineral content and may even result in an accrual of bone. However, withdrawal of estrogen therapy results in a rapid loss of bone mineral content, with values four years after cessation of therapy indistinguishable from those in women who had never received estrogen therapy.

The present study has confirmed the bone-conserving effects of 17β-estradiol implants. The overall 1.8% gain in midshaft bone mineral content and the 0.8% maintenance of the distal shaft areas is compatible with other studies that have used single photon absorptiometry. For example, Christiansen et al. using rectilinear scanning, showed a bone gain of 1.2% in women treated with an estrogen/progestogen combination and a loss of 1.9% in those receiving a placebo over a three-year period. In the present study, we observed an improvement in bone mineral content with both dosage schedules—25 mg and 50 mg of

Figure 1. The plasma insulin response to a glucose load before and six months after the insertion of either 25-mg (closed circles) or 50-mg (open circles) pellets in surgically menopausal women. Values are measured in μU/mL (mean ± SD); numbers in parentheses represent the total insulin area under the curve (μU/mL/minute) for the respective groups.

Figure 2. Monthly plasma estradiol and free testosterone levels after the injection of either 25-mg (closed circles) or 50-mg (open circles) 17β-estradiol estrogen pellets. Values are mean ± SD.
17\beta-estradiol. These doses resulted in serum estradiol values between 67-92 and 120-131 pg/mL, respectively—levels equivalent to those in the early, midfollicular, and late luteal phases of normal premenstrual women.\(^1\) The estradiol/estrone ratios of 1.42 for the lower dosage and 1.59 for the 50-mg dosage reflect the physiologic estrogen milieu achieved with the implants.

The menopause, whether induced surgically at an early age or achieved biologically during the middle years, is associated with a dramatic change in the lipoprotein moiety; high-density lipoprotein cholesterol concentrations decrease and low-density lipoprotein cholesterol increases.\(^2\) Fahraeus et al.\(^1\) in a study of postmenopausal women receiving either percutaneous estradiol cream or oral micronized estradiol, found that high-density lipoprotein cholesterol was increased after the oral estrogen but unaffected by the estrogen cream. Using 50 mg of 17\beta-estradiol implants, Farish et al.\(^1\) found only minimal alterations in the lipoprotein metabolism, with a slight rise in high-density lipoprotein due primarily to an increase in the nonatherogenic HDL\(_3\) subfraction. This result is consistent with our findings. Some studies have found that estrogen raises serum triglycerides and lowers cholesterol. Our results showed a modest decrease in serum cholesterol—14 mg in patients receiving 25 mg of estradiol and 11 mg in those given 50 mg of estradiol—and a maintenance of the serum triglycerides. Burger et al.\(^1\) treated 17 patients with a combination of 50 mg of estradiol and 100 mg of testosterone pellets, and observed no changes in triglycerides, total cholesterol, or their subfractions.

Exogenous estrogens exert a biphasic effect on carbohydrate metabolism; a deterioration in glucose tolerance is found in about 40\% of menopausal women on estrogen therapy.\(^1\) Although the insulin levels are usually normal, the response to oral estrogen is frequently delayed, with the peak insulin value shifted to the right. The mechanism for this is not known. The qualitative response to a glucose stimulus, as assessed by the shape of the curve for both glucose and insulin, improved in both groups of subjects. The six-month results reflect this in the return to the fasting levels of the two-hour blood glucose levels, the shift in the peak insulin values to 30 minutes in the 25-mg group subjects, and the lowering of the two-hour plasma insulin values in the 50-mg group. The insulinogenic index was unaffected by the pellet implants at all three time intervals. In view of the progressive improvement in the glucose tolerance curve and the normal insulinoergic indices, one may conclude that estrogen implants are unlikely to have either glucogenic potential (reversible elevation in glucose) or diabetogenic potential (permanent alteration in glucose tolerance).

Despite the recognized association between oral contraceptive usage and myocardial infarction and stroke in older premenopausal women, a similar relationship has not been noted in postmenopausal women on hormone replacement therapy.\(^1\) It was reassuring to note that both the 25- and 50-mg dosages of 17\beta-estradiol had an imperceptible effect on our subjects' coagulation-anticoagulation profile. This is consistent with earlier work from our laboratory and may be explained by the following: 1) The menopause per se appears to induce a relative anticoagulant/fibrinolytic state\(^1\); 2) the type of estrogen used is "natural" and is much less potent than that used in oral contraceptives; 3) parenterally administered estrogens are less likely to stimulate factors synthesized by the liver; and 4) the blood levels of estrogen obtained were within the range expected for premenopausal women.

Although orally administered estrogens are known to stimulate renin substrate, prospective studies have shown oral estrogens to cause, variously, no effect or a slight increase in blood pressure, or even moderate hypotension.\(^2\) The blood pressure response in both of our study groups was unaffected throughout the period of observation. A slight increase in weight (± 1.35 kg) was noted with both implant dosages; although this conflicts with the experience with oral estrogen usage, an increase in weight has been reported with combination hormone therapy. The cause for the weight increase in our subjects was not established, and did not require specific therapy.

Both the 25- and the 50-mg estradiol implants resulted in plasma levels of 17\beta-estradiol equivalent to those of normal premenopausal women.\(^1\) The total and free testosterone values were significantly reduced, but both alterations were within our laboratory's range of normal. Furthermore, all subjects exhibited an excellent symptomatic response, with 42\% actually reporting an improvement in libido. Because decreased libido is a frequent menopausal complaint, some authors routinely combine estrogen with testosterone pellets.\(^5\) Our subsequent clinical experience has confirmed that 17\beta-estradiol pellets alone improve libido and generally enhance sexuality and sexual enjoyment.

The main indication for the long-term usage of estrogen replacement therapy is to prevent osteoporosis. It would be inaccurate and inappropriate to extrapolate the experience of 12 women treated with estrogen implants over a two-year period. Nevertheless, this detailed prospective study has shown that estrogen replacement with subcutaneous implants maintains the bone mineral content of the radius over
a limited period and is free from the potential metabolic disturbances and cardiovascular complications sometimes associated with oral estrogen therapy.

References


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THE BRAIN


- HRT and particularly ERT plays an efficacious role in preventing neurodegenerative conditions.
- 17B Estradiol reduced risk for Alzheimer’s disease.
- Minimizes cognitive decline in otherwise healthy women
- Estradiol (E2) protects against B-amyloid induced degeneration. Progestins may actually dampen this affect.
- Compared to E2 users vs Non-users E2:

For avg. 15 years had increased cerebral blood flow
Review Article

Hormone Replacement Therapy and Risk for Neurodegenerative Diseases

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Over the past two decades, there has been a significant amount of research investigating the risks and benefits of hormone replacement therapy (HRT) with regards to neurodegenerative disease. Here, we review basic science studies, randomized clinical trials, and epidemiological studies, and discuss the putative neuroprotective effects of HRT in the context of Alzheimer’s disease, Parkinson’s disease, frontotemporal dementia, and HIV-associated neurocognitive disorder. Findings to date suggest a reduced risk of Alzheimer’s disease and improved cognitive functioning of postmenopausal women who use 17β-estradiol. With regards to Parkinson’s disease, there is consistent evidence from basic science studies for a neuroprotective effect of 17β-estradiol; however, results of clinical and epidemiological studies are inconclusive at this time, and there is a paucity of research examining the association between HRT and Parkinson’s-related neurocognitive impairment. Even less understood are the effects of HRT on risk for frontotemporal dementia and HIV-associated neurocognitive disorder. Limits to the existing research are discussed, along with proposed future directions for the investigation of HRT and neurodegenerative diseases.

1. Introduction

Hormone replacement therapy (HRT), defined here as use of various types of estrogen alone or in conjunction with progestins (synthetic or exogenous progestogen), has long been studied as a possible prophylactic against Alzheimer’s disease. While the association between HRT and Alzheimer’s disease has been explored through several observational and randomized clinical trials to date, the relationship between HRT and other neurodegenerative diseases has received relatively little attention. In this review, we explore the body of research on HRT as a prophylactic against various neurodegenerative conditions, including Alzheimer’s disease, Parkinson’s disease, frontotemporal dementia, and HIV-associated neurocognitive disorder. In reviewing observational studies, randomized clinical trials, and basic science studies, we find evidence that some forms of HRT are neuroprotective, resulting in preservation of cognitive abilities in healthy postmenopausal women, improvement of Parkinson’s symptoms, and variably altering risk of neurodegenerative disease.

2. Alzheimer’s Disease

Alzheimer’s disease (AD) represents the most common neurodegenerative disease, accounting for more than 50% of all dementia types [1]. Within the United States alone, national prevalence estimates indicate that AD affects 2.4 million individuals aged 70 and older [1, 2]. With increasing age, AD progressively affects more individuals, affecting 2.5% of those aged 71–79, 18% of those aged 80–89, and 30% of those aged 90 and older [1, 2].

Cognitive decline in AD is characterized by insidious onset and gradual progression over a course of several years [3–5]. Clinical research has identified subtle losses
2.1. Estrogen and Risk for AD—Observational Studies. Incidence rates indicate the risk of AD among women is double that of men after the age of 80, even after controlling for protective factors such as education [11]. The higher incidence rate of AD among women have led to explorations on the association between estrogen deficiency and AD.

Observational studies have examined both HRT and estrogen replacement therapy (ERT), or estrogen alone, in relation to incidence of AD (see Table 1). For instance, in a sample of 514 women enrolled in the Baltimore Longitudinal Study of Aging, Kawasaki et al. found that ERT was associated with significantly reduced risk for AD [12]. Although the duration of use ranged between 1–15 years, the data did not show a significant effect for duration of ERT in addition, no effect was observed for age of menopause. In another observational study reported by Tang et al., ERT was also associated with significantly reduced risk for AD in a sample of 1124 women enrolled in the Manhattan Study of Aging [13]. Here, however, an inverse relationship was observed for duration of use and risk for AD, with the lowest risk noted for women taking estrogen for longer than one year. Other observational studies have provided moderate support for decreased AD risk with ERT and the importance of duration of use. Using retrospective data on a sample of 355 women, Paganini-Hill and Henderson found that ERT was associated with moderately reduced risk for development of AD [14]. An inverse relationship was seen for duration of ERT and risk for all-cause dementia (AD as well as other causes of dementia), with those on ERT for seven or more years having the lowest risk for AD. While findings from these observational studies suggest that ERT may reduce risk of AD, given the nature of observational studies the findings may be affected by several biases. Specifically, the women who decided to take ERT for several years may have been healthier to begin with; they may have also been more proactive in seeking early postmenopausal treatment due to higher education and/or availability of resources. An additional criticism is the lack of controls in the studies; for instance, all observational studies described above involved varied ERT regimens among all participants rather than a uniform ERT regimen. Thus, the findings of the observational studies present with several limitations.

Although all of the studies examined above have included women who underwent natural menopause, recent observational studies have examined the differences between women who underwent natural versus surgical menopause [15]. In one, women who had surgical menopause demonstrated an increased long-term risk for cognitive impairment compared to women with natural menopause. In another paper based on the same data, the same researchers reported a linear trend, with increased risk seen with younger age at oophorectomy (bilateral or unilateral) [16]. These findings suggest that earlier age of surgical menopause increased risk of cognitive impairment and that estrogen deficiency may initiate risk for neurological diseases such as AD. Notably, the researchers also found increased risk of depression and cardiovascular disease among women with history of bilateral oophorectomy, suggesting that the relationship between surgical menopause and cognitive impairment may be multifactorial [17, 38].

2.2. Randomized Clinical Trials of HRT in Healthy and At-Risk Women. While observational studies generally support a neuroprotective role for ERT against AD, the results of randomized clinical trials (RCTs) have been equivocal. To date, the largest study has been the Women’s Health Initiative Memory Study (WHIMS), an ancillary study of the Women’s Health Initiative (WHI), a prospective study that enrolled 7479 postmenopausal women [39, 40]. A total of 4532 women with natural menopause (intact uterus) were randomized into a trial comparing conjugated equine estrogen (CEE) + medroxyprogesterone (MPA) versus placebo [40]. However, the trial was discontinued before completion due to unexpected health risks. Despite the early termination, data revealed that women who received CEE + MPA demonstrated greater cognitive decline compared to the placebo group [40]. Additional analyses revealed that risk for dementia was doubled for women who received CEE + MPA compared to the placebo group [39]. Taken together, data from the WHIMS demonstrated a higher incidence of dementia and greater cognitive decline among hormone users relative to placebo groups.

Although the WHIMS has been considered one of the largest and longest randomized studies examining HRT and cognitive deficits, generalizability of the findings is affected by several limitations. First, external validity of the WHIMS findings has come into question, as the participants in the treatment group were at high risk for cardiovascular and cerebrovascular disease; thus the higher rates of dementia may have been attributed to vascular disease. Second, in their analyses of the WHIMS data, the researchers included all dementia types into an “all-cause” dementia that included AD, vascular dementia, dementia due to Parkinson’s disease, and frontotemporal dementia, thus limiting the interpretation of results. Third, a methodological limitation included the unavailability of baseline cognitive measures prior to
treatment; thus, participants may have already been cognitively impaired prior to beginning HRT. Still another criticism has been the age of the participants; participants were age 65 or older, at least a decade past the average age of menopause. Together, these limitations have called into question the validity of the WHIMS findings, suggesting that the WHIMS may not be the best model for understanding the effect of HRT on Alzheimer’s disease.

Another limitation in the generalizability of the WHIMS involves the type of HRT that was used. Specifically, it has been pointed out that CEE does not contain the hormone 17β-estradiol, [41] the estrogen compound that has been shown in basic science studies to be neuroprotective [42–44]. In addition, the greater rates of dementia seen among participants of the CEE + MPA study trial of the WHIMS suggest that simultaneous use of MPA may present additional risk [45]. Indeed, consistent with WHIMS findings, a recent randomized-controlled study by Maki et al. found that women receiving CEE + MPA for four months demonstrated mild declines in verbal memory compared to women receiving placebo [21]. Additionally, a recent comparison of several different HRT types has provided some insight into which treatment provides the most cognitive benefit. Using functional neuroimaging as an outcome measure, Silverman et al. compared the cerebral metabolic activity associated with three hormone regimens over the course of one year: 17β-estradiol (E2), CEE, and CEE + progesterin [24]. Results revealed that the E2 group performed significantly better on verbal memory than the CEE group. This group also demonstrated higher metabolism in the receptive language and auditory association areas. Additionally, the CEE + progesterin group demonstrated lower metabolism in areas associated with long-term memory storage (i.e., mesial and inferior lateral temporal regions) compared to the CEE group. Taken together, these findings suggest that E2-based therapies may provide the most beneficial neuroprotective effect. In addition, the Silverman et al. study suggests that combination therapies that include progestin may actually dampen the beneficial effects of estrogen.

Since the discontinuation of the WHIMS trials, the case for ERT in reducing the risk for AD and improving the cognitive functioning of postmenopausal women has continued to gain at least modest support through further RCTs. Indeed, results from several RCTs published in the past few years have demonstrated support that E2 formulations are associated with a reduced amount of decline in verbal memory among healthy postmenopausal women when compared to controls. The benefits of these treatments have been observed in trials with durations ranging from three months to two years (see Table 2) [18, 22–24]. In contrast, at least one study has found no benefit on verbal memory associated with E2 compared to placebo [20]; however, it was noted that the women in that study used E2 for only two months. Thus, it is possible that the effects of E2 on verbal memory may be evident only after three months or more. In a separate study, Joffe et al. found that E2 was not associated with an improvement in verbal memory scores but rather decreased likelihood for errors during the memory tasks [19]. Specifically, women on E2 demonstrated less perseverative errors during recall tasks compared to women on placebo. These women, as a group, were also less likely to demonstrate an interference effect when retaining previously learned information. Thus, although E2 was not found to enhance verbal memory scores per se, the authors concluded that E2 enhanced verbal information processing by decreasing the forgetfulness of a response already given [19].

2.3. Neuropathological and Neurophysiological Studies of HRT: Relevance to AD. While results of recent RCTs show modest support for a beneficial effect, evidence from histopathological and neurophysiological studies has provided stronger support for estrogen’s neuroprotective effects, particularly for the neurodegenerative disease process thought to underlie AD [46–48]. Neuroimaging and autopsy results have

### Table 1: Observational studies of ERT and risk for dementia.

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Sample description</th>
<th>Overall findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paganini-Hill and Henderson [14]</td>
<td>355 postmenopausal women (165 users; 190 nonusers) with a mean age of 86.5 years at death; retrospective data from the Leisure World, Laguna Hills cohort</td>
<td>ERT (not specified) for 1–7 years was associated with reduced risk for AD (OR: 0.67, CI 95% 0.38–1.17) compared to nonusers. Risk for AD decreased with longer duration of use.</td>
</tr>
<tr>
<td>Tang et al. [13]</td>
<td>1124 healthy postmenopausal women (156 users; 968 nonusers), with a mean age of 74.2, enrolled in the Manhattan Study of Aging</td>
<td>After controlling for age, education, and ethnicity, ERT (majority used CEE) for 6–8 years was associated with lower risk for AD (OR 0.50, 95% CI, 0.25–0.90) compared to nonusers. Risk for AD decreased with longer duration of use.</td>
</tr>
<tr>
<td>Kawas et al. [12]</td>
<td>514 healthy postmenopausal women (230-users; 242-non-users), with a mean age of 65.5, enrolled in the Baltimore Longitudinal Study of Aging</td>
<td>After controlling for education, ERT (not specified) for 1–10 years was associated with lower risk for AD (OR: 0.46, 95% CI, 0.21–0.99) compared to non-users. No effect was observed for duration of use.</td>
</tr>
<tr>
<td>Rocca et al., [15–17]</td>
<td>813 women with unilateral oophorectomy, 676 women with bilateral oophorectomy, and 1,472 women who did not undergo oophorectomy.</td>
<td>Women who underwent oophorectomy (unilateral or bilateral) before onset of menopause were at increased risk for cognitive impairment or dementia (OR: 1.46, 95% CI, 1.13–1.90) compared to women who did not undergo oophorectomy.</td>
</tr>
</tbody>
</table>
**Table 2: Randomized clinical trials of HRT and verbal memory.**

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Hormone treatment used</th>
<th>Sample size</th>
<th>Age</th>
<th>Outcome measure</th>
<th>Overall findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagger et al., [18]</td>
<td>E2 2 mg + varied progestins versus placebo for 2 years</td>
<td>261</td>
<td>54.1</td>
<td>Cognitive screening task</td>
<td>Followup study of women randomized 5, 10 and 15 years earlier to HRT or placebo during clinical trials. Logistic regression showed that for women who received HRT for 2-3 years, the relative risk for cognitive impairment was significant decreased by 64% compared to the never users. Long-term/current users of HT also demonstrated a decreased risk of 66% compared to the never users. Women on E2 had fewer perseverative errors during verbal recall when placebo-treated women. Women on E2 also showed greater retention of new information without interference.</td>
</tr>
<tr>
<td>Joffe et al. [19]</td>
<td>E2 0.5 mg versus placebo for 12 months</td>
<td>52</td>
<td>40–60</td>
<td>Verbal memory; Functional MRI</td>
<td>Women on estrogen therapy did not show higher cognitive performance on verbal memory tasks compared to women on placebo.</td>
</tr>
<tr>
<td>LeBlanc et al., [20]</td>
<td>Estradiol 2 mg versus placebo for 2 months (CEE) + medroxyprogesterone acetate (MPA) versus placebo for 4 months</td>
<td>32</td>
<td>53.26 (treatment) 52.08 (placebo)</td>
<td>Verbal memory</td>
<td>Modest negative effects on verbal memory (short- and long-term recall) were found in the HRT versus placebo group.</td>
</tr>
<tr>
<td>Maki et al., [21]</td>
<td>E2 0.5 mg versus placebo for 12 months</td>
<td>158</td>
<td>51.9 (treatment) 52.4 (placebo)</td>
<td>Verbal memory</td>
<td>All women were administered the antimuscarinic drug scopolamine (SCOP) or placebo. E2 pretreatment significantly decreased the anticholinergic drug-induced impairments on verbal memory task for the younger group only compared to the older group.</td>
</tr>
<tr>
<td>Dumas et al. [22]</td>
<td>E2 2 mg versus placebo for 3 months</td>
<td>22</td>
<td>50-62 (younger) 70–81 (older)</td>
<td>Verbal memory</td>
<td>Women on E2 who scored at or above average showed less decline in delay verbal memory compared to women on placebo.</td>
</tr>
<tr>
<td>Tierney et al. [23]</td>
<td>E2 1 mg versus placebo for 2 years</td>
<td>142</td>
<td>61–87</td>
<td>Verbal memory</td>
<td>Women on E2 had significantly higher verbal memory than CEE and showed higher metabolism in Wernicke’s and auditory association. E2 was also associated with higher metabolism in mesial and inferior lateral temporal regions and inferior frontal cortex compared to PE.</td>
</tr>
<tr>
<td>Silverman et al. [24]</td>
<td>17β-estradiol (E2) versus conjugated equine estrogen (CEE) versus CEE + P for 1 year</td>
<td>53</td>
<td>50–65</td>
<td>Verbal memory; FDG-PET</td>
<td>---</td>
</tr>
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</table>

indicated that β-amyloid and tau proteins are involved in the structural changes that lead to AD pathology, particularly in the hippocampus and other medial temporal regions, as well as the parietal and frontal cortical regions [49]. Evidence has shown that estrogen (particularly E2) provides protection against β-amyloid-induced damage and tau-related changes [50]. Observational and RCT studies that also utilized neuroimaging outcomes have also been supportive of the benefits of 17β-estradiol, particularly in the brain regions that show preclinical abnormalities in individuals who are at risk for AD. For instance, as mentioned earlier, E2 has been associated with higher metabolism in language processing and auditory association areas compared to other HRT regimens (CEE or CEE + MPA) [24]. However, observational studies and RCTs have also demonstrated support for varied ERT regimens. Compared to nonusers, long-term ERT (E2 or CEE for an average of 15 years) has been associated with increased cerebral blood flow to the hippocampus and left superior temporal gyrus at a two-year followup [51]. Further, compared to placebo, a four-month trial of ethinylestradiol and progestin was associated with increased activation in brain regions associated with the left middle/superior frontal cortex, and left inferior parietal cortex during verbal memory encoding tasks on functional magnetic resonance imaging [52]. Finally, in another study, long-term users of ERT (E2 or CEE for an average of 18 years) demonstrated higher
density of muscarinic receptors in the hippocampus and prefrontal cortex than individuals who had never used ERT, suggesting that one of the neuroprotective effects of E2 or other ERT regimens could also include the maintenance of the cholinergic system in the hippocampus and frontal cortex [48].

A recently proposed explanation may explain the inconsistent results of the aforementioned observational studies and RCTs. Known as the “healthy-cell bias” [53], the hypothesis is that E2 may selectively benefit healthy neurons. In the context of human studies, based on the findings from observational studies and RCTs, this hypothesis predicts that E2 can be protective if initiated before or during times of neuronal stress, but harmful if given after the cells have progressed toward degeneration. In their study, Chen et al. administered E2 to rat hippocampal neurons exposed to β-amyloid, using varied doses and dose schedules (acute versus continuous versus intermittent). Data indicated that neurodegeneration was prevented when E2 was administered before or during β-amyloid exposure, and a continuous dose was found to demonstrate the strongest effects. In contrast, exposure to higher doses of E2 actually worsened neuronal death when β-amyloid was present. Additionally, E2 administered after β-amyloid exposure exacerbated neuronal death. It was concluded that the best E2 dosing was pretreatment and continuous exposure to prevent degeneration. Consistent with the “healthy-cell bias” hypothesis, Dumas et al. demonstrated a selective benefit of 17β-estradiol toward cognitively intact women [22]. A group of 142 postmenopausal women (age range: 61–87) were randomized to receive E2 (n = 70) or placebo (n = 72) for two years. Verbal memory was assessed at baseline and at 1-year and 2-year followup. Results revealed that women who received E2 and who performed at or above average on verbal recall at baseline demonstrated higher scores at the 1-year and 2-year followup compared to the placebo group. In contrast, women who received E2 and performed below average on verbal recall at baseline showed no significant difference compared to the placebo group. Dumas et al. concluded that these findings provided support to the healthy cell bias hypothesis, as they considered it improbable that women with a normal score or better had significant neurodegenerative changes [22]. Notably, the women who benefitted from estrogen exposure were age 70 (average) and approximately 20 years postmenopause, suggesting that older women who have intact verbal memory can benefit from a new regimen of ERT late in life, as long as they have not demonstrated memory impairment. Interestingly, basic science research has supported the biased neuroprotective effect of E2 toward healthy individuals; in fact, the presence of apolipoprotein E4 (APOE4) genotype has been found to reduce the neuroprotective role of E2 in an animal model [54]. Thus, an alternative explanation for the findings of Dumas et al. could be that the women who demonstrated lower than average recall at baseline may have had the APOE4 genotype; in turn, they may have not experienced the neuroprotective effects of ERT. The healthy cell bias hypothesis also helps explain the finding, reported in most observational studies, of an inverse relationship between length of HRT treatment and risk for AD.

Other investigators have hypothesized that there may be a “critical period” for postmenopausal women during which 17β-estradiol selectively provides a beneficial effect for younger as opposed to older women with an intact uterus [41, 55]. This hypothesis has also received support from at least one RCT. For example, LeBlanc et al. randomized 22 postmenopausal women to receive either E2 or placebo for 3 months [20]. At the end of the trial, the antimuscarinic drug scopolamine (SCOP) was administered before a verbal task to initiate anticholinergic-induced memory impairment. Results showed that E2 pretreatment significantly decreased the anticholinergic-induced impairment on the verbal memory task for the younger group (age 50–62); however, the benefit of E2 was not observed in the older group (age 70–81). Interestingly, the beneficial effects of E2 were only observed during the anticholinergic challenge with SCOP and not during the placebo challenge. LeBlanc et al. concluded that younger women benefit from E2 more than older women, and that the benefits of E2 in younger women may be observed only when the cholinergic system is temporarily disrupted. Consistent with this finding is that younger women have a higher density of muscarinic receptors than older women, and thus may be more sensitive to cholinergic changes [48]. Thus, it is plausible that the women in the aforementioned WHIMS may have been past the “critical period” for the beneficial effects of E2.

2.4. Summary—AD. Taken together, the findings from studies employing a variety of methods demonstrate that some forms of ERT are neuroprotective, resulting in preservation of cognitive abilities and reduced risk of AD. While some studies have affirmed that young and healthy postmenopausal women may benefit the most from estrogen exposure, other studies have suggested that older and healthy women with intact verbal memory can also benefit from estrogen. The consistent findings from the observational studies reviewed above seem to be that ERT (most commonly CEE), with a minimal duration of at least one year, is beneficial in reducing risk for AD among healthy postmenopausal women. Although benefits have been observed among varied regimens (CEE, CEE + P, E2) [48, 51, 52]; the most beneficial estrogen formulation seems to be E2 unopposed by progestin [24, 50]. Randomized clinical trials in healthy, postmenopausal women have suggested that E2 has been most beneficial in reducing cognitive decline, particularly verbal memory, which is the predominant symptom of early AD [18, 22–24]. Additionally, both observational and RCT studies utilizing neuroimaging outcomes have been supportive of the benefits of E2, particularly in the brain regions that show preclinical abnormalities in individuals who are at risk for AD [21, 24, 51, 52].

3. Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative disorder after AD, with an estimated
prevalence of 0.3% in the general population. Risk increases with age, with a prevalence of 1% in those over 60, and 4% in those 80 years and older [56]. Many, but not all, studies have reported higher risk for PD and younger age of onset in males [57–66]. This observation, along with the fact that the neuropathological process underlying PD commonly begins before menopause, suggests that estrogen may play a modulatory role. In addition, estrogen has a direct modulatory affects on dopaminergic functioning [67]. Together, these observations suggest a potential protective effect of estrogen against PD, or ameliorative impact on symptoms.

3.1. Estrogen and PD Symptoms. A variety of studies have addressed the impact of estrogen on PD. Perhaps the most indirect are observational studies of PD symptoms during the menstrual cycle. Early studies in the 1980s reported that some female patients with PD had fluctuations in motor symptoms that paralleled presumed fluctuations in endogenous estrogen levels [68, 69], with presumably lower levels of estrogen associated with greater motor symptoms. However, more recent studies have shown mixed results. Kompoliti et al. did not find significant correlation between endogenous hormone levels and motor examination in the "off" state (a state of decreased mobility as a result of nonresponsiveness to medication) among female PD patients examined at various times during their menstrual cycle [70].

A small number of prospective studies of ERT and PD have also been reported, with mixed results (Table 4). Strijks et al. did not find a significant dopaminergic effect in their 8-week placebo-controlled, randomized, double-blind trial of estrogen (E2) in 12 postmenopausal female patients under the age of 80 [35]. However, an 8-week double-blind, parallel-group, prospective study using Premarin (CEE) versus placebo in PD patients with motor fluctuations showed a statistically significant improvement in "off" times (i.e., when dopamine agonist medications have diminished efficacy) among the estrogen treated group [36]. Further, another double-blind, placebo-controlled crossover study of high-dose transdermal E2 in 8 postmenopausal women with mild-to-moderate PD demonstrated a slight anti-Parkinsonian effect without significantly worsening dyskinesias [34].

Although the overall symptomatic effect of ERT on PD remains unclear, these early studies raised the possibility that some forms of estrogen may mitigate the symptoms of PD. Despite this early optimism, a more recent multicenter, randomized, double-blind, placebo-controlled, pilot trial of CEE in postmenopausal women with PD experiencing motor fluctuations did not find any benefit of ERT in ameliorating symptoms [37]. In that study, 23 women received either 0.625 mg/day of CEE or matching placebo for 8 weeks. None of the outcome measures, including changes from baseline to study completion in Unified PD Rating Scale scores, "on" time (i.e., duration that dopamine agonist medication is effective), dyskinesia ratings, and results from neuropsychological testing, were significantly different between the placebo and treatment groups, although the authors emphasized a nonsignificant trend of improvement on the total and motor scores of the Unified PD Rating Scale. It is conceivable that the null findings were due to the small sample size; however, the existing literature on ERT and PD symptoms remains equivocal at this time.

3.2. HRT and Observational Studies of PD Risk. Epidemiological studies of the protective effects of HRT against PD have been mixed as well (Table 3). The relationship between lifetime reproductive events and PD was examined by Martignoni et al. Comparing a large sample of women diagnosed with PD to healthy controls, they found that the duration of reproductive life was similar between the two groups [28]. Time and mode of menopause onset were also similar between the groups; however, women with PD reported less access to HRT. In addition, the PD group overall reported more premenstrual symptoms, fewer deliveries and abortions, and less use of contraception, indicating a relationship between PD and reproductive events. Benedetti et al. reported a case-control study in which women with PD had an earlier reported age of menopause, a higher frequency of hysterectomies, and lower occurrence of HRT [27]. Further, Currie et al. found that ERT in postmenopausal women was associated with a significantly reduced risk of developing PD [29], and Ragonese et al. found that factors reducing estrogen stimulation during life were associated with development of PD [30]. Specifically, PD was significantly associated with shorter fertile life lengths (<36 years) and a longer cumulative length of pregnancies (>30 months). This group later reported a significant correlation between age of PD onset and both age at menopause and fertile life duration [32]. Despite these findings, others have found contrary results. Popat et al. found that the association of postmenopausal HRT and PD risk depended on the type of menopause [31]. Among women with history of hysterectomy (with or without an oophorectomy), ERT use was associated with a 2.6-fold increased risk for PD, and a trend for additional risk was noted for increasing duration of estrogen use. Conversely, among women with natural menopause, no increased risk of PD was observed with HRT (ERT alone or in conjunction with progesterin). Contrary to the findings of Benedetti et al., earlier age of menopause was associated with reduced risk of PD. Further, Simon et al. recently reported results of a 22-year prospective study of 244 participants in the Nurses’ Health Study who developed PD [33]. Among their sample, risk of PD was not significantly associated with reproductive factors or HRT use. However, they did find that use of HRT may modify the associations of smoking and caffeine with PD risk; specifically, the inverse relationship between caffeine use and risk of PD was observed only in non-HRT users. Further, whereas the researchers also reported an inverse relationship between pack-years of smoking and risk of PD for both HRT users and nonusers, risk was reduced more in the latter group. As such, HRT use appeared to attenuate the observed beneficial effects of caffeine use and tobacco smoking. Of note, this study did not separately analyze the data based on type of HRT.
<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Sample description</th>
<th>Overall findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marder et al. [25]</td>
<td>87 women with Parkinson’s disease without dementia (PDND), 80 women with Parkinson’s disease with dementia (PDD), and 989 nondemented healthy women.</td>
<td>ERT reduced risk of dementia among the PD-only sample (OR = 0.22, 95% CI: 0.05–1.0), and also when PDD patients were compared to healthy controls (OR = 0.24, 95% CI: 0.07–0.78). ERT did not affect the risk of PD.</td>
</tr>
<tr>
<td>Fernandez and Lapane [26]</td>
<td>Data from 10,145 elderly women with PD available via the Systematic Assessment in Geriatric drug use via Epidemiology (SAGE) database. Included 195 women with PD who received estrogen and 9950 who did not receive estrogen.</td>
<td>Independent of age, estrogen users had better cognitive functioning and were more independent with regards to activities of daily living. More estrogen users were depressed and likely to be taking antidepressant medications.</td>
</tr>
<tr>
<td>Benedetti et al. [27]</td>
<td>72 women with PD and 72 healthy women.</td>
<td>The PD group had undergone hysterectomy (with or without unilateral oophorectomy) more than the control group (OR = 3.36; 95% CI: 1.05–10.77). The PD group had more frequent occurrence of early menopause (&lt; or = 46 years) (OR = 2.18; 95% CI: 0.88–5.39). The PD group used ERT for at least 6 months after menopause less frequently than the control group (14%; OR = 0.47; 95% CI = 0.12–1.85). The PD group did not have earlier menopause than the control group.</td>
</tr>
<tr>
<td>Martignoni et al. [28]</td>
<td>150 women with idiopathic PD and 300 healthy women, all postmenopausal.</td>
<td>Duration of reproductive life was similar between women with PD and those without PD. Women with PD reported less access to HRT. The PD group also reported more premenstrual symptoms, fewer deliveries and abortions, and less use of contraception, indicating a relationship between PD and reproductive events. 50% of women in the control group took ERT, as compared to 25% of women in the PD group. Women who had taken postmenopausal ERT were less likely to develop PD than those who had not (odds ratio, 0.40; 95% CI: 0.19–0.84). Among women with PD, postmenopausal ERT was not associated with age at onset.</td>
</tr>
<tr>
<td>Currie et al. [29]</td>
<td>68 women with PD and 72 healthy women, all postmenopausal.</td>
<td>PD was significantly associated with a fertile life length of less than 36 years (OR 2.07; 95% CI: 1.00 to 4.30). PD was also associated with a cumulative pregnancy length of longer than 30 months (OR 2.19; 95% CI: 1.22 to 3.91). There was an inverse association between PD and surgical menopause (OR 0.30; 95% CI: 0.13 to 0.77).</td>
</tr>
<tr>
<td>Ragonese et al. [30]</td>
<td>131 women with idiopathic PD and 131 healthy women.</td>
<td>Among women with history of hysterectomy (with or without an oophorectomy), ERT use was associated with a 2.6-fold increased risk for PD, and a trend for additional risk was noted for increasing duration of estrogen use. Among women with natural menopause, no increased risk of PD was observed with HRT (ERT alone or in conjunction with progestin). Earlier age of menopause was associated with reduced risk of PD.</td>
</tr>
<tr>
<td>Popat et al. [31]</td>
<td>178 women with PD and 189 healthy women.</td>
<td>A significant correlation was found between age at PD onset and age at menopause, and also between age at PD onset and fertile life duration.</td>
</tr>
<tr>
<td>Ragonese et al. [32]</td>
<td>145 women with PD.</td>
<td>Women who underwent either unilateral or bilateral oophorectomy had an increased risk of parkinsonism compared to referent women (HR: 1.68; 95% CI: 1.06–2.67). This risk increased with younger age at oophorectomy.</td>
</tr>
<tr>
<td>Rocca et al. [16, 17]</td>
<td>1,252 women with unilateral and 1,075 women with bilateral ophorectomy, and 2,368 referent women.</td>
<td>Risk of PD was not significantly associated with reproductive factors or HRT. The association of smoking and caffeine with PD risk was modified by HRT, however. Based on a very small sample (4), women using progestin only hormones had increased risk for PD.</td>
</tr>
<tr>
<td>Simon et al. [33]</td>
<td>22-year prospective study of 244 women with PD enrolled in the Nurses’ Health Study.</td>
<td></td>
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</table>

Table 3: Case-control and epidemiological studies of HRT and Parkinson’s disease.
Table 4: RCTs of ERT and Parkinson’s disease.

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Hormone treatment used</th>
<th>Sample Size</th>
<th>Outcome measure</th>
<th>Overall findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blanchet [34]</td>
<td>High-dose transdermal E2. Cross-over design with 2 weeks on E2, 2 weeks washout, and 2 weeks on placebo</td>
<td>8</td>
<td>Therapeutic threshold for levodopa.</td>
<td>All but one participant had levodopa-induced dyskinesia at start of study. After 10 days of E2 treatment a significant reduction was observed in the anti-parkinsonian threshold dose of intravenous levodopa without significantly worsening dyskinesias</td>
</tr>
<tr>
<td>Strijks et al. [35]</td>
<td>17β-estradiol (E2) versus placebo for 8 weeks</td>
<td>12</td>
<td>Motor score from the Unified Parkinson’s Disease Rating Scale (UPDRS); patient report of subjective changes.</td>
<td>No differences in outcome measures between E2 and placebo.</td>
</tr>
<tr>
<td>Tsang et al. [36]</td>
<td>CEE versus placebo for 8 weeks</td>
<td>40</td>
<td>UPDRS, timed tapping score, Hamilton Depression Scale, patient self-report.</td>
<td>“On” and “off” times, and motor score on the UPDRS improved with estrogen.</td>
</tr>
<tr>
<td>The Parkinson Study Group Poetry I Investigators [37]</td>
<td>CEE versus Placebo for 8 weeks</td>
<td>23</td>
<td>Primary outcome was ability to complete the trial. Other outcome measures included adverse events, UPDRS, “on” time, dyskinesia ratings, and neuropsychological functioning</td>
<td>The estrogen group showed a trend for improvement on the total and motor UPDRS scores.</td>
</tr>
</tbody>
</table>

In one of the largest observational studies to date, Rocca et al. examined 1,252 women with unilateral oophorectomy, 1,075 women with bilateral oophorectomy, and 2,368 controls for development of PD. Data for the participants were collected until death or the termination of the study using direct or proxy interviews, neurologic examinations, medical records, and/or death certificates. The authors found that women who underwent either unilateral or bilateral oophorectomy before the onset of natural menopause, thereby decreasing endogenous estrogen levels, had an increased risk of parkinsonism compared with referent women. Further, risk increased with younger age at oophorectomy. The findings were similar regardless of unilateral or bilateral oophorectomy. Importantly, while the authors reported a trend, the surgical menopause group was not at increased risk for PD.

Although these studies might appear to provide conflicting results, complex factors are at play. The indication for HRT (posthysterectomy, posthysterectomy + oophorectomy, natural menopause), the specific type of HRT (CEE, E2, estrogen/progestin combinations), and other variables may combine in ways yet unknown to increase or decrease PD risk. Clearly, further study is necessary.

3.3. Studies of HRT and Dementia due to PD. PD is also associated with cognitive decline, with anywhere between 24–31% becoming demented [71]. PD dementia is considered a subcortical dementia, with associated deficits ranging from simple motor ability to higher-order cognitive functions [72]. Despite the high incidence of neurocognitive dysfunction in PD, the relationship between HRT and dementia in those with Parkinson’s disease has received considerably less attention. Only two case-control studies were found. Marder and colleagues investigated risk of PD both with and without dementia among a sample of 1156 women. They reported that ERT protected against development of PD-associated dementia, but not against PD itself [25]. Similarly, Fernandez and Lapane found that estrogen use was associated with better cognitive functioning and greater independence in activities of daily living among a large sample of elderly women living in nursing homes [26]. They also noted that estrogen users were more depressed and likely to be on an antidepressant as compared to nonusers. One-year death rates were comparable between estrogen users and nonusers.

3.4. Mechanisms of Estrogen Action in PD. While epidemiologic, observational, and experimental studies of ERT and PD have produced equivocal results, the biological mechanisms for a beneficial effect of estrogen upon dopaminergic functioning are less so. There are two general mechanisms of action through which estrogen might influence PD: symptomatic and neuroprotective. Estrogen receptors have been located in the nuclei of nigral dopaminergic (DA) neurons, including estrogen receptor alpha (ERα) and beta (ERβ) [73, 74], suggesting that estrogen might therefore directly influence DA functioning. ERα has also been found in midbrain glial cells [75], and ERβ in striatal medium spiny neurons [74]. Novel surface membrane estrogen receptors have also been described [76, 77]. Perhaps related to these, administration of exogenous conjugated estrogens results in an increase in binding of the DA transporter ligand TRODAT in otherwise healthy postmenopausal women [78]. It has also been shown that, in the absence of nigral neuroprotection, central E2 synthesis limits striatal DA loss caused by 6-OHDA in male rodents, implicating a modulatory effect on DA function [79]. These studies provide evidence that
Estrogens may upregulate the nigrostriatal pathway, either pre- or postsynaptically, by an effect on nuclear or surface membrane estrogen receptors.

Estrogen’s neuroprotective actions have been well established. In PD, there are animal models that are exquisitely specific for nigral cell death, of which the 6-hydroxydopamine (6-OHDA) and MPTP/MPP+ models are perhaps the best known [80, 81]. There is ample evidence that both endogenous and exogenous estrogen ameliorate the 6-OHDA animal model [79, 92–96], a methamphetamine model [97–100], and a wide range of other relevant animal models [101–103]. The exact mechanisms of neuroprotection, however, are not clear. Studies have shown a role for binding of estrogen to the nuclear estrogen receptor [104], the ERα subtype, [105] ERα with a glial contribution, [75] ERα + ERβ [106], and ER-independent mechanisms [88]. This has implications for potential therapeutic agents, as some estrogen analogues lack activity at one or both nuclear receptors; while others, such as the “inactive” enantiomer E2, may have no ER binding activity at all. E2 has been shown in the MPTP model to have neuroprotective properties [101], and has been investigated as a possible neuroprotective agent [107].

It is important, however, to recognize the imperfect nature of these preclinical models. First, while PD is a chronic, slowly progressive disorder, the aforementioned animal models use agents that cause acute toxicity. Second, despite the wide use of these models over the past two decades and the demonstration in preclinical models that many agents are neuroprotective against 6-OHDA, MPTP, or both, none of these agents have proven neuroprotective in human subjects with PD. There may be a simple explanation for this. We now know that neurodegeneration in most cases of familial PD is due to impaired ubiquitin-proteosomal function and alpha-synuclein protein aggregation [108]. Although the relationship between these abnormalities and those replicated by the 6-OHDA and MPTP models are complex, it appears likely that any agent that will be neuroprotective in humans with idiopathic PD will need to act to reduce alpha-synuclein aggregation. This can occur either by reducing its synthesis, reducing protein aggregation, enhancing its elimination, or reducing the toxic effects of excessive alpha-synuclein. Only recently has evidence been found that estrogen has the ability to act on alpha-synuclein in a beneficial manner. Hirohata et al. found a variety of sex hormones, including estradiol, estradiol, estrone, androstenedione, and testosterone to exert significant antiaggregation and fibril-stabilizing effects on alpha-synuclein in vitro. Estradiol was especially effective [109]. Further, Marwarha et al. showed that activation of ERβ, in conjunction with inhibition of LXRβ, may reduce progression of PD by slowing α-synuclein accumulation.

4. HIV-Associated Neurocognitive Disorder (HAND)

Internationally, an estimated 33 million individuals have HIV/AIDS, [110] and in many areas women comprise the majority of those infected [111]. Aggressive intervention with a regimen of multiple antiretroviral drugs (combined antiretroviral therapy, or cART) has successfully increased lifespan and attenuated some of the most dire neurological effects of HIV infection. However, cART cannot eradicate HIV, and it has attenuated, not eliminated, the most common neurological complication of HIV, or HIV-associated neurocognitive disorder (HAND) [112]. In this section, we discuss what is known about estrogen and HAND from observational studies in humans, studies in animal models, and in vitro studies. No relevant human clinical trials of estrogen for HAND have been published.

HAND is a constellation of cognitive impairments caused by HIV infection [112]. Because of the lack of diagnostic biomarkers, HAND remains largely a clinical diagnosis, made when an HIV+ individual experiences neurocognitive decline, sometimes with concomitant deficits in day-to-day functioning, and only after other conditions that might cause this decline have been ruled out. The severity of HAND ranges between mild neurocognitive impairment with no impact on day-to-day functioning to a debilitating HIV-associated dementia [112]. While the incidence of new cases of HAD has declined dramatically [113, 114], the prevalence of milder forms of HAND has actually increased along with the longevity of the cART-treated HIV+ population [113]. This phenomenon has been variously ascribed to several explanations, including the presence of irreparable CNS damage pre-cART [115], the failure of many cART regimens to adequately penetrate and treat the CNS [116], persistent low levels of HIV despite treatment [117], and to persistent CNS inflammation [118], among others. The latter is particularly relevant to the putative therapeutic benefit of estrogen, as it appears that cART does not always reduce and in some cases may increase, the CNS inflammation [119] that is associated with HAND [120]. Estrogen has significant anti-inflammatory and neuroprotective properties [121–123] and can potentially counteract inflammation in the HIV+ brain, as discussed in more detail below.

There are several other important reasons for investigating the use of estrogen as an adjunctive treatment in
HIV and HAND. First, estrogen and other gonadal steroids have significant effects on the course and presentation of HIV disease itself. For example, women are at increased risk for acquiring HIV compared to men, and this vulnerability may be affected by gonadal hormones [124]. Further, in a macaque model of HIV infection, progestogen-based hormonal contraceptives increased the risk of acquiring simian immunodeficiency virus (SIV), increased disease progression, and increased genital shedding of SIV; whereas treatment with estrogen lowered risk of acquiring SIV [125]. Results of natural history studies suggest a gender role in disease progression, possibly due to hormonal differences. For example, women have lower HIV RNA viral loads at seroconversion compared to men [126], and when adjusted for CD4+ count, women have lower viral loads throughout the course of their infection [127]. While one study found a lower risk of clinical progression to AIDS among HIV+ women versus HIV+ men treated with cART [128], others have found no differences in clinical outcome by gender [129]. A possible explanation for such gender disparity, should it turn out to be valid, is estrogen, which decreases HIV replication in peripheral blood mononuclear cells [130]. However, all such studies must be interpreted with caution because of the reported gender differences between HIV+ men and women in socioeconomic status, risk behavior, substance abuse, and access to care [131], which also affect progression to AIDS [132, 133]. With regards to HAND, whether women develop HAND at the same rate as men or if there are different clinical manifestations of HAND in men and women remains a controversial topic. In part, this is because so few studies had sufficient numbers of females to evaluate. A sub-study of the Women's Interagency HIV Study is beginning to address this problem [134].

There is neurobiological reason to expect a reduction of HIV-related neuropathological changes with ERT. Firstly, microglia are the resident immune cells of the CNS, and these cells play an important role in driving inflammation in many neurodegenerative diseases, thus representing an important target for therapy [135]. In HIV infection, microglia can be infected and/or activated; they are major sources of complete HIV virions, individual neurotoxic viral proteins, proinflammatory substances, and other potential mechanisms that drive neurotoxicity, neuroinflammation, oxidative stress, and neurodegeneration. Microglia express endogenous estrogen receptors [136], and treatment with estrogen is anti-inflammatory provided it is administered early in the course of an insult [121, 123]. Secondly, estrogen's anti-inflammatory effects may directly counteract the neuroinflammation caused by HIV proteins. HIV-infected cells can generate both replication-competent virions and excess viral proteins, which are shed or secreted into the extracellular space. The HIV coat protein, gp120, is the binding protein for viral entry [137] and acts as an indirect neurotoxin via its effects on microglia, macrophages, and astrocytes, initiating a cascade of events that damage neurons. Estrogen has been reported to have a broad anti-inflammatory effect on microglia [121]. Estrogen reduces the neuroinflammatory responses to gp120 and exerts neuroprotective effects on gp120-exposed neurons, by raising the levels of neurotrophins, decreasing apoptotic factors, and antioxidant properties [138]. Zemlyak et al. reported two different beneficial effects of estrogen in the amelioration of gp120-induced toxicity: a major effect of attenuating the neurotoxicity of factors released by gp120-treated microglial cultures, and a minor effect of enhancing the ability of neuronal cultures to survive exposure to neurotoxic factors [122]. Another neurotoxic HIV protein, tat, the nuclear trans-activating protein, is essential in promoting the transcription and replication of HIV. tat can act both directly to harm neurons [139], and indirectly by stimulating macrophages, microglia, and astrocytes to synthesize harmful substances such as proinflammatory cytokines [140], and by increasing free radicals and oxidative stress [141]. In cell culture, $17\beta$-estradiol suppressed tat-activated transcription of HIV in astrocytes [142]. $17\beta$-estradiol also attenuated the tat-induced release of pro-inflammatory mediators in endothelial cells [143], prevented oxidative stress and cell death associated with combined gp120 and tat neurotoxicity in vitro [144], and prevented gp120/tat-induced loss of dopamine transporter function [144].

These observations have led to the proposal that serum estradiol levels be maintained in HIV+ women as a possible neuroprotective agent against HAND [145]. Despite this, there is little clinical information about estrogen and HAND in HIV+ women. A single retrospective study from the pre-cART era, of 84 older (age 40+) years HIV+ women, reported that hormone replacement therapy (HRT) was associated with a significantly decreased risk of mortality [146]. Of interest, there were six women in the cohort who were diagnosed with HIV-associated dementia, none of whom reported taking HRT. This study has been interpreted by some to indicate a neuroprotective effect of HRT; however, this was not a prospective study that examined cognition in an organized or standardized fashion. However, based on this last report and on the neuroprotective role of estrogen in other inflammatory and degenerative conditions, the role of estrogen and other hormones in HAND has become an area of growing interest among basic scientists.

No studies of HAND or neurocognitive functioning in HIV+ persons have considered hormonal status or use of exogenous hormones. The preponderance of evidence to date indicates that HIV+ men and women develop neurocognitive impairment at a similar rate, when issues such as access to care, education, and substance abuse history are similar. While some have reported a higher occurrence of HIV-associated dementia among women [147], others have not found this [148, 149]. More recently, Martin et al. studied a large well-matched group of adult male and females, stratified by HIV status, all with a history of substance dependence [150]. Participants were abstinent at the time of testing. Whereas the performance of HIV+ men did not differ from HIV-negative counterparts of measures of motor skill and probabilistic learning, the HIV+ women performed worse than their seronegative counterparts, suggesting that women might be more vulnerable to the effects of HIV. However, due to the absence of a nonsubstance-dependent control group, they could not exclude the possibility that the observed differences were due to gender-related differences in the
cognitive effects of addiction. Another study reported no gender difference in rate of neurocognitive decline over time [151]; and still another found that while rates of impairment were similar between men and women, there were some differences in the neurocognitive profiles [148]. Whether this is related to estrogen or other gonadal hormones remains to be determined.

4.1. Summary—HIV/HAND. HAND shares many features with other neurodegenerative diseases, including microglial activation and neuroinflammation. Preliminary studies in animal and in vitro models indicate that, like many other neurodegenerative diseases, the effect of HIV on the brain may be blunted by treatment with 17β-estradiol, and possibly other gonadotrophic hormones. This would have to be balanced against the risks of adding estrogen to the regimens of HIV+ patients, both male and female. However, there is a pressing need to determine if HRT may benefit patients with AIDS who remain at risk for HAND even when treated with HAART.

5. Frontotemporal Dementia

Frontotemporal dementia, or FTD, is the most common form of a group of related neurodegenerative diseases that primarily affect the frontal and/or temporal lobes. The others include semantic dementia and progressive nonfluent aphasia. Collectively, these have been called frontotemporal lobar degenerative diseases [152], and they are believed to account for an estimated 20% of dementia cases with presenile onset [153].

Only one study to date has addressed the relationship between HRT and FTD. Levine and Hewett reviewed the medical files of all women seen at an Alzheimer’s disease center (ADC) in Central California and found that 70% of women diagnosed with FTD had been taking HRT (exact regimen unspecified) when evaluated, as compared to an estimated 24% of the surrounding population [154]. While one easy interpretation would be that women exhibiting cognitive impairment would have been more likely to be placed on HRT before coming to the ADC, only 20% of women diagnosed with AD at the same center had been taking HRT, so it is therefore unlikely that HRT was administered as a result of preclinical cognitive problems. The women diagnosed with FTD were also similar in age to women entering the center with AD (average age of symptom onset was 65, average age of initial evaluation was 70). While poor diagnostic accuracy and estrogen’s beneficial effects on mood were cited as possible reasons for the findings, a more compelling reason offered was a marked upregulation of tau in response to E2 administration, as evidenced in vitro [155]. The neuropathological correlates of many FTD cases appear to be tau-related, and in some cases directly linked to mutations in the tau gene [156]. In such cases, E2 may increase risk of FTD by increasing production of mutated forms of tau. However, while the role of tau in FTD has been well established, it is now known that it does not account for all forms of FTD [157]. Still the relationship between tau and E2 is a compelling reason to further study the influence of ERT on risk for FTD.

6. Summary and Conclusions

In summarizing the evidence discussed above, HRT, in particular ERT, appears to play an efficacious role in treating and preventing several neurodegenerative conditions. Figure 1 depicts putative neurobiological and neurobehavio-ral sequelae resulting from 17β-estradiol use, based on studies reviewed in this paper. The case for a neuroprotective role of HRT and AD is supported by research from epidemiological and RCT studies, which have shown that estrogen, specifically E2 (17β-estradiol), can reduce the risk for AD and minimize cognitive decline in otherwise healthy women, particularly verbal memory. Based on basic science research, the mechanisms for this neuroprotection may involve E2’s protection against β-amyloid-induced degeneration and may even include the maintenance of the cholinergic system in the hippocampus and frontal cortex. In addition, at least one study has demonstrated that the presence of progestins in combination therapies may actually dampen the beneficial effects of estrogen [24].

Similarly, in vitro and non-human in vivo experiments have demonstrated E2’s neuroprotective effects in dopaminergic neurons and animal models of PD. In addition, E2’s modulation of alpha-synuclein indicates a specific mechanism through which the hormone may reduce risk for PD and/or mitigate symptoms. To date, results of clinical and epidemiological studies of ERT alleviating motor symptoms in PD patients have been mixed and warrant further investigation. The effects of HRT on the neurocognitive symptoms of PD have received little attention, with the two case-control studies to date indicating that ERT reduces risk of cognitive impairment in women with PD.

Preliminary studies in animal and in vitro models indicate that treatment with E2, and possibly other gonadotrophic hormones, may reduce the effect of HIV on the brain. To date, much research on the neuroprotective effects for HIV neurodegenerative changes has been conducted on animal models and has yet to extend to humans. Nonetheless, preliminary research has suggested that development of HAND may be alleviated by HRT pretreatment. Conversely, and contrary to the findings from other neurodegenerative diseases, there is some evidence that E2 may actually augment risk for FTD via its action on tau.

Additional research is needed to further delineate the molecular mechanisms through which E2 and other estrogens act to delay or prevent neuropathological progression, or possibly cause progression in the case of FTD. Large-scale observational studies that accurately document HRT regimen and control for factors such as depression, education, and medical comorbidities (e.g., vascular risk factors) will also help to elucidate the role of ERT in the neurodegenerative disease etiology. While observational studies and RCTs examining ERT and AD have demonstrated long-term beneficial effects of varied ERT regimens (E2 or CEE), future studies may include long-term followup (5–10 years) of E2-based therapies alone on cognitive measures.
and neuroimaging outcomes, as such would provide helpful information on the duration of the benefits of E2 following discontinuation.

Notably, possible medical risks should be considered in study of HRT and neurocognitive functioning [45]. For instance, breast cancer is often a substantial concern that is linked with HRT. In fact, it is claimed that combined HRT with estrogen plus progestin is a cause for breast cancer. However, while followup analysis approximately three years after termination of the WHI study demonstrated an increased risk for “all-cause cancer” for participants in the CEE + MPA trial compared to the placebo group [158], the risk for breast cancer and other types of cancer did not differ between groups. Similarly, recent retrospective analyses of the WHI data found insufficient evidence that estrogen plus progestin increased risk of breast cancer [159]. Another study using the WHI data found that among women in the CEE + MPA trial, increased breast cancer risk was especially pronounced among women with breast tenderness [160]. In fact, new onset of breast tenderness after HRT initiation was associated with increased breast cancer risk among women assigned to the CEE + MPA trial, but not among women assigned to CEE-alone. In contrast, an additional followup analyses after the termination of the WHI data demonstrated that participants in the CEE-alone trial did not demonstrate increased risk for breast cancer [161]. Although the available information is insufficient at this time to support a clear link between HRT and increased risk for breast cancer, at least one study from the WHI has reported an increased risk of breast cancer among users of estrogen plus progestin with new onset of breast tenderness. This is an issue that requires continued investigation.

In clinical settings, the financial cost will need to be considered when recommending E2-based therapies for prevention of AD or other neurodegenerative diseases. Patients and their physicians will have to determine whether the potential cognitive benefit associated with E2 will outweigh the financial cost, as well as the above-mentioned medical risks.

References


Psychoneuroendocrinology 2010; 36:502-513, Silverman et al looked at

- 17B Estradiol vs CEE vs CEE plus progestin on 17-B Estradiol performed the best on verbal memory (an early warning of Alzheimer’s disease) by more than 3 standard deviations.
Differences in regional brain metabolism associated with specific formulations of hormone therapy in postmenopausal women at risk for AD

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KEYWORDS
PET; Estrogen formulations; Postmenopausal; Hormone therapy; AD; Verbal memory

Summary  Differential cerebral metabolic effects of various hormone therapy formulations, and their associations with cognitive status, remain to be established. The principal aim of the current study was to assess relationships between regional cerebral metabolism and estrogen-based hormone therapies. Postmenopausal women (n = 53) at elevated risk for Alzheimer’s disease (AD) were on estrogen-containing hormone therapy for at least one year prior to enrollment in a prospective, randomized clinical trial. Subjects underwent an FDG-PET scan, along with neuropsychological, medical, and demographic assessments at time of enrollment, to be repeated one year following randomization to hormone therapy continuation versus discontinuation, and results from analyses of the baseline assessments are reported here. Across all subjects, years of endogenous estrogen exposure correlated most closely with metabolism in right superior frontal gyrus (p < 0.0005). Women taking 17β-estradiol (E) performed three standard deviations higher in verbal memory than women taking conjugated equine estrogen (CEE), and their verbal memory performance positively correlated with metabolism in Wernicke’s (p = 0.003) and auditory association (p = 0.002) areas. Women taking progesterone-plus-estrogen had lower metabolism than women taking unopposed estrogen within the mesial and inferior lateral temporal regions (p < 0.0005) and the inferior frontal cortex, contralateral to Broca’s area (p < 0.0005). In conclusion, particular areas of relatively preserved metabolism were seen in women with more years of endogenous estrogen exposure, as well as in women taking estradiol-based formulations or estrogen therapies unopposed by progesterone, together suggesting regionally specific neuroprotective estrogenic effects.

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1. Introduction

Sex hormones exert a wide variety of effects upon brain development, aging, and function. Though estrogen influences have been the focus of particularly intensive investigation in this regard for decades, the nature of their impact upon the mature human brain remains a subject of substantial controversy. Answers to even some of the most basic questions—such as, whether the net influence of exogenous estrogen exposure on neurologic function is more harmful than beneficial—have been elusive. This in part reflects the complexity of estrogen-related effects, such that obtaining meaningful answers may require asking more specific questions: for example, whether the effects of estrogen hormone therapy (HT) exposure are influenced by the duration of endogenous estrogen exposure (i.e. length of reproductive life), whether the type of estrogen formulation (17β-estradiol (E) compared to conjugated equine estrogens (CEE)) influences brain function, and whether the use of concurrent progesterone has an effect on brain function or a moderating effect on the influence of estrogen on brain function.

Experimentally addressing these more focused questions calls for prospective investigation of subpopulations characterized by greater degrees of homogeneity, and who are studied by more sophisticated neurological tools, than has typified previous clinical research in this field.

Support for neuroprotective roles of estrogen comes both from human epidemiologic studies (see below), and from experimental models of brain function in vitro and in vivo. Estrogen has long been known to influence several neurotransmitter systems, including those that are cholinergic (Luine, 1985; Dominguez et al., 2004; Kompoliti et al., 2004; Bora et al., 2005; Bartholomeusz et al., 2008; Ping et al., 2008), serotonergic (Kendall et al., 1981; Halbreich et al., 1995; Rubinow et al., 1998; Archer, 1999; Lasiuk and Hegadoren, 2007), adrenergic (Sar and Stumpf, 1981; Ungar et al., 1993; Wang et al., 2006), or dopaminergic (Roy et al., 1990). Hormones may act through inducing temporary changes in neuronal microstructure, such as dendritic spine formation (Woolley et al., 1990), affecting neurotransmitters and receptors (McEwen, 1981; Arnold and Bredlove, 1985; Meusburger and Keast, 2001), altering cell membranes (McEwen et al., 1991) and modifying cerebral glucose metabolism and blood flow (Namba and Sokoloff, 1984; Nehlig et al., 1985; Bishop and Simpkins, 1995; Eberling et al., 2000).

Evidence of effects of HT on human brain aging and cognition currently is mixed. Observational studies support a decreased risk of clinically diagnosed AD for women on HT (Maki, 2006). Surgical menopause has been associated with increased risk of cognitive impairment dementia later in life (Rocca et al., 2007). Surgically menopausal HT users have also been reported to have higher verbal memory performance compared to non-users in clinical trials with randomized, placebo-controlled design (Phillips and Sherwin, 1992), as well as cross-sectional studies (Nappi et al., 1999; Verghese et al., 2000). On the other hand, the large randomized control trial (RCT), Women’s Health Initiative Memory Study (WHIMS), has reported a nearly doubled risk for all-cause dementia for women on HT compared to women not receiving HT (Shumaker et al., 2004) and estrogen plus progesterin therapy in the form of CEE and medroxyprogesterone acetate (MPA) specifically diminished verbal memory performance (Resnick et al., 2006; Maki et al., 2007). More recently it was found that unopposed CEE initiated in postmenopausal women aged 65 years and older did not diminish verbal memory performance, though it was associated with somewhat lower spatial rotational ability (Resnick et al., 2009a). Relative metabolic effects of CEE vs. E also remain controversial (Maki and Resnick, 2001).

Evidence from functional brain imaging studies, on the other hand, has been more consistent. Neuroimaging studies in healthy aging women have demonstrated enhanced function of medial temporal structures, including the hippocampus, amygdala, and entorhinal cortex among estrogen users vs. non-users (Maki and Resnick, 2001). Studies of specific neurochemical systems in living human brain suggest that postmenopausal hormone therapy positively modulates both cortical serotonin binding (Moses et al., 2000) and cholinergic receptor density (Smith et al., 2001). Moreover, in postmenopausal women at genetic risk for AD, HT is associated with attenuated metabolic decline in cortical regions especially affected by AD pathology, such as posterior cingulate, superior temporal, and lateral temporal cortical regions (Rasgon et al., 2001, 2005, 2008a,b).

The present study is part of an ongoing larger prospective randomized longitudinal clinical trial, in which postmenopausal women at increased risk for eventual development of dementia and on HT at time of study enrollment have been randomly assigned to continue or discontinue HT for at least 2 years. Cognitive status and regional cerebral metabolic rate for glucose (CMRglc) were assessed at baseline and 2 years after randomization. An interim analysis of the data analyzed from the first 25 subjects to complete that trial has thus far indicated that women randomized to continue HT: (1) experience less significant decline of their posterior cortical metabolism (which is most marked in right inferior parietal cortex in women who discontinue HT), and (2) preserve anterior cortical metabolism relative to overall brain metabolism in the medial prefrontal area, which is not seen in their demographically matched counterparts who discontinue HT (Rasgon et al., 2008a,b). In the study reported here, we describe the regional cerebral metabolism for the entire cohort of subjects for whom PET scans were obtained at time of enrollment, for a relatively homogeneous group of postmenopausal women on HT. We specifically examine the relationships between brain metabolism and three hormonal factors potentially affecting geriatric cognitive decline: length of prior endogenous estrogen exposure, whether the HT regimen includes a progesterone compound, and type of estrogen formulation being taken at time of enrollment.

2. Methods

The study in its entirety was approved by the Stanford University Human Research Protection Program and the Institutional Review Board at the University of California, Los Angeles (UCLA). Cognitively normal postmenopausal women ages 50–65 at risk for AD and receiving estrogen-containing hormone therapy (HT) for at least one year were enrolled. All subjects were receiving either estrogen therapy opposed or unopposed by progesterone through any route of administration (i.e. oral, transdermal, vaginal), and no changes in...
their HT regimen were implemented by the investigators prior to randomization or thereafter. Assessment of endocrine reproductive markers included gathering information on age at menarche and menopause, parity, use of hormonal contraception during reproductive years, duration of peri-menopausal transition in relation to time of start of HT use, and type of menopausal symptoms. All subjects had a screening and baseline visit. The screening visit included psychiatric, physical, and neurological examination, and laboratory blood measures to determine eligibility for the study. During the physical and neurological examination, subjects were screened for Parkinson disease using the motor examination (items 18–31) of the Unified Parkinson’s Disease Rating Scale (Fahn et al., 1987). After the screening visit, subjects underwent positron emission tomography (PET) scan and neuropsychological testing.

The study required the following inclusion criteria: willingness to sign human subject consent prior to enrollment into the study; willingness to be randomized to continuation/discontinuation of HT, women ages 50–65 years of age; ≥1 year post complete cessation of menses; ≥1 year current HT use; ≥8 years of education, and adequate visual and auditory acuity to allow neuropsychological testing. In addition, all subjects were required to be at elevated risk for eventual development of dementia, as defined by one or more of the following risk factors: personal history of mood disorder; personal history of hypothyroidism; family history of AD; documentation of the apolipoprotein (APOE) allele ε4, conferring increased risk for AD.

Because cognitive decline may be caused by a wide variety of conditions having different cerebral metabolic signatures, we excluded subjects with impairment from numerous causes (e.g., vascular disease, etc.), to enrich for those at increased risk specifically for AD. Volunteers with a history of TIAs, carotid bruits, or lacunes on MRI scan were excluded. Other exclusion criteria included evidence of current depression as determined by a score of ≥8 on the 17-item Hamilton Depression Rating Scale (Hamilton, 1960), history of drug or alcohol abuse, contraindication for MRI scan (e.g., metal in body, claustrophobia), history of mental illness (excluding mood disorders), or significant cognitive impairment, as evidenced by impairment in daily functions and/or MMSE < 24 (Folstein et al., 1975), history of myocardial infarction within the previous year or unstable cardiac disease, significant cerebrovascular disease, as evidenced by neurological examination, uncontrolled hypertension (systolic BP > 170 mmHg or diastolic BP > 100 mmHg), history of significant liver disease, clinically significant pulmonary disease, diabetes, or cancer. Subjects were excluded if they already had possible or probable AD (McKhann et al., 1984) or any other dementia (e.g., vascular, Lewy body, frontotemporal), or evidence of neurologic or other physical illness that could be expected to imminently produce cognitive deterioration. Subjects were also excluded if they used drugs with potential to significantly affect psychometric test results, including centrally active beta-blockers, narcotics, clonidine, anti-Parkinsonian medications, antipsychotics, benzodiazepines, systemic corticosteroids, medications with significant cholinergic or anticholinergic effects, anticonvulsants, warfarin, or sporicidal use of phytoestrogen-containing products, which may produce estrogen-like agonist and antagonist effects (Polkowski and Mazurek, 2000; Vincent and Fitzpatrick, 2000).

Hormone levels were assayed at baseline. Follicle-stimulating hormone (FSH) was measured by immunoassay. Estradiol was measured using the enzyme-linked immunosorbent assay (Bio-Quant Inc., San Diego, CA). Limits of detection were 0.10 mIU/mL for FSH, and the minimum detectable concentration of the estradiol assay was 2 standard deviations plus mean of a zero standard, which was estimated to be 0.4 pg/mL.

2.1. PET scan acquisition and analysis

Participants were required to fast 4–6 h for the PET imaging studies. The [F-18] fluorodeoxyglucose (FDG) PET method was used for the determination of patterns of regional cerebral metabolism. An intravenous line was placed 10–15 min prior to injection of 370 MBq FDG. Uptake of FDG proceeded while subjects were supine with low ambient lighting and sound, eyes and ears unoccluded. Scans were performed 40 min post FDG injection using a CTI/Siemens (Siemens Corp, Hoffman Estates, IL) HR+ tomograph (63 image planes).

We analyzed PET data by both a standardized volume of interest method as well as the statistical parametric mapping method developed by Friston et al. (1995a,b). Briefly, images from all subjects were co-registered and reoriented into a standardized coordinate system using the SPM2 software package courteously provided by the members of the image analysis team at the Wellcome Department of Cognitive Neurology, Functional Imaging Laboratory (London, UK). Data were spatially smoothed, and normalized to mean global activity as previously described (Silverman et al., 2007), with the exception that a 12 mm (full-width half-maximum) smoothing filter was applied to images prior to statistical analysis. The set of pooled data were then assessed with the t-statistic on a voxel-by-voxel basis, to identify the profile of voxels that significantly covaried with parameters characterizing each subject.

In addition, relative quantification of regional brain activity was performed using software originally developed at UCLA dedicated to the visual display and quantitative analysis of brain PET data, which has been cleared for those purposes by the U.S. Food and Drug Administration and is commercially available as the clinical package NeuroQ™ (Syntermed Inc., Atlanta). The software implements an algorithm for automatically measuring, after correction for tissue-based attenuation, the number of radioactive events emitted by a positron source (gamma-ray lines of coincidence) per second detected by the PET scanner, emanating from pixel locations assigned by a computerized reconstruction algorithm as falling within each standardized region of interest (sROI). Mean pixel activity values were calculated within each of 240 sROI’s defined throughout those transaxial planes across the field of view in which brain tissue was represented, following the transformation of each PET scan to a template space by a method previously described by Tai et al. (1997). The sROI’s were then automatically grouped into 47 clusters of regions falling within structurally defined boundaries corresponding to distinct neuroanatomical (e.g. left inferior parietal lobule) or functional (e.g., Broca’s area) standardized volumes of interest (sV0I’s), and the mean activities for each of these volumes calculated. Finally, all mean activity values were automatically normalized to the
mean pixel activity measured throughout that brain scan, or to the mean activity of an individual user-specified reference sVOI within that scan.

For the purpose of guiding interpretations of statistical analyses, a priori hypotheses were established for six specific cortical volumes bilaterally, based on their known association with physiologic memory processing and/or pathologic involvement in neurodegenerative disorders: left and right medial temporal sVOI’s, including amygdala and hippocampus/parahippocampal areas, were chosen for their established role in "new learning" (Squire et al., 1992; Stern et al., 1996; Tulving et al., 1996; Gabrieli et al., 1997). Two sVOI’s of the dorsolateral prefrontal cortex (DLPFC) were chosen for known involvement in both encoding and working memory/retrieval. The posterior cingulate cortex was chosen for its role in encoding (Shallice et al., 1994) and retrieval (Tulving et al., 1996; Fletcher et al., 1998).

Finally, the parietotemporal and inferior lateral temporal cortical VOI’s were assessed due to their importance in language, semantic memory and related memory deficits in AD (Martin and Fedio, 1983; Wiggs et al., 1999). The selected volumes have also been implicated as biomarkers of impending or actual cognitive decline in functional imaging studies. Consistent patterns of hypometabolism and hypoperfusion in the parietal, temporal, and posterior cingulate cortices (Small et al., 1989, 1995; Smith et al., 1992; Reiman et al., 1996; Ibáñez et al., 1998) have been observed in asymptomatic persons at increased genetic risk for AD, as well as in patients with AD.

Results were reported in terms of locations of the most significant effects (regionally, and/or in x, y, z Talairach-style millimeter coordinates). The probability of finding by chance an effect in any volume containing a voxel of maximal significance by statistical parametric mapping, or in any sVOI, was assessed after a Bonferroni-type adjustment for multiple comparisons, with a correspondingly less harsh correction required for the 12 volumes (6 left, 6 right) specified by a priori hypotheses, and effects at each region of the brain were considered significant if that adjusted probability was less than 0.05. For large areas, corrected values based upon the number of contiguous voxels achieving a pre-specified level of significance were additionally provided. For sVOI analyses, based on the number of regions, effects in the sVOI’s specified a priori were considered significant for pre-adjusted \( p \leq 0.004 \), while effects in other sVOI’s were considered significant for pre-adjusted \( p \leq 0.001 \); sVOI analyses serving specifically to support another pre-established result were considered corroborative for \( p < 0.05 \). Analyses were also controlled for examination of multiple effects by using analysis of variance (ANOVA) methods with all three main hormonal effects entered into the statistical model (see below).

2.2. Neuropsychological assessment

An extensive neuropsychological evaluation was conducted at the baseline and final visits by the study neuropsychologist. Tests in the neuropsychological battery were chosen based on prior studies that indicated their ability to predict cognitive decline (Hänninen et al., 1995) and sensitivity to predict cognitive change (Small et al., 1995). The battery included the Auditory Consonant Trigrams (ACT), Benton Visual Retention Test (BVRT), Boston Naming Test (BNT), Color Trail Making Test (Color Trails 1 & 2), Delis Kaplan Executive Function System (DKEFS), Rey-Osterrieth Complex Figure Test (RCFT), Wechsler Adult Intelligence Scale 3rd Edition (WAIS-III) — Digit Span, Symbol Coding, and Letter Number Sequencing subtests, and the Wechsler Memory Scale-III (WMS) — Logical Memory I & II subtests. The Wechsler Abbreviated Scale of Intelligence was used to characterize intelligence (IQ). Individual test scores were z-transformed and parcelled into the cognitive domains of attention, verbal memory, visual memory, word finding and category fluency, and executive functioning. Lastly, the Memory Function Questionnaire (MFQ) was administered to assess subjective memory functioning.

2.3. Reproductive endocrine variables

Information was obtained from all subjects and supported by documentation from their primary health care providers on the duration of endogenous and exogenous exposure to reproductive hormones. Duration of endogenous exposure was calculated by subtracting age at menarche from the age at menopause, duration and type of hormone therapy was recorded as well as type of menopause, parity and past use of steroidal contraception.

3. Results

Eighty-one subjects were initially recruited; baseline data from 53 subjects were included in the final analysis; the other 28 subjects had incomplete data sets caused by schedule conflicts, technically limited MRI or PET imaging, or due to a variety of reasons not noted during initial screening (hysterectomy at a young age, discontinued HT prior to randomization, TIA, claustrophobia emerging during imaging, severe mood episode with menopause transition, congenital ovarian agenesis, progesterone-only therapy). Demographic and personal characteristics of these 53 subjects are presented in Table 1. All subjects had MMSE and cognitive performance scores within the normal range for persons of their same age and educational level. There was a history of depression in 41 subjects and 26 were currently taking antidepressants.

3.1. Endogenous estrogen exposure

As a potential factor related to cognitive status in postmenopausal women, duration of pre-menopausal endogenous estrogen exposure was explored by statistical parametric mapping for associations with regional cerebral metabolism. The total duration of endogenous estrogen exposure (i.e. age at menopause minus age at menarche) positively correlated most closely with metabolism in the posterior part of the right superior frontal gyrus \(( t = 5.64, p < 0.0005 \) at voxel of peak significance: 22, 32, 58), remaining significant after full correction for multiple comparisons \(( p = 0.005 \) ), as well as after adjustment for variance in age and education \(( t = 5.19, p < 0.0005; p = 0.024 \) after multiple comparison correction). The sVOI analyses corroborated SPM analyses in the right superior frontal gyrus \(( r = 0.27, p = 0.047 \) ). Again adjusting for age and education, years of endogenous estrogen exposure also positively correlated with activity in the...
right peri-insular area at the axial level of the anterior commissure (48,16,0; \( t = 5.29, p < 0.0005, p = 0.017 \) after multiple-comparison correction); an area of similar size and significance was seen in the contralateral hemisphere but centered slightly more superiorly and anteriorly, in the left inferior frontal gyrus overlapping with Broca’s area (57,20,14; \( t = 5.28, p < 0.0005, p = 0.017 \) after multiple-comparison correction). Endogenous estrogen exposure also tended to positively correlate with metabolism in the left parahippocampal gyrus (−28,−30,−20; \( t = 4.05, p < 0.0005 \), area in the vicinity of the subgenual anterior cingulate (−44,−14; \( t = 4.25, p < 0.0005 \)), and an area of the left superior lateral temporal cortex (−48,−24,10; \( t = 4.58, p < 0.0005 \)) (Fig. 1). The sVOI analyses also corroborated the correlation with metabolism in the left superior lateral temporal cortex (\( r = 0.28, p = 0.037 \). Not surprisingly, age of menopause, which was highly correlated with the duration of endogenous estrogen exposure (\( r = 0.92, p < 0.00001 \), also positively correlated with metabolism in these regions.

### Table 1  Study sample demographics and clinical characteristics (\( n = 53 \)).

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<th>Mean</th>
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<td>23.7</td>
<td>22.7, 112.2</td>
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n

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<th>Surgical</th>
<th>33, 20</th>
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<td>Family history of AD/FamH × AD</td>
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<td>FamH × AD- = 28</td>
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<tr>
<td>Type of HT</td>
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<td>E + MPA = 5</td>
<td>CEE + MPA = 9</td>
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<tr>
<td>Vasomotor symptoms</td>
<td>Present = 38</td>
<td>not present = 15</td>
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</table>

All times are expressed in years. HT: hormone therapy, E: estradiol, CEE: conjugated equine estrogen, P: progesterone, MPA: medroxypregesterone acetate, AD: Alzheimer’s Disease.

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Fig. 1  Metabolic correlation with years of endogenous estrogen exposure. All voxels positively correlating with endogenous estrogen exposure at \( p < 0.001 \) are shown in color. Years of endogenous estrogen exposure correlated with metabolism in areas within the right superior frontal gyrus (red arrow), right peri-insular region (dark blue), left inferior frontal gyrus (light blue), left parahippocampal gyrus (not visible from these surface rendering views), left subgenual anterior cingulate (yellow arrow), left superior lateral temporal gyrus (green arrow) (\( p < 0.0005 \)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
3.2. Hormone replacement therapy formulations

3.2.1. 17β-Estradiol and conjugated equine estrogen formulations

Among 53 subjects, 35 subjects were taking formulations of 17β-estradiol (E) and 18 subjects were taking conjugated equine estrogen (CEE) at time of recruitment. Groups were similar with respect to years of education (av \( \pm 1.6 \) vs. 16 \( \pm 1.9 \)), age at menarche (13 \( \pm 1.6 \), 13 \( \pm 1.7 \)), and length of HT (9 \( \pm 5.7 \), 11 \( \pm 5.7 \)). Approximately 2/3 of the subjects in the E and CEE groups had opposed therapy (24 of 35, and 11 of 18, respectively). The proportion of subjects taking E and CEE were also similar for the natural and surgical menopause groups, constituting 65% E for surgical group (13 of 20), and 67% E for the women who underwent natural menopause (22 of 33).

3.2.1.1. Group differences in cerebral metabolism. Analyses by statistical parametric mapping directly comparing E-users to CEE-users showed that levels of metabolism in the left cerebral hemisphere, and the superior temporal gyrus in the auditory association area of the right cerebral hemisphere, were significantly greater in E-users \( (t = 4.41 \) and 4.67, respectively; \( p < 0.0005 \) (Fig. 2).

3.2.1.2. Neuropsychologic performance and cerebral metabolic correlates. In examining associations with neuropsychologic performance, we found that E-users attained scores for the verbal memory cognitive domain that were 3 SD higher than those of CEE-users \( (p = 0.007) \). We therefore examined associations of verbal memory performance with cerebral metabolism.

Across all subjects, statistical parametric mapping analyses indicated that verbal memory performance positively correlated most strongly with the magnitude of cortical metabolism in the right superior frontal gyrus \( (t = 3.60, p < 0.0005) \) at voxels of peak significance: 10,32,54, in the left Wernicke’s area \( (t = 2.86, p = 0.003) \) at voxel of peak significance: -50,-80,34 and with metabolism of the right auditory association area \( (t = 3.00, p = 0.002) \) at voxel of peak significance: 70, -36,14. When adjusting for age, education, and years of endogenous estrogen exposure, similar correlations were seen \( (t = 2.95, p = 0.002; t = 2.50, p = 0.008; t = 2.58, p = 0.007, respectively) \). The correlations tended to be somewhat stronger among E-users with respect to metabolism in both the Wernicke’s \( (t = 3.66, p < 0.0005; t = 3.48, p < 0.001) \) and auditory association \( (t = 3.30, p = 0.001; t = 2.94, p = 0.003) \) areas, while CEE-users alone demonstrated the most significant correlation with metabolism of the superior frontal region \( (t = 4.37, p < 0.0005) \).

3.2.2. Estrogen—progesterone versus unopposed estrogen replacement therapies

Among the 53 subjects taking estrogen-based hormone replacement therapy, 18 women were taking unopposed estrogen and 35 were taking progesterone-plus-estrogen replacement therapies, of whom 14 were taking MPA. Groups were similar in years of education \( (av \pm 1.8\) vs. 16 \( \pm 2.2 \)), age at menarche (13 \( \pm 1.8\), 13 \( \pm 1.5 \)), age at recruitment (58 \( \pm 5.6\), 58 \( \pm 4.4 \)), and length of HT (10 \( \pm 5.7\), 9 \( \pm 5.7 \)) respectively. They differed in age at menopause (43 \( \pm 7.0\), 49 \( \pm 3.7 \)), (with the younger average age of the unopposed estrogen group primarily reflecting prematurely induced menopause secondary to surgical removal of uterus and/or ovaries in these subjects), and thus in mean number of years of endogenous estrogen exposure \( (31 \pm 6.4\), 36 \( \pm 3.9 \)). A similar proportion were taking E versus CEE: 69% were on estradiol, 24 of 35 in the progesterone-plus-estrogen group, and 61% were on estradiol in the unopposed group (11 of 18).

Analyses by statistical parametric mapping showed that postmenopausal women taking progesterone-plus-estrogen had lower metabolism than postmenopausal women taking unopposed estrogen within the right mesial temporal region \( (t = 4.51, p < 0.0005) \) at voxel of peak significance: 38,20, -36) and left inferior lateral temporal cortex \( (t = 4.64, p < 0.0005) \) at voxel of peak significance: -48,22, -34); the voxel of peak significance within the right mesial temporal region was in the largest cluster \( (3085 \text{ contiguous voxels at } p < 0.01, p_{\text{corrected}} = 0.002) \) (Fig. 3). After adjusting for years of endogenous estrogen exposure, the right and left temporal relative hypometabolism in progesterone-plus-estrogen users remained
significant \((t = 4.01, p < 0.0005; t = 4.42, p < 0.0005)\), respectively. The sVOI analyses corroborated SPM analyses, demonstrating that progesterone-plus-estrogen users had lower mean metabolism in corresponding temporal regions than unopposed estrogen users in the right mesial temporal cortex \((rMT) t = 2.61, p = 0.01\), and a trend towards lower metabolism in the left inferior lateral temporal cortex \((liLT) t = 1.72, p = 0.09\) (which became more significant after adjusting for endogenous estrogen exposure; see below.) Only 2 of 35 progesterone-plus-estrogen users had rMT metabolism levels greater than mean rMT metabolism of unopposed estrogen users and, conversely, only 5 of 18 unopposed estrogen users had rMT metabolism levels lower than mean rMT metabolism of progesterone-plus-estrogen users (Fig. 4).

Analyses by statistical parametric mapping also demonstrated that women taking progesterone-plus-estrogen had lower metabolism than women taking unopposed estrogen in the right inferior frontal cortex \((t = 3.65, p < 0.0005)\) at voxel of peak significance: 24,26,−14. The significance of this difference again persisted when statistical correction for the disparate number of years of endogenous estrogen exposure was taken into account \((t = 3.69, p < 0.0005)\) and occurred in the largest regional cluster \((1747 \text{ contiguous voxels at } p < 0.005, p_{\text{corrected}} = 0.005)\) (Fig. 5). The sVOI analyses corroborated SPM analyses, with progesterone-plus-estrogen users having lower mean metabolism than unopposed estrogen users in the right posterior inferior frontal gyrus \((rpIFG)\), contralateral to Broca’s area in the left hemisphere \((av/C6 SD; 1.14 ± 0.03 vs. 1.16 ± 0.03, t = 2.29, p = 0.026)\), a difference that became more significant following statistical correction for number of years of endogenous estrogen exposure: \(F = 9.39, p = 0.004\). Only 3 of 18 unopposed estrogen users demonstrated levels of metabolism in this region that were lower than the mean metabolism of progesterone-plus-estrogen users and, conversely, only five of the 35 progesterone-plus-estrogen users had metabolic levels that were greater than the mean metabolism of unopposed estrogen users.

Potential interactions of opposed versus unopposed estrogen with the other major variables discussed above (years of endogenous estrogen exposure and E vs. CEE) relative to rates of metabolism in the standardized VOI’s were examined with ANOVA. Taking into account endogenous estrogen exposure, sVOI analyses corroborated significantly lower metabolism in right mesial temporal, and left inferior lateral temporal cortex, in women taking opposed estrogen regi-

![Fig. 3](image3.png)

**Fig. 3** Posterior temporal areas of decreased metabolism in progesterone-plus-estrogen users compared to unopposed estrogen users. SPM analyses demonstrated right mesial temporal \((t = 4.51, p < 0.0005)\) and left inferior lateral relative hypometabolism \((t = 4.64, p < 0.0005)\) in progesterone-plus-estrogen users compared to unopposed estrogen users. Voxels with significance \(p < 0.01\) are shown above in color. The crosshairs intersect at voxel of peak significance \((38,20,−36)\).

![Fig. 4](image4.png)

**Fig. 4** Lower right mesial temporal metabolism in progesterone-plus-estrogen users compared to unopposed estrogen users. In the individual-subject plot above, 35 postmenopausal women on progesterone-plus-estrogen therapy are represented by blue diamonds and the average right mesial temporal metabolism, assessed by sVOI analysis, is shown in light blue \((0.797)\). 18 postmenopausal women on unopposed estrogen therapy are represented by red squares and the average right mesial temporal metabolism is shown in orange \((0.815)\). Progesterone-plus-estrogen users had a lower mean rate of metabolism than unopposed estrogen users \((p = 0.012)\), a difference that became more significant after correcting for years of endogenous estrogen exposure \((p = 0.007)\). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
mens ($F = 7.788, p = 0.007; F = 4.199, p = 0.046$, respectively). Simultaneously taking into account years of endogenous estrogen exposure and E vs CEE use, sVOI analyses again corroborated significantly lower metabolism in these regions ($F = 7.624, p = 0.008; F = 4.122, p = 0.048$).

Looking at the other direction, women taking unopposed estrogen did not have any areas demonstrating significantly lower metabolism after correction, than women taking progesterone-plus-estrogen formulations.

4. Discussion

Relative regional cerebral hypometabolism of 53 postmenopausal women on HT selected to be at heightened risk of eventually developing dementia was associated with a presumptive historical risk factor for cognitive decline: shorter endogenous estrogen exposure. Years of endogenous estrogen exposure positively correlated most robustly with metabolism in the right superior frontal gyrus, and also in right peri-insular area, left inferior frontal gyrus, left parahippocampus, subgenual anterior cingulate, and left superior lateral temporal cortex. Additionally, in the present analysis E-users had greater metabolism than CEE-users in the Wernicke’s area and the auditory association area. Verbal memory was better in postmenopausal women taking E than CEE, and cognitive differences were associated with metabolic differences in these receptive language and auditory association areas for E-users and in right superior frontal gyrus for CEE-users. Postmenopausal women taking estrogen-plus-progesterone users had lower metabolism than postmenopausal women taking unopposed estrogen in the right mesial temporal region, left inferior lateral temporal cortex, and right inferior frontal cortex. Overall, particular areas of relatively preserved metabolism were seen in women with more years of endogenous estrogen exposure, as well as in women taking estradiol-based formulations or estrogen therapies unopposed by progesterone, together suggesting regionally specific neuroprotective estrogenic effects. The results presented here provide support for estrogen having a neuroprotective role in brain regions affected during aging.

Several of the regions in the current study have also been implicated in a number of previous studies on hormonal influences involving neuroimaging. Relevant to our identification of hormone related activity in superior and inferior frontal gyri, similar or overlapping regions of frontal cortical involvement have been identified as positively correlating with HT: orbital gyrus of the frontal cortex (Eberling et al., 2004), right superior frontal gyrus (Ottowitz et al., 2008), left middle/superior frontal cortex (Persad et al., 2009) and left medial frontal cortex (Maki and Resnick, 2000; Persad et al., 2009). Initiation of E/HT has also been shown to result in more effective activation of the prefrontal cortex during fMRI visual working memory tasks, suggesting functional plasticity in these frontocortical working memory systems among postmenopausal women that can be altered by E/HT (Smith et al., 2006). In a recent double-blind, placebo-controlled
study, the inferior frontal region was also an area that increased during fMRI tests of verbal and spatial working memory in E/HT users, suggesting improved executive function (Joffe et al., 2006). In addition to increases in inferior frontal activation, Joffe et al. (2006) also found parietal activation (bordering Brodmann’s area 40, near the Wernicke’s region) in E/HT users (40–60 yrs) during a verbal recall task. This finding is pertinent to our observation of hormonal effects in Wernicke’s area and its correlation with verbal memory in all subjects, especially E-users. In a recent, double-blind placebo-controlled study, postmenopausal women (56–60 yrs) randomized to hormone therapy had increased activation in the frontal cortex and left inferior parietal cortex (Brodmann’s Area 40) during memory encoding (Persad et al., 2009). These findings suggest that estrogen may provide cognitive benefits and protect language-related brain areas when given to younger women shortly after or during the menopausal transition.

We also observed hormonal effects in the mesial temporal structures including hippocampal and parahippocampal regions. Functional neuroimaging studies utilizing PET and fMRI in healthy aging women, have consistently reported greater metabolic activity in mesial temporal structures, including the hippocampus, amygdala, and entorhinal cortex, among users of E/HT vs. non-users (Resnick et al., 1998; Shaywitz et al., 1999; Eberling et al., 2000; Maki and Resnick, 2000, 2001; Rasgon et al., 2001; Gleason et al., 2006). Regional brain volumes were recently assessed in structural MRI images of the brains of women who had been previously enrolled in WHIMS (Resnick et al., 2009b). Overall, mean hippocampal volumes tended to be smaller among women randomized to hormone therapy, by an average of \( -0.10 \text{ cm}^3 (p = 0.05) \); in the group with at least 2 cm\(^3\) ischemic burden, the effect was more pronounced, with hippocampal volumes smaller in the HT arm by an average of \( -0.16 \text{ cm}^3 (p = 0.005) \). Hence, those data are consistent with the WHIMS findings of detrimental cognitive effects associated with opposed CEE therapy. The fact that both types of HT were associated with increase in hippocampal neuronal firing could be due to predominant effects of the estrogen component, rather than the presence of the progesterone. In addition, Ottowitz et al. found that estradiol increased connectivity between the right hippocampus and right prefrontal cortex suggesting that estradiol may enhance verbal memory performance by means of recruiting a bilateral cooperation between prefrontal and hippocampal systems during verbal memory tasks (Ottowitz et al., 2008).

Overall, basic science analyses using both in vitro and in vivo model systems have also indicated that estrogen—typically 17β-estradiol but also in some instances conjugated equine estrogens—protect neurons against insults associated with AD (Brinton, 2005). Moreover, these same estrogens in the same model systems can activate biochemical, genomic, cellular and behavioral mechanisms of memory (Singh et al., 1994; Toran-Allerand, 2000; Frye et al., 2007). An important aspect of these studies, and of virtually all of the basic science in vitro and in vivo analyses, is that neurons were healthy prior to estrogen exposure and prior to exposure to neurodegenerative insults or lesions (Brinton, 2005). In human studies, beyond the neuroimaging results discussed above, data on the effects of estrogen or postmenopausal cognition have been consistent. Recent reviews of human observational studies and clinical trials have emphasized lack of benefits or likely harm of hormonal therapies in older postmenopausal women and/or insufficient evidence for benefit in younger women (Genazzani et al., 2007; Barrett-Connon and Laughlin, 2009) of particular note, results from WHIMS indicated significant increases in risk for probable dementia in the combination HT subtrial alone and in the combined analysis of combination HT and CEE-alone subtrials (Shumaker et al., 2003, 2004) and poorer performance on the 3MS (Rapp et al., 2003; Espeland et al., 2004), after initiation of HT in postmenopausal women aged 65 and older. On the other hand, in the Multi-Institutional Research in Alzheimer Genetic Epidemiology case—control study, the protective association of HT was modified by age and was seen among younger, but not older, postmenopausal women (Henderson et al., 2005).

It remains an open question, whether the neuroprotective effects of estrogen documented in preclinical studies can be replicated in randomized controlled trials performed with the most suitable patient group under appropriate conditions. On a path to accomplish these goals, variables to be examined as our data suggest, include both the formulations of estrogen and progestogens (progesterone versus MPA) employed in human studies and clinical trials, dosage and levels of exposure, timing of hormone therapy exposure, duration of hormone therapy, as well as substrate differences (i.e., neuronal health status and risk for or presence of degenerative disease). One clinical trial which takes these variables in the account is on its way (KEEPS) (Miller et al., 2009), but results are not yet being made available. Other limitations of the current work that future studies will be able to address, include assessing the potential effect of testosterone in hormone replacement regimens (present in the HT of 5 of the 53 subjects in the study, too small a subgroup to separately assess) and any differences in effects of MPA vs. micronized progesterone that may exist.

Further along these lines, since many demographic factors and other types of patient characteristics may potentially affect cognitive performance and corresponding patterns of cerebral metabolism, greater homogeneity among subjects can be expected to produce greater statistical power, in analysis of cognitive and metabolic data from a subject pool of given size. In the present investigation, all of our subjects are postmenopausal women with well-documented pharmacologic status with respect to hormonal (and other) therapies, all are middle age (between 50 and 65 years old), and initiated hormone therapy perimenopausally. In addition, the detailed information obtained by self-report and recorded from the medical records with respect to estrogen status—both historical (ages at menarche and menopause, etc.) and current (specifics regarding types of estrogen used, and whether they were progesterone-containing or unopposed, etc.)—allows for subgroup and correlational analyses of cerebral metabolism that is generally not feasible in analysis of brain PET data obtained in other investigations. These advantages are purchased at the cost of an inevitable corresponding disadvantage: an inherent limitation is that the findings cannot be assumed to be generalizable beyond the particular kind of populations comprising our subject pool. In the current study, evidence is presented for relative preservation of metabolism in specific brain regions in middle age women at increased risk for future development of AD.
being associated with greater endogenous estrogen exposure, and unopposed or estradiol-based HT formulations initiated in the perimenopausal period. While these findings suggest a potential neuroprotective role for estrogen under these conditions, future studies including other subpopulations will be needed to examine the degree of generalizability of the findings reported here.

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Contributors

Natalie Rasgon, Daniel Silverman, Heather Kenna, and Tonita Wroolie designed the study; Natalie Rasgon, Heather Kenna, Tonita Wroolie, and Bevin Powers acquired the data; Natalie Rasgon, Heather Kenna, Katherine Williams, Cheri Geist, Daniel Silverman, and John Brooks critically revised the manuscript for intellectual content; Natalie Rasgon and Daniel Silverman obtained funding; Natalie Rasgon, Heather Kenna, Katherine Williams, Daniel Silverman, and Tonita Wroolie provided supervision; Natalie Rasgon, Heather Kenna, Katherine Williams, Bevin Powers, and John Brooks provided administrative, technical, or material support. Cheri Geist wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

Authors Daniel H.S. Silverman, MD PhD, Cheri L. Geist, BS, Heather A Kenna, MA, Katherine Williams, MD, Tonita Wroolie, PhD, Bevin Powers, BA, and John Brooks, MD, PhD have no disclosures or potential conflict of interest including any financial, personal, or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence, or be perceived to influence, their work.

Natalie Rasgon, MD, PhD, would like to disclose that she is a consultant for Wyeth. She certifies that all of her affiliations with or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in manuscript are completely disclosed above.

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CEREBRAL METABOLIC ACTIVITY


- Both Estrogen and Testosterone have neuro-protective roles. Women with lower E2 levels have an even greater risk of AD.
- There is overwhelming evidence that E and T help decrease apoptosis. Protective effect of both hormones decreases beta amyloid deposition.
Protective actions of sex steroid hormones in Alzheimer's disease

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1. Introduction

As expertly described in the accompanying review articles, sex steroid hormones are potent regulators of neuron survival in multiple CNS regions and across a variety of circumstances ranging from normal development to neural injury. A compelling, and as yet largely unrealized, promise of sex steroid hormones is the translation of their neuroprotective properties into efficacious strategies for the treatment and or prevention of age-related neurodegenerative disorders such as Alzheimer's disease (AD). Despite this unfulfilled therapeutic potential, abundant experimental, epidemiological and clinical evidence suggest that neural actions androgens, estrogens, and perhaps even progestogens can reduce the risk for AD.

AD is an age-related neurological disease that is the leading cause of dementia. Neuropathologically, AD is characterized by brain region-specific deposition of β-amyloid protein (Aβ) which creates senile plaques, hyperphosphorylation of the cytoskeletal protein tau that forms lesions called neurofibrillary tangles and neuropil threads, glial activation which is associated with inflammatory responses, and both synaptic and neuronal loss [3,37,139,140,196]. Although the mechanisms of AD pathogenesis remain to be fully resolved, the leading hypothesis posits that the disease is initiated and driven by prolonged elevation of Aβ levels [140]. Aβ is a proteolytic byproduct of the metabolism of amyloid precursor protein, a widely expressed protein with numerous functions ranging from axonal transport to gene transcription [336]. As a consequence of amyloid precursor protein expression, Aβ is normally found as a soluble protein at low levels in fluids and tissues throughout the body. In theory, alterations in either the production or clearance of Aβ that sway Aβ homeostasis towards increased neural levels will promote the development of AD [140]. The accumulation of Aβ encourages its abnormal assembly into oligomeric species that exhibit an altered structural conformation and can induce a range of neurodegenerative effects [136]. Consequently, enormous effort has been expended on identifying factors that regulate Aβ accumulation and or affect its neurodegenerative properties. One such class of factors is sex steroid hormones.
In this review, we will discuss the neuroprotective properties of sex steroid hormones as they relate to AD pathogenesis, focusing largely on their effects on Aβ accumulation and its associated neurodegeneration. Estrogens are the most thoroughly studied steroid hormones in terms of AD. We will cover the epidemiological and clinical evidence that suggests depletion of ovarian hormones at menopause increases the risk of AD in post-menopausal women, a danger some studies suggest may be mitigated by estrogen-based hormone therapy (HT). Consistent with a protective role against AD, experimental studies demonstrate that estrogens not only reduce neuron loss induced by AD-related insults but also act to reduce Aβ levels. However, in the Women's Health Initiative (WHI) trial, the most exhaustive clinical evaluation of HT thus far, HT was associated with increased rather than decreased risk of AD. Analysis of experimental work reveals several key limitations of estrogen's neuroprotective actions that may contribute to this clinical observation, including loss of neural estrogen respon-
siveness with age and interactions with progestogens that may limit estrogen neuroprotection. A more recent and still emerging literature suggests a parallel relationship in men with their primary sex steroid hormone, testosterone. That is, normal age-re-
lated testosterone is associated with increased risk of AD in aging men. Like estrogens, androgens also exert neuroprotective proper-
ties relevant to AD, including promotion of survival in neurons challenged with AD-related insults and reduction of Aβ levels. Finally, we consider future directions in this field, emphasizing the clinical potential of sex steroid hormones in prevention rather than treatment of AD and the emerging promise of selective estrogen receptor and androgen receptor modulators.

2. Menopause, hormone therapy, and Alzheimer's disease

Converging lines of evidence indicate a potentially important role of estrogens in regulating AD pathogenesis. Preliminary clues sug-
ggest this possibility stemmed from reports of sex differences in AD risk, with women showing higher prevalence and incidence. Although sex differences in AD are difficult to interpret due to gender differences in life expectancy, many studies of various cohorts indicate that women are at greater risk of AD [9, 17, 39, 93, 101, 137, 180, 221, 281, 290]. Further, there is some evidence that AD patho-
genesis may be more severe in women as indicated by sex differ-
cestrogens in cognitive deficits and neuropathology [21, 49, 66, 150], although other studies indicate men have higher levels of tau pathology [293, 294]. Further, there is a stronger association between the apolipoprotein E ε4 allele and sporadic AD in women compared to men [66, 84, 165] and the ε4 allele has been shown to be associated with greater hippocampal atrophy and memory impairments in women compared to men [98]. When considered together, these epide-
miological and neuropathological studies indicate sex differences in AD, suggesting that women may be more vulnerable to AD than men.

Several transgenic mouse models exhibit sex differences in AD-like neuropathology that appears to parallel that observed in human AD cases. For example, at both 15 and 19 mo of age, female Tg2576 mice display a higher plaque load burden and higher levels of both soluble and insoluble Aβ40 and Aβ42 than age-matched males [51]. Similarly, female APPswPS1 transgenic mice have higher Aβ load burden and plaque number than age-matched males [350]. The same pattern of greater Aβ deposition in female versus male mice is also observed in the 3xTg-AD triple transgenic mouse [156]. These studies in transgenic mouse models of AD sug-
gest that the female brain may be more vulnerable to AD pathogenesis.

The increased risk of AD in women is presumed to be associated with the precipitous loss of estrogens and progestosterone at meno-
pause. Consistent with this position, plasma levels of the estrogen 17β-estradiol (E2) [212] are reported to be lower in women with AD in comparison to age-matched controls. If the depletion of ovarian hormones at menopause contributes to women's increased risk of AD, then one would predict that estrogen-based hormone therapy (HT) would be effective in the prevention and or treatment of AD. This critical issue remains unresolved with persuasive argu-
ments both for and against the use of HT for AD. An early study of this issue found that AD risk was lower in women who used HT relative to nonusers and this risk decreased significantly as both dose and duration of HT use increased [247]. Similarly, findings from several other case control and prospective studies suggest that post-menopausal women with a history of HT use were at reduced risk of AD [52, 151, 184, 248, 329, 351, 382]. Further, a meta-analysis of studies found that HT was associated with decreased risk of cog-
nitive dysfunction [158, 197, 241, 305]. Collectively, these studies suggest a potential protective role of estrogen against the develop-
ment of AD. Despite indications of benefits, the potential protective of HT against AD remains controversial. Arguing against a protective role, several studies found that HT use was not associated with reduced risk of AD [reviewed in [146]] or failed to yield significant cognitive benefits [7, 22, 34, 40, 119, 152, 228, 266, 349, 369]. One possible expla-
nation for this discrepancy is suggested by findings from the Cache County Study, which demonstrated that the association between HT use and reduced risk of AD was strengthened in long-term HT users [382]. Interpretations of these findings include the concept that HT may have a largely preventative role against AD and or the hypothesis that early initiation of HT is essential as women who took HT for longer periods likely began treatment nearer the time of menopause.

The notion that estrogen-based HT can effectively reduce the risk of AD and improve age-related deficits in cognition has been challenged by findings from the Women's Health Initiative Memory Study (WHIMS). WHIMS was a randomized, multi-center, double-blind, placebo-controlled study of ~4500 women between 65 and 79 yrs of age that evaluated effects of HT consisting of conjuga-
ted equine estrogen (CEE) alone or CEE with the progestin medroxyprogesterone acetate (MPA). This study reported that neither CEE nor CEE + MPA significantly improved cognition versus placebo in women showing cognitive decline associated with nor-
mal aging [91, 273] or dementia [312, 311]. In the CEE alone arm, there was no significant difference in dementia incidence between HT or placebo groups although there was a non-significant trend towards increased risk in the HT group [91, 312]. In the CEE + MPA arm, they found that women receiving HT had a higher risk of probable dementia [311]. HT use was also associated with increased incidence of stroke and breast cancer, suggesting that the risks of HT may outweigh its benefits. Further, the important dif-
terences between the CEE alone arm and the CEE + MPA arm raise several issues about the inclusion of a progestin component.

Although the WHIMS findings raise serious concerns over HT use, many challenge the interpretation that these data dismiss the potential efficacy of HT in reducing the risk of AD [75, 118, 149, 209, 265, 276]. A variety of issues have been identified that may have affected HT outcomes in the WHIMS compared to the many observational studies such as differences in methodolog-
tical techniques, outcome measures, hormone exposures, meno-
pausal symptoms, and the timing of hormone use [149]. In particular, neural sensitivity to sex steroid hormones may diminish during the menopause transition, resulting in a critical window in which to initiate HT in order to realize benefits (reviewed in [75]). Because HT was initiated many years after menopause in the WHIMS study, the study's design may have inadvertently focused on an age group in which estrogen is minimally active in brain and thus was unlikely to detect potential cognitive benefits. In addition, as suggested by prior epidemiological and clinical find-
ings, estrogen may be most effective in preventing rather than treating AD. In this case, the relatively advanced age of WHIMS subjects would also be biased against positive outcomes. Additional issues include HT formulation, route of administration, and treatment regime (e.g., cyclic versus continuous hormone delivery). In order to unravel this conundrum, a greater understanding of the neuroprotective actions of estrogens and progestogens are needed, as well as their limitations in the context of aging.

In the following sections, we examine the neuroprotective effects of estrogen, focusing on its abilities to increase neuronal resilience against AD insults and to antagonize AD pathogenesis by reducing Aβ accumulation. Importantly, we also discuss experimental evidence that addresses how these protective actions of estrogen are affected by the concerns raised by WHIMS.

3. Estrogen neuroprotection and Alzheimer’s disease insults

An established neural action of estrogens that may contribute to a protective role against AD is promotion of neuron viability. Estrogen is neuroprotective against a variety of insults in several cell culture and rodent paradigms of injury and neurodegenerative disease. Of particular interest is the ability of estrogen to protect against neuronal loss induced by Aβ, which is thought to be primary neurodegenerative agent in AD. Reports from several groups demonstrate that estrogen can protect cultured neurons and neural cell lines from Aβ mediated toxicity [26,124,132,135,222,259].

Estrogen may potentially protect against Aβ-induced neurotoxicity at several steps in the degenerative process. The leading theory of Aβ toxicity posits a pathologic assembly of Aβ involving adoption of a β-sheet conformation, resulting in a change in protein structure that is associated with a toxic gain of function (reviewed in [347]). Consistent with this working hypothesis of Aβ neurotoxicity, our prior work has shown that Aβ is toxic only in an assembled state [263,261]. Assembled Aβ in the form of soluble oligomers and insoluble fibrils can induce neuronal death [72,348,373] degeneration of neurites [169,262] and synaptic disruption [145,182,288,348] leading to impaired learning and memory [61,198]. Interaction of Aβ assemblies with neurons initiates a cascade of upstream signaling mechanisms associated with cell death, including calcium dysregulation [216,356], oxidative stress [25,123,214], and activation of pro-inflammatory pathways promoting chronic glialis [89,264]. Most evidence suggests that the plethora of upstream signaling cascades elicited by Aβ ultimately mediate neurotoxicity by downstream activation of neuronal apoptosis pathways [71,73]. In particular, we find that Aβ-induced neuronal apoptosis involves activation of JNK signaling and consequent dysregulation of the Bcl-2 family of apoptosis-related proteins [374].

Since neuronal apoptosis is an important downstream mediator of Aβ neurotoxicity, regulation of apoptosis is predicted to be a key mechanism of estrogen protection from Aβ. Consistent with this hypothesis, estrogen has been implicated in the regulation of Bcl-2 family members in neurons [86,110,191,234,254,259,316,320]. The Bcl-2 family includes both proteins that promote cell survival (e.g., Bcl-2, Bcl-xL, and Bcl-w) and others that antagonize it (e.g., Bax, Bad, Bak, Bik, Bid, BNIPI3, and Bim) (reviewed in [12,70]). We have found that physiological levels of E2 inhibit neuronal apoptosis at least in part by increasing expression of anti-apoptotic Bcl-xL [259,320] and Bcl-w [375] while down-regulating expression of the pro-apoptotic Bim [375]. The observed effects of E2 on Aβ mediated apoptosis were found to be mediated ER-dependent mechanisms, since the anti-apoptotic effects of E2 were blocked by pre-treatment with an ER antagonist [259,375]. Supporting this, it has been previously demonstrated that both ERα and ERβ are crucial in regulating Bcl-2 expression and neuronal survival [388] and recently it has been shown that E2 can also increase Bcl-2 through Akt-dependent CREB activation [381].

Interestingly, estrogen dependent regulation of the Bcl-2 family of proteins has also been implicated in neuroprotection against excitotoxicity, a form of neuronal injury implicated in AD neurodegeneration [reviewed in [29,280]]. Evidence suggests that in AD, Aβ toxicity and glutamate excitotoxicity may cooperatively activate pathways leading neuronal death. Glutamate-induced excitotoxic injury is potentiated by Aβ. In cell culture paradigms, the combination of sub-lethal concentrations of glutamate combined with sub-lethal levels Aβ yields robust neuronal loss [189,215]. Such degenerative interactions are predicted to occur in AD because the AD brain exhibits both Aβ accumulation and evidence of glutamate injury. Whether upstream and or downstream pathways of Aβ and glutamate action are responsible for their synergistic toxic effects is unclear. Glutamate excitotoxicity leads to calcium dysregulation and oxidative stress, and excitotoxic neuron death is mediated in part by apoptosis (reviewed in [641]).

Several studies have demonstrated that estrogen reduces excitotoxic neuronal death induced by glutamate agonists in cell culture [43,236,275,317,315,319]. For example, Dorsa and colleagues found that estrogen inhibited neuronal death in murine cortical cultures following excitotoxic insult, an effect that could be pharmacologically blocked with the ER antagonist, tamoxifen [315]. Further, Brin-ton and colleagues found estrogen to promote intracellular Ca++ accumulation in neuronal cultures treated glutamate at physiological doses, while inhibiting intracellular Ca++ accumulation following treatment with excitotoxic glutamate doses [236]. Similar observations of estrogen neuroprotection following excitotoxic challenge have been reported by several groups [124,275,354].

Estrogen has also been shown to regulate the extent of excitotoxic injury in rodent models [15,14,55,286]. For example, administration of exogenous E2 to ovariectomized (OVX) rats has been reported to protect against kainate-induced neuronal loss [14]. Additionally, depletion of endogenous estrogen levels may increase susceptibility to excitotoxicity, with pronounced kainate-induced neuronal loss observed in intact rats during proestrus or following OVX [15]. Similarly, we observe neuroprotection against kainate lesion following administration of estrogen to OVX rats [55,286].

Interestingly, estrogen neuroprotection against excitotoxic injury shares mechanistic similarities with protection against Aβ-in-duced apoptosis. That is, estrogen regulation of the Bcl-2 family is implicated in protective actions against glutamate-related injury [232,234,316,388]. Brion and colleagues found that estrogen mediated neuroprotection against glutamate excitotoxicity by promoting mitochondrial Ca++ sequestration, and this was associated with increased expression of Bcl-2 [232]. Estrogen dependent modulation Bcl-2 following excitotoxicity may be mediated by rapid, non-genomic ER-dependent signaling mechanisms [387,388]. Estrogen activates an ER-dependent Src/ERK/CREB signaling pathway that leads to upregulation of Bcl-2 [364]. In contrast, others suggest that estrogen may regulate Bcl-2 family expression through a direct genomic mechanism. Supporting this we have described an estrogen responsive element (ERE) on the Bcl-x gene [259], while others describe ERs on Bcl-2 [83,256]. These pathways are summarized in Fig. 1.

In addition to regulation of the Bcl-2 family of proteins, estrogen has also been implicated in neuroprotection against many other prominent features of the AD-neurodegenerative cascade including inflammation and oxidative stress. Many studies indicate that Aβ may contribute to AD-related oxidative stress [25,123,214] and deposition of Aβ may activate microglia and promote inflammation [291,338]. Abundant evidence indicates that estrogen is a potent inhibitor of oxidative damage [340] and hydrogen peroxide mediated neuronal death [27,26,124]. Estrogen mediates these antioxi-
The glycogen synthase kinase-3 (GSK-3β) is involved in the pathogenesis of Alzheimer's disease (AD) through its role in neuroprotection against Aβ toxicity. However, the therapeutic potential of estrogen in AD treatment remains controversial.

Estrogens, including 17β-estradiol (E2), activate neuroprotective pathways that may attenuate Alzheimer's disease. Estrogens may suppress reactive gliotic responses associated with end-stage AD and that specific allele differences in ERα levels in humans and transgenic mice. In addition, recent studies have demonstrated that ERα levels in the frontal cortex correlate with mini-mental state examination scores in women with end-stage AD and that specific allele differences in ERα are correlated with an increased risk for AD in women with Down syndrome. Also, ERβ immunoreactivity is reportedly increased in the hippocampus compared to age-matched controls. Taken together, these studies suggest that the expression of ERα/β in the AD brain may play an integral role in the neuroprotective actions of E2. These effects are discussed in several recent reviews.

Both ER subtypes ERα and ERβ are implicated in mediating estrogen neuroprotection, although their relative contributions have been incompletely defined. Selective expression of ERα versus ERβ in neural cell lines has suggested a more important role of ERα in mediating neuroprotection in some studies but significant contributions from both ERα and ERβ in others.

Cell culture studies utilizing selective ERα (propylpyrazole triol, PPT), and ERβ agonists (diarylpropionitrile; DPN) to study estrogen neuroprotection typically report similar levels of protection from both agonists, although some evidence suggests greater activity of the ERα agonist PPT. Our studies in primary neuron culture indicate comparable levels of neuroprotection against Aβ from E2, PPT, and DPN, suggesting potential contributions from both ERα and ERβ in estrogen neuroprotection. However, our data also suggested potential differences between ER subtypes in terms of protective mechanisms, with PPT but not DPN inducing PKC-dependent neuroprotection.

Brinton and colleagues find that PPT and DPN closely mimic E2 protection from glutamate toxicity and a gene called dickkopf-1 as well as through the protein kinase A pathway as well as through the protein kinase A pathway. These results demonstrate some of the first insights into the mechanism behind estrogen neuroprotection in tau-related disorders.

The described neuroprotective actions of estrogen against AD-related insults are largely mediated by activation of estrogen receptors (ER). It has been well established that both ER subtypes are widely distributed in the brain, including in brain regions affected in AD such as the hippocampus, frontal cortex, and amygdala. Recently, changes in the subcellular distribution of ERs in hippocampal neurons have been implicated in AD pathogenesis. Specifically, the shift of ERs to the cytoplasm may decrease the development of AD pathology in humans and transgenic mice. In addition, recent studies have demonstrated that ERα levels in the frontal cortex correlated with mini-mental state examination scores in women with end-stage AD and that specific allele differences in ERα are correlated with an increased risk for AD in women with Down syndrome. Also, ERβ immunoreactivity is reportedly increased in the hippocampus compared to age-matched controls.

Taken together, these studies suggest that the expression of ERα/β in the AD brain may play an integral role in the neuroprotective actions of E2. These effects are discussed in several recent reviews.
AD-related insults but that each may mediate protection by preferentially activate different signaling pathways.

4. Estrogen regulation of β-amyloid accumulation

In addition to increasing neuronal resistance to AD-related insults, estrogen may also protect against AD by preventing the key initiator of AD pathogenesis, accumulation of Aβ. Steady state levels of Aβ are influenced by opposing pathways of Aβ production and Aβ clearance, both of which appear to be regulated by estrogen. Estrogen regulation of Aβ was first suggested by cell culture experiments focused on Aβ production. Early studies demonstrated that estrogen modulates processing of amyloid precursor protein (APP), the transmembrane parent protein of Aβ [106].

The majority of APP is metabolized by two competing pathways, the amyloidogenic and non-amyloidogenic pathways. In the amyloidogenic pathway, thought to occur following endocytosis of cell-surface APP, APP is first cleaved by β-secretase (BACE) to liberate β-APPs. The C-terminal fragment (C99/β-CTF) is left embedded in the membrane and is cleaved by the γ-secretase enzyme liberating the Aβ40/Aβ42 peptides. It is thought that another fragment is also released termed the APP intracellular domain (AICD), which can translocate to the nucleus and activate gene transcription. In the non-amyloidogenic pathway, which is the predominant pathway, APP is cleaved within the Aβ domain by α-secretase to liberate a neuroprotective, secreted form of APP (α-APPs). A C-terminal fragment (C83/scTF) is left embedded in the membrane for further cleavage into non-amyloidogenic fragments [297,343].

Estrogen appears to regulate Aβ levels at least in part by promoting the non-amyloidogenic cleavage of APP, precluding production of the Aβ peptide. In the human kidney 293 cell line, E2 has been shown to reduce the level of Aβ peptide in a concentration-dependent manner [59]. Further, some preliminary results suggest that in a clinical setting, short-term E2 treatment is able to reduce plasma levels of Aβ in post-menopausal women naive to HT [20], although the significance of plasma Aβ in terms of both AD pathogenesis and diagnostic value remains controversial.

How estrogen regulates APP processing is not clear, although multiple pathways have been implicated. First, most data suggest that estrogen increases the α-secretase pathway of APP processing [171,213,366,385]. There is evidence that estrogen can promote the α-secretase pathway via activation of extracellular-regulated kinases 1 and 2 (ERK1 and ERK2) signaling [213], a well-established estrogen signaling pathway [319,335,353]. The action of estrogen on the ERK components of mitogen-activated protein kinase (MAPK) signaling pathway and APP generation are rapid and may be ER independent [213]. Estrogen may also regulate APP processing through protein kinase C (PKC)-dependent pathways. PKC signaling is a strong activator of non-amyloidogenic APP processing [74,190,242]. Further, estrogen is a significant activator of PKC in both neuron culture [69,68] and in brain [11,268,299]. Consistent with this possibility, a recent cell culture study has shown that estrogen activation of α-secretase APP processing is blocked by PKC inhibitors [385]. However, not all studies demonstrate such straightforward results. For example, an in vitro study demonstrated that E2 is capable of increasing the production of sAPPα but not reducing the release of Aβ in cortical neurons over-expressing APPα [344].

Some evidence also suggests that estrogen may promote non-amyloidogenic APP processing by altering APP trafficking. Specifically, Greenfield et al. reported that estrogen promoted the secretion of APP containing vesicles from the primary site of amyloidogenic APP processing, the trans golgi nucleus, thereby decreasing available APP substrate for Aβ formation [134]. In addition to promoting the non-amyloidogenic APP processing pathway, estrogen has also been implicated in the modulation of APP levels through the regulation of alternative splicing [333] and APP overexpression post-injury [307], thereby altering substrate for APP processing and subsequent Aβ production.

Importantly, subsequent studies demonstrated that estrogen also functions as a regulator of Aβ in animal models. For example, the depletion of endogenous estrogen by OVX in guinea pigs increased the levels of soluble Aβ in brain, an effect partially reversed by E2 treatment [258]. This estrogen action may be sex-dependent since androgen but not E2 treatment reduced elevated Aβ levels resulting from orchietomy (ORX) of adult male rats [271]. A similar pattern of E2 regulation of Aβ has been observed in several transgenic mouse models of AD. That is, OVX is associated with increased Aβ and E2 treatment with reduced Aβ levels in Tg2576 [380,390], APPα [199], Tg2576P51 [368,390], and 3xtg-AD [53,54] mice. The mechanism by which estrogen regulates Aβ in vivo has yet to be elucidated. In the APPα transgenic mouse model of AD, E2 treatment was associated with increased sAPPα, indicating increased α-secretase APP processing [199]. However, in wild type guinea pigs, experimental manipulation of estrogen status was not associated with corresponding changes in sAPPα levels [258]. Increased levels and activity of BACE observed in arotamate knock-out mice also suggests a potential role for estrogen in the regulation of secretase expression and/or activity [380]. Further work will be needed to elucidate whether estrogen regulation of Aβ in animals involves APP processing and, if so, to define the relevant upstream signaling components (e.g., MAPK, PKC).

Curiously, estrogen levels are associated with Aβ accumulation only in some but not all transgenic mouse models of AD [120,133,148,380]. In the Tg2576, PDAPP, and APPαP51 mice models, OVX was not associated with increased Aβ levels and E2 treatment did not reduce Aβ levels. Discrepancies in the effects of estrogen on Aβ levels across transgenic mouse models may reflect several differences, ranging from molecular design of the transgenic lines to variability in methodological parameters such as the timing and dosing of hormone manipulations. One potentially important methodological difference across the studies is their varying techniques for Aβ quantification, each of which preferentially measures different pools of Aβ ranging from soluble monomeric Aβ to oligomeric and deposited forms. Whether estrogen differentially regulates these various Aβ pools is currently unknown. In our laboratory, we found that estrogen decreases Aβ accumulation in the 3xtg-AD mouse model as assessed by the immunoreactive load method [53,54], which detects relatively insoluble intra- and extra-cellular Aβ. Future studies will be needed to determine exactly which Aβ pool(s) estrogen is capable of regulating and whether this contributes to observed differences in estrogen actions across models.

Discrepancies between studies on the role of estrogen as a regulator of Aβ accumulation also may indicate differences in brain levels of estrogen. Recent work suggests that OVX has limitations as a strategy to fully deplete brain estrogens. For example, in the estrogen-responsive element-luciferase mouse model, which was engineered to express the non-mammalian luciferase protein in regions of the brain estrogen activity in comparison to other body regions [65]. Genetic differences between transgenic mouse models may reflect several differences, ranging from molecular design of the transgenic lines to variability in methodological parameters such as the timing and dosing of hormone manipulations. One potentially important methodological difference across the studies is their varying techniques for Aβ quantification, each of which preferentially measures different pools of Aβ ranging from soluble monomeric Aβ to oligomeric and deposited forms. Whether estrogen differentially regulates these various Aβ pools is currently unknown. In our laboratory, we found that estrogen decreases Aβ accumulation in the 3xtg-AD mouse model as assessed by the immunoreactive load method [53,54], which detects relatively insoluble intra- and extra-cellular Aβ. Future studies will be needed to determine exactly which Aβ pool(s) estrogen is capable of regulating and whether this contributes to observed differences in estrogen actions across models.
regulation of brain Aβ accumulation. This hypothesized importance of brain hormone levels is supported by the finding that brain levels of E2 are lower in female AD patients in comparison to age-matched control cases [380], a finding we have recently replicated [287]. In addition, it has recently been shown that long-term OVX in female mice significantly lowers E2 levels in the hippocampus while increasing serum levels of Aβ [105]. Thus, estrogen regulation of Aβ may depend primarily upon brain estrogen levels, which may be dependent on both ovarian and brain steroid production.

In addition to modulating the production of Aβ, estrogen may also promote Aβ clearance. One mechanism of Aβ clearance is through microglial degradation [282]. Estrogen has been shown to promote microglial phagocytosis [47,267] and E2 treatment increases microglial internalization of Aβ in microglia of both murine [143] and human origin [200]. Estrogen treatment has also been found to reduce Aβ accumulation in rats following intracerebroventricular Aβ injection [143]. Correspondingly, increased Aβ burden and impaired microglial Aβ clearance has been reported in an estrogen deficient transgenic mouse model of AD [380]. Estrogen has also been implicated in the regulation of levels of two major Aβ degrading enzymes, insulin degrading enzyme and neprilysin. Both of these enzymes are significant regulators of Aβ levels and their regulation and activities are implicated in AD pathogenesis [331,337]. A few recent studies suggest that estrogen depletion by OVX can decrease neprilysin activity in female rat brain, an effect reversed by E2 replacement [163]. Thus, estrogens may influence Aβ clearance through regulation of neprilysin, although this pathway may involve an androgen responsive element on the neprilysin gene [365]. Estrogen pathways of Aβ-lowering are illustrated in Fig. 1. Given the significance of Aβ accumulation to AD pathogenesis, future studies must clearly define the role of estrogen in regulating both Aβ production and clearance pathways and how they are affected by differences in E2 brain levels.

5. Aging effects on estrogen responsiveness

Whether the described neuroprotective effects of estrogen prove to have therapeutic relevance to age-related neural diseases including AD will depend in part on the brain’s responsiveness to estrogen with advancing age. One of the primary criticisms of WHIMS and other clinical studies of estrogen-based HT is that the intervention may have been initiated beyond a critical window of opportunity [118,265,276]. This notion refers to the possibility that the aging brain age may lose responsiveness to sex steroid hormones after an extensive period of low hormone levels, such as occurs following menopause. According to this argument, HT may exert estrogenic effects only if begun near the time of menopause. Since most participants in the WHIMS study were many years beyond menopause, the critical window hypothesis could explain in part the absence of beneficial neural actions of HT. Consistent with this position, several clinical studies have noted that HT is associated with positive neural effects in women showing menopause symptoms (e.g., flushing), suggesting retained estrogen responsiveness [154,153,157,306,305]. Although this issue remains to be fully evaluated, results from the Multi-Institutional Research on Alzheimer Genetic Epidemiology study show that HT was associated with reduced AD risk only in post-menopausal women that initiated treatment at a relatively younger age [154].

The critical window notion of an age-related loss in brain responsiveness to estrogen is supported by studies in animal models. One experimental approach to address this issue is the “gap paradigm” in which OVX animals are treated with E2 replacement after short versus long periods of time, a design that assesses the effects of prolonged hormone deprivation on subsequent hormone exposure. In general, results from gap studies indicate diminished estrogen responses following many months of hormone absence. For example, E2 and E2 plus progesterone replacement was associated with improved spatial memory on the delayed match task when administered within 3 months post-OVX, but not when hormone treatment was delayed 10 months post-OVX [115]. Further, E2 given immediately after OVX in rats improved spatial memory performance on radial arm maze while E2 given after 5 months of OVX did not [78]. Recently, it was reported that E2 given immediately after OVX but not after a 5 months delay was able to increase hippocampal ChAT protein levels in middle-aged OVX wild type rats [35]. Although this topic requires further work to elucidate the key factors and underlying mechanisms, the data generated thus far suggest that in laboratory animals, the brain can show reduced hormone responsiveness after an extended period of hormone depletion.

Another approach to evaluate the role of aging in neural estrogen responsiveness has been to simply compare the effects of estrogen in young adult versus aging female rodents. This type of study is critical in determining the efficacy of female sex steroid hormones before and after the onset of reproductive senescence, the result of normal reproductive aging in female rodents (reviewed in [58]). While reproductive aging in rodents does not mimic menopause, rodents do experience a pattern of estrus cycle irregularity followed by reproductive senescence that is similar in some respects with the perimenopause period in women [95]. Age-related reproductive changes in female rodents typically become apparent between 9 and 11 months of age, depending upon species and strain. For example, the age-related estrus cycle changes have been well characterized in the C57Bl6 mouse strain [95]. Like women, these female mice demonstrate a large range of variability in cycle irregularity and hormone levels during middle and old age. These mice experience cycle cessation between 11 and 16 mo of age during which a substantial proportion enters a period of persistent vaginal cornification lasting 2–4 months. After this variable period, all mice enter an irreversible final stage of permanent diestrus characterized by low E2 and progesterone levels and elevated luteinizing hormone levels [114], ovarian follicle depletion, and loss of reproductive capability [126].

In estrogen neuroprotection studies, middle-aged female rodents undergoing reproductive senescence show diminished effects in some studies but retained protection in others. First, several reports suggest that the neural effects of OVX and E2 treatment are diminished in aging female rodents. Studies by Sohrabi and colleagues suggest that reproductive senescence in middle-aged female rats reduces protective estrogen actions. For example, in assessing estrogen regulation of neurotrophin expression, they found that E2 treatment in OVX young adult female rats (age 3 mo) increased levels of BDNF, trkA, and trkB in olfactory bulb and diagonal band of Broca, whereas E2 treatment of middle-aged (17 months) OVX reproductively senescent, female rats showed either no increase or decreased expression of neurotrophins [175]. Similar studies by this research group found that, in comparison to young adult female rats, reproductively senescent female rats show several alterations in estrogen-mediated effects including cytokine and growth factor responses following injury [176,237] and blood–brain-barrier permeability [18]. In another paradigm, Finch and colleagues reported differences between young adult (3 mo) and middle-aged (18 mo) female rats on estrogen regulation of compensatory neuronal sprouting following entorhinal cortex lesion. In comparison to young rats, older rats no longer exhibited an OVX-induced decrease in sprouting and showed differences in regulation of GFAP mRNA [321]. However, some neural actions of estrogen appear to remain relatively robust during aging. For example, E2 treatment in middle-aged OVX rats enhanced performance on hippocampal-dependent spatial
memory tasks [78] as well as altered the hippocampal expression of several genes [1].

In neural injury paradigms, there is evidence of both retained and altered estrogen neuroprotection. In reproductively senescent female rats, both E2 and progesterone reduced infarct size in a model of stroke injury [5]. Wise and colleagues reported similar neuroprotective effects of E2 treatment in OVX young (3–4 mo) and middle-aged (9–12 mo) rats in the middle cerebral artery occlusion (MCAO) model of stroke [85,360]. In our laboratory, we have found that reproductively senescent female rats show evidence of altered but not completely diminished estrogen neuroprotection. In young adult (3 mo) female rats, an excitotoxic lesion to hippocampus induced by systemic application of the glutamate agonist kainate is significantly worsened in animals OVX for 2 weeks prior to the lesion. In a similar paradigm, we find that E2 treatment, initiated at the time of OVX, significantly increases the number of viable hippocampal neurons following kainate lesion [286]. However, in reproductively senescent (14 mo) rats, OVX was not associated with further cell loss, although E2 replacement still resulted in a significant increase in neuron survival [55]. In a model of spinal cord injury, the effects of E2 treatment in young (2 mo) versus middle-aged (12 mo) sham-OVX and OVX female rats were investigated. Treatment with E2 protected against several indices of injury in both young and aging OVX rats, however in ovari-intact rats E2 neuroprotection was lost in middle-aged rats [60].

Interestingly, emerging data suggest that patterns of reproductive aging in female rats may contribute to altered estrogen responsiveness with age. A recent study compared estrogen protection using the MCAO stroke model in middle-aged female rats stratified by their stage of reproductive aging: reproductively senescent rats that had entered a persistent acyclic state, and rats ever in ovary-intact rats E2 neuroprotection was lost in middle-aged rats [60].

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Why the brain shows is less responsive to estrogen with age is unclear, but age-related decreases in ER expression as well as E2 binding to ERs in aged rat brain have been reported [58,289,361]. Estrogen actions also show age-related changes in other estrogen responsive tissues, including uterus, bone and heart. For example, the uterus becomes less responsive to estrogen with increasing age, showing smaller OVX-induced decreases in uterine weight [367] and uterotrophic effects of estrogen only when treated soon after OVX [78]. Further, while most studies demonstrate that the trophic effects of E2 on bone extend through middle-age, some studies suggest that as aging progresses, this effect is altered from E2-mediated one [168]. Interestingly, increased ERx predominance has also been suggested to underlie age changes in neural estrogen responsiveness [19].

Taken together, available experimental research suggests that although estrogen neuroprotection is often observed in aging rats, it can also be significantly diminished. Still uncertain is how the many AD-related facets of estrogen neuroprotection may be impacted by aging. Additional studies are necessary to determine the extent to which observed age-related changes in estrogen responsiveness may be delayed, prevented and or reversed. This phenomenon has important implications for the future of HT in post-menopausal women. Ongoing clinical and animal studies promise to shed new insight into this issue in the next few years.

6. Progesterone interactions with estrogen in regulation of Alzheimer’s disease

Estrogen actions must also be considered with respect to the second major class of ovarian hormones, progestogens. Progestrone has long been recognized as a regulator of estrogen, particularly in the female reproductive system (reviewed in [130]), where it often antagonizes estrogen action. Clinically, progestogens are typically a key element of HT that are thought to minimize deleterious effects of estrogen. Perhaps most importantly, in experimental paradigms progestrone can inhibit human endometrial cancer cell growth [77,79] and in clinical studies progestogens are associated with reduced risk of endometrial cancer [129,257].

In clinical studies of AD and dementia, most results indicate similar outcomes with both estrogens alone (ie., CEE) and estrogens in combination with progestogens (ie., CEE + MPA). In the WHIMS trial, a comparison between the CEE alone and CEE + MPA arms of the study raised the question that the clinical efficacy of HT may be dependent upon the hormone constituents within. Both the CEE and CEE + MPA arms failed to demonstrate a protective effect and both actually increased the risk of dementia compared to women receiving placebo [311]. Notably, the CEE alone arm had a negative impact on global cognitive function, however this negative impact was worsened when pooled with the data from the CEE + MPA arm [91]. Interestingly, short-term HT treatment in women with existing AD was associated with some benefits on psychiatric symptoms in the CEE group but not in the CEE + MPA group [162]. These and related issues suggest that the inclusion of a progestogens may influence the effects of CEE alone on cognitive outcomes and risk of dementia in post-menopausal women [75].

Despite the common use of the progestogen MPA in current HT paradigms, researchers have only relatively recently begun to investigate the effects of progestrone on the CNS in regards to aging and neurodegenerative diseases. Although compelling experimental evidence indicates numerous protective actions of estrogen and progesterone when delivered independently [44,295], comparatively less is known about interactions between estrogen and progesterone when they are administered together. Interestingly, accumulating observations indicate that progesterone often antagonizes rather than synergizes with estrogen-mediated neuroprotective actions. This incomplete understanding highlights the need to determine the interactive effects of these hormones in the brain.

Findings from an increasing body of research have begun to provide insight into how beneficial neural actions of estrogen are affected by interactions with progesterone. Findings from both in vitro and in vivo paradigms suggest that progesterone treatment can antagonize estrogen neuroprotection. For example, long-term progesterone treatment in aged (23–24 mo) OVX female rats blocked estrogen upregulation of brain derived neurotrophic factor, nerve growth factor, and neurotrophin 3 [32]. In a similar paradigm of hormone treatment in middle-aged OVX rats, estrogen-induced improvement in spatial memory performance was blocked by co-administration of progesterone [33]. More specific to neuron viability though are recent studies from our laboratory demonstrating that progesterone blocks estrogen neuroprotection from excitotoxic injury in female rats. In both young adult (3 mo) [286] and middle-aged (14 mo) [55] OVX
rats, continuous E2 treatment reduced kainate-induced neuron loss in hippocampus CA2/3 whereas continuous progesterone did not significantly affect neuron viability. Importantly, when progesterone was included with E2, estrogen neuroprotection was no longer observed [55,286]. Similarly, Brinton and colleagues recently reported that E2 and progesterone administered independently to wild type rats enhanced several markers of brain mitochondrial function but, when replaced in combination, the hormones showed attenuated rather than enhanced responses [167].

While the mechanisms behind this antagonistic property remain unknown, our laboratory has begun to investigate the possibility that progesterone modulates estrogen function in part by regulating ER expression. We have observed in primary neuron culture that low physiological concentrations of progesterone rapidly downregulate mRNA levels of both ERα and ERβ [174]. Functionally, this progesterone-mediated decrease in ER expression was associated with inhibition of both ERE-mediated transcriptional activity and estrogen neuroprotection against apoptosis [174]. Although progesterone treatment by itself did not significantly affect apoptosis in this paradigm, abundant evidence demonstrates that progesterone can activate several neuroprotective cell signaling pathways, including Akt [318] and ERK [233,232] signaling and upregulation of the anti-apoptotic protein Bcl-2 [232]. Further, as reviewed in an accompanying article, progesterone can significantly protect neurons against numerous insults [44,295]. Thus, independently estrogen and progesterone can exert protective actions, but in combination they can inhibit each other and thus fail to protect. Perhaps not unexpectedly, the interactive neuroprotective effects of the two female sex steroid hormones are not quite so straightforward. Besides antagonistic effects, progesterone can also synergistically interact with estrogen to promote beneficial neural effects including increased spine density [100,313]. In OVX female rats, acute progesterone treatment (2–6 h) was observed to augment the estrogen-induced increase in spine density, whereas prolonged progesterone treatment (18 h) blocked the estrogen effect [362].

Thus, a key factor in understanding estrogen neuroprotection and perhaps its relevance to HT in post-menopausal women is elucidation of the interactions between estrogen and progesterone.

A similar progesterone regulatory relationship also appears to affect estrogen protection from indices of AD-like neuropathology. In recent studies, our laboratory has begun to investigate how progesterone interacts with estrogen in regulating Aβi accumulation in the 3xTg-AD transgenic mouse model of AD. As discussed above, we found that 3 months following OVX of young adult female 3xTg-AD mice, there were robust increases in Aβi accumulation and tau phosphorylation and impaired performance in spontaneous alternation behavior in comparison to sham OVX 3xTg-AD mice [54]. Continuous E2 treatment during the 3 month OVX period largely prevented the worsening of AD-like neuropathology and behavioral performance, however continuous progesterone by itself did not affect OVX-induced changes in either Aβi accumulation or behavior. Consistent with an antagonistic role of progesterone, we observed that co-treatment with progesterone antagonized the beneficial effect of estrogen in lowering Aβi accumulation [54]. However, estrogen and progesterone co-treatment did show a beneficial effect in reducing levels of tau hyperphosphorylation, suggesting positive estrogen-progesterone interactions. Consistent with a beneficial role of progesterone, Frye and colleagues report that long-term progesterone treatment following OVX in another transgenic mouse model of AD was associated with some cognitive benefits [104]. Taken together, these studies provide the first information to date on the interactive effects of estrogen and progesterone on AD-like neuropathology and demonstrated the potential for both positive and negative outcomes in terms of protection from AD-related neuropathology.

Despite evidence of antagonistic neural effects of progesterone on estrogen neuroprotective actions, progestogens are still deemed a necessary component of HT in women with a uterus. Therefore, HT may need to be optimized to maximize the benefits and minimize the unwanted consequences associated with estrogen-progestogen interactions. One possible strategy is the use of cyclic hormone delivery rather than the continuous, combined treatment that is currently common to HT. Initial clinical evaluation of cyclic progestogen exposure has been completed and more is underway. Several clinical HT trials have incorporated a comparison between continuous versus cyclic progestogen in post-menopausal women on osteoporosis and cognitive function. For example, two completed studies have both demonstrated that long-term HT with a cyclic progestogen dose was able to increase bone mineral density in post-menopausal women [239,56]. Similarly, a continuous estrogen plus cyclic progestogen paradigm is currently employed in the KEEPS (Kronos Early Estrogen Prevention Study) Cognitive and Affective Study, a randomized, placebo-controlled, double-blind study investigating the effects of HT in post-menopausal women who are within 36 months of their final menstrual period [357]. This and similar new trials promise to provide important insights on the efficacy of cyclic hormone delivery and the hypothesized importance of a critical window of hormone intervention.

Experimental studies in animal models lend support for the use of cyclic rather than continuous progesterone to optimize estrogen neuroprotection. For example, Gibbs have demonstrated that short-term treatment with E2 and progesterone can improve cholinergic function [116], with maximal benefit resulting from cyclic administration of estradiol and progesterone and the least benefit from continuous combined treatment [115]. In our laboratory, we have begun investigating the potential utility of cyclic progesterone against AD neuropathology by comparing cyclic versus continuous progesterone in the presence and absence of continuous E2 in OVX 3xTg-AD mice. Our results show that whereas continuous progesterone largely inhibits estrogen protection from AD-related neuropathology, cyclic progesterone appears to significantly increase estrogen protection against Aβi accumulation, tau phosphorylation, and working memory deficits (unpublished observations). These exciting new findings support the hypothesis that estrogen–progesterone interactions can yield additive neuroprotection and that cyclic hormone delivery may be a critical parameter.

7. Age-related androgen depletion and Alzheimer’s disease

In parallel to the relationships between age-related estrogen loss in women and increased AD risk, testosterone is depleted as a normal consequence of aging in men and is linked with elevated risk of AD. As discussed above, a significant biological event in women that contributes to the role of aging in AD is menopause and the resultant loss of the sex steroid hormones estrogen and progesterone. Although men do not experience menopause per se (i.e., a cessation of reproductive activity, nearly complete loss of sex steroid hormones), men do experience a somewhat similar process termed androgen deficiency in aging males (ADAM). ADAM refers to normal, age-related depletion of testosterone and the corresponding constellation of symptoms that reflect dysfunction and vulnerability to disease in androgen-responsive tissues including brain [24,50,96,125,179,183,217,224,302]. The decline in testosterone levels begins in the 3rd decade and continues at an annual rate of 0.2–1% for total testosterone and 2–3% for bioavailable testosterone [94,131,227]. Unlike menopause, aging men do not experience comparable levels of andropause. That is, although all men exhibit significant age-related testosterone loss typically beginning in the third decade of life, men vary in the extent of testosterone loss and the corresponding severity of clinical manifestations [226,326]. It
is estimated that 30–70% of men aged 70 and older are hypogonadal, resulting in at least 5 million aging men in the US suffering the consequences of andropause and only a small minority of those receive hormone treatment [142,225]. ADAM is associated with increased risk of sarcopenia, osteoporosis, falls, frailty, and all cause mortality.

The brain is a highly androgen responsive tissue where androgens induce several beneficial actions. For example, androgens have been shown to improve mood and promote select aspects of cognition, including spatial abilities [127,172] and verbal fluency [4]. Men with a higher free testosterone index have been found to perform better on visual and verbal memory and exhibited better long-term memory [23], while those with a low free testosterone index can show decreased visual memory, visuomotor scanning, verbal memory, and visuospatial processing [219]. ADAM has been associated with impaired cognitive performance in some but not all studies [141,219].

One recently established consequence of ADAM is an increased risk for the development of AD. Several [161,160,159,274,352] but not all [255] studies have identified a relationship between low circulating levels of testosterone and a clinical diagnosis of AD. In these studies, the relationship between testosterone and AD appears to be strongest when circulating levels of free rather than total testosterone are examined, and when mean ages are under 80 years [161,160,159,249]. The relationship between testosterone and AD may be influenced by the presence of at least one apolipoprotein ε4 allele, a genetic risk factor for AD [322]. Specifically, men with at least one ε4 allele had lower levels of testosterone than men without an ε4 allele [160]. Animal studies support a link between apolipoprotein E, testosterone, and AD [269,270].

Although the majority of studies have identified a relationship between low testosterone and increased AD risk in men, most were unable to determine whether low testosterone contributes to the disease process or is merely a result of it. However, two complementary studies suggest low testosterone occurs prior to or in the early stages of AD pathogenesis, and thus likely acts a risk factor. The first study compared clinical diagnosis of dementia with blood levels of testosterone in the prospective Baltimore Longitudinal Study on Aging [220]. Male subjects were followed for 4–37 years with a mean of 19 years per subject and were diagnosed as clinically normal at the time of their first testosterone measurement. Subjects that eventually received a clinical diagnosis of AD showed lower circulating levels of free testosterone. Interestingly, those men with AD, testosterone levels were reduced at check-ups 5–10 years prior to diagnosis [220], suggesting androgen loss occurred well before clinical manifestations of the disease. Consistent with this study are findings from a study by our laboratory in which we linked low brain levels of testosterone with increased risk of AD in men [284]. First, using human post-mortem brain tissue from neuropathologically normal men, we found that levels of testosterone but not E2 show a significant age-related decline. When we examined changes in brain levels of hormones across cases stratified by neuropathological status, we found significantly decreased brain levels of testosterone in AD cases as compared with neuropathologically normal cases even after controlling for the age-related hormone loss [284]. We also measured brain levels of androgens in cases with mild neuropathological changes, consistent with the earliest stages of AD. These cases also exhibited low testosterone levels [284], again indicating that testosterone loss occurs prior to robust pathology and thus may contribute to the development of AD. Taken together, these results suggest age-related testosterone depletion in men is a risk factor for AD.

Unlike the numerous clinical studies that have evaluated the efficacy of HT use in treating and or preventing AD in post-menopausal women, comparatively few studies have examined testosterone therapy in men for protective roles against age-related cognitive decline and development of dementia. Androgen therapies have been approved and used for the treatment of hypogonadism in men and are typically associated with reduced fat mass and improved muscle mass and bone density as well as increased mood, libido, and overall quality of life [31,327,332]. However, clinical evaluation of beneficial cognitive effects of testosterone therapy have been mixed. For example, a study of hypogonadal and eugonadal men found that testosterone increased verbal fluency [4]. Others report improvements in spatial cognition and working memory following testosterone treatment [62,173,172]. In contrast, some studies did not find significant changes in cognition following testosterone therapy [30,90,141,210,339].

A few studies have evaluated the effects of testosterone therapy in subjects with AD. In a small clinical study of men recently diagnosed with AD, testosterone treatment for up to 1 year contributed to improvement in both overall cognitive ability and visual spatial skills [328]. In contrast, other studies have not reported significant benefits of testosterone therapy in men with mild cognitive impairment and AD [63,206]. As with the observed inconsistencies in the literature of HT use in women, there are likely several factors that contribute to the observed differences between testosterone studies, including cognitive domains, treatment type and duration, and the age and other characteristics of the subjects.

8. Testosterone neuroprotection and Alzheimer’s disease insults

One beneficial action of androgens that is hypothesized to contribute to a role in reducing risk of AD is neuroprotection. Androgens are established promoters of neuron viability during neural development as well as in adult brain following mechanical injury and disease-related toxicity. One target of androgen neuroprotection is motorneurons following axotomy [178]. In this paradigm, testosterone treatment accelerates the rate of nerve regeneration and attenuates neuron loss [164,178,177,193,194,195,330,377,379]. Similarly, following facial nerve crush in male hamsters, testosterone increased the rate of axonal growth and functional recovery [193,192]. These effects are true not only for testosterone but also its potent androgen metabolite dihydrotestosterone (DHT) [378,377,379]. In addition, the anti-androgen flutamide was able to block testosterone’s neuroprotective effects on motor neurons [195], corroborating the role of androgen versus estrogen pathways. The mechanism behind this neuroprotective action appears to be through androgen regulation of trophic factors [378,379]. Recently, studies have found that in addition to long-term treatment, short-term testosterone, DHT, and estrogen treatments are protective and suggest a more direct mechanism of hormone action [164].

In addition to neuroprotective effects on motor neurons, androgens have also been found to promote neuron survival in brain regions vulnerable to neurodegenerative diseases such as Alzheimer’s disease. These areas include the hippocampus and cortical regions, which are both affected in AD and rich in androgen receptors [314]. In a study by Garcia Segura and colleagues, acute testosterone treatment attenuated neuron loss in the hilus of the dentate gyrus following excitotoxic lesion in ORX male mice [16]. Interestingly, acute treatment of E2 was also protective while DHT treatment did not protect against neuron loss in androgen-depleted mice. Furthermore, the protective effect of testosterone was blocked by an aromatase inhibitor, suggesting that in this model of acute hormone treatment, estrogen is responsible for testosterone neuroprotection [16]. A study from our laboratory investigated the effect of long-term hormone replacement on neuronal death induced by excitotoxic lesion. In this study, depletion of endogenous androgens as a consequence of ORX resulted in increased hippo-
campal neuron loss following kainate lesion in comparison with sham ORX rats. However, in ORX rats treated for two weeks with DHT, this increase in cell loss was blocked [272]. In contrast to the results of Garcia Segura and colleagues, we found that rats treated with E2 did not exhibit decreases in cell loss following kainate lesion. This suggests that androgen regulation of neuroprotection in this paradigm is a result of androgen rather than estrogen pathways. Behavioral responses to seizures were measured and no differences were observed between groups suggesting that the observed androgen neuroprotection was not a result of decreased seizure severity [272]. Although we did not observe an androgen-mediated effect on seizure severity, other studies have found a significant effect of androgens on seizure severity. In studies by Frye and colleagues, testosterone decreased neuron loss by inhibiting seizure activity [102,103]. Subsequent studies found that protection was due to DHT metabolism to 5α-androstane-3α,17β-diol, an androgen that acts on GABAA receptors. GABA activation reduces excitatory signaling, which in turn attenuates seizure activity thereby minimizing lesion severity [87,88,277].

While in vivo models provide valuable insight into androgen neuroprotection, neuron culture models of toxicity have proven valuable in defining the underlying molecular mechanisms. Cell culture models of neural injury have demonstrated testosterone protection against serum deprivation [45,138], Aβ toxicity [229,260,384], and oxidative damage [2]. Testosterone neuroprotection against serum deprivation-induced apoptosis requires activation of an androgen receptor (AR) dependent mechanism [138]. Specifically, the anti-androgen flutamide attenuated protection while an aromatase inhibitor had no effect on neuron viability [138]. Consistent with this androgen-mediated mechanism of androgen neuroprotection is an early study from our laboratory, which found that testosterone neuroprotection against toxicity induced by extracellular Aβ results from DHT not E2 [260]. DHT treatment in this paradigm was equally as protective as testosterone, but use of an anti-estrogen droloxifene failed to block protection, suggesting androgen pathways are responsible for neuroprotection [260]. Further, we observe androgen neuroprotection in PC12 cells transfected with AR but not in either untransfected PC12 cells or those transfected with empty vector [229].

There are several potential mediators of androgen neuroprotection downstream of AR. Some evidence suggests androgen neuroprotection may be mediated through attenuation of oxidative stress [2]. Another potential mechanism involves a classic genomic mechanism, increased expression heat shock proteins. Androgen attenuation of Aβ induced toxicity was associated with elevated levels of heat shock protein 70, which is known to participate in protective responses against cellular stress and neurodegeneration [384]. Recent work from our lab identified a non-genomic, AR-dependent mechanism involving activation of a mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway [229]. We observed that physiological levels of testosterone and DHT rapidly and transiently activated MAPK/ERK signaling in cultured hippocampal neurons. Downstream of ERK, we found that androgens activated p90 kDa ribosomal S6 kinase (Rsk), which in turn phosphorylates the pro-apoptotic protein Bad. Phosphorylation of Bad results in its inactivation of Bad, thereby inhibiting the balance of apoptosis towards increased cell viability. Pharmacological inhibition at any step of this pathway prevents both phosphorylation of Bad and androgen neuroprotection [229]. Confirming the non-genomic nature of this pathway, we found that in this neuron culture paradigm the classic anti-androgens flutamide and cyproterone acetate mimicked neuroprotection against apoptotic insults afforded by testosterone and DHT even though they also blocked classic genomic actions of androgens [231]. Further, protective actions of flutamide and cyproterone acetate were only observed in AR-containing cell lines. Whether activation of protective androgen pathways requires membrane AR, intracellular AR, or both is unclear. We observed that testosterone conjugated to albumin – which is designed to prevent cell entry and thus permit only activation of membrane receptors – was ineffective in activating protective MAPK/ERK androgen signaling [229]. Similarly, Gatz and colleagues reported that while DHT phosphorlates both ERK and Akt, albumin-conjugated DHT resulted in dose-dependent suppression of ERK signaling in glioma cells expressing AR [113]. Pathways of androgen neuroprotection are summarized in Fig. 2.

In contrast to these observations of androgen neuroprotection, there appear to be circumstances in which androgens fail to protect against neural injury and can even exacerbate insults. For example, Dluzen and colleagues found that E2 but not testosterone reduces methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) nigrostriatal dopaminergic toxicity [82]. Further, testosterone has been found to exacerbate neuron loss following middle cerebral artery occlusion (MCAO) [372,371]. In this model removal of testosterone 6 h prior to MCAO reduces lesion volume by as much as half [372,371]. It is not clear whether the absence of androgen neuroprotection in these paradigms reflects roles of brain region, insult, and or other factors. One important issue in at least some studies may be androgen concentration. That is, at physiological low nanomolar levels testosterone can protect against excitotoxicity in cultured neurons, but worsens cell death when present at supraphysiological micromolar levels [244]. Similarly, micromolar but not nanomolar concentrations of testosterone were associated with increased calcium signaling and neuronal apoptosis [92,112]. Thus, although there is ample evidence of androgen neuroprotection against AD-related insults, androgen effects on neuron viability may depend on a number of factors, including brain region, type of insult, and hormone concentration.

In addition to direct neuroprotection, androgens also protect against another form of neuropathology directly relevant to AD, hyperphosphorylation of tau. Abnormal, excessive phosphorylation of the cytoskeletal protein tau in the form of neurofil thread and neurofibrillary tangles is a defining neuropathological characteristic of AD and several other neurodegenerative disorders [166,355,358]. Although relatively little research has examined the relationship between androgens and tau hyperphosphorylation, available evidence does indicate that androgens are protective against this pathology. Papasozomenos and colleagues have examined the effects of testosterone and E2 on tau hyperphosphorylation using a model of heat shock-induced phosphorylation. In this paradigm, testosterone but not E2 prevents tau hyperphosphorylation [252,250,251]. In addition to affecting phosphorylation of tau, recent evidence suggests that androgens also regulate tau cleavage [253]. Specifically, testosterone prevented calpain-mediated tau cleavage preventing the generation of the 17-kDa tau fragment [253].

9. Testosterone regulation of β-amyloid accumulation

In addition to classic neuroprotective actions, androgens may also protect the brain from AD by regulating accumulation of Aβ. Initial work suggesting a relationship between androgens and Aβ came from a small study evaluating men treated with anti-androgen therapies for prostate cancer. Gandy and colleagues found that within several weeks following initiation of anti-androgen therapy (consisting of leuprolide and flutamide), circulating levels of testosterone and E2 were largely depleted whereas plasma levels of Aβ were significantly elevated [107]. Martins and colleagues similarly reported an association between androgen depletion and elevated Aβ levels in males receiving anti-androgen therapy for the treatment of prostate cancer [6] and in older males suffering from memory loss or dementia [117]. To what extent these observations reflect effects of androgen versus estrogen pathways or perhaps re-
flect associated changes in gonadotropins remains incompletely resolved [13,283].

Consistent with the observations in aging men, experimental work in rodents also indicates that androgens function as endogenous negative regulators of 

As reviewed in this article, there are numerous neuroprotective actions of estrogens and androgens that have direct relevance to Alzheimer’s disease [238]. Male 3xTg-AD mice were depleted of androgens by ORX at age 3 mo, a time prior to the development of significant AD-like pathology [238]. Mice were exposed immediately to either DHT or vehicle, treatments that were maintained continuously for the next 4 months. After the treatment period, we observed significant intracellular accumulation of presumably insoluble Aβ in gonadally intact, sham ORX 3xTg-AD mice (age 7 mo) in the CA1 hippocampus, subiculum, and amygdala [285]. In comparison to the sham ORX animals, the ORX mice showed significantly higher levels of Aβ in all three brain regions as well as significantly poorer behavioral performance on a spontaneous alternation task [285]. The elevated pathology and exacerbated behavioral impairments in the ORX group were blocked in ORX mice treated with DHT, suggesting a preventive effect of androgens in regulation of AD-related pathology. Confirming our findings in animal models, a recent study reported that levels of Aβ in plasma and cerebrospinal fluid were significantly elevated following ORX in guinea pigs, an effect prevented by testosterone replacement 1 week following ORX [346].

There are likely several mechanisms that contribute to androgen regulation of Aβ (Fig. 2). One obvious possibility is that aromatase-mediated conversion of testosterone to E2 in brain allows activation of the several estrogen pathways of Aβ regulation discussed above. In fact, cell culture studies are consistent with indirect testosterone activation of estrogen-mediated regulation of APP processing. The first study to examine testosterone regulation of APP and Aβ in culture found that prolonged testosterone treatment was associated with increased α-secretase cleavage of APP and reduced Aβ [128]. Unclear was whether these findings involved direct androgen pathways, indirect activation of estrogen pathways, or both. In a subsequent culture study, testosterone was also observed to promote proteolysis of APP by α-secretase, however this effect was blocked in the presence of aromatase inhibitors suggesting estrogen dependence [121]. In our study of male rats in which androgens were found to regulate brain levels of soluble Aβ, we did not observe detectable differences in either full-length APP or APPα across androgen treatment groups [271], perhaps indicating that alterations in APP processing are not the only mechanism by which androgens affect Aβ levels.

In addition to regulating Aβ generation via effects on APP proteolysis, androgens may also decrease Aβ levels by promoting endogenous clearance pathways. Recent evidence from our group demonstrates that androgens reduce Aβ levels as a consequence of regulating expression of the Aβ-catabolizing enzyme neprilysin [376], a critical enzyme in homeostasis of brain Aβ [170]. In neural cell cultures, we found that androgens robustly increase protein levels of neprilysin. A classic, AR-dependent genomic mechanism is implicated since (i) the neprilysin gene contains androgen response elements [304], (ii) neprilysin regulation was blocked by anti-androgens flutamide and cyproterone acetate, and (iii) androgens only increased neprilysin in AR-containing cells [376]. Over-expression of human APP in cultured cells resulted in elevated levels of soluble Aβ that were reduced by testosterone and DHT. Further, we found that pharmacological inhibition of either AR or neprilysin blocked the ability of androgens to reduce Aβ levels, suggesting that the Aβ-lowering actions of androgens is mediated by AR-dependent regulation of neprilysin expression [376]. Importantly, these observations were replicated in male rats. We found that ORX-induced androgen depletion resulted in decreased levels of NEP and elevated Aβ, and DHT replacement restored NEP and Aβ to levels observed in sham GDX animals [376].

10. Emerging strategies of hormone-related therapies in Alzheimer’s disease

As reviewed in this article, there are numerous neuroprotective actions of estrogens and androgens that have direct relevance to
AD pathogenesis and compelling potential to prevent and possibly treat the disease. However, the promise of estrogen-based and androgen based HTs in reducing AD risk have yet to be realized. As findings and directions from clinical and basic science research become increasingly integrated, it is anticipated that critical parameters affecting HT efficacy will be optimized, including age of HT initiation as well as the formulation, regimen, and delivery of HT. As ongoing research continues to address these crucial and immediate concerns, an emerging area of investigation is the development of natural and synthetic hormone mimetics that will preferentially activate estrogen and androgen neuroprotective mechanisms while minimizing deleterious consequences in other tissues.

Estrogen compounds that show tissue-selective agonist actions are termed selective estrogen receptor modulators (SERMs). Currently the most studied and clinically relevant SERMs are tamoxifen and raloxifene, synthetic compounds that exhibit tissue-dependent ER agonist and antagonist actions. As a potent antagonist of estrogen action in breast tissue, tamoxifen is best recognized as an antiestrogen used to treat breast cancer although it can exert agonist ER effects on bone and lipids [48,300]. Interestingly, low concentrations of tamoxifen can protect cultured neurons from toxicity due to Aβ and glutamate [243], suggesting the potential for a protective role against AD. However, tamoxifen has also been observed to block E2-mediated protection in cultured neurons [57,383]. Potential benefits of tamoxifen use in post-menopausal women for prevention or treatment of AD has not been well studied. Some studies indicate increased risk of cognitive deficits in tamoxifen users [246,308], whereas another study suggested tamoxifen may reduce AD risk [41]. Like tamoxifen, raloxifene antagonizes estrogen actions in breast but has agonist actions on bone [48,81]. In brain, raloxifene mimics some but not all protective estrogen actions. Raloxifene increases choline acetyltransferase in hippocampus of OVX rats [363] and in cultured neurons it increases neurite outgrowth [235] and can reduce Aβ toxicity [243]. Conversely, E2 but not raloxifene was effective in attenuating Aβ-induced inflammatory reaction in OVX rats [334]. In post-menopausal women, raloxifene use has been linked with reduced risk of cognitive impairment and development of AD [370].

Currently, effort is being focused on next generation SERMs that exhibit more robust and specific neuroprotective actions [42,303]. For example, Brinton and colleagues recently developed a synthetic SERM with both estrogenic and antioxidant potential that protects cultured neurons from cell death [389]. Evaluation of such compounds in animal models of AD is only beginning. Towards this end, our laboratory has begun to investigate two particular SERMs, propylpyrazole triol (PPT) and diarylpropionitrile (DPN), which show relative specificity for ERα and ERβ, respectively. Although we observe that both compounds protect cultured neurons from Aβ toxicity [67], PPT but not DPN treatments effectively mimicked E2 in reducing Aβ accumulation and improving behavioral deficits in OVX 3xTg-AD mice [53].

In parallel to the ongoing research on SERMs, there has been a recent growth in the interest and development of tissue-specific selective androgen receptor modulators (SARMs) [108,147,186]. Given the prevalence of ADAM and its widespread deleterious consequences, androgen based therapy is of considerable interest. However, prostate cancer, which is the second leading cancer amongaging men in terms of both prevalence and cause of death [99], is androgen-dependent and typically treated by androgen deprivation therapy. Consequently, the use of testosterone therapy with its potential to increase risk and or progression of prostate tumorigenesis has been controversial [183] and promoted the development of SARMs that lack significant androgen action in prostate but exert agonist effects in selected androgen-responsive tissues of interest, including brain, muscle, and bone.

Several strategies of SARM design are currently been pursued [46,108,359]. One strategy is to develop novel steroidal compounds that are not substrates for the enzyme 5α-reductase or yield reduced metabolites with minimal androgenicity. Testosterone is converted to DHT by the actions of 5α-reductase, an enzyme localized in specific target tissues such as prostate. Prostate growth depends largely on the actions of DHT rather than T because DHT exhibits ~10-fold greater net potency, which reflects both a higher binding affinity for AR and a slower dissociation rate from AR [359]. SARMs that are not 5α-reductase substrates or form DHT-like derivatives with weak androgenic activity have low androgen action in prostate [108,188,201,245,301,342]. A promising SARM in this category is 7α-methyl-19-nortestosterone, commonly called MENT [223,301,342], which was developed by the Population Council and is currently in clinical trials as an androgen therapy for hypogonadal men [10,345]. MENT shows low androgen activity in prostate but is more potent than T in other peripheral androgen-responsive tissues including bone [76,301,342] and muscle [76,301,342]. The effects of MENT on neural function are virtually unknown, but it has been shown to mimic the ability of testosterone to induce sexual behavior in castrated rats [223] and is a robust regulator of the hypothalamic–pituitary–gonadal axis, suggesting neural efficacy.

Another SARM design strategy is the development of non-steroidal synthetic AR ligands. One such SARM is flutamide, which mimics the abilities of T and DHT to increase hippocampal spine density in both male and female rats [207,208]. In cultured neurons, we have found that flutamide antagonizes classic genomic actions of T and DHT but mimics rather than blocks the nongenomic neuroprotective actions of these androgens [230]. Of particular interest are novel compounds that bind AR but have altered interactions with AR binding pocket side chains that underlie tissue specificity. Recent research has determined that although synthetic SARMs must closely mimic the rigid backbone core structure of the AR ligand binding domain, there can be extensive variation in how they interact with amino side chains in the binding pocket [359]. Thus, SARMs are predicted to exert tissue-specific effects dependent in part upon how they interact with amino acid sidechains in the AR binding pocket [36,46,201]. Recent research indicates success in developing SARMs with desired tissue specificity [245]. As with SERMs, the development of brain-specific SARMs that exert neuroprotective actions associated with reduction of AD pathogenesis is a key topic of ongoing research.

Acknowledgments

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- Multiple benefits including men given aromatize-able testosterone
- Increase blood flow to the coronary arteries (even in patients with C.A.D.)
- Decrease plaque in the coronary arteries
- Decrease inflammation in the coronary arteries

_Coupled with the lipid data above is impressive and safe way to reduce the number one killer of men and women in America._
sponses to acetylcholine. Although this reference group had normal coronary angiograms, referral due to chest pain sets these patients apart from a purely voluntary group with normal coronary arteries.

Much has been written about vasodilatation of diseased and "control" human coronary arteries using an intravascular Doppler mounted catheter of the Millar Velocimeter (Houston, Texas) type. Because of its catheter-based configuration, only proximal straight segments of the coronary arteries may be safely and easily accessed with this technique. This technique is also subject to a greater likelihood of artifact and poorer signal quality secondary to the size and relative inflexibility of the catheter and difficulty in ensuring coaxial positioning. With use of this technique, endothelium-dependent and endothelium-independent vasodilation has been reported in control subjects defined variously. In some cases, mild coronary disease in the study vessel and/or severe disease in another vessel were allowed. Hypertension, hyperlipidemia, hyperglycemia, and current tobacco use are commonly seen in previously reported control subjects.

Our study describes coronary relaxation properties in a referral normal population, which we defined simply as normotensive nondiabetic subjects without angiographic coronary artery disease. Our study suggests that an optimal intracoronary acetylcholine bolus infusion is 16 µg. Similarly, an optimal intracoronary acetylcholine infusion rate appears to be 30 µg/min. We found that 39% of referral normal subjects had evidence of endothelial dysfunction, defined as reduced endothelium-dependent relaxation (<150% increase in coronary blood flow above baseline).

In a referral normal cardiac population, endothelium-independent coronary relaxation is nearly always normal, but endothelium-dependent relaxation may be depressed in a significant proportion of patients. Further study of the natural history of referral normal subjects with endothelial dysfunction is necessary to assess the potential cardiovascular risk of this finding in a presumed low-risk population.


Testosterone Decreases Lipoprotein(a) in Men
Joseph M. Zmuda, MS, Paul D. Thompson, MD, Roberta Dickenson, BS, Linda L. Bausserman, PhD

Lipoprotein(a) [Lp(a)] has recently emerged as an important risk factor for atherosclerotic cardiovascular disease. Lp(a) levels are largely under genetic control and have proved remarkably resistant to therapeutic manipulations. Of the commonly used lipid-lowering medications, only high-dose niacin appears to reduce Lp(a) levels. Others have demonstrated that estrogen and the anabolic-androgenic steroid stanozolol reduce Lp(a) concentrations in postmenopausal women, but to our knowledge, the effect of androgenic hormone administration on Lp(a) in men has not been examined. Testosterone is normally aromatized to estradiol, and peripheral aromatization of testosterone is the major source of circulating estrogen in men. We recently examined the effect of testosterone aromatization on serum lipid and lipoprotein levels in men by administering testosterone alone or in combination with the aromatase inhibitor testolactone. Recent reports that
estrogen decreases Lp(a) prompted us to examine Lp(a) levels from our study to determine the effects of testosterone and its aromatization to estradiol on Lp(a) levels.

Fourteen healthy nonsmoking male weightlifters (aged 27 ± 7 years; mean ± SD) provided written informed consent and completed the study. Subjects weighed 85.4 ± 21.0 kg before the study; body fat, estimated from the sum of 3 skinfold measurements, was 11.0 ± 6.0%. All of the men had been weightlifting for 5.7 ± 5.6 years and exercised ≥3 times per week. No subject averaged >1 alcoholic beverage daily or took regular medications, and all denied current and prior use of androgen. Baseline urinalysis confirmed that the subjects had not recently used either anabolic-androgenic steroids or testosterone. The subjects were instructed to maintain their habitual level of physical activity and avoid altering their dietary habits during the study. Subjects were reimbursed for their participation as approved by the Miriam Hospital Clinical Research Review Board.

Subjects were randomly assigned to a counterbalanced cross-over design involving 3 treatments: testosterone enanthate (E.R. Squibb & Sons, Inc, Princeton, New Jersey), 200 mg/wk intramuscularly (IM); oral testolactone (E.R. Squibb & Sons, Inc), 250 mg 4 times daily (QID); and both testosterone enanthate, 200 mg/wk IM and testolactone, 250 mg QID. This testosterone dose has been recently studied as a male contraceptive and is twice the minimum dose used to treat male hypogonadism. Each treatment lasted 3 weeks, and treatments were separated by a 4-week washout period. Venous blood was obtained before and after each 3-week treatment, between 6 and 9 A.M., after a 12-hour fast, and before any testosterone injection. Samples were stored at −70°C until analysis.

Lp(a) levels were measured in duplicate with an enzyme-linked immunosorbent assay (MACRA, Strategic Diagnostics, Newark, Delaware). Measurement techniques for triglycerides and total low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol have been described. Intraassay variation for all lipid and Lp(a) determinations was avoided by analyzing all samples for an individual subject in a single assay or autoanalyzer run. Intraassay coefficient of variation for Lp(a) was <5%. Serum testosterone and estradiol were assayed in duplicate with radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, California).

Data were analyzed with a treatment-by-time repeated measures analysis of variance. When interaction effects were significant, the effect of time for each treatment and the differences between treatments at each measurement point were tested statistically. A modified Bonferroni procedure was used to adjust for multiple comparisons. Because Lp(a) demonstrated a skewed distribution, statistical analyses were performed on log-transformed values. Nontransformed Lp(a) values are presented in the report. Spearman correlation coefficients were used to examine the relation between initial Lp(a) values and its subsequent change. Results are presented as mean ± SD.

Pretreatment serum testosterone levels were in the normal physiologic range for young adult men. Serum testosterone levels increased by 39% when testosterone was given alone and by 105% when testosterone and testolactone were combined (p < 0.01 for both; Table I). Testosterone also produced a 47% increase (p < 0.01) in serum estradiol levels, an effect that was not observed when testosterone and testolactone were administered together. Testolactone alone did not significantly change either testosterone or estradiol levels. These results indicate that testolactone inhibited aromatase activity and blocked the conversion of exogenous testosterone to estradiol.

Average Lp(a) values decreased by 37% during treatment with testosterone, by 28% when testosterone and testolactone were combined (p < 0.01 for both), but did not change significantly during treatment with testolactone alone (Table I and Figure 1). Lp(a) levels were similar after the 2 testosterone treatments. These results suggest that most of the reduction in Lp(a) during testosterone treatment resulted from an androgenic effect and not from aromatization of testosterone to estradiol.

The absolute change in Lp(a) levels during testosterone and testosterone plus testolactone treatment was inversely related to initial Lp(a) concentrations (r = −0.77 and r = −0.84, respectively; p < 0.01 for both). Thus, men with the highest initial Lp(a) values experienced the greatest reduction in Lp(a) during testosterone treatment. Lp(a) concentrations were similar before each treatment, indicating that the 4-week washout period was sufficient to eliminate any residual drug effect.

As previously reported, testosterone treatment also reduced HDL by 16%, an effect that was slightly but nonsignificantly greater when testosterone and testolactone were combined (−20%; p < 0.01 for

| TABLE I Testosterone and Estradiol Levels During Three Drug Conditions |
|-----------------|-----------------|-----------------|
| Treatment       | Baseline        | Week 3          |
| Testosterone    | 18.7 ± 5.5      | 26.0 ± 4.9*     |
| Testolactone    | 18.7 ± 5.2      | 22.9 ± 6.9      |
| Testosterone + testolactone | 18.4 ± 3.8  | 37.8 ± 11.1 *   |
| Estradiol       | 133 ± 32        | 195 ± 75*       |
| Testolactone    | 133 ± 32        | 118 ± 50*       |
| Testosterone + testolactone | 130 ± 44 | 113 ± 22*       |

*Significant (p < 0.01) difference from baseline within treatment.
Significant (p < 0.01) difference from testosterone treatment.
Significant (p < 0.01) difference from testolactone treatment.
Results have been published previously and are included to facilitate evaluation of Lp(a) changes.

Values are expressed as mean ± SD.
TABLE II: Lipid, Lipoprotein, and Lipoprotein(a) Concentrations During Three Drug Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>176 ± 33</td>
<td>173 ± 27</td>
</tr>
<tr>
<td>Testolactone</td>
<td>178 ± 29</td>
<td>171 ± 28</td>
</tr>
<tr>
<td>Testosterone + testolactone</td>
<td>187 ± 25</td>
<td>177 ± 28</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>108 ± 27</td>
<td>110 ± 21</td>
</tr>
<tr>
<td>Testolactone</td>
<td>108 ± 24</td>
<td>103 ± 22</td>
</tr>
<tr>
<td>Testosterone + testolactone</td>
<td>119 ± 22</td>
<td>118 ± 24</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>50 ± 15</td>
<td>42 ± 13*</td>
</tr>
<tr>
<td>Testolactone</td>
<td>48 ± 15</td>
<td>46 ± 15</td>
</tr>
<tr>
<td>Testosterone + testolactone</td>
<td>50 ± 14</td>
<td>40 ± 12*</td>
</tr>
<tr>
<td>Lipoprotein(a) (mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>9.4 ± 1.6</td>
<td>5.9 ± 1.7*</td>
</tr>
<tr>
<td>Testolactone</td>
<td>7.4 ± 0.8</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td>Testosterone + testolactone</td>
<td>7.2 ± 0.9</td>
<td>5.2 ± 0.9*</td>
</tr>
</tbody>
</table>

*Significant (p < 0.01) difference from baseline within treatment.
†Significant (p < 0.01) difference from testosterone treatment.

Values are expressed as mean ± SD.

This report adds testosterone to the brief list of interventions known to affect Lp(a) levels. Neither hydroxymethylglutaryl coenzyme A reductase inhibitors, bile acid sequestrants, diet, nor vigorous exercise appear to be effective in reducing Lp(a) levels. Recent reports suggest that estrogen and high-dose niacin lower Lp(a) concentrations. One prior study reported a 65% decrease in Lp(a) in postmenopausal women treated for 6 weeks with the nonaromatizable androgen stanozolol. Consequently, it appears that androgens can reduce Lp(a) in both men and women.

The original aim of this study was to examine the effects of testosterone, with or without aromatization to estradiol, on HDL cholesterol levels. The observation that estrogen decreases Lp(a) prompted us to examine the effects of testosterone, testolactone, or the combination on Lp(a) levels. Because supraphysiologic doses of testosterone increase estrogen levels, the use of testolactone provided an opportunity to examine the androgenic effects of testosterone with and without its aromatization to estradiol. Testosterone alone increased serum estradiol levels by 47%, whereas estradiol levels did not change significantly when testosterone and testolactone were combined. Lp(a) decreased 37% during testosterone and 28% during testosterone and testolactone. These decreases were not significantly different, suggesting that most of the decrease in Lp(a) is mediated by testosterone and not by its conversion to estradiol.

The decrease in Lp(a) during testosterone and testosterone plus testolactone treatment was greatest in men with the highest initial Lp(a) levels and largely confined to the 7 men with baseline Lp(a) levels >5 mg/dl. These results suggest that the effect of testosterone may be most pronounced in subjects with the highest pretreatment Lp(a) levels.

The mechanisms by which testosterone treatment reduced Lp(a) concentrations are not clear. LDL cholesterol levels did not change in the present study, suggesting that the effect of testosterone on Lp(a) was independent of changes in LDL metabolism. Lp(a) synthesis, rather than catabolism, is thought to be the primary metabolic determinant of Lp(a) concentrations in humans, and testosterone has recently been documented to reduce apo(a) gene expression in a transgenic mouse model expressing the human apo(a) gene. Thus, it is possible that testosterone lowers Lp(a) levels by decreasing apo(a) synthesis.

Androgens have been assumed to increase atherosclerotic disease risk by reducing HDL-C. The
present results suggest that the effect of testosterone on vascular disease risk factors is more complex, and that its deleterious effect on HDL cholesterol may be offset by potentially beneficial effects on Lp(a). Testosterone, like estrogen,\(^1\) may also have favorable effects on coronary vasomotor function, because exogenous testosterone decreases exercise-induced ST segment depression in men with angina pectoris\(^1\) and vasodilates rabbit\(^1\) coronary arteries. Such results may have important implications for the prolonged use of testosterone in hypogonadism\(^1\) and as a male contraceptive,\(^1\) and to prevent frailty as men age.\(^2\)

In summary, the results of the present study indicate that testosterone reduces Lp(a) concentrations in normal men primarily by an androgenic effect and not by its conversion to estradiol.


Meta-Analysis of the Use of Low-Dose Beta-Adrenergic Blocking Therapy in Idiopathic or Ischemic Dilated Cardiomyopathy

Dawn G. Zarembski, PharmD, Paul E. Nolan, Jr., PharmD, Marion K. Slack, PhD, and Charles Y. Lui, MD

Several compensatory neurohormonal systems are stimulated in congestive heart failure (CHF) in an attempt to maintain cardiac function. The sympathetic nervous system is activated with resultant increases in circulating plasma norepinephrine levels that produce tachycardia, vasoconstriction, and increased force of contraction.\(^3\) The renin-angiotensin-aldosterone system is also activated, leading to sodium and water retention and further increases in vasoconstriction secondary to angiotensin II synthesis.\(^1\) Eventually, as the disease progresses these compensatory mechanisms become inadequate to maintain ventricular function.\(^1\) In addition, the increase in plasma norepinephrine levels observed in CHF can be directly correlated to severity and mortality of CHF.\(^4\) Contemporary treatment of CHF is focused on altering these neurohormonal systems. Therapy aimed at attenuating excess sympathetic nervous system activity through β-adrenergic blocking agents has been proposed in a number of small clinical trials.\(^5\) Given the small sample size in each of the trials, the ability of these trials to impact medical practice has been limited. To optimize the available data, we conducted a meta-analysis designed to assess the ability of β blockers to improve quality of life and hemodynamic indexes in patients with idiopathic or ischemic cardiomyopathy.

Prospective, randomized, placebo-controlled trials were gathered from a review of the reports. Current Contents: Clinical Practice was reviewed and MEDLINE files from 1960 to November 1995 were searched.

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THE HEART

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- Increase blood flow to the coronary arteries (even in patients with C.A.D.)
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- Decrease inflammation in the coronary arteries

Coupled with the lipid data above is impressive and safe way to reduce the number one killer of men and women in America.
The effect of supraphysiologic doses of testosterone on fasting total homocysteine levels in normal men

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Abstract

Elevated total homocysteine (tHcy) levels are associated with increased risk for atherosclerotic cardiovascular disease. tHcy levels are higher in men than in women, and estrogen replacement therapy may reduce tHcy levels in postmenopausal women. The effect of androgenic hormones on tHcy levels in men has not been examined. The present study determined the effect of supraphysiologic doses of testosterone, with or without its aromatization to estradiol, on fasting tHcy levels in 14 normal male weightlifters aged 19–42 years. Subjects received testosterone enanthate (200 mg/week intramuscularly), the aromatase inhibitor, testolactone (1 g/day orally), or both drugs together in a crossover design. Each treatment lasted 3 weeks and each treatment was separated by a 4-week washout. Both testosterone regimens increased serum testosterone levels, whereas estradiol increased only during testosterone alone. Mean tHcy levels were not significantly altered when testosterone was given alone or together with testolactone. Testolactone did not significantly influence tHcy levels. We conclude that short-term, high-dose testosterone administration does not affect fasting tHcy levels in normal men. © 1997 Elsevier Science Ireland Ltd.

Keywords: Estrogen; Homocysteine; Men; Testosterone

1. Introduction

Homocysteine is a sulphur-containing amino-acid formed by the demethylation of dietary methionine. Homocysteine may promote atherosclerosis by injuring the vascular endothelium, [1] and elevated total homocysteine (tHcy) levels are associated with increased risk for atherosclerotic cardiovascular disease [2]. The observation that fasting tHcy concentrations are higher in men than in women has led to the suggestion that sex steroid hormones may influence tHcy levels [3]. Evidence to support this idea comes from studies showing that estrogen plus progestin replacement therapy [4] and the estrogen agonist tamoxifen [5] reduce tHcy concentrations in postmenopausal women. Others have documented an inverse correlation between serum estradiol concentrations and postmethionine tHcy levels in premenopausal women, [4] but to our knowledge the effect of androgenic hormones on tHcy concentrations in men has not been examined.

The androgenic hormone testosterone is normally aromatized to estradiol in liver, muscle, and adipose tissue, and peripheral aromatization of testosterone is the major source of circulating estrogen in men [6]. We have previously examined the effect of testosterone aromatization on serum lipids [7] in men by administering testosterone alone or in combination with the aromatase inhibitor testolactone. Because of the observations that tHcy concentrations are higher in men than in women [3] and that female sex hormones may affect tHcy levels [4,5], we used stored plasma samples from this prior study to determine the effects of testosterone and its aromatization to estradiol on fasting tHcy levels in men.


2. Materials and methods

2.1. Study subjects

Fourteen healthy non-smoking men between 19 and 42 years of age (Mean ± S.D.; 27.1 ± 7.4 years) provided written informed consent and completed the study. Their mean body weight was 86.6 ± 20.6 kg before the study, and body fat estimated from the sum of three skinfold measurements [8] was 12.7 ± 6.9% (Table 1). All men had been weightlifting for approximately 6 years and exercised at least three times per week. None of the subjects had a history of renal, hepatic, or vascular disease, and no subject averaged more than one alcoholic beverage daily or took regular medications. All men denied current and prior androgen use. Baseline urinalysis confirmed that subjects had not recently used either anabolic–androgenic steroids or testosterone [7]. The subjects were instructed to maintain their habitual level of physical activity and to avoid altering their dietary habits and nutritional supplement use during the study. Subjects were reimbursed for participation as approved by The Miriam Hospital Clinical Research Review Board.

2.2. Study design

Subjects were randomly assigned to a counterbalanced cross-over design involving three treatments: testosterone enanthate (E.R. Squibb and Sons, Princeton, NJ), 200 mg/week intramuscularly (i.m.); oral testolactone (E.R. Squibb and Sons), 250 mg four times daily (QID); and both testosterone enanthate, 200 mg/week i.m. and testolactone, 250 mg QID. This testosterone dose has been recently studied as a male contraceptive [9]. Each treatment lasted 3 weeks, and treatments were separated by a 4-week washout period. Blood samples were obtained from an antecubital vein before and after each 3 week treatment, between 06:00 and 09:00, after a 12-h fast, and before testosterone injections. Plasma samples were collected in pre-chilled evacuated tubes that contained heparin as an anticoagulant and were immediately placed on ice. These samples were separated by centrifugation usually within 1 h (maximum 2 h), and frozen and stored at −70°C until analyzed.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of study subjects</th>
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<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.6</td>
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<tr>
<td>Body fat (%)</td>
<td>12.7</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>19.0</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>145.7</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

2.3. Biochemical assays

Fasting tHcy levels were measured in plasma with the fluorimetric method of Vester and Rasmussen [10], except that 20% methanol was used in buffer B in the high-performance liquid chromatography (HPLC) procedure. Interassay variation for tHcy determinations was avoided by analyzing all samples for an individual subject in a single assay or autoanalyzer run. Intra-assay coefficient of variation for tHcy was less than 6.0%. Serum testosterone and estradiol were assayed in duplicate using radioimmunoassay kits (Diagnostic Products, Los Angeles, CA).

2.4. Statistical analysis

Data were analyzed with a treatment-by-time analysis of variance with repeated measures on both factors. When interaction effects were significant, the effect of time for each treatment and the differences between treatments at each measurement point were tested statistically. A modified Bonferroni procedure was employed to adjust for multiple comparisons [11]. Pearson simple and partial correlation coefficients were computed to examine the associations between baseline serum testosterone and estradiol levels and fasting tHcy concentrations with and without adjustment for baseline age and body weight.

3. Results

Pretreatment serum testosterone concentrations were in the normal physiologic range for young adult men. Serum testosterone levels increased by 38% when testosterone was given alone and by 102% when testosterone and testolactone were combined (P < 0.01 for both; Fig. 1). Testosterone also produced a 43% increase (P < 0.01) in serum estradiol levels, an effect that was not observed when testosterone and testolactone were administered together (Fig. 1). Testolactone alone did not significantly change either testosterone or estradiol levels. These results indicate that testolactone inhibited aromatase activity and blocked the conversion of exogenous testosterone to estradiol.

Mean pretreatment tHcy levels (5.0 ± 1.1 μmol/l; range, 3.3–7.0 μmol/l) were lower than previously reported values for men of a similar age [12]. We suspect that most of this difference is due to habitual use of B-vitamin-containing supplements, a common practice among athletes. Fasting tHcy concentrations were not significantly altered when testosterone was given alone or when testosterone and testolactone were combined (Table 2). Testolactone did not significantly affect plasma tHcy levels. Baseline serum testosterone (r = −0.10; P = 0.74) and estradiol (r = −0.13; P = 0.65) con-
centrations were not strongly associated with tHcy levels. Similar correlation coefficients were observed after controlling for baseline age and body weight. These results indicate that testosterone, with or without its aromatization to estradiol, does not influence fasting tHcy levels in men.

4. Discussion

The original aim of this study was to examine the effects of testosterone, with or without aromatization to estradiol, on high-density lipoprotein cholesterol levels in men [7]. Recent reports suggesting that estrogens may decrease the atherogenic amino-acid tHcy in postmenopausal women [4,5] prompted us to examine the effects of testosterone, testolactone or the combination on fasting tHcy levels. Because supraphysiologic doses of testosterone increase estrogen levels in men, the use of testolactone provided an opportunity to examine the androgenic effects of testosterone with and without its aromatization to estradiol. Testosterone alone increased serum estradiol levels by 43%, whereas estradiol levels did not change significantly when testosterone and testolactone were combined. Fasting tHcy levels were not significantly altered during either testosterone condition, however, suggesting that testosterone and its aromatization to estradiol does not affect tHcy concentrations in eugonadal men.

The observation that fasting tHcy concentrations are higher in men than in women [3] has led to the suggestion that sex steroid hormones may explain the gender difference in tHcy levels [3]. Support for this idea comes from studies showing that estrogen plus progestin replacement therapy [4] and the estrogen agonist tamoxifen [5] reduce tHcy concentrations in postmenopausal women. Others have documented an inverse correlation between serum estradiol concentrations and post-methionine tHcy levels in premenopausal women [4]. The present study is the first, to our knowledge, to examine the effects of androgenic hormones on tHcy levels in men. Serum testosterone and estradiol levels were not strongly associated with pretreatment tHcy levels. Furthermore, testosterone administration, with or without its aromatization to estradiol, did not significantly alter tHcy levels. These results suggest that neither testosterone nor estradiol have an important influence on tHcy levels in normal young men, and raise the possibility that factors other than testosterone contribute to higher fasting tHcy levels in men than in women.

A more likely explanation for the gender difference in fasting tHcy levels may relate to the fact that creatine–creatinine production is directly coupled to $s$-adenosylhomocysteine generation from $s$-adenosylmethionine [13], and that lean body mass and creatine–creatinine production tend to be higher in men than in women. Indeed, plasma tHcy levels correlate directly with serum creatinine concentrations in men and women [14]. Moreover, the gender difference in tHcy levels disappeared in one recent study when men and women were matched for serum creatinine concentrations [14]. These results suggest that the higher mean fasting tHcy levels in men compared with women are most likely a result of the direct relationship between homocysteine production and creatine–creatinine synthesis [14].

The present study has several limitations. First, a longer treatment duration and larger sample size may be required to reveal a testosterone effect on mean fasting tHcy levels. Two previous reports demonstrating that female sex hormones decrease plasma tHcy levels in women included at least 27 study subjects [4,5], and a significant decline in tHcy in one study was not detectable until after 3–4 months of treatment [5]. Therefore, the present study may have underestimated the true magnitude of testosterone’s effect on tHcy levels. Since fasting tHcy levels are only weakly related to the tHcy response to methionine loading [15], we also cannot exclude the possibility that testosterone administration influences post-methionine tHcy levels.

Finally, all of our study subjects had normal total testosterone levels, so it is possible that men with hypogonadism would have experienced a change in...
Table 2
Plasma homocysteine concentrations before and after 3 weeks of testosterone, testolactone, and testosterone plus testolactone

<table>
<thead>
<tr>
<th>Testosterone</th>
<th>Testolactone</th>
<th>Testosterone + Testolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine (μmol/l) Before</td>
<td>4.7 ± 1.1</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td>After</td>
<td>4.6 ± 0.9</td>
<td>4.9 ± 1.1</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. There were no significant differences within or between treatments.

they with testosterone treatment. Additional studies are needed to examine these possibilities.

Acknowledgements

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References

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Circulation. 1999; 100: 1690-1696,

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Effects of Testosterone on Coronary Vasomotor Regulation in Men With Coronary Heart Disease

Carolyn M. Webb, PhD; John G. McNeill, DCRR; Christopher S. Hayward, MB, BS, FRACP; Dominique de Zeigler, MD; Peter Collins, MD, FRCP

Background—The increased incidence of coronary artery disease in men compared with premenopausal women suggests a detrimental role of male hormones on the cardiovascular system. However, testosterone has direct relaxing effects on coronary arteries in animals, as shown both in vitro and in vivo. The effect of testosterone on the human coronary circulation remains unknown.

Methods and Results—We studied 13 men (aged 61±11 years) with coronary artery disease. They underwent measurement of coronary artery diameter and blood flow after a 3-minute intracoronary infusion of vehicle control (ethanol) followed by 2-minute intracoronary infusions of acetylcholine (10⁻⁷ to 10⁻⁵ mol/L) until peak velocity response. A dose-response curve to 3-minute infusions of testosterone (10⁻¹⁰ to 10⁻⁷ mol/L) was then determined, and the acetylcholine infusions were repeated. Finally, an intracoronary bolus of isosorbide dinitrate (1000 µg) was given. Coronary blood flow was calculated from measurements of blood flow velocity using intracoronary Doppler and coronary artery diameter using quantitative coronary angiography. Testosterone significantly increased coronary artery diameter compared with baseline (2.78±0.74 mm versus 2.86±0.72 mm [P=0.05], 2.87±0.71 mm [P=0.038], and 2.90±0.75 mm [P=0.005] for baseline versus testosterone 10⁻⁷ to 10⁻⁵ mol/L, respectively). A significant increase in coronary blood flow occurred at all concentrations of testosterone compared with baseline (geometric mean [95% CI]: 32 [25, 42] versus 36.3 [27, 48] [P=0.006], 35.3 [26, 47] [P=0.029], 36.8 [28, 49] [P=0.002], and 37 [28, 48] [P=0.002] mL/min for baseline versus testosterone 10⁻¹⁰ to 10⁻⁷ mol/L, respectively). No differences existed in coronary diameter or blood flow responses to acetylcholine before versus after testosterone.

Conclusions—Short-term intracoronary administration of testosterone, at physiological concentrations, induces coronary artery dilatation and increases coronary blood flow in men with established coronary artery disease. (Circulation. 1999;100:1690-1696.)

Key Words: coronary arteries ■ testosterone ■ blood flow

In recent years, sex hormones, particularly estrogen, have emerged as important factors for modulating cardiovascular disease.¹,² Although the incidence of coronary heart disease increases in women after menopause, postmenopausal women have a lower incidence of coronary heart disease and myocardial infarction than men of a similar age. It has been proposed that testosterone may predispose to coronary artery disease and may partially explain the sex difference. However, no direct evidence exists linking testosterone to an increased incidence of coronary heart disease and myocardial infarction.

In men without prior myocardial infarction who are referred for coronary angiography, a significant inverse correlation was found between plasma testosterone levels and extent of coronary artery disease, demonstrating that men with low testosterone levels may be at increased risk for coronary atherosclerosis.² Some reports suggest that testosterone therapy in men has a beneficial effect on angina pectoris³,⁴ and on exercise-induced ST-segment depression in patients with angina pectoris.⁵,⁶ A double-blind study was performed in 50 men who had ST-segment depression after exercise.⁶ After 4 to 8 weeks of treatment with testosterone or placebo, a significant decrease in the exercise-induced extent of ST-segment depression occurred with testosterone when compared with placebo. The mechanism(s) by which testosterone decreased postexercise ST-segment depression was not established.

The direct effect of testosterone on coronary circulation in men is unknown. Testosterone induces relaxation in precontracted rabbit coronary arteries and aorta in vitro, with or without endothelium.⁷ A high-cholesterol diet and environmental tobacco smoke have detrimental effects on endothelial function in male animals; this effect was exacerbated by testosterone at physiological concentrations.⁸ Short-term in-
intrapranocoronary infusions of testosterone dilate male and female canine coronary arteries in vivo and increase coronary blood flow, partially by an endothelium-dependent mechanism. ATP-sensitive potassium channels are also involved in the dilator response.

These data suggest a beneficial effect of testosterone on the coronary circulation. We therefore investigated the effects of testosterone on the coronary circulation of men with atherosclerotic coronary artery disease.

Methods

Patients
Men aged 35 to 70 years who had angiographically proven coronary artery disease at diagnostic angiography for investigation of stable angina pectoris were enrolled in the study. Patients with primary valvar heart disease, complete heart block, or uncorrected hypokalemia were excluded. All patients gave written informed consent in accordance with the ethical requirements of the Royal Brompton Hospital Ethics Committee.

Study Design
Cardiac medication was withheld for at least 24 hours before cardiac catheterization, and caffeinated beverages were prohibited during this time. After diagnostic coronary angiography and full heparinization, a 0.014-inch Doppler flow wire (Cardiometrics Inc) was positioned in the proximal portion of an unobstructed coronary artery (no lesion >50% occlusive) from which continuous traces of average peak blood flow velocity were recorded. Arterial pressure, heart rate, and ECG were displayed continuously.

Intracoronary Infusions
A 3-minute intracoronary infusion of vehicle control (ethanol) was given, followed by investigation of endothelium-dependent coronary responses by increasing concentrations of intracoronary acetylcholine (estimated concentrations, 10^{-7} to 10^{-3} mol/L) for 2 minutes each or until peak velocity response. After this, a dose-response curve to testosterone was performed for 3 minutes at each concentration into the right coronary artery with an infusion rate of 1 mL/min (estimated concentration of testosterone: 2.3, 23, 230, and 2300 ng/min) and of 1.5 mL/min into the left coronary artery (estimated concentration of testosterone: 3.45, 34.5, 345, and 3450 ng/min). These doses are approximately equal to the 10^{-6} to 10^{-3} mol/L concentrations of testosterone, respectively, achieved in the coronary blood. Acetylcholine infusions were then repeated. All infusions were given at a rate of 1 mL/min into the ostium of the right coronary artery or 1.5 mL/min into the ostium of the left coronary artery. The study protocol was completed with an intracoronary bolus of 1000 g/mL isosorbide dinitrate, a non-endothelium–dependent vasodilator. Coronary angiograms were performed at baseline and then immediately after the peak velocity response to each dose of vasoactive substance. A second baseline angiogram was positioned in the proximal portion of an unobstructed coronary bolus of 1000 g/mL isosorbide dinitrate, a non-endothelium–dependent vasodilator. Coronary angiograms were performed at baseline and then immediately after the peak velocity response to each dose of vasoactive substance. A second baseline angiogram was positioned in the proximal portion of an unobstructed coronary artery (no lesion >50% occlusive) from which continuous traces of average peak blood flow velocity were recorded. Arterial pressure, heart rate, and ECG were displayed continuously.

Testosterone Dilutions
The testosterone dilutions were calculated to give a dose of testosterone in the coronary artery of 10^{-10} to 10^{-7} mol/L (normal range, 10^{-8} to 5\times10^{-4} mol/L [3 to 10 ng/mL]) using an assumed coronary blood flow of 80 mL/min in the left coronary arterial tree and 40 mL/min in the right coronary artery. Stock solutions of 2300 g/mL testosterone and vehicle control (ethanol) were provided by Columbia Laboratories (Paris, France). We diluted 0.1 mL of 2300 g/mL testosterone in 10 mL of the patient’s blood to give a 23 g/mL (10^{-6} mol/L) testosterone concentration. This was then diluted further to give testosterone concentrations of 10^{-7} to 10^{-10} mol/L (2.3 g/mL to 2.3 ng/mL) in the coronary blood.

Testosterone is lipid-soluble and was prepared in 60% ethanol. Vehicle control was prepared for intracoronary infusion at 1 concentration, which was equivalent to the concentration given at the greatest concentration of testosterone (0.01 mL of 60% ethanol in 10 mL of blood).

Quantitative Coronary Angiography and Calculation of Flow
Coronary angiograms were acquired and analyzed digitally using a real-time digital image acquisition and analysis system (Digitron III VACT, Siemens AG), as previously described. Measurement of diameter and velocity were made at baseline and at peak velocity change. Diameter was measured ~4 mm distal to the tip of the Doppler wire at the sample volume site by an independent observer. Care was taken to measure diameter at an identical position after each infusion. The wire position did not change in any of the patients studied. A quantitative estimate of coronary blood flow was calculated from Doppler flow velocity and diameter 4 mm distal to the Doppler wire tip using the following equation:

\[
Q = \pi (D/4) (APV/2)(0.6)
\]

where Q is flow (mL/min), D is vessel diameter (mm), and APV is average peak velocity (cm/s).

In addition to measuring local changes in diameter, global diameter changes throughout the entire artery were measured by an independent observer using quantitative coronary angiography. With this analysis, changes in mean coronary diameter were measured, as were responses at the sites of defined focal narrowing and/or dilatation.

Statistical Analysis
Baseline 1 versus baseline 2 comparison was performed using a paired t test or a Wilcoxon matched pairs test when the data were not normally distributed. All other analyses were performed using a 2-way ANOVA with patient and time as factors. In Results, we present comparisons of baseline 1 versus acetylcholine responses before testosterone, baseline 2 versus testosterone, and baseline 2 versus acetylcholine responses after testosterone. We also compared responses to respective doses of acetylcholine before versus after testosterone. The following assumptions were tested: normality of residuals by the Shapiro–Wilk test and equality of variances in the time groups by Bartlett’s test. Data are presented as mean±SD or as geometric mean (95% confidence interval [CI]) where data have been log-transformed to normality. P<0.05 was considered significant.

Results

Patients
Thirteen men were enrolled in the study; they had a mean age of 61±11 years. Patient characteristics are described in Table 1. Seven patients had 1 significantly diseased coronary artery (stenosis >70%), and 6 patients had 2-vessel disease. The study vessel of all patients was irregular but not significantly obstructed on angiography. The left anterior descending coronary artery was studied in 2 patients, the left circumflex...
TABLE 1. Patient and Control Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n=13)</th>
<th>Controls (n=8)</th>
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<td>Age, y</td>
<td>61±11</td>
<td>57±9</td>
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<td>Previous MI, PTCA, or CABG</td>
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<td>2</td>
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<tr>
<td>Hypertension</td>
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<td>2</td>
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<tr>
<td>Cigarette smoking, n</td>
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<td>Current</td>
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</tr>
<tr>
<td>Ex</td>
<td>7</td>
<td>2</td>
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</tr>
<tr>
<td>Never</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>No. of diseased coronary arteries</td>
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<td>3</td>
<td></td>
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<tr>
<td>Total cholesterol, mmol/L</td>
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<td>5.1±0.9</td>
<td>4–6.5</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
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<td>0.8±0.2</td>
<td>1.1–2</td>
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<tr>
<td>Baseline testosterone, mmol/L</td>
<td>11±6</td>
<td>13±10</td>
<td>11–44</td>
</tr>
<tr>
<td>Baseline 17β-estradiol, pmol/L</td>
<td>188±77</td>
<td>174±36</td>
<td>&lt;174</td>
</tr>
<tr>
<td>Baseline FSH, IU</td>
<td>7±7</td>
<td>6±6</td>
<td>1–12</td>
</tr>
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</table>

Values are mean±SD. Normal ranges are from our biochemistry laboratory for men. MI indicates myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery bypass graft surgery; FSH, follicular stimulating hormone; and HDL, high-density lipoprotein.

in 1 patient, and the right coronary artery in 10 patients. Ten patients had focal narrowing in the study vessel between 10% and 40%, with a mean lesion severity of 23±10%.

Plasma Hormone Concentrations

Plasma testosterone levels in the femoral artery were significantly increased at the greater concentrations of infused testosterone (11±6 versus 14±4, 16±5, 29±16, and 35±15 nmol/L for baseline versus 10⁻⁵ to 10⁻⁷ mol/L testosterone, respectively; P=0.40, 0.12, <0.001, and <0.001, respectively). The mean baseline plasma estradiol level was 188±77 pmol/L (range, 112 to 358 pmol/L).

Coronary Artery Responses to Testosterone

Testosterone (10⁻⁵ to 10⁻⁷ mol/L) significantly increased coronary diameter (Table 2) by 3.1% (P=0.05), 3.5% (P=0.038), and 4.5% (P=0.005) respectively, compared with baseline 2 (Figure). A significant increase occurred in coronary blood flow at all concentrations of testosterone compared with baseline 2 (Table 2). Blood flow was increased by 15.9% (P=0.006), 11.9% (P=0.029), 16.3% (P=0.002), and 17.4% (P=0.002) at levels of 10⁻¹⁰ to 10⁻⁷ mol/L testosterone, respectively (Figure). Velocity was not affected by testosterone at any concentration (Table 2).

Coronary Artery Responses to Acetylcholine Before and After Testosterone

Coronary artery diameter at the Doppler wire tip did not change after acetylcholine infusion compared with baseline, and no difference existed in this diameter response to acetylcholine before and after testosterone (Table 2). Coronary velocity and flow were significantly increased by 10⁻⁶ and 10⁻⁵ mol/L acetylcholine compared with baseline 1 (all P<0.001; Table 2), but no difference existed in the velocity or flow responses before and after testosterone.

Mean Coronary Artery Diameter Changes

The mean diameter of the entire study vessel was measured in response to the maximum dose of acetylcholine infused in 11 patients. Acetylcholine (10⁻⁵ mol/L) induced a significant increase in mean diameter compared with baseline (3.01±0.68 versus 3.14±0.63 mm for baseline 1 versus acetylcholine 10⁻⁵ mol/L; P<0.05); however, no difference existed in this response before and after testosterone infusion (3.14±0.63 versus 3.07±0.64 mm). In 8 areas of focal narrowing (mean severity, 23±10%), the diameter response to acetylcholine (10⁻⁵ mol/L) was unchanged before and after testosterone (2.46±0.7 mm versus 2.46±0.86 mm). In 11 areas of dilatation to acetylcholine (10⁻⁵ mol/L), the dilator response was the same before and after infusions of testosterone (4.01±0.82 mm versus 3.85±0.79 mm).

Coronary Artery Responses to Vehicle Control and Isosorbide Dinitrate

Vehicle control did not change coronary artery diameter or velocity or blood flow compared with baseline 1 or baseline 2 (Table 2). Isosorbide dinitrate significantly increased coronary velocity, diameter and flow compared with baseline 2 (P<0.001, P=0.005, and P<0.001, respectively; Table 2).

Systemic Hemodynamics

Table 2 shows that mean arterial pressure and heart rate did not change significantly throughout the study.

Controls

Characteristics

Eight controls were enrolled (mean age, 57±9 years), and all had coronary atherosclerosis (Table 1). Three controls had 1-vessel disease, and 5 had 2-vessel disease. The left circumflex coronary artery was studied in 1 control patient, and in 7 controls, the right coronary artery was studied. No differences existed between the patients and controls with respect to age, baseline plasma testosterone concentration, or factors that might affect endothelial function, such as lipid profile, blood pressure, heart rate, or coronary atherosclerosis (Table 1). Seven controls had focal narrowing in the study vessel of between 10% and 40%, with a mean lesion severity of 21±9%.

Coronary Artery Vasoreactivity

Vehicle control did not affect coronary velocity, diameter, or blood flow compared with baseline (Table 3). No differences existed in the velocity, diameter, or blood flow responses to acetylcholine before and after vehicle control (Table 3). Mean diameter was measured in response to the maximum dose of acetylcholine infused in 8 patients. Acetylcholine (10⁻⁵ mol/L) did not significantly change mean diameter compared with baseline 1 (3.22±0.56 mm versus 3.31±0.6 mm), and no differences existed in this response before and after infusions of vehicle control (3.31±0.6 mm versus 3.27±0.58 mm). In 7 areas of focal narrowing (mean severity, 21±9%), the diameter response to acetylcholine (10⁻⁵ mol/L) was unchanged before and after vehicle control.
In 8 areas of dilatation to acetylcholine (10^{-7} mol/L), the dilator response was the same before and after infusions of vehicle control (4.6±1.2 mm versus 4.5±1.23 mm). Systemic blood pressure and heart rate did not change after infusion of vehicle control compared with baseline (Table 3).

**Discussion**

We demonstrated that testosterone, administered acutely at physiological (and greater) concentrations, induces coronary artery dilatation (up to 4.5%) and increases coronary blood flow (up to 17.4%) in men with coronary atherosclerosis. Testosterone did not alter acetylcholine-induced increases in coronary blood flow or areas of constriction or dilatation induced by acetylcholine, which suggests that it had no measurable effect on stimulated endothelial nitric oxide. These findings are similar to those found in vitro in which testosterone-induced coronary relaxation was shown to be independent of the endothelium, with the possible involvement of potassium channel modulation. The effect of testosterone on coronary artery diameter and blood flow are approximately half that demonstrated after intracoronary diltiazem administration, where diameter was increased by 10% and flow by 30%. Nicorandil, a potassium channel activator, exerts its vasodilator actions through a mechanism similar to that of testosterone, via activation of ATP-sensitive potassium channels. In a study in patients with normal coronary arteries and coronary artery disease, this agent increased proximal coronary artery diameter by 13%. The dose of nicorandil infused into these coronary arteries was 0.5 mg, a pharmacological dose.

In an animal model, short-term intracoronary infusions of testosterone (10^{-7} and 10^{-6} mol/L) increased coronary blood flow, similar to the findings of the present study. Inhibition of nitric oxide synthase by L-NAME significantly attenuated testosterone-induced increases in blood flow (P<0.04), indicating an endothelium-dependent effect of testosterone on this calculated parameter. However, a high concentration of testosterone was used to achieve this effect (10^{-6} mol/L), and the attenuation of cross-sectional area and velocity were not statistically significant at the 5% level (both P=0.06). These authors could not distinguish between a direct effect of

| TABLE 2. Effects of Testosterone on Coronary Vasoreactivity and Systemic Hemodynamics |
|---------------------------------|---------|---------|---------|---------|---------|
|                                 | Diameter, mm | Velocity, cm/s | Flow, mL/min | Mean Arterial Pressure, mm Hg | Heart Rate, beats/min |
| Baseline 1                      | 2.93 (0.72) | 16 (10)   | 40 (13)   | 79 (9)   | 62 (9)   |
| Vehicle control                 | 2.89 (0.74) | 20 (16, 25) | 38 (30)   | 81 (9)   | 63 (8)   |
| ACh 10^{-7} mol/L               | 3.04 (0.77) | 23 (5)    | 52* (22)  | 79 (9)   | 61 (8)   |
| ACh 10^{-6} mol/L               | 3.01 (0.78) | 34‡ (8)   | 75.9†‡ (51) | 80 (10)  | 63 (15)  |
| ACh 10^{-5} mol/L               | 2.92 (0.85) | 49‡ (13)  | 89‡ (39)  | 80 (10)  | 60 (9)   |
| Baseline 2                      | 2.78 (0.74) | 20 (7)    | 35 (15)   | 79 (11)  | 61 (9)   |
| Testosterone 10^{-12} mol/L     | 2.82 (0.71) | 22 (9)    | 40† (21)  | 79 (10)  | 63 (9)   |
| Testosterone 10^{-11} mol/L     | 2.86* (0.72) | 21 (9)   | 39* (21)  | 79 (10)  | 59 (9)   |
| Testosterone 10^{-10} mol/L     | 2.87† (0.71) | 22 (9)   | 41† (20)  | 80 (9)   | 63 (12)  |
| Testosterone 10^{-9} mol/L      | 2.9† (0.75) | 10 (10)   | 41† (20)  | 81 (9)   | 59 (11)  |
| ACh 10^{-7} mol/L               | 2.85 (0.72) | 22 (6)    | 48* (22)  | 81 (9)   | 60 (10)  |
| ACh 10^{-6} mol/L               | 2.88 (0.67) | 28‡ (8)   | 68‡ (32)  | 81 (9)   | 60 (11)  |
| ACh 10^{-5} mol/L               | 2.92 (0.72) | 49‡ (8)   | 103‡ (41) | 82 (8)   | 60 (9)   |
| Isosorbide dinitrate            | 3.1† (0.71) | 43‡ (18)  | 98‡ (60)  | 82 (8)   | 65 (17)  |

Values are mean (SD). ACh indicates acetylcholine. *P<0.05, †P<0.01, and ‡P<0.001 compared with most recent baseline.
Testosterone on nitric oxide synthase or a flow-mediated effect of testosterone on the endothelium. Testosterone-induced changes in coronary velocity and flow were significantly attenuated in resistance vessels by glibenclamide \((P<0.03\) and \(P<0.02\), respectively), indicating inhibition of ATP-sensitive potassium channels by testosterone. Although acetylcholine had no effect on epicardial coronary artery diameter in the present study, it did increase blood flow velocity and blood flow volume in a dose-dependent manner, suggesting that functional endothelium exists in resistance vessels. The fact that this response was not different after administration of testosterone does not preclude involvement of the endothelium in testosterone-induced increases in blood flow. In vitro\(^2\) and in vivo\(^13\) animal data would suggest that testosterone stimulates epicardial coronary dilatation, independent of the endothelium, possibly by effects on ion channels on the vascular smooth muscle plasma membrane, such as ATP-sensitive potassium channels. However, it is impossible to rule out an indirect flow-mediated endothelium-dependent effect of testosterone on blood flow response without the use of an inhibitor of nitric oxide.

We assessed the mean coronary artery diameter response to acetylcholine throughout the length of the study arteries and found no significant difference in mean diameter response to acetylcholine before versus after the testosterone infusions. Areas of focal narrowing and areas that dilated to acetylcholine before exposure to testosterone did not react differently before versus after exposure to testosterone. This reinforces our suggestion that testosterone does not enhance endothelial function in sites of constriction or dilatation to acetylcholine.

Testosterone is converted to 17\(\beta\)-estradiol by the enzyme aromatase. It is possible that estradiol may account for the vascular effects of testosterone; however, the evidence to date does not support this potential mechanism. Inhibition of both the testosterone and estrogen receptors does not affect testosterone-induced coronary relaxation in vitro\(^7\) or coronary dilatation and increases in blood flow in vivo.\(^13\) Androgen receptors have been identified in ventricular and atrial myocytes, endothelial cells, and vascular smooth muscle cells of some mammalian species\(^14–16\); however, at present, no information exists regarding the presence of androgen receptors in the coronary arteries of humans. Also, it has been shown in a

**TABLE 3. Effects of Vehicle Control on Coronary Vasoreactivity and Systemic Hemodynamics**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter, mm</th>
<th>Velocity, cm/s</th>
<th>Flow, mL/min</th>
<th>Mean Arterial Pressure, mm Hg</th>
<th>Heart Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>2.91</td>
<td>16.1</td>
<td>36</td>
<td>77</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>(0.47)</td>
<td>(5.8)</td>
<td>(13)</td>
<td>(14)</td>
<td>(7)</td>
</tr>
<tr>
<td>ACh 10(^{-7}) mol/L</td>
<td>2.97</td>
<td>23</td>
<td>52</td>
<td>78</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>(0.38)</td>
<td>(12)</td>
<td>(33)</td>
<td>(13)</td>
<td>(8)</td>
</tr>
<tr>
<td>ACh 10(^{-6}) mol/L</td>
<td>2.9</td>
<td>34</td>
<td>72†</td>
<td>74</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>(0.41)</td>
<td>(21)</td>
<td>(52)</td>
<td>(14)</td>
<td>(9)</td>
</tr>
<tr>
<td>ACh 10(^{-5}) mol/L</td>
<td>2.82</td>
<td>49</td>
<td>99†</td>
<td>80</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>(0.43)</td>
<td>(26)</td>
<td>(69)</td>
<td>(13)</td>
<td>(6)</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>2.89</td>
<td>17</td>
<td>35</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(0.5)</td>
<td>(5.6)</td>
<td>(19)</td>
<td>(12)</td>
<td>(5)</td>
</tr>
<tr>
<td>ACh 10(^{-7}) mol/L</td>
<td>2.86</td>
<td>22</td>
<td>44</td>
<td>79</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(0.42)</td>
<td>(10)</td>
<td>(27)</td>
<td>(18)</td>
<td>(7)</td>
</tr>
<tr>
<td>ACh 10(^{-6}) mol/L</td>
<td>2.77</td>
<td>28</td>
<td>55</td>
<td>76</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>(0.51)</td>
<td>(10)</td>
<td>(31)</td>
<td>(10)</td>
<td>(9)</td>
</tr>
<tr>
<td>ACh 10(^{-5}) mol/L</td>
<td>2.85</td>
<td>49</td>
<td>104</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>(0.48)</td>
<td>(23)</td>
<td>(79)</td>
<td>(13)</td>
<td>(12)</td>
</tr>
<tr>
<td>Isosorbide dinitrate</td>
<td>3.12</td>
<td>37*</td>
<td>94*</td>
<td>77</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>(0.57)</td>
<td>(14)</td>
<td>(55)</td>
<td>(14)</td>
<td>(9)</td>
</tr>
</tbody>
</table>

Values are mean (SD). ACh indicates acetylcholine.

*\(P<0.01\), †\(P<0.001\) compared with baseline.
number of studies that estrogen does not have a direct relaxing effect on human coronary arteries in vivo.9,17

We demonstrated direct coronary effects of testosterone at physiological concentrations (adult male normal range is \(10^{-9} \text{ to } 10^{-8} \text{ mol/L}\)) in humans. Previous studies (described above) showed significant effects of testosterone at pharmacological concentrations in vitro (\(10^{-6} \text{ and } 10^{-5} \text{ mol/L}\)) and near-physiological concentrations in vivo (\(10^{-7} \text{ mol/L}\)).13 Interestingly, the mean baseline testosterone level of the men included in the present study was at the lower end of the normal range (mean, 11±6 nmol/L; range, 1 to 26 nmol/L), which reinforces the observation of Phillips et al2 that low plasma testosterone may be a risk factor for coronary heart disease. Indeed, 7 of the 13 men had plasma testosterone levels <11 nmol/L. Serum testosterone decreases with age,19 and bioavailable testosterone is decreased in older men.20 Concurrently, an apparent stimulation of gonadotrophin release occurs, with increased levels of follicular-stimulating hormone and luteinizing hormone in elderly men.21 The patients in the current study demonstrated relatively low testosterone levels for men of a younger age; therefore, it would be plausible that, in our study group, coronary artery disease was not simply a function of age but may also be related to serum testosterone levels. Separate analyses of flow responses in our subjects with normal and low baseline testosterone levels showed no significant correlation between baseline plasma testosterone levels and flow response to infused testosterone (data not shown). This may be due to small numbers (n=7 and n=6, respectively) or to the fact that the normal group had testosterone levels at the lower end of the normal range.

Dose is an important consideration regarding long-term testosterone administration. High concentrations of testosterone have detrimental effects on atheroma progression,22 plasma lipids,23 and hemostatic factors,2,24,25 and they increase the risk of myocardial infarction and stroke.26 However, recently published data show a beneficial effect of testosterone on atheroma development in rabbits.27 The natural androgens testosterone and dehydroepiandrosterone, at physiological concentrations, produced this effect, which was only partially mediated through a beneficial effect on lipid profile. The results of the present study are important because they show the effects of low-dose, physiological levels of testosterone on coronary vasomotion in humans. The results are also important because testosterone is present in both men and women and, therefore, the results may be pertinent to both sexes. Further studies will be needed to investigate the effects of long-term, low-dose testosterone administration on coronary reactivity and risk factors for coronary artery disease in men and the role of testosterone in coronary physiology and pathophysiology in women.

Limitations

We found no effect of testosterone on acetylcholine-induced increases in blood flow, indicating a lack of effect of testosterone on endothelium-dependent responses. To prove a direct effect of testosterone on nitric oxide synthase, however, experiments inhibiting endothelium-derived nitric oxide synthesis would need to be performed. These further experi-


• Protected by the use of estradiol and testosterone
• Clinical and non-human primate studies suggest androgens inhibit mammary epithelial proliferation and breast growth. Estrogen, particularly in oral form, stimulates SHBG and reduces free testosterone. Testosterone is being used worldwide as a treatment for breast cancer.
Review

Androgens and the breast

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Abstract

Androgens have important physiological effects in women while at the same time they may be implicated in breast cancer pathologies. However, data on the effects of androgens on mammary epithelial proliferation and/or breast cancer incidence are not in full agreement. We performed a literature review evaluating current clinical, genetic and epidemiological data regarding the role of androgens in mammary growth and neoplasia. Epidemiological studies appear to have significant methodological limitations and thus provide inconclusive results. The study of molecular defects involving androgenic pathways in breast cancer is still in its infancy. Clinical and nonhuman primate studies suggest that androgens inhibit mammary epithelial proliferation and breast growth while conventional estrogen treatment suppresses endogenous androgens. Abundant clinical evidence suggests that androgens normally inhibit mammary epithelial proliferation and breast growth. Suppression of androgens using conventional estrogen treatment may thus enhance estrogenic breast stimulation and possibly breast cancer risk. This is due to suppression of gonadotropins by exogenous estrogen treatment, resulting in globally reduced ovarian steroidogenesis, so both endogenous estrogen and androgen production are reduced, but only estrogens are provided by the treatment regimens. Additionally, estrogens, particularly in oral form, stimulate the hepatic production of sex hormone binding globulin (SHBG), which binds testosterone with high affinity, reducing androgen bioavailability. As a result of this dual effect, total and bioavailable testosterone levels are significantly reduced in women taking oral contraceptives or estrogen supplementation for ovarian insufficiency [4].

This literature review compares the findings that androgens in women promote the risk for breast cancer versus the evidence that androgens protect the mammary gland from hormone-induced stimulation, increased proliferation and neoplasia.

Introduction

Treatment of women with physiological testosterone supplementation to remedy hypoactive sexual desire disorder is an area of great interest at present [1]. While it seems evident that testosterone treatment increases sexual activity, the risk-benefit ratio for such treatment remains unclear. Androgen receptors are found in virtually every tissue in women as well as men, including breast, bone and brain, indicating that androgens and their metabolites may play an important role in normal tissue homeostasis and possibly in pathologies like breast cancer, osteoporosis, libido and cognitive decline. Thus, testosterone treatment to improve sexual function may have unintended effects in diverse tissues. A continuing area of concern is the notion that excess androgen exposure may increase the risk of breast cancer in women [2].

Experimental data suggest that conventional estrogen treatment regimens, both as oral contraceptives (OCs) and hormone therapy (HT) [3], upset the normal estrogen/androgen balance and promote ‘unopposed’ estrogenic stimulation of mammary epithelial proliferation and, hence, potentially breast cancer risk. This is due to suppression of gonadotropins by exogenous estrogen treatment, resulting in globally reduced ovarian steroidogenesis, so both endogenous estrogen and androgen production are reduced, but only estrogens are provided by the treatment regimens. Additionally, estrogens, particularly in oral form, stimulate the hepatic production of sex hormone binding globulin (SHBG), which binds testosterone with high affinity, reducing androgen bioavailability. As a result of this dual effect, total and bioavailable testosterone levels are significantly reduced in women taking oral contraceptives or estrogen supplementation for ovarian insufficiency [4].

This literature review compares the findings that androgens in women promote the risk for breast cancer versus the evidence that androgens protect the mammary gland from hormone-induced stimulation, increased proliferation and neoplasia.

Normal breast development: estrogens and androgens

Estrogens stimulate, while androgens inhibit breast development independently of genetic sex. Breast tissue is similar in prepubertal boys and girls. Pubertal rises in estrogen levels cause breast growth in girls and frequently in boys (transiently). Estradiol levels are significantly higher in girls with premature thelarche than in normal prepubertal girls. An
association between expression of a high activity isoform of the testosterone metabolizing CYP3A4 and the early onset of thelarche has been documented, suggesting that decreasing testosterone levels may also trigger early breast growth [5]. Conversely, androgen excess due to adrenal tumor or hyperplasia suppresses normal breast development in girls, despite apparently adequate estrogen levels [6]. In castrated male-to-female transsexuals, feminizing estrogen therapy stimulates breast growth with full acinar and lobular formation and estrogen-treated genetically male breast tissue exhibits normal female histology. Estrogens taken to treat prostate cancer also lead to breast development in men with suppressed gonadal function and reduced testosterone levels. Conversely, androgen use by female athletes and female-to-male transsexuals leads to breast atrophy.

Supporting the normal inhibitory role of endogenous androgens on breast growth, androgen receptor (AR) blockade with flutamide causes gynaecomastia and rarely breast adenocarcinoma [7]. Males may also develop gynaecomastia when the estrogen/androgen ratio is increased due to decreased androgen production or increased aromatization.

The balance between stimulatory effects of the estrogens and inhibitory effects of the androgens is the critical factor that regulates mammary cell proliferation both in normal and in cancer tissues [8]. It has not been possible to identify specific estrogen/androgen ratios predictive of breast stimulation or inhibiting effects for several reasons. Estradiol and testosterone assays have not been very sensitive nor accurate in the lower ranges, while both hormones bind to SHBG, so total values may not be as informative as ‘free’ or bioavailable hormone [4]. Moreover, single hormone measurements may not be very informative about tissue exposure over time. Both estradiol and testosterone levels vary hourly in response to diurnal rhythm, diet, stress and exercise, so a single value may be inadequate to assess true tissue exposure. In addition, estradiol and testosterone may be synthesized locally in peripheral tissues from circulating precursors such as the sulfate of dehydroepiandrosterone (DHEA-S) and androstenedione [9]. The conjugated products of steroid metabolism find their way into the circulation after peripheral action and provide evidence as to the proportion of the precursor pools of steroids utilized as androgen or estrogen. Analysis of these metabolites by Labrie and colleagues [9] and Sasano and colleagues [10] suggested that the major proportion of androgen effectors in women derive from such an endocrine mode of action, which will not be detected by assays of circulating testosterone or dihydrotestosterone (DHT). Interestingly, while circulating levels of testosterone and DHT are five- to ten-fold higher in men than women, the abundance of androgen metabolites is less than two-fold higher in men, suggesting that local tissue production and action of androgens in women may be more significant than historically suspected.

The mammary gland is capable of synthesizing both estradiol and testosterone. All the steroidogenic enzymes necessary for the formation of androgens and estrogens from steroid precursors - steroid sulfatase, 17β-hydroxysteroid dehydrogenases (17β-HSDs), 3β-HSDs, 5α-reductases and aromatase - have been reported in normal mammary tissues, breast cancer specimens or cell lines [10]. Androgens stimulate or inhibit the growth of breast cancer cells in vitro depending on the cell line and clone under study according to former data [11]. Breast cancer cell lines and tissue specimens express the enzymes involved in DHT as well as estradiol synthesis. In a histochemical study, expression of 5α-reductase was significantly correlated with androgen receptor expression and 17β-HSD and 3β-HSD immunoreactivities and the abundance of this androgenic molecular assembly was inversely correlated with tumor size, histological grade and proliferative index [12], suggesting an inhibitory role for DHT in tumor growth.

**Androgen receptor**

Androgen agonists such as testosterone and DHT function through binding to the intracellular AR. This is a member of the nuclear hormone receptor super-family comprising classic DNA-binding, hormone binding and activation domains (Figure 1). AR expression is abundant in normal mammary epithelium and in the majority of breast cancer specimens and cell lines. The AR is co-localized with estrogen and progesterone receptors in epithelial cells but not detected in mammary stroma or myoepithelium [13]. The co-expression of estrogen receptor (ER) and AR in mammary epithelial cells suggests that the effects of estrogen and androgen on mammary epithelial proliferation are integrated within the mammary epithelial cell. The AR gene is located on the X chromosome with no corresponding allele on the Y, so it functions solely as a single copy gene, as shown by the complete loss of androgen effect in XY individuals with an inactivating mutation of the AR [14].

Binding of testosterone or DHT triggers a cascade of signaling events, including phosphorylation and conformational changes in the receptor, which dissociates from cytoplasmic proteins and migrates to the cell nucleus. Ligand-activated AR regulates gene expression through binding to androgen response elements located in a gene’s enhancer or promoter region. As with other similar receptors, the AR functions in transcriptional regulation in concert with a host of nuclear proteins, which may serve as co-activators or co-repressors. Interestingly, the BRCA1 gene product has been identified as an AR co-activator [15]. The BRCA1 protein binds to the AR and potentiates AR-mediated effects, suggesting that BRCA1 mutations may blunt androgen effects. However, other studies have not confirmed these findings [16].

AR has a highly polymorphic CAG repeat in exon 1 that encodes a polyglutamine stretch (Figure 1). There is evidence that longer CAG repeats are associated with earlier age of
breast cancer onset [16]. However, other studies have not confirmed this finding [17]. In a study nested within the Nurses’ Health Study cohort [18], no relation was found between AR genotype and breast cancer risk among postmenopausal Caucasian women overall, but an increased risk was observed when analysis was limited to those individuals with a first-degree family history of breast cancer. Germ-line mutations in the AR gene conferring variable degrees of androgen insensitivity have been associated with the occurrence of breast cancer in men [19]. Another study [20] provides evidence that the association with the long AR-CAG was observed only in postmenopausal and not premenopausal women, which may explain the insignificant results in studies restricted to young women. In other studies, reduced risk was observed with another trinucleotide repeat, GGC, in young women. AR repeat length might be partly responsible for the increased risk of early onset breast cancer in women using oral contraceptives or HT [21].

Emphasis should be given to the fact that none of these studies had sufficient statistical power to implicate or exclude specific AR defects in breast cancer risk. A recent epidemiological meta-analysis concludes that there is no association between AR genetic variations and breast cancer risk among Caucasian women [17].

AR-CAG repeat length was inversely associated with testosterone levels in both pre- and postmenopausal normal women [22]. Lillie and colleagues [23] evaluated the association between AR-CAG repeat length and mammographic density, a strong breast cancer risk factor. They found that in postmenopausal estrogen/progesterone users, carriers of the less active AR had a higher mean percentage of density than carriers of the more active AR. This suggests that AR genotype modifies hormone-induced proliferation as reflected in mammographic density and explains the mechanism by which estrogen/progesterone use increases breast cancer risk. However, the exact mechanisms and metabolic paths in which AR participates in normal tissues are still obscure. The role of AR in oncogenesis or breast tumor proliferation remains unclear. It is possible that the steroid receptor contributes differently in healthy compared to cancerous breast tissue; thus, a number of unanswered questions remain and further studies are needed before safe conclusions are drawn.

The hypothesis that androgens are directly involved in breast carcinogenesis is based on the presence of ARs in the majority of breast cancers. It is proposed that androgens, through binding to their receptors, act independently to produce tumors with specific clinical behaviors [24]. Clinical data support that a significant number of poorly differentiated breast carcinomas are ER-negative and progesterone receptor (PR)-negative but AR-positive, or patients with AR-positive tumors experience a better disease-free survival. These associations constitute important clinical and pathologic prognostic information. Recently, AR expression in a tumor is considered as an indicator of lower malignancy potential; this provides a new range of therapeutic targets for poorly differentiated cancers [25].

Androgens and breast cancer: epidemiological data

Long-term estrogen treatment increases the risk of breast cancer in both males and females through estrogenic stimulation of mammary epithelial proliferation. Additional carcinogenic effects by estrogen metabolites have been proposed [26]. The most widely accepted risk factor for breast cancer is the cumulative dose of estrogens that breast epithelium is exposed to over time. However, it has been difficult to correlate breast cancer risk with isolated serum estrogen levels in epidemiological studies, probably secondary to problems using single random hormone levels for the evaluation of tissue-specific exposure as discussed above.

Attempts to correlate adrenal precursor steroids with breast cancer incidence have been relatively successful, or at least consistent, perhaps reflecting the importance of local tissue conversion. Many years ago, reduced 17-ketosteroid excretion was noted in the urine of pre-menopausal women with

Figure 1

Schematic design of the androgen receptor gene (top) and protein (below). The polymorphic trinucleotide repeat site (CAG) is indicated in green at the left. Trans-activating function (TAF), DNA-binding (DBD) and ligand-binding domains (LBD) are labeled.
breast cancer and subsequent studies have documented reduced DHEA and its sulfate (DHEA-S) in the serum of premenopausal breast cancer patients. In the first prospective study in this field, levels of androgen metabolites in urine were found to be abnormally reduced in premenopausal women who subsequently developed breast cancer [27], indicating a protective role of androgens on the breast. In contrast, in a recent prospective study of pre-menopausal women [28], no association was found between plasma adrenal androgen levels and risk of breast cancer. In the Nurses’ Health Study II, no correlation between DHEA and DHEA-S levels and breast cancer risk overall was found but, interestingly, among premenopausal women there was a positive association, especially for tumors that express both ERs and PRs [29]. Also, among premenopausal women, higher levels of testosterone and androstenedione were associated with increased risk of invasive ER+/PR+ tumors, although with a non-statistically significant increase in overall risk of breast cancer [29].

Several epidemiological studies have examined the correlation of circulating androgens, such as testosterone, and risk for breast cancer. A major limitation of these studies was that the androgen assays used were developed primarily to measure the higher levels found in men and lack reliability in the low ranges found in normal women [4]. Testosterone and androstenedione levels demonstrate substantial daily variability, while most of the epidemiological data are based on a single blood sample collected at non-standard times. Another problem using serum testosterone levels to gauge androgenic effects at the tissue level is that most of the circulating testosterone is tightly bound to SHBG, while only the free hormone is bioactive. SHBG, and thus total testosterone levels, vary widely based on genetic, metabolic and endocrine influences, and it is now accepted that measurement of free or bioavailable testosterone predicts androgenic effects more accurately than total testosterone levels [4]. Finally, most androgenic activity in women originates from the peripheral conversion of precursors such as DHEA into androgens within the cells of target tissues, and this activity will not be detected by measurement of circulating androgens.

In a recent study [30], levels of testosterone and DHEA-S in saliva were statistically significantly lower in breast cancer patients compared to controls and these differences were more profound in postmenopausal women. Breast cancer patients, when compared to controls, presented with an androgen insufficiency and a relative imbalance of sex steroid hormones in favor of estrogens.

Several studies have revealed, however, that adrenal androgens are increased in postmenopausal women with breast cancer [31]. A possible explanation regarding the divergence between pre- and postmenopausal findings is that one adrenal ‘androgen’, androstenediol, also known as ‘hermaphrodiol’, is a weak ER agonist. In the presence of high estrogen levels in premenopausal women, androstenediol could exhibit anti-estrogenic effects, while in the hypoestrogenic postmenopausal milieu, the agonist effect may predominate. This view remains speculative, and other possibilities still exist. It is possible that the high estrogen environment in premenopausal women promotes androgenic enzyme and AR expression in mammary tissue, allowing androgenic effects by DHEA metabolites, while in postmeno-

pausal women, an estrogen-deficient tissue microenvironment may favor estrogenic effects.

Genetic variation in CYP19 and SHBG genes was found to contribute to the variance in circulating hormone levels in postmenopausal women, but none was statistically significantly associated with breast cancer risk [32].

In some prospective epidemiological studies, age-adjusted mean values of total and free testosterone and estradiol were significantly higher pre-diagnostically in postmenopausal breast cancer cases compared with controls, and estradiol and total testosterone were elevated in other case-control studies of postmenopausal breast cancer. It was observed that elevated serum levels of both estrogens and androgens contribute to a greater risk of breast cancer [33] and a meta-analysis of nine prospective studies revealed that breast cancer risk increases with increasing concentrations of almost all sex hormones [34].

None of these studies manage, however, to disconnect the risk associated with increased estradiol levels from the androgen component, and since androgens are the obligate precursors for estradiol synthesis, this is a major confounding factor in assessing the role of androgen independently of the known cancer-promoting estrogen effect. In line with these observations, a recent study [35] concluded that increased breast cancer risk with increasing body mass index among postmenopausal women is largely the result of the associated increase in estrogens. The association of androgens with breast cancer risk did not persist after adjustment for estrone, the estrogen most strongly associated with the risk. Other authors conclude that conversion of DHEA to estrogens, particularly estradiol, is required to exert a mitogenic response [36]. These results suggest that the contribution of androgens to breast cancer risk is largely through their role as substrates for estrogen production.

Other studies have found no association between androgens and breast cancer [37,38]. Recent epidemiological studies on the association between androgen levels and breast cancer risk are summarized in Table 1.

The above mentioned observations indicate the difficulty separating potential direct effects of circulating testosterone from its potential to be aromatized into estradiol. An interesting research topic would be to investigate levels of testosterone and DHT metabolites in these studies in order to assess more
Elevated levels of androgens (and estrogens) are associated with increased risk of breast cancer

Tamimi et al. 2006 [47] Prospective cohort study in Nurses' Health Study with over a million person-years studied: women receiving postmenopausal hormones with testosterone had a 17.2% increased risk of breast cancer per year of use

Micheli et al. 2007 [54] Breast cancer patients (n = 194) with high testosterone had significantly lower event-free survival than those with low testosterone (P = 0.004) and a significantly higher risk of breast cancer events with an adjusted hazard ratio of 1.77 (95% CI, 1.06 to 2.96)

The Endogenous Hormones and Breast Cancer Collaborative Group 2002 [34] Meta-analysis: breast cancer risk increases statistically significantly with increasing concentrations of almost all sex hormones

Tworoger et al. 2006 [31] Prospective nested case-control study within the Nurses' Health Study II: adrenal androgens are positively associated with breast cancer among predominately premenopausal women (for example, for DHEA: RR, 1.6; 95% CI, 0.9 to 2.8; P = 0.09)

Eliassen et al. 2006 [29] Nurses' Health Study II, nested: higher levels of testosterone and androstenedione in 18,521 premenopausal women are associated with insignificant overall increase in breast cancer risk, but increased risk of invasive and ER+PR+ cancers (for example, RR = 2.9; CI = 1.4 to 6.0)

Androgen levels acting with protective patterns

Hofling et al. 2007 [51] Randomized, double-blind, placebo-controlled study: testosterone use inhibited exogenous estrogen-induced breast tissue proliferation in 99 postmenopausal women (P < 0.001)

Dimitrakakis et al. 2004 [48] Retrospective, observational study that followed 508 postmenopausal women receiving testosterone in addition to usual hormone therapy: incidence of breast cancer in testosterone users was substantially less than in women receiving estrogen/progesterin in the WHI study and in the Million-woman study

Suzuki et al. 2001 [55] Intratumoral dihydrotestosterone inhibits cancer cell proliferation in hormone-dependent human breast carcinoma

Haiman et al. 2002 [18] A case-control study nested within the Nurses’ Health Study cohort (cases, n = 727; controls, n = 969): longer CAG repeat alleles of AR increases breast cancer risk (odds ratio, 1.70; 95% CI, 1.20 to 2.40; P = 0.04)

MacLean et al. 2004 [19] Forty-one male breast cancers were studied: incidence of longer CAG repeats in AR was significantly higher in the breast cancer group than in the normal population (P < 0.05)

Ogawa et al. 2008 [25] In 227 primary breast cancers, AR expression was significantly higher in breast tumors with favorable characteristics

Dimitrakakis et al. 2009 [30] Testosterone and DHEA-S salivary levels were statistically significantly lower in breast cancer patients compared to controls (n = 541)

No association between serum concentrations of androgens and breast cancer risk

Ness et al. 2009 [46] A group of postmenopausal participants in the WHI study used testosterone combined with estrogens: testosterone addition had no statistically significant effect on breast cancer occurrence

Cox et al. 2006 [17] Among postmenopausal women, common variants of the AR gene are not associated with risk of breast cancer

Page et al. 2004 [28] Prospective observational study: no relationship between serum DHEA or DHEA-S and subsequent breast cancer in middle-aged women

Olson et al. 2007 [32] No association with breast cancer risk was detected for individual variants of CYP19 mutation in 750 cases

Adly et al. 2006 [37] Serum levels of steroids in 331 women: androgen levels were not independently associated with increased risk of breast cancer

Beattie et al. 2006 [38] Case-cohort design including 135 postmenopausal women with and 275 without breast cancer enrolled in the NSABBP P-1 trial: risk of breast cancer was not associated with sex hormone levels

AR, androgen receptor; CI, confidence interval; DHEA, dehydroepiandrosterone; DHEA-S, DHEA sulfate; ER, estrogen receptor; NSABBP, National Surgical Adjuvant Breast and Bowel Project; PR, progesterone receptor; RR, relative risk; WHI, Women’s Health Initiative.

directly tissue exposure to androgens. As noted above, a single serum hormone measurement seems unlikely to be informative about a woman’s true long-term exposure to that hormone or her specific risk of developing breast cancer. Nor does it seem to be a biologically plausible mechanism that androgens acting as androgens could promote breast cancer, since virtually all clinical data suggest just the opposite. If elevated androgen levels directly contribute to breast cancer, then women with clinically evident long-term hyperandrogenism - for example, polycystic ovary syndrome and congenital adrenal hyperplasia - should experience increased rates of breast cancer, but this is not the case [39]. Moreover, androgen levels are chronically elevated in men, who have a breast cancer risk less than 1% of that of women. This is despite the fact that lifetime estradiol
levels are not much lower in men than in women. In fact, decreased androgen levels, for example, in Kleinfelter’s syndrome and other hypogonadal syndromes increase the risk of breast cancer in males [40]. Epidemiological studies in men indicate that low urinary androstenedione and serum free testosterone levels are related to early onset of breast cancer, a much higher relapse rate and a worse response to endocrine therapy [41].

**Hormone therapy and androgens**

Exposure to endogenous and exogenous estrogens is thought to contribute to increased breast cancer risk. Since the introduction of combined OCs 40 years ago, many changes in doses and their biochemical structures have taken place and the possibility that OCs may increase the risk of breast cancer has been the subject of intense research. Although many epidemiological studies in the past have not linked OC use to breast cancer risk, several more recent studies have found an association, either overall or especially in subgroups of women. A large meta-analysis on previously published studies [42] calculated a small but significant increase in relative risk of breast cancer (RR = 1.24) in current OC users while other publications [43] have not associated current or former OC use with an increased risk of breast cancer. However, because pill users are young, this represents a very small increase in absolute risk. It is not yet known if lower dose and variable OC formulations are associated with a similar increase in risk, making comparisons very difficult.

The bulk of the currently available evidence supports a causal relationship between the use of HT and breast cancer. Recent and long-term users of HT are associated with higher risk. The effect of concurrent progestin use appears to further increase risk above that with estrogens alone. The most important randomized clinical trial providing information about this issue is the Women’s Health Initiative (WHI) study [44] reporting increased risk of breast cancer in women who took estrogen plus progestin but not in women who took estrogen alone [45]. The results from observational studies are generally consistent with those of the WHI trial, reporting no significant variation in the risk of breast cancer with use of different estrogens, progestins, doses, or routes of administration.

A group of postmenopausal participants in the WHI study used testosterone combined with estrogens. In this group, testosterone addition for a period of a year had no statistically significant effect on breast cancer occurrence, suggesting at least that androgen induction did not increase the number of breast cancer cases in this trial [46]. In the same study, rates of breast cancer were lower in longer-term compared to shorter-term users of estrogen plus testosterone. On the other hand, in a prospective study of over a million person-years with 24 years of follow-up within the Nurses’ Health Study, current users of estrogen plus testosterone have shown a 2.5-fold increased risk of developing breast cancer compared to menopausal women who used estrogen-only therapy or to women who never used postmenopausal hormone formulations [47].

If androgens are protective against breast cancer as many studies suggest, then conventional HT may promote breast cancer not only by increasing estrogen exposure but also by decreasing endogenous androgen activity. Oral estrogen therapy reduces free androgens by stimulating hepatic production of SHBG and through suppression of luteinizing hormone, thus inhibiting ovarian androgen production [4]. Thus, institution of pharmacological estrogen therapy at menopause may result in a drastic reduction in the testosterone/estradiol ratio, which is normally maintained at relatively high levels throughout a woman’s lifespan (Figure 2).

Pertinent to these results, our published article [48] provides important information about the addition of testosterone to the HT regimen. In our evaluation of 508 postmenopausal women in Australia receiving testosterone in addition to usual HT, the incidence of breast cancer in testosterone users was substantially less than in women receiving estrogen/progestin in the WHI study and in the Million-woman study [49]. Breast cancer rates in the testosterone users was closer to that reported for HT never users, and their age-standardization rate was the same as for the general population in South Australia. These observations suggest that the addition of physiological doses of androgen to OCs and HT could protect the breast from ‘unopposed’ estrogenic effects.

Suppression of normal endogenous androgen may be an adverse consequence of pharmacological estrogen therapy, if
androgens are indeed protective against estrogen-induced mammary proliferation. We have shown that addition of low physiological doses of testosterone (producing serum levels in the mid-normal range for women as well as rhesus monkeys) to estrogen therapy in ovariectomized rhesus monkeys significantly inhibits HT-induced mammary epithelial proliferation [3] (Figure 3). Additionally, testosterone treatment significantly reduced mammary epithelial estrogen receptor expression, thus suggesting a potential mechanism for the growth inhibitory effect. Moreover, we have found that treatment of intact cycling monkeys with the AR antagonist flutamide resulted in a significant increase in mammary epithelial proliferation [3], adding to the burden of evidence that endogenous androgens normally limit mammary proliferation and, hence, cancer risk. Other studies on primates also suggest that inclusion of testosterone with estrogen/progesterone use may counteract breast cell proliferation [50]. In a recent randomized, double-blind, placebo-controlled study, testosterone use inhibited exogenous estrogen-induced breast tissue proliferation in postmenopausal women [51]. There is also evidence that testosterone does not influence mammographic breast density like conventional HT [51,52]. The antiproliferative effects of androgens on breast tissue may occur either indirectly, via downregulation of other receptors like PRs, or directly, through breast AR stimulation.

Women, and particularly postmenopausal women, have been treated with testosterone for female sexual dysfunction for almost six decades. The exact biological role of androgens in the restoration of libido in hypoactive sexual desire disorder in females is still unclear. The main safety concern for women who have undergone years of this therapy has been breast and endometrial cancer risk related to androgens. In a recent trial of 814 sexually hypoactive women, the results for breast cancer risk were inconclusive [1]. Nevertheless, current experience does not confirm a positive correlation between testosterone use and breast cancer occurrence; thus, androgens do have a place in female sexual dysfunction treatment.

**Conclusion**

This review focuses on the role of androgens with regard to breast growth and neoplasia. Measurement of circulating sex
steroids and their metabolites demonstrates that androgen activity is normally abundant in healthy women throughout their entire lifetime. Epidemiological studies investigating testosterone levels and breast cancer risk have major theoretical and methodological limitations and do not provide consensus. The molecular epidemiology of defects in pathways involved in androgen synthesis and activity in breast cancer hold great promise, but investigation of these is still in the early stages. Clinical observations and experimental data indicate that androgens inhibit mammary growth, and they have been used in the past with success to treat breast cancer. It is of concern that current forms of estrogen treatment in OCs and for ovarian failure result in suppression of endogenous androgen activity considering that the addition of testosterone to the HT regimen ameliorates the stimulating effects of estrogen/progesterin on the breast. Research addressing the role of androgens in breast cancer prevention and the efficacy of hormonal supplementation with physiological androgen to maintain estrogen/androgen ratios typical of normal women is warranted.

Competing interests
The authors declare that they have no competing interests.

References


Rebecca Glaser M.D., a renowned breast cancer surgeon: Testosterone pellets reduce significantly tumor volume in an active breast cancer patient. Patient achieved physiologic testosterone blood levels.
CASE REPORT

Rapid response of breast cancer to neoadjuvant intramammary testosterone-anastrozole therapy: neoadjuvant hormone therapy in breast cancer

Rebecca L. Glaser, MD,1,2 and Constantine Dimitrakakis, MD, PhD3,4

Abstract

Objective: Experimental and clinical data support the inhibitory effect of testosterone on breast tissue and breast cancer. However, testosterone is aromatized to estradiol, which exerts the opposite effect. The aim of this study was to determine the effect of testosterone, combined with the aromatase inhibitor anastrozole, on a hormone receptor positive, infiltrating ductal carcinoma in the neoadjuvant setting.

Methods: To determine clinical response, we obtained serial ultrasonic measurements and mammograms before and after therapy. Three combination implants—each containing 60 mg of testosterone and 4 mg of anastrozole—were placed anterior, superior, and inferior to a 2.4-cm tumor in the left breast. Three additional testosterone-anastrozole implants were again placed percutaneously 48 days later.

Results: By day 46, there was a sevenfold reduction in tumor volume, as measured on ultrasound. By week 13, we documented a 12-fold reduction in tumor volume, demonstrating a rapid logarithmic response to intramammary testosterone-anastrozole implant therapy, equating to a daily response rate of 2.78% and a tumor half-life of 23 days. Therapeutic systemic levels of testosterone were achieved without elevation of estradiol, further demonstrating the efficacy of anastrozole combined with testosterone.

Conclusions: This novel therapy, delivered in the neoadjuvant setting, has the potential to identify early responders and to evaluate the effectiveness of therapy in vivo. This may prove to be a new approach to both local and systemic therapies for breast cancer in subgroups of patients. In addition, it can be used to reduce tumor volume, allowing for less surgical intervention and better cosmetic oncoplastic results.

Key Words: Testosterone – Anastrozole – Breast cancer – Intramammary – Neoadjuvant

The biological effect of testosterone on the androgen receptor (AR) prevents the proliferation of breast tissue and inhibits the growth of breast cancer cells.1-4 Since the 1940s, testosterone and testosterone pellet implants have been used to treat advanced breast cancer as well as menopausal symptoms in breast cancer survivors.5-6 However, in breast cancer tumors, both malignant epithelial cells and surrounding fibroblasts overexpress aromatase, increasing local estrogen production, which subsequently stimulates cancer cell growth.7,8

We have previously demonstrated that two 6.4 × 3.1-mm subcutaneous implants—each containing 4 mg of anastrozole combined with 60 mg of testosterone—were able to provide therapeutic levels of testosterone without elevation of estradiol (E2) levels in breast cancer survivors.9 We have also documented both the safety and the efficacy of this combination therapy in breast cancer survivors and in women without breast cancer, including a reduction in the incidence of breast cancer.10,11

This combination testosterone-anastrozole (T + A) implant therapy, delivered locally (intramammary/percutaneously) in the neoadjuvant setting, has several theoretical advantages, including higher doses of both testosterone and the aromatase inhibitor anastrozole being delivered directly to the tumor. With increasing monetary constraints on funding of large trials, the neoadjuvant/presurgical setting will probably become increasingly useful in evaluating clinical response to novel therapies.12
METHODS

The patient is a 90-year-old G3P3 woman with a history of menarche at age 14, natural menopause at age 43, and a family history of breast cancer (grandmother). She was found to have a suspicious left breast lesion on CT scan of the chest, which was ordered after a fall.

Subsequent mammogram on December 7, 2012 revealed a 2.4-cm-deep obscured mass in the posterior central left breast, consistent with CT findings. Ultrasound (US) of the left breast revealed a 2.4 × 2.3 × 1.7–cm (ie, tumor volume\textsuperscript{a} of 5.12 cm\textsuperscript{3}) suspicious mass in the subareolar 3-o’clock position. US-guided core biopsy was performed, revealing grade 2 infiltrating ductal carcinoma (estrogen receptor [ER]–positive, progesterone receptor [PR]–positive, AR-positive, and HER2-negative).

An oncology consultation recommended 20 mg of tamoxifen daily. The patient refused surgical intervention but complied with tamoxifen therapy from December 2012 through March 2013.

She was seen and evaluated at the Millennium Clinic on March 1, 2013. The patient was alert and oriented and in good health, with no history of diabetes or cardiovascular disease. Medical history was significant for depression, mildly elevated blood pressure, postherpetic pain syndrome, and elevated lipid levels. There was a slight thickening in the left breast at the 3-o’clock position at the areolar border, extending under the nipple-areola complex. There was no palpable axillary adenopathy and no skin or nipple changes. Initial medication list was extensive and included duloxetine HCl, lisinopril, atorvastatin, omeprazole, temazepam, aspirin, pregabalin, tamoxifen, and supplements.

US performed in the office reconfirmed the presence of a 2.3-cm-deep left subareolar mass, unchanged from her previous US on December 7, 2012.

A written informed consent form was obtained, and the patient agreed to intramammary placement of T + A implants. In addition, the patient was informed that the combination T + A implant was prescribed by a physician for a purpose for which it has not been specifically approved (ie, “off-label” use).

Through a 5-mm lateral incision, three compounded 60 mg T + 4 mg A pellets were implanted into the breast tissue surrounding the tumor approximately 1 cm superior to, 1 cm inferior to, and anterior to the subareolar tumor through a disposable trocar (Fig. 1). Tamoxifen was discontinued.

RESULTS

Follow-up examination of the left breast 2 weeks after intramammary T + A pellet implantation revealed a marked decrease in tumor size on physical examination and office US. The periareolar “thickening” was no longer palpable. By week 4, the patient’s (previously unreported) left breast pain had subsided.

On April 16, 2013, 46 days after intramammary T + A therapy, follow-up left breast mammogram and US, performed at the original imaging center by the same radiologist, revealed a significant decrease in tumor mass size. On US, the tumor measured 1.6 × 1.1 × 0.8 cm, with a tumor volume of 0.74 cm\textsuperscript{3}, indicating a sevenfold reduction in tumor volume compared with 5.12 mL on December 7, 2012.

Three additional implants (ie, total dose of 180 mg T + 12 mg A) were again placed peritumorally in the left breast on April 18, 2013.

Follow-up mammogram (Fig. 2) and US (Fig. 3) on week 13, again performed at the same radiology facility, revealed that the size of the carcinoma had continued to decrease, measuring 1.5 × 0.8 × 0.6 cm on US, with a tumor volume of 0.42 cm\textsuperscript{3}. This 12-fold reduction in tumor volume from the original measurement equates to a 2.78% decrease per day (after therapy) and a half-life\textsuperscript{b} of 23 days. The logarithmic response of the carcinoma to T + A therapy is evidenced by an $R^2$ value greater than 0.99 (Fig. 4).

In addition, many of the patient’s symptoms, including memory loss, physical fatigue, urinary incontinence, sleep disturbance, depression, and pain, improved with testosterone therapy. Adequate serum levels of testosterone, without elevation of E\textsubscript{2}, were confirmed on day 7 postinsertion (testosterone, 473 ng/dL; E\textsubscript{2} <5 pg/mL), day 46 postinsertion (testosterone, 366 ng/dL; E\textsubscript{2} <5 pg/mL), and again on day 7 after the second intramammary insertion procedure (testosterone, 345 ng/dL; E\textsubscript{2} <5 pg/mL). Interestingly, the patient was able to discontinue several medications in addition to tamoxifen, including duloxetine HCl, lisinopril, and atorvastatin. There have been no adverse drug events with therapy. The patient “feels better than

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\textsuperscript{a}Ellipsoid formula: $4/3\pi (a/2 \times b/2 \times c/2)$.  
\textsuperscript{b}Logarithmic half-life equation: $x = \log(0.5) / \log(1 - 0.0278)$.  

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she has in years,” is no longer using a walker, and is driving her car again. She continues to refuse any surgical intervention.

Follow-up US and mammogram are scheduled for September 2013, 6 months from the initial intramammary T + A pellet insertion. If a residual mass is present, an US-guided core biopsy will be performed to reevaluate tumor characteristics and hormone receptor status. The patient will be continued on intramammary therapy (180 mg T + 12 mg A) for a minimum of 1 year after complete clinical response. She will then continue to be treated with T + A implant therapy, possibly at alternate insertion sites.

DISCUSSION

Although there was virtually no response to oral tamoxifen therapy between December 2012 and March 2013 (as evidenced by tumor measurements on office US), our patient manifested a rapid clinical response to intramammary T + A therapy by 46 days, continuing through week 13. This was particularly evident when we compared three-dimensional tumor volumes over time, calculated using the ellipsoid formula, which has been shown to be superior to using only the greatest (tumor) diameter.

This innovative pharmacologic combination (T + A), unique delivery method (sustained-release implant), and unique delivery location (intramammary and peritumoral) in the neoadjuvant setting has many potential advantages both for research and for the local and systemic therapy of breast cancer.

The combination of testosterone delivered simultaneously with anastrozole allows the beneficial effects of testosterone on the AR and simultaneously prevents aromatization to E2. Testosterone is beneficial for immune function and treats many systemic symptoms, adverse effects of cancer therapies, and the breast cancer itself.1-4,9,13,14 The sustained-release combination implant ensures continuous, simultaneous delivery...
and absorption of both active ingredients locally at the tumor site while maintaining consistent therapeutic systemic levels of testosterone without elevating E₂. We have previously published data on the safety and efficacy of these doses of testosterone and have shown that pharmacologic doses of testosterone—as evidenced by serum levels several fold higher than endogenous levels measured on week 4 (299.36 ± 107.34 ng/dL) and before reimplantation (171.43 ± 73.01 ng/dL)—produce a physiologic (therapeutic) effect and are not associated with adverse events. The only expected androgenic adverse effect in our patients has been a slight increase in facial hair. The safety of the 180-mg testosterone implant has also been confirmed in historic studies, where doses from 150 to 225 mg were routinely prescribed and doses up to 800 to 1,800 mg were safely used in the long term to treat metastatic breast cancer and female-to-male transgender patients. Contrarily, there is a lack of evidence to support that serum ranges from endogenous production should be

![FIG. 3. Left breast subareolar biopsy-proven malignancy. Baseline ultrasound performed on December 7, 2012 (top) compared with follow-up ultrasound performed on May 31, 2013 (bottom) demonstrates a significant reduction in tumor size 13 weeks after testosterone-anastrozole (T + A) implant therapy. Baseline tumor size was reconfirmed on office ultrasound before therapy on March 1, 2013 (image not shown).](image)

![FIG. 4. Logarithmic decline in tumor volume (cm³) measured by ultrasound (R² > 0.99).](image)
extrapolated to exogenous therapy or used to determine implant dosing in lieu of "clinical efficacy" and "adverse effects." In addition to testosterone, the androgen precursors dehydroepiandrosterone sulfate (DHEAS) and androstenedione, found in much greater concentrations (up to 10^{-5}-fold) than testosterone, also decline with age. We have found that higher serum levels of testosterone (supplied by the implant) are necessary to provide adequate amounts of testosterone to the AR, replacing not only testosterone but also the significant contributions of DHEAS and androstenedione to bioavailable testosterone.  

Unlike oral medication, patient compliance is not an issue with implanted pellets. Furthermore, the implant allows for lower, more efficient, and effective dosing of anastrozole. Subcutaneous delivery avoids the gastrointestinal tract and subsequent gastrointestinal adverse effects, including nausea, vomiting, and pain. There is no first-pass effect and no adverse effect on the liver or clotting factors.

The intramammary location is particularly unique in that it allows differential dosing. High doses of both testosterone and anastrozole are delivered locally, directly targeting the tumor, whereas lower therapeutic systemic levels are achieved. This is in direct contrast to neoadjuvant therapy by other delivery methods, including oral or intravenous, where higher systemic levels are necessary to deliver adequate dosing to the breast tissue/tumor site, resulting in greater systemic adverse effects and toxicities and less effective local therapy.

The neoadjuvant/presurgical setting also allows identification of responders (vs nonresponders) and early evaluation and documentation of the effectiveness of therapy. Moreover, in larger tumors, this therapy may allow for breast-conserving surgical operation, with improved oncologic cosmetic results.

This patient and several others who have responded to T + A therapy were AR-positive, consistent with testosterone effect on the AR.  

Interestingly, an 81-year-old patient with a clinical stage 2, ER-positive, PR-positive, AR-positive tumor, subsequently treated with T + A intramammary therapy, is also responding, although at a slower rate (1.87% per day, half-life of 37 d), with a logarithmic decline in tumor volume ($R^2 = 0.96$). In addition, her palpable adenopathy is also responding to therapy.

In the future, it would be interesting to monitor response to T + A therapy among "triple-negative" (ER-negative, PR-negative, and HER2-negative) patients, who may be AR-positive. Contraindications to this therapy have not been recognized; however, caution should be exercised in ER-negative, HER2-positive patients; one study has shown that AR positivity was "associated" with poorer prognosis. Although an AR-negative tumor may not respond as well to this therapy, patients may still benefit from testosterone's anabolic effects on bone density, as well as testosterone's immunostimulatory, anti-inflammatory, and pain-relieving properties. Unlike higher-dose oral aromatase inhibitors, which can secondarily increase gonadotropin-releasing hormone and subsequently stimulate the ovary to reproduce estrogens, low-dose parenteral anastrozole, in combination with testosterone, may also prove to be appropriate in postmenopausal hormone receptor-positive patients. However, until further studies are performed, older postmenopausal patients with hormone-positive tumors are considered to be the most appropriate candidates for this novel treatment.

The combination implant is also cost-effective, providing 2 to 3 months of therapy with a single, minimally invasive, 2-minute procedure at a cost of approximately US$2.00 to US$3.00 per day. In the future, this therapy may be used in conjunction with other endocrine therapies (such as tamoxifen) or sulfatase inhibitors, which prevent hydrolysis of estrone sulfate and DHEAS, both of which can be reduced to steroids with estrogenic properties in a low-estrogen environment.

**CONCLUSIONS**

We have shown that this combination T + A therapy was well tolerated by this 90-year-old patient and effectively treated her AR-positive breast cancer. Neoadjuvant endocrine therapy for a grade 2, hormone receptor-positive breast cancer using intramammary T + A therapy resulted in a rapid clinical response (ie, 12-fold reduction in tumor volume within 3 mo).

A neoadjuvant study option allows the observation and documentation of clinical response and, if followed by surgical excision, the biology of tumor response. In addition, this therapy may allow for fewer surgical operations and improved oncologic results. Further research is needed to delineate the role of sustained-release T + A in both the prevention and the treatment of breast cancer. In the future, this combination may have the potential for both systemic and local therapies for breast cancer in subgroups of patients, possibly eliminating surgical operation, radiation therapy, and adverse effects of oral medication.

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