

Differential effects of alternatives to hormone replacement therapy in postmenopausal healthy women

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This review has been accepted as a thesis with seven previously published papers by the University of Copenhagen, October 2004, and defended on April 15, 2005.

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Dan Med Bull 2005;52:64-81.

INTRODUCTION

The postmenopausal phase of a woman's life may be regarded as an endocrinological deficiency syndrome caused by ovarian failure. Postmenopausal hypogonadism increases the risk of osteoporosis (1) and climacteric symptoms (2) and may increase the risk of atherosclerosis (3). Logically, the intervention against such conditions has been estrogen replacement, which can be used unopposed in hysterectomized women or in combination with a progestogen in women with an intact uterus. Due to the universal influence of these therapies, a side effect regarded by the woman as more severe than the symptoms experienced will often lead to discontinuation before occurrence of a protective effect, since the treatment required is long-term. In addition, not all postmenopausal women experience symptoms or develop diseases related to estrogen deficiency, suggesting that additional mechanisms are involved. Therefore, these treatments should be individualized, but this is difficult with regard to hormone replacement therapy because of the need for giving both estrogen and progestogen in women with an intact uterus and because of the numerous target organs and tissues influenced.

A mechanistic understanding of tissue-specific responses to estrogen deficiency as well as to hormone replacement therapy would be the ideal basis for diagnosis and intervention in postmenopausal women. To establish a dose-response relationship on target organ risk markers of standard hormone replacement therapy combinations would be a helpful operational starting point. Because of the multiple effects of these therapies, it is unrealistic that they would be safe or tolerable for all women in need for treatment. Accordingly, additional treatment modalities have shown up. Bisphosphonates, calcitonin and calcium in combination with vitamin D (1) as well as parathyroid hormone (4) have been found to be efficacious in the treatment of osteoporosis, but these compounds do not seem to have substantial influence on other aspects of postmenopausal estrogen deficiency than osteoporosis.

Hormone replacement therapy alternatives or selective estrogen receptor modulators (SERMs) are interesting because of the possibility of modulating the dose-response relationship of hormone replacement therapy on different targets. Thus, one could imagine treatments that would have beneficial effects on bone, the cardiovascular system, the brain and tissues involved in the symptomatology of climacteric symptoms such as the vagina, but at the same time were inhibitory in the breast and endometrium. Moreover, treat-

ments could be envisioned to target either of these organs/tissues, the central biochemical basis for such theories being the estrogen receptor. This review summarizes the effects of tibolone and raloxifene in postmenopausal healthy women. These compounds are chosen, because they are at present the only known alternatives to hormone replacement therapy with an acceptable safety profile for long-term use in healthy, postmenopausal women. Tibolone reduces climacteric complaints similarly to hormone replacement therapy (5). In contrast, raloxifene does not have this potential, but increases the incidence of mild hot flushes (6). Whereas tibolone is a synthetic steroid with estrogenic, progestogenic and androgenic activities (5), raloxifene is a non-steroidal benzothiophene or a SERM (6). For a positive gold standard hormone replacement therapy is described. The primary target tissues chosen are bone and the cardiovascular system, because osteoporosis and atherosclerosis are the principal conditions for which a preventive therapy should be efficacious. For a safety description, the breast and the endometrium are evaluated. For tibolone and raloxifene, randomised, placebo-controlled trials with more than one dose level and with relevant endpoints are included. With regard to HRT, randomised, placebo-controlled trials involving both traditional and low-dose regimens comparing as well sequential and continuous regimens are included. For the in vivo studies, randomised, placebo-controlled studies in the rabbit model investigating tibolone, HRT and raloxifene were included.

BONE

THE IMPORTANCE OF ESTROGEN ON BONE PHYSIOLOGY

Estrogen deficiency

The major influence of postmenopausal estrogen deficiency on skeletal physiology is an increase in bone resorption. Two main pathways lead to this result, a direct effect on bone cells and an indirect effect via calcium metabolism. A strong evidence for the direct effect is the identification of estrogen receptors on osteoblasts and osteoclasts (7, 8) and both receptor isoforms are present in bone (9). A decrease in endogenous estrogen leads to an increase in osteoclast recruitment and activity, which is partially mediated by a decrease in osteoblast production of nitric oxide and transforming growth factor β (10) and by an increase in cytokine production by bone marrow stromal and mononuclear cells (11). Indirect effects comprise a negative change in calcium balance by a decrease in intestinal calcium absorption and an increase in urinary calcium loss (12). The increase in bone resorption leads to a secondary, more retarded increase in bone formation. The level of bone resorption markers after menopause is twice the level seen in premenopausal women, whereas the level of bone formation markers is about 50% higher after than before menopause (13). It is the relatively higher level of bone resorption as compared to bone formation, which causes the postmenopausal bone mass deficit and it is the sharp increase in bone resorption at menopause, which leads to the increased bone loss seen in the first 5-10 years after menopause (14).

Estrogen presence

The effects of estrogen on bone tissue have been found to be an antagonism of processes seen in estrogen deficiency, thus an inhibition of bone resorption (1, 15). This is in accordance with data showing that estrogen induces osteoclast apoptosis (16). Due to the coupling between bone resorption and bone formation, a secondary and more retarded decrease will be observed in bone formation until a new steady state in bone turnover is reached. Therefore, after initiation of therapy, a time interval of a relatively higher level of bone formation as compared to bone resorption is seen and an increase in bone mass is expected followed by a new steady state in bone mass, which will be stable for as long as treatment is continued, if the treatment regimen used is effective in reducing bone turnover to premenopausal levels (14).

OSTEOPOROSIS

Osteoporosis is defined as a metabolic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk (1).

The two most important risk factors for developing osteoporosis are the bone mass by the time of the menopause and the subsequent bone loss following the increase in bone resorption after menopause. To assess these variables as osteoporosis risk markers, indirect measures of bone mass can be performed by densitometry and indirect measures of bone turnover by biochemical estimates of bone resorption and formation (1).

Osteoporosis occurs worldwide, but is most prevalent in white and Asian women. A robust estimate of the prevalence in the United States based on the WHO criteria concludes that the total population at risk compares to that of hypertension (17) and the increasing age of the world population combined with the exponential age-related increase in fracture risk indicate that the size of the problem is growing. Although there is an increased mortality following both fracture at the hip and spine, the worst consequence is not mortality but the morbidity, loss of independence and the decrease in quality of life (1).

METHODS FOR THE EVALUATION OF OSTEOPOROSIS

Bone mass

Bone mass may be measured by densitometry in specific areas as well as in the total skeleton. In untreated individuals, bone mass is considered to be the optimum single predictor of bone strength (18), which is reflected by the selection of bone mass by WHO for the diagnosis of osteoporosis (19). Whereas the predictive value of bone mass at any site is roughly equivalent in the prediction of all fractures, a specific site has a slightly higher predictive value for a fracture at that specific site (20). For this reason a bone mass measurement of the hip is preferred for diagnosis, because the most debilitating osteoporotic fracture occurs at the hip. However, an optimised scientific evaluation of response to therapy includes both an axial assessment (the spine) as well as appendicular measurements (for example the hip or the forearm), all being areas of particular interest because of their susceptibility to osteoporotic fractures. Another reason for the choice of these sites, lies in the different ratios of trabecular and cortical bone in the 3 areas. Trabecular bone constitutes about 20% of the skeleton but accounts for about 80% of the bone turnover, whereas the inverse holds true for cortical bone (21). Therefore, the response to antiresorptive therapies may differ between these 2 types of bone. The content of trabecular bone is highest in the spine, intermediate in the hip and lowest in the forearm. Thus, follow-up of these areas during therapy enables assessments of the response in bone with predominantly trabecular content and also in bone with predominantly cortical content. Finally, therapies, which lead to only small increments in bone mass, may just lead to skeletal redistribution of bone mass. Under such circumstances, inclusion of a measurement of total body bone mass is preferred. The measurements of bone mineral utilize a gamma or X-ray source that scans the area of interest and calculates bone mass from the attenuation of the ionizing beam. They are standard accepted assessments (22) and precision is followed by daily phantomscans with a weekly system calibration. For the multicenter trial, the same phantoms were used in all centers and cross-calibration were performed by a quality assurance center, whose employees remained blinded throughout the study.

The precision error of a bone mass measurement is in the order of magnitude of the average change in bone mass over one year, both in untreated and treated postmenopausal women ($CV\% \approx 1-3\%$) (22). Therefore, the observation period in studies should be at least 1 year and preferably longer. Because the least significant change in the individual is in the order of 4% (23), a monitoring interval of 2 years is recommended. Thus, an observation period of at least 2

years in a clinical trial may be estimated to provide the best foundation for a clinical evaluation.

The primary basis for including bone mass measurements in clinical trials lies in the ability of this surrogate marker to predict a change in fracture risk. Such a relationship is well-established for antiresorptive therapies (24).

Bone turnover

Although bone presents as a rigid tissue, bone is continuously remodelled for the maintenance of calcium homeostasis and for shape and repair. Thus, bone remodelling is the predominant metabolic process regulating bone structure and function with the osteoclast playing a key role. Osteoclasts are specialized cells derived from the monocyte/macrophage haematopoietic lineage that develop and adhere to bone matrix, then secrete acid and lytic enzymes that degrade it in a specialized, extracellular, sealed compartment between the basolateral domain of the osteoclast and bone matrix. By endocytosis, the degradation products (solubilized calcium, phosphate and collagen fragments) are then incorporated and processed within the osteoclast. Finally, the products are released into the general circulation and filtered and excreted by the kidneys. The osteoclast may then migrate to initiate a new cycle of bone resorption (25). Most biochemical bone resorption markers measure collagen degradation products in serum and urine (such as c- or n-telopeptide fragments of collagen 1 degradation or more old-fashioned methods such as urinary hydroxyproline) (14). Bone resorption markers exhibit a diurnal variation with a peak in the early morning, a nadir in the afternoon followed by an increase over the night (26). This variation reflects that bone resorption is regulated by food intake – thus bone resorption increases during prolonged fasting and decreases acutely after food intake (27). This is most likely controlled by a gastrointestinal hormonal regulation and ensures availability of calcium when calcium supplies are low such as during fasting (28). The clinical implication of this fact is that the sampling during intervention must be standardized in order to reduce variation unrelated to the influence of therapy. We proposed – in parallel with sampling conditions for lipids – to collect samples for bone resorption markers as a fasting morning specimen (27) and this procedure was followed in all studies. Under these optimised conditions, the inter-individual variation is less than 10% (29). The results of the urinary samples were corrected for creatinine excretion.

The osteoblast is the bone cell responsible for the production of matrix constituents. It originates from a local mesenchymal stem cell that has differentiated and occurs in clusters at a resorption pit, where an osteoclast previously degraded bone. The osteoblasts are present lining the bone matrix they are producing, until mineralization occurs. At finalization of bone matrix production, the osteoblast turns into a lining cell or an osteocyte, which is then followed by mineralization (25). Biochemical formation markers assess proteins or enzymes synthesized by osteoblasts (such as osteocalcin or bone specific alkaline phosphatase) during bone matrix formation and made available for serum assessment as a result of spillover to the general circulation from the production in bone (14). There is only a very slight diurnal variation on bone formation markers (27). Also for formation markers, the inter-individual variation is around 10% or less (29). Osteocalcin may be evaluated as the intact molecule or as the N-terminal mid-segment, which may be more stable to freeze-thaw cycles (30).

The sampling schedule for all bone markers should reflect the fact, that the response in bone markers to anti-resorptive therapy is faster than that of bone mass. Thus, the first 6 months after initiation of therapy were closely monitored in all studies.

There are several reasons why it is important to assess bone turnover in a clinical study. The use of the markers enables the scientist to separate the influence on bone resorption and formation as well as time points for monitoring may be identified (29). But the most promising reason lies in the ability of some markers to predict the

change in fracture risk independently of the response in bone mass (31).

HORMONE REPLACEMENT THERAPY

From the numerous studies on the influence of combinations of estrogen and progestogen on bone mass and bone markers it is evident that traditional regimens lower bone turnover and increase bone mass (1). The effect is independent of type of estrogen, thus conjugated equine estrogens, estradiol, ethinyl estradiol and piperazine estrone sulphate are all efficacious (32-35). With regard to dose finding of the estrogen component, almost no randomised, controlled studies find regimens to be non-efficacious in terms of protection against bone loss. There is, however, some indication of therapies with a borderline efficacy, thus 0.5 mg estradiol and 0.3 mg conjugated equine estrogens do not prevent all treated women against bone loss (36, 37). Therefore, we selected a daily dose of 1 mg estradiol as a low, but efficacious therapy and the traditional dose level of 2 mg estradiol as a positive comparator for our study. Gestodene, a 19-nortestosterone progestogen, was added in 2 regimens on each estrogen dose level. This enabled us to evaluate as well whether gestodene influenced bone and whether a continuous regimen differed from a sequential (Table 1, study I). In addition, our study included assessments already 2 weeks after randomisation and was extended to 3 years of duration. This is important because it is rarely seen that the response to hormone replacement therapy is assessed before 3 months or after 2 years.

We found a universal bone response to hormone replacement therapy in our study of 278 healthy, early postmenopausal women (33). Thus, the bone loss seen in the placebo group was significantly inhibited at all sites measured, but it varied by site with the greatest increase in the spine, smaller in the hip and again smaller in the forearm (33). When the groups with 1 mg estradiol were pooled and the groups with 2 mg estradiol were pooled, a significant difference was seen between these 2 estrogen dose levels. A significant response to 1 or 2 mg estradiol in bone resorption markers was seen within 2 weeks (33) (Figure 1A). This response was fully expressed from 6 months and as long as treatment was continued with a 50% decrease on 1 mg estradiol and a 65% decrease on 2 mg estradiol (Figure 1A). Bone formation markers demonstrated a 30-35% decrease on therapy (Figure 1B). During the study, 2 mg estradiol increased spinal bone mineral density with 7% and 1 mg estradiol with 5%, whereas a loss of 2% was seen in the placebo group (Figure 1C). We did not identify a response in bone to the gestodene dose levels studied. This is in accordance with previous results on medroxyprogesterone acetate (32) but in contrast to studies using norethisterone acetate,

which enhances the effect of estrogen on bone mass (38). Whether the occurrence of an independent progestogenic effects on bone is exclusive for norethisterone acetate, remains to be investigated.

No study has evaluated the influence of hormone replacement therapy with clinical fracture as the primary endpoint. However, 2 small randomised studies have found a protective effect on vertebral fractures with about a 50% risk reduction – one of transdermally administered 100 µg/day of estradiol (39) and another of oral mestranol 23 × 3 µg/day (40). Moreover, a randomised 5-year study of a sequential treatment with estradiol and norethisterone acetate versus placebo in 2,016 early postmenopausal Danish women demonstrated a significant 55% reduction in the risk of forearm fracture, but only a borderline significant reduction in overall fracture risk (41). In contrast, a study in 2,763 postmenopausal women randomised to either a continuous combination of 0.625 mg conjugated equine estrogens and 2.5 mg medroxyprogesterone acetate or placebo did not reveal differences in fracture risk between treatment groups after 4.1 years of follow up (42). The population in this study was however selected to be at risk of atherosclerotic but not osteoporotic events. In addition, the study did not apply a rigorous method such as spine x-ray for fracture evaluation, but fracture endpoints were collected as adverse events data only. After 5.2 years follow-up in a study of 16,608 healthy postmenopausal women randomised to a continuous combination of 0.625 mg conjugated equine estrogens and 2.5 mg medroxyprogesterone acetate or placebo, active therapy lead to a significant 34% reduction in the risk of hip fracture and of clinical vertebral fractures (43).

In conclusion, during regimens of estradiol and gestodene a dose-response relationship is observed with a slightly greater response on 2 mg estradiol as compared to 1 mg estradiol, which is effective to reduce bone turnover markers and to increase bone mass. Lower doses may not demonstrate an acceptable efficacy in all women. The addition of gestodene does not significantly influence bone parameters. Combining results from several randomised studies indicate that hormone replacement therapy reduces the risk of vertebral fracture (39, 40, 43), forearm fracture (41) and hip fracture (43).

TIBOLONE

Prior to our study, only 2.5 mg tibolone per day had been evaluated for its effect on bone and fracture data were not available. We therefore decided to explore a lower dose in a target population for fracture prevention. Thus, 91 healthy women at least 10 years after menopause were randomised to double-blind daily intervention with either tibolone 2.5 mg, 1.25 mg or placebo in addition to 400 mg calcium (Table 1, study II) (44).

Table 1. Participants in human studies.

Study	Screened	Included	Randomisation (dose of medication and calcium is per day)	Duration (years)	Completers	Age (years)	BMD-spine (g/cm ²)	BMI (kg/cm ²)
I	471	278	1) 2 mg estradiol sequentially combined with 25 µg gestodene (days 17-28) (n = 55)	3 (1993-1997)	24	54±3	0.98±0.11	25±4
			2) 2 mg estradiol sequentially combined with 50 µg gestodene (days 17-28) (n = 56)		26	53±3	0.99±0.16	24±3
			3) 1 mg estradiol sequentially combined with 25 µg gestodene (days 17-28) (n = 56)		30	53±3	0.95±0.13	26±3
			4) 1 mg estradiol continuously combined with 25 µg gestodene (n = 55)		32	54±3	0.96±0.13	26±4
			5) Placebo (n = 56)		41	54±3	0.95±0.12	26±4
II	144	91	1) Tibolone 2.5 mg + calcium 400 mg (n = 35)	2 (1992-1994)	28	66±8	0.85±0.16	23±3
			2) Tibolone 1.25 mg + calcium 400 mg (n = 36)		29	66±7	0.86±0.15	25±4
			3) Placebo + calcium 400 mg (n = 20)		13	68±6	0.90±0.12	25±3
III	823 ¹	601 ²	1) Raloxifene 150 mg + calcium 500 mg (n = 147)	2 (1994-1996)	100	55±3	0.94±0.10	26±4
			2) Raloxifene 60 mg + calcium 500 mg (n = 152)		119	55±3	0.94±0.12	26±4
			3) Raloxifene 30 mg + calcium 500 mg (n = 152)		114	55±3	0.93±0.11	26±4
			4) Placebo + calcium 500 mg (n = 150)		119	55±4	0.94±0.11	25±4

1) 378 women were screened in Center for Clinical and Basic Research, Ballerup, Denmark.

2) 248 women were included in Center for Clinical and Basic Research, Ballerup, Denmark.

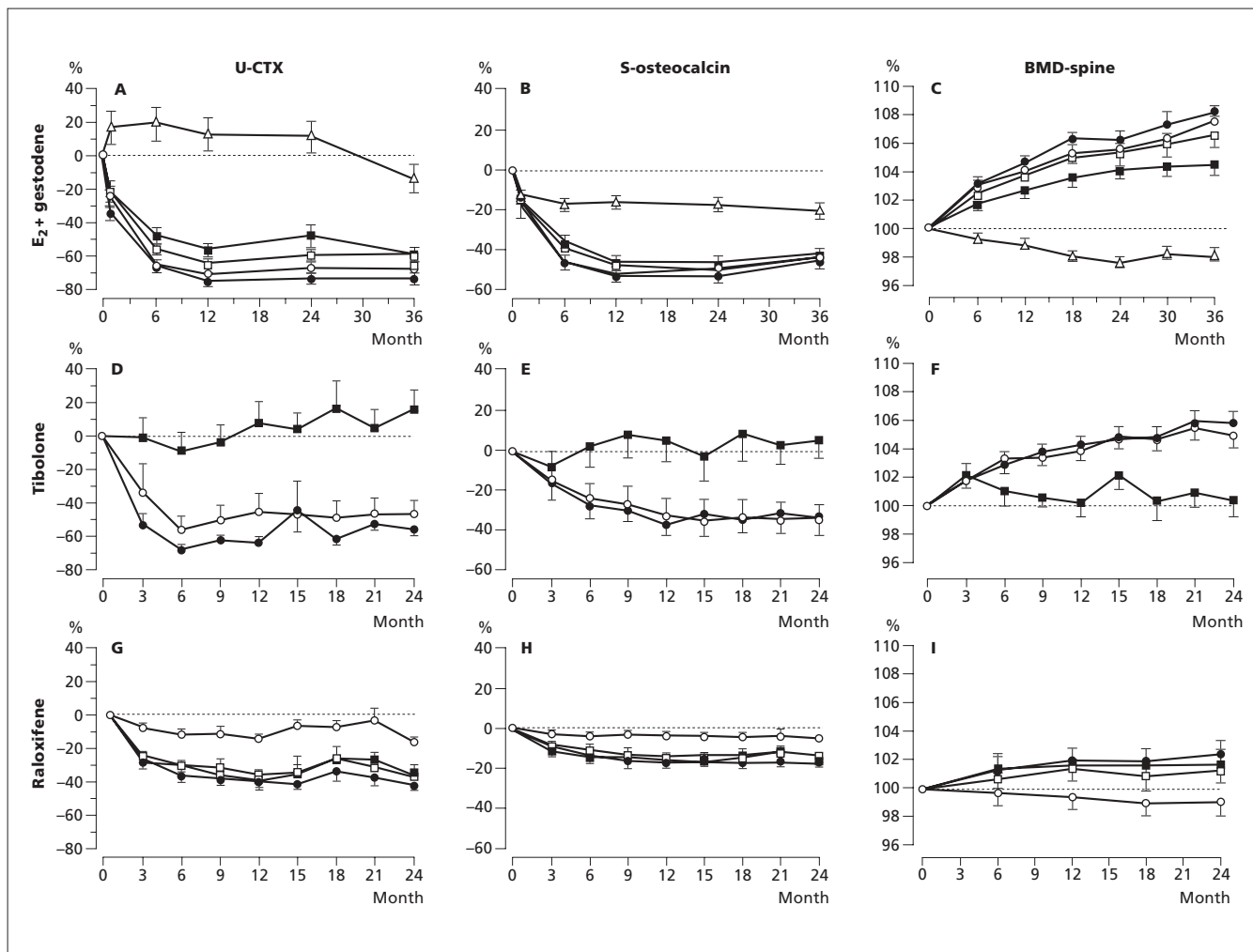


Figure 1. The response in bone turnover and bone mass during treatment. As a resorption and formation marker, urinary c-telopeptide fragments corrected for creatinine excretion and serum osteocalcin are shown. Bone mass is visualized by lumbar spine BMD. Please note that these results origin from 3 different studies [33, 44, 49]. **A-C:** show the response to hormone replacement therapy (adapted from *Bjarnason et al*, 2000 [33] with permission). 2 mg estradiol in sequential combination with 25 mg gestodene (days 17-28): -○-; 2 mg estradiol in sequential combination with 50 mg gestodene (days 17-28): -●-; 1 mg estradiol in sequential combination with 25 mg gestodene (days 17-28): -□-; 1 mg estradiol continuously combined with 25 mg gestodene (days 1-28): -■-; Placebo: -△-. **D-F:** show the response to tibolone treatment (adapted from *Bjarnason et al*, 1996 [44] with permission). 2.5 mg tibolone in combination with 400 mg calcium: -○-; 1.25 mg tibolone in combination with 400 mg calcium: -●-; Placebo in combination with 400 mg calcium: -■-. **G-I:** show the response to raloxifene therapy (adapted from *Delmas et al*, 1997 [49] with permission). 150 mg raloxifene in combination with 400-600 mg calcium: -●-; 60 mg raloxifene in combination with 400-600 mg calcium: -■-; 30 mg raloxifene in combination with 400-600 mg calcium: -□-; Placebo in combination with 400-600 mg calcium: -○-.

During active therapy a reduction in bone turnover was found, with a pattern similar to that seen during hormone replacement therapy – a fast decrease in resorption markers, which was fully expressed at 6 months (Figure 1D) and a slower change in formation, which was not fully expressed until about 12 months (Figure 1E). No difference between 1.25 mg and 2.5 mg tibolone was detected in any parameter. The decreases in c-telopeptide fragments and serum osteocalcin in tibolone treated women were 50% ($p < 0.001$) and 30% ($p < 0.001$), respectively. The response over 2 years in spinal bone mass was about 5.5% ($p < 0.001$) (Figure 1F) and in the forearm, the increase was about 2% with a difference of 4% relative to placebo ($p < 0.001$). These results are in accordance with those seen in early postmenopausal women (45, 46).

Data from two randomised, double-blind, placebo-controlled 2-year studies of tibolone 0.3, 0.625, 1.25 and 2.5 mg in a total of 770 early postmenopausal American women revealed that 0.3 mg counteracted but did not arrest bone loss, 0.625 mg arrested bone loss and slightly increased bone mass while bone mass was increased using 1.25 and 2.5 mg (47). The increases on the top doses were comparable with increases seen during hormone replacement therapy. Because the influence of tibolone 1.25 mg and 2.5 mg was compar-

able in early and late postmenopausal women, it may be estimated that this would also be the case for 0.625 mg although this dose has not been studied in elderly women.

In conclusion, we estimated that 1.25 mg tibolone is a low but effective dose on bone mass and bone turnover in healthy postmenopausal women. As a consequence, this regimen is presently being evaluated in a vertebral fracture outcome study. Based on the reduction in bone turnover and the increase in bone mass seen during this therapy, the reduction in fracture risk may be estimated to be comparable with the reduction seen during hormone replacement therapy.

RALOXIFENE

Results from 8-week experiments in healthy, early postmenopausal women had revealed significant decreases in biochemical markers of bone metabolism during raloxifene therapy up to 600 mg daily (48). Based on these data, we designed a study of long-term raloxifene intervention (Table 1, study III). Here, 601 healthy women 2-8 years after menopause were randomised to double-blinded treatment with raloxifene 30 mg, 60 mg, 150 mg or placebo in addition to a supplementation of 500 mg calcium (49).

The sequence of changes in bone parameters during raloxifene therapy was parallel to those seen during hormone replacement therapy and tibolone treatment (Figure 1G-I). However, the size of the changes was smaller and for the dose levels studied, there was no significant dose-response relationship, except for a trend in the total body bone mass, which was measured in our center only. Furthermore, the response in bone mass in all sites measured was in the same order of magnitude. For 60 mg raloxifene the reduction in urinary c-telopeptide fragments and osteocalcin was about 35% and 25%, respectively (Figure 1G-H). The increase in bone mass over 2 years was in the order of 1.5% regardless of measurement site compared with a loss of about 1% in the placebo group ($p < 0.001$ for all comparisons). 3- and 5-year follow up revealed comparable results (50, 51), as did a study in women with osteopenia or osteoporosis (52).

In 7,705 elderly, osteoporotic women randomised to 3 years of daily double-blinded intervention with either raloxifene 60 mg, 120 mg or placebo in addition to 500 mg calcium and 400 units of vitamin D, similar results were found with regard to bone turnover and bone mass. These results translated into a 30% reduction in radiographical vertebral fractures during therapy with 60 mg raloxifene and 50% for 120 mg raloxifene (53). Whereas the difference between the efficacy of 60 and 120 mg raloxifene upon vertebral fracture incidence in the subset of women with prevalent vertebral fracture was statistically significant, no differences were found between the 2 dose levels on bone mass or bone markers. Although a trend was found for protection against non-vertebral, clinical fractures, this was not significant, in particular there was no reduction in the risk of hip fracture during raloxifene therapy (53). These results were confirmed in 4-year follow up from the same study (54).

In conclusion, the dose-response relationship of raloxifene on bone is broad – in general there is no difference in the bone marker response in the dose range from 30-600 mg, in the bone mass response from 30-150 mg and in the vertebral fracture response from 60-120 mg in osteoporotic women with no prevalent vertebral fractures. In contrast, 120 mg was significantly more protective than 60 mg in women who had prevalent vertebral fractures in addition to a low bone mass. On vertebral fracture protection, the lowest effective dose may be 60 mg, but 120 mg is more effective in severely diseased women. Raloxifene does not reduce non-vertebral fractures.

CARDIOVASCULAR SYSTEM

THE IMPORTANCE OF ESTROGEN ON CARDIOVASCULAR PHYSIOLOGY

Estrogen deficiency

Men has a 2.5 to 4.5-fold increased risk for cardiovascular death as compared to women in both areas with high and low incidence of cardiovascular disease. In general, women are diagnosed with cardiovascular disease 10 years later than men, but the gender difference in risk diminishes with age. One theory has been that women lose protection due to estrogen deficiency after occurrence of menopause. But only observational studies can evaluate this hypothesis, and these studies are in general not capable of separating effects of menopause from those of age alone. It has also been suggested that the similarities in the risk of cardiovascular disease in older men and women rather reflect that the male risk declines during middle age (55). Thus, the influence of estrogen deficiency on the cardiovascular system is controversial, as the available data is observational and may be subject to unknown confounders and various epidemiological approaches (56).

A negative influence of estrogen deficiency on biochemical risk markers for cardiovascular disease has been found. An increase in LDL-cholesterol and triglycerides is observed, whereas there is a decrease in HDL-cholesterol in postmenopausal women compared to premenopausal women (57, 58). However, other data have suggested that the adverse influence on cardiovascular risk markers is rather associated with age than with menopause (55). Finally, cross-sectional studies have failed to demonstrate an association between

endogenous estradiol levels and risk factors for cardiovascular disease in women, although this does not exclude a threshold effect (55). The menopausal transition may be accompanied by a change in markers of fibrinolysis towards decreased fibrinolysis and increased thrombosis (59, 60). In one study, fibrinogen was identified as a predictor of heart disease in women (61). Thus, in postmenopausal women compared to premenopausal women increases in fibrinogen and antithrombin III are observed together with higher levels of plasminogen activator inhibitor 1 and tissue plasminogen activator inhibitor activity (59). Most of the studies are cross-sectional, but also longitudinal data exists and statistical methods have been applied to correct for the increment in age between pre- and postmenopausal women, thereby indicating that menopause may have an individual influence on these risk markers.

In randomised experiments, ovariectomized monkeys have more atherosclerotic arteries than sham-operated animals of similar age (62). The difference between ovariectomized and sham-operated rabbits in similar experiments is less pronounced (63), a possible explanation being the fact that in contrast to monkeys, female rabbits do not exhibit a regular menstrual cycle but ovulate during mating.

Estrogen presence

Estrogen influences cardiovascular physiology through both systemic and direct pathways. One example of systemic influence is the influence on serum lipoproteins (58, 64). Estrogen-receptor mediated effects on hepatic expression of apoprotein genes lead to changes in serum lipids and lipoproteins. Oral estrogen reduces the activity of hepatic lipase, leading to higher levels of high density lipoprotein (HDL) cholesterol. It increases low density lipoprotein (LDL) receptors in the liver, which is followed by a decrease in the serum concentration of LDL-cholesterol and it increases the production of triglyceride-rich very low density lipoprotein (VLDL) cholesterol, thereby increasing serum triglycerides. This response may be influenced by progestogen addition, depending on type and dose. These changes in lipids and lipoproteins have been demonstrated in numerous studies (58). Until recently, possible beneficial effects of estrogens have been attributed principally to the hormone's effect on serum lipid concentrations. However, data suggest that direct actions of estrogen on blood vessels may contribute. Both isoforms of the estrogen receptor are present in vessels, both in endothelial cells and in smooth-muscle cells as well as in myocardial cells (64). But the direct influence of estrogen on vascular cells and tissues are not only caused by estrogen-receptor mediated gene transcription. Several experiments have found very acute vasodilatory responses to estrogen indicating, that a genomic pathway is highly unlikely to be the only mechanism accounting for the effect. Two mechanisms for this non-genomic phenomenon have been identified; an influence of estrogen on calcium-activated potassium channels leading to smooth muscle relaxation (65) and release of nitric oxide from endothelial cells leading to smooth muscle relaxation and inhibition of platelet activation (66). Genomic effects include an increase in the expression of genes for vasodilatory enzymes such as nitric oxide synthase and prostacyclin and induction of re-endothelialization through expression of growth factors during vascular injury. Preliminary data indicate that estrogen is protective against vascular injury in both estrogen receptor α knockout and in estrogen receptor β knockout mice (64).

Hormone replacement therapy has been associated with both inhibition of coagulation (32, 67) and increase in fibrinolysis (60). Additionally, estrogen influences systemically the hepatic modulation of haemostatic factors and the renin-angiotensin system with a net effect towards vasodilatation (64).

ATHEROSCLEROSIS

Atherosclerosis is the underlying pathology of cardiovascular disease. The first step in the development of atherosclerosis is endothelial dysfunction and denudation. This covers increased endothe-

lial permeability to lipoproteins, which is mediated by nitric oxide, prostacyclin, platelet-derived growth factor, angiotensin II and endothelin. In addition, leukocyte adhesion molecules and endothelial adhesion molecules are up-regulated and migration of leukocytes into the arterial wall starts. Possible causes of endothelial dysfunction include increased LDL-cholesterol; free radicals caused by smoking, hypertension and diabetes; elevated homocysteine concentrations and genetic alterations (68). When macrophages in the arterial wall internalise and oxidise LDL-particles, these cells are transformed to foam-cells and a fatty-streak lesion occur. This process, which is initiated already in childhood, represents a major cause of injury to the endothelium and the underlying smooth muscle. Interestingly, this process may be regarded as an inflammatory response (68). T-cells are activated in the fatty-streaks and smooth muscle cells can migrate and proliferate within the lesion, which is then transformed into an intermediate lesion (68). As the lesion grows the arterial wall may compensate transiently by gradual dilation ensuring an unaltered lumen. The final event in the change to the advanced, complicated lesion is the formation of a fibrous cap that separates the plaque from the lumen. Within the lesion, a necrotic core may develop. Rupture or ulceration of the fibrous cap can lead to plaque instability resulting in thrombosis and occlusion of the artery, which may result in a clinical significant event such as myocardial infarction or stroke (69).

METHODS FOR THE EVALUATION OF ATHEROSCLEROSIS

Lipids

Triglyceride and cholesterol levels in the blood are well-known risk factors for atherosclerosis (70). These lipids are transported in plasma as lipoproteins, a lipid core surrounded by a surface layer of apoproteins. As the lipoproteins during the circulation deliver lipids but retain protein, their density increases. Lipoproteins are classified based on their densities as demonstrated by ultracentrifugal separation. In order of increasing density these particles include: chylomicrons and VLDL-cholesterol (density ≤ 1.006 g/ml), which carries triglyceride, Intermediate Density Lipoprotein (IDL)-cholesterol (1.007 g/ml \leq density ≤ 1.019 g/ml), which is an intermediate stage of minor human occurrence in the break-down of VLDL-cholesterol, LDL-cholesterol (1.02 g/ml \leq density ≤ 1.063 g/ml), being the principal carrier of to peripheral tissues and HDL-cholesterol (density > 1.063 g/ml), which is responsible for reverse cholesterol transportation, i.e. from peripheral tissues to the liver for excretion. The most abundant apolipoproteins include apolipoprotein A1, which is the major apolipoprotein of HDL-cholesterol and apolipoprotein B, which is the major apolipoprotein of LDL-cholesterol. During excess of LDL-cholesterol, the particle may be taken up by monocyte-derived macrophages in the arterial wall. When these macrophages become cholesterol-overloaded, they are converted to foam cells, a classic component of atheromatous plaques. By contrast, HDL-cholesterol acquires cholesterol from peripheral cells and from other lipoproteins, and this cholesterol is transferred to the liver for excretion. This is in accordance with observational evidence for a high LDL-cholesterol or a low HDL-cholesterol level being a risk factor for atherosclerosis, whereas a low LDL-cholesterol and a high HDL-cholesterol level is known to be protective. Large clinical studies have indicated that a decrease in LDL-cholesterol during statin therapy is associated with a reduction in cardiac events and/or mortality (71), however it is at present unknown whether pharmacologically induced changes by other therapies or in other lipoproteins will result in a change in cardiovascular risk.

A serum lipid assessment (total cholesterol, HDL-cholesterol and triglycerides) is a standard analysis assessed enzymatically using autoanalysis. LDL-cholesterol is then calculated according to Friedewalds formula:

$$\text{LDL-cholesterol} = \text{total cholesterol} - (\text{HDL-cholesterol} + 0.45 \times \text{triglycerides})$$

that leads to a robust estimate for triglyceride levels below 4.5 mmol/l (72). This assumption is valid in healthy humans, whereas higher values may present in vivo models. Hence, ultracentrifugation may be preferred for the analyses of serum lipids in animal studies, where serum lipid levels is usually much higher, and this methods was chosen for the rabbit studies. Briefly, serum samples were adjusted to the abovementioned densities by solution. Upon finalized centrifugation, tube slicing separated the fractions, allowing measurement of cholesterol content in the individual fractions. Although ultracentrifugation is regarded as the reference method, it still depends on the enzymatical analysis of cholesterol.

The importance of assessing lipids and lipoproteins in clinical trials lies in the ability to evaluate the influence of the estrogen and progesterone and to compare with available regimens. In addition, lipids are part of the biochemical safety evaluation.

Haemostasis

An occlusive arterial event requires atheromatosis and thrombus formation. Whereas circulating lipid levels are related to atheroma formation, haemostasis factors may be evaluated for the assessment of thrombus formation risk.

Coagulation

By its conversion to fibrin, fibrinogen is the ultimate natural substrate of the coagulation system. It is synthesized in the liver, belongs to the acute phase proteins and has been found to be a risk marker of cardiac events (73). The plasma concentration of fibrinogen is associated with other risk factors for cardiovascular disease such as age, body mass index and smoking. Additionally, plasma fibrinogen is elevated in postmenopausal women. Fibrinogen was measured by a functional photometrical assay just after sampling (74). Antithrombin III is responsible for the conversion of thrombin to fibrinogen. Antithrombin III is measured spectrophotometrically (75).

Fibrinolysis

Free tissue plasminogen activator is involved in the primary activation of plasminogen to plasmin, which degrades fibrin. Tissue plasminogen activator is released from vascular endothelium, circulates in an inactive complex with plasminogen activator inhibitor 1, but is active at the vessel wall (evaluated as plasminogen activator inhibitor activity). This is supported by the clinical observation that a high level of tissue plasminogen activator and a low level of plasminogen activator inhibitor activity have been associated with an increased risk of myocardial infarction (73, 76). All assessments of fibrinolysis markers requires sampling with minimal stasis after 10 minutes of supine rest and must be spinned cooled within 30 minutes of sampling. Tissue plasminogen activator, plasminogen activator inhibitor 1 and plasminogen activator inhibitor activity are assessed by ELISA (77-79).

The idea in measuring haemostasis markers has to do with the wish to evaluate thrombosis risk. We chose coagulation markers characterising the last steps of coagulation. Particularly fibrinogen is known to be an independent predictor of risk and this marker is influenced by hormone replacement therapy. With regard to markers of fibrinolysis, we chose markers, that have been found to be independent predictors of risk and which are directly involved in the transformation of plasminogen to plasmin, which represents a central step in fibrinolysis.

The cholesterol-fed rabbit – an in vivo model of atherosclerosis

In vivo models are relevant because of their potential to study mechanistic factors involved in atherosclerosis and to serve as a fast screening tool in the identification of interventions, which need clinical trial investigations. The possibility to use rigorous control and the ability to bridge surrogate markers to relevant endpoints are important advantages of in vivo experiments. The principal disadvantage of in vivo experiment is the uncertainty with regard to

extrapolation of effect to humans. Recognized models include cholesterol-fed experimental animals, such as cynomolgus monkey, baboon and rabbit. Unlike monkeys, who experience a regular menstrual cycle, rabbits ovulate during mating. However, with regard to the effect of sex steroids, there has generally been agreement between the models, which are known to be sensitive to sex steroid intervention (80-83). In these models, estrogen deficiency is mimicked by ovariectomy. The experiment is then based on the development of atherosclerotic plaques during cholesterol feeding (80). Because soy protein in the animal chaw may influence atherosclerosis (84) and thereby have the potential to introduce a bias in the response to treatment, it is important to include relevant control groups. We included both placebo (a negative control) and estradiol (a positive control) in all experiments. Additionally, a sham operated control group (an intact estrogen production control) were included in our first experiment. Rabbits metabolise both estrogen and raloxifene to a greater extent than women do. Therefore, the dose-levels of the therapies had to be chosen in order to ensure comparability of the drug concentrations in the blood samples. Table 2 outlines the rabbit experiments. The primary endpoint parameter was the cholesterol content in the portion of aorta between the heart and the first intercostals arteries. After isolation of intima with part of the media, the tissue was minced and the lipid phase extracted. The lipid content could then be determined enzymatically (85) and area and protein content were also determined. The cholesterol content in aorta may be corrected for size by adjusting for the amount of protein or for the surface area. These methods are equally justifiable and are generally comparable. The serum lipids were determined after ultracentrifugation as described above. For the evaluation of influence on other estrogen-sensitive tissues, the uterus was removed, its weight determined and the endometrium sampled. After homogenisation and centrifugation, the cytosolic and nuclear estrogen and progesterone receptors were assessed using steroid binding assays and the values normalized for protein content (86).

The inclusion of the rabbit model served as a fast method to further evaluate the influence on cardiovascular risk factors of alternatives to hormone replacement therapy. The results of the studies formed part of the basis for the design of the clinical trial, which evaluates the response to raloxifene during raloxifene therapy in women with established atherosclerosis and with risk factors for atherosclerosis.

HORMONE REPLACEMENT THERAPY

Postmenopausal women

In our study, LDL-cholesterol was lowered 11% during therapy with 1 mg estradiol and 15.5% during therapy with 2 mg estradiol

(33). The addition of gestodene did not influence LDL-cholesterol (Figure 2A). The effects on LDL-cholesterol were mirrored in the response in apolipoprotein B. This was in contrast to the responses in HDL-cholesterol and triglycerides, which were a function of both estradiol and gestodene therapy. On these markers, the estradiol-induced increases were increasingly counteracted with increasing gestodene doses (Figure 2B-C), where the responses in apolipoprotein A1 followed HDL-cholesterol (33). On the sequential regimens with 2 mg estradiol, we found a cyclical variation in HDL-cholesterol with a significantly higher level mid-cycle than end-cycle. The variation on the regimens with 1 mg estradiol could not be distinguished from random variation.

Our results are in accordance with studies on different types and doses of oral estrogens and progestogens on lipids in postmenopausal women. Thus, LDL-cholesterol is lowered by all oral estrogen therapies (58) and this lowering seems to be independent of addition of progestogens such as medroxyprogesterone acetate (32) and norethisterone acetate (34, 35). The evaluation of HDL-cholesterol and triglycerides is more complex, because these markers are influenced by both estrogen and progestogen and conclusions about the relative influence requires dose finding of both hormones as well as comparisons of sequential and continuous regimens. However, data on the combination of conjugated equine estrogens and medroxyprogesterone acetate are in accordance with our findings (32, 58). An important consequence of the opposite actions of estrogens and progestogens upon HDL-cholesterol and triglycerides is that these lipids may exhibit cyclic variation during sequential therapy. This has previously been shown for sequential regimens combining estradiol or estradiol valerate with levonorgestrel, desogestrel or medroxyprogesterone acetate (87, 88), but remains to be studied for other sequential regimens.

In a study investigating conjugated equine estrogens alone or in combination with progesterone or medroxyprogesterone acetate, there was a slight increase in fibrinogen in the placebo group, but no difference from baseline was seen in the active groups (32). Recently, 6 months continuous combination of conjugated equine estrogens 0.625 mg and medroxyprogesterone acetate 2.5 mg or placebo was studied in healthy, postmenopausal women (89). In this trial, there was a significant decrease of 30% to hormone replacement therapy in plasminogen activator inhibitor 1, but no change in fibrinogen. Another study found that this effect of oral therapy was expressed already after 1 month and that there was no difference in responses between conjugated equine estrogen alone or combined with medroxyprogesterone acetate (90). Based on these few data, an increase is observed in fibrinolysis during hormone replacement therapy, which is independent of progestogen addition. In general, there

Table 2. Experimental animals in in vivo studies.

Rabbits	Randomisation (dose of medication is per day)	Cholesterol amount per day	Soy protein	Duration, weeks	Com- pleters
Study IV (n = 100)	1) Raloxifene 3.5 mg (weeks 8-17), 35 mg (weeks 18-45) (n=25)	200 mg (weeks 8-17), 125 mg (weeks 18-45)	~13%	45 (1994-1995)	23
	2) Estradiol 4 mg (weeks 8-45) (n=25)				24
	3) Sham operated, placebo (weeks 8-45) (n=25)				24
	4) Placebo (weeks 8-45) (n=25)				25
Study V (n = 24)	1) Raloxifene 35 mg (weeks 8-9) (n=6)	3 20 mg (weeks 4-5), 125 mg (weeks 6-10)	~13%	10 (1996)	6
	2) Raloxifene 105 mg (weeks 8-9) (n=6)				6
	3) Raloxifene 210 mg (weeks 8-9) (n=6)				6
	4) Placebo (weeks 8-9) (n=6)				6
Study VI (n = 80)	1) Raloxifene 70 mg (weeks 8-48) (n=20)	200 mg (weeks 8-17), 125 mg (weeks 18-48)	4.2%	48 (1996-1997)	19
	2) Raloxifene 210 mg (weeks 8-48) (n=20)				20
	3) Estradiol 4 mg (weeks 8-48) (n=20)				19
	4) Placebo (weeks 8-48) (n=20)				19
Study VII (n = 80)	1) Raloxifene 210 mg (weeks 20-58) (n=20)	240 mg (weeks 5-19), 80 mg (weeks 20-58)	0%	58 (1997-1999)	20
	2) Estradiol 4 mg (weeks 20-58) (n=20)				18
	3) Placebo (weeks 20-58) (n=20)				19
	4) Baseline control (n=20)				20

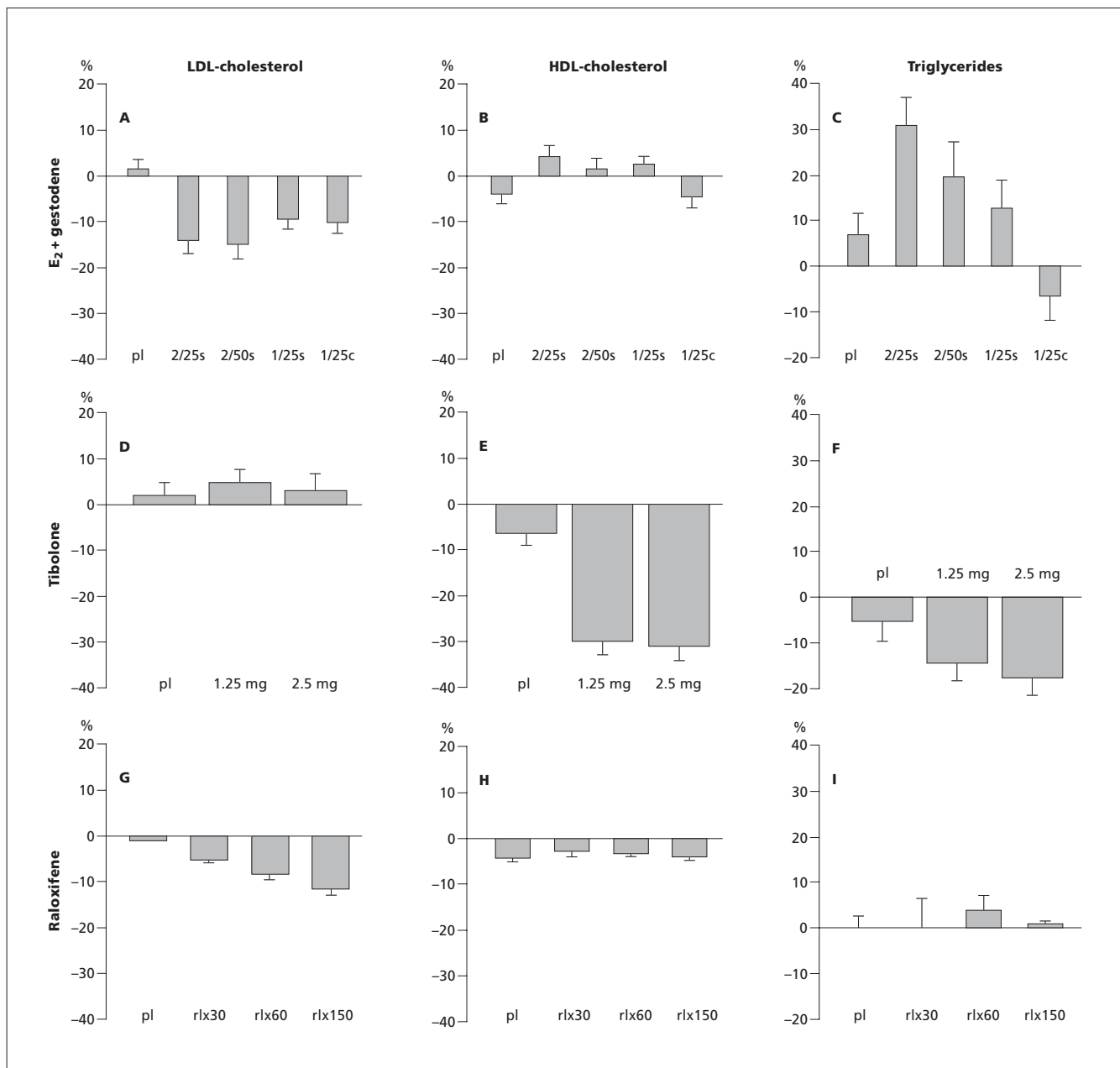


Figure 2. The response in lipids and lipoproteins during treatment. Please note that these results origin from 3 different studies [33, 96, 49]. **A-C:** show the total response to hormone replacement therapy (average of mid- and endcycle relative to baseline calculated from cycles 1, 7 and 26) (adapted from *Bjarnason et al*, 2000, Table 3A [33] with permission). 2/25s: 2 mg estradiol in sequential combination with 25 mg gestodene (days 17-28); 2/50s: 2 mg estradiol in sequential combination with 50 mg gestodene (days 17-28); 1/25s: 1 mg estradiol in sequential combination with 25 mg gestodene (days 17-28); 1/25c: 1 mg estradiol continuously combined with 25 mg gestodene (days 1-28); pl: placebo. **D-F:** show the 2 year responses to tibolone treatment (adapted from *Bjarnason et al*, 1997 [96] with permission); 2.5 mg: 2.5 mg tibolone; 1.25 mg: 1.25 mg tibolone; pl: placebo. **G-I:** shows the 2-year response to raloxifene treatment (adapted from *Delmas et al*, 1997, Table 3; [49] with permission); rlx150: 150 mg raloxifene; rlx60: 60 mg raloxifene; rlx30: 30 mg raloxifene; pl: placebo.

does not seem to be a meaningful effect on coagulation, but data on both fibrinolysis and coagulation needs further investigation. This is important because hormone replacement therapy increases the risk of venous thrombosis (42, 43).

In conclusion, oral therapy with 1 and 2 mg estradiol lowers LDL-cholesterol dose-related in healthy postmenopausal women independent of dose and regimen of concomitant gestodene addition. The influence of oral estradiol on HDL-cholesterol and triglycerides is a dose-related increase, which is counteracted in a dose-related fashion by addition of gestodene. The observed cyclic variation in HDL-cholesterol during sequential gestodene intervention has been found for regimens containing desogestrel, levonorgestrel and medroxyprogesterone acetate (87, 88). Thus, it is likely to occur often during sequential therapy. Because the variation detected is higher

than the analytic precision error in most laboratories, it is important to consider the timing for sampling in women undergoing oral sequential therapy, particularly in smoking women, who have been reported to experience exaggerated cyclic variations (88). The most correct lipid profile will be assessed if samples are drawn both mid-cycle (before progestogen addition) and end-cycle, but the simplest schedule to implement would be to sample all women end-cycle.

Ovariectomized, cholesterol-fed rabbits

Analysis of aortic cholesterol content after a follow up of 40 weeks revealed a significant reduction to one half to one third of placebo during estradiol treatment 4 mg/day with a non-significant trend for a reduction on sham-operated animals (63) (Table 2, study IV), equivalent with the results seen in other experiments (80, 81). Re-

gression analyses have shown that although serum total cholesterol and LDL-cholesterol decrease during estradiol therapy, this only partially explains the effect on the aorta (63, 80). Moreover, experiments in rabbits with pre-induced atherosclerosis have demonstrated comparable effects (91, 92) (Table 2, study VII).

In our studies estradiol exhibits a comparable efficacy in primary and secondary intervention studies. In rabbits clamped to cholesterol-levels higher than those in our experiments, the influence of estrogen on atherosclerosis progression seem to be partially dependent on an intact endothelium (93), which may be a possible key finding in the understanding of the influence of estrogen on atherosclerosis. This would theoretically indicate that estrogen could be expected to be more beneficial in primary prevention as compared to secondary prevention. This is however not supported by data from randomised controlled studies combined hormone replacement therapy, which found no benefit in women with symptomatic atherosclerosis (42) but a harmful effect in healthier women (43). It may be suggested that this difference is related to the choice of progestogen. Unfortunately medroxyprogesterone acetate is the only progestogen, which have been evaluated in a study with clinical endpoints. In the rabbit model, some progestogens have been found to counteract (progesterone) (94) or enhance (norethisterone acetate) (95) the influence of estrogen on the rabbit vessel wall. This remains to be studied during dose finding of other progestogens.

TIBOLONE

Postmenopausal women

In parallel with the results on bone, we observed no difference on cardiovascular risk parameters between the two dose levels of tibolone studied in late postmenopausal women (96). Tibolone therapy induced neither changes in LDL-cholesterol (Figure 2D) nor in apolipoprotein B (96). However, a major decrease of 30% was seen in HDL-cholesterol ($p < 0.001$) (Figure 2E). This change was mirrored in apolipoprotein A1 and total cholesterol (96). Whereas triglycerides decreased significantly during tibolone treatment (Figure 2F) ($p < 0.01$), no change was observed in Lipoprotein(a) (96). Tibolone lowered plasminogen activator inhibitor 1, plasminogen activator inhibitor activity and tissue plasminogen activator ($p < 0.001$ for all). With regard to coagulation, a borderline decrease was seen for the low dose tibolone group in fibrinogen, whereas no response to tibolone was seen in antithrombin III. These data are in agreement with available data on tibolone except that others have found a decrease in lipoprotein(a) in non-controlled (97), non-randomised and open (98) or in cross-sectional (99) studies. In the combination of two randomised, double-blind placebo-controlled study of a total of 770 healthy early postmenopausal American women, dose-related decreases were observed in HDL-cholesterol and Lp(a) but not in total cholesterol (47). The changes in these studies were generally greater at 12 months than at 24 months (47).

Experiments with tibolone in advanced atherosclerosis have not been performed.

In conclusion, whereas tibolone has no influence on LDL-cholesterol, a decrease is seen in both HDL-cholesterol and triglycerides. Based on biochemical markers, tibolone promotes fibrinolysis, but does not seem to influence coagulation. On the 2 dose levels studied, we found an equal response on all parameters whereas a dose-relationship for HDL-cholesterol but not for total cholesterol was observed by others (47).

Ovariectomized, cholesterol-fed rabbits

Tibolone was investigated in the rabbit model by Zandberg et al. (100) in a 20-week experiment with a cholesterol regimen, which was high relative to that used in our experiments (63, 101). Tibolone in dose levels of 2, 6 and 18 mg per day significantly reduced aortic cholesterol accumulation to about 10-50% of placebo, almost eliminated fatty streak formation and reduced advanced lesion formation to 50% of placebo in cholesterol-fed ovariectomized rabbits (100).

Regression analyses revealed that the aortic effects were largely independent of changes in serum lipids. Although the authors claim that tibolone did not influence serum triglycerides, there was evidence of a lowering on the lowest tibolone dose (2 mg), no change on the mid dose (6 mg) and an increase on the high dose of tibolone (18 mg). The results of this study are in accordance with an additional study in cholesterol-fed rabbits (102) but in contrast with a study in cholesterol-fed, oophorectomized monkeys, which did not identify differences between tibolone in 2 doses and control (103).

In a rabbit study with tibolone, there was a significant and comparable effect of tibolone and injectable estrogen but unexpectedly no effect of oral estrogen (100). In another recent rabbit study and in a monkey study with tibolone, a positive effect was seen during estrogen therapy (102, 103). Because the many experiments in this model have found an effect of oral estrogen (63, 80, 91, 94, 95, 101), it may be concluded that the effect of tibolone in the rabbit model is qualitatively equivalent with the influence seen with estrogen. In the monkey model, however, no difference between control and tibolone was found (103). Possible reasons for conflicting results in rabbit and monkey models are discussed in the following section about raloxifene.

RALOXIFENE

Postmenopausal women

In our study of long-term raloxifene intervention, treatment over 2 years induced a significant dose-related decrease in LDL-cholesterol (9% decrease on raloxifene 60 mg, $p < 0.05$) (Figure 2G), which was mirrored in the response in total cholesterol (5% decrease on raloxifene 60 mg, $p < 0.05$) (49). Changes were observed neither in HDL-cholesterol (Figure 2H) nor in triglycerides (Figure 2I). These results were confirmed by 3- and 5-year data (50, 51).

In a 6 months study of cardiovascular risk markers, raloxifene 60 and 120 mg was compared to placebo and 0.625 mg conjugated equine estrogens in continuous combination with 2.5 mg medroxyprogesterone acetate. In this study, raloxifene therapy lead to a 12% decrease in fibrinogen, whereas no change in plasminogen activator inhibitor 1 was observed (89). This investigation added a significant decrease in lipoprotein(a), which was intermediate to the decrease found during hormone replacement therapy. Finally, this study also showed that raloxifene does not influence C-reactive protein, which was increased during combined hormone replacement therapy (89).

In conclusion, raloxifene lowers LDL-cholesterol, but influences neither HDL-cholesterol nor triglycerides. Raloxifene inhibits coagulation, but does not change fibrinolysis based on biochemical markers of haemostasis. In contrast to the results on bone, there was a tendency for a dose response relationship on total and LDL-cholesterol in the range from 30-150 mg raloxifene.

Ovariectomized, cholesterol-fed rabbits

In our first rabbit study (Table 2, study IV), suboptimum dose levels of raloxifene had induced a reduction in aortic cholesterol content to two thirds of placebo ($p < 0.05$), which was significantly less than the effect seen with estradiol alone ($p < 0.01$) (63). The rabbits had initially been treated with 3.5 mg raloxifene per day for 11 weeks, which lead to undetectable plasma raloxifene concentrations. At this point, we chose to increase the raloxifene dose to 35 mg per day for the following 27 weeks. This regimen produced measurable plasma raloxifene levels, which however were low relative to on-treatment levels seen in postmenopausal women. After a pharmacokinetic study in hypercholesterolemic rabbits treated with raloxifene 35, 105 and 210 mg per day for 2 weeks (101) (Table 2, study V), the long-term experiment was repeated with 70 and 210 mg raloxifene/day (101) (Table 2, study VI). These raloxifene regimens lead to significant reductions in aortic cholesterol content compared to placebo ($p < 0.05$ for both) and the responses to raloxifene and estradiol were not significantly different. After correcting the ral-

oxifene responses to estradiol responses in the two long-term studies (63, 101), a trend for a dose-response relationship was revealed (Figure 3). By relating plasma raloxifene C_{max} in rabbits to on-treatment levels seen in postmenopausal women during raloxifene intervention, it was estimated that a clinically relevant raloxifene treatment regimen would result in about two thirds of the effect of a clinically relevant estradiol treatment regimen in the cholesterol-fed, ovariectomized rabbit (Figure 3). In a third long-term rabbit study where mild atherosclerosis was induced over 15 weeks, we found a reduction in the progression of atherosclerosis as compared to placebo (92) (Table 2, study VII). During the time period in which the 4 rabbit studies were conducted, hypotheses concerning a preventive effect of soy protein contents upon atherosclerosis were raised (84). Therefore, we chose rabbit chaw with the lowest content of soy protein, which was available at the time of study initiation (Table 2). However, the fact that the difference between active treatment and placebo was in the same order of magnitude in all studies, indicated that the presence of relevant control arms had eliminated soy protein content as a potential bias (63, 92, 101). In all studies, analyses of covariance relating serum lipids and lipoproteins to aortic cholesterol content showed that only part of the effect of raloxifene and estradiol could be attributed to the lipid lowering (63, 92, 101).

A study in ovariectomized, cynomolgus monkeys on development of atheroma formation in coronary arteries was not able to demonstrate an effect of raloxifene, whereas treatment with conjugated equine estrogens was effective in reducing plaque formation (104). Reasons for this inconsistency between rabbit and monkey are unknown, but have been under discussion (105). The consistency in 3 long-term rabbit studies supports our results. The effect of raloxifene in this model is always less than that of estrogen, therefore a failure of raloxifene to demonstrate effect in a single monkey experiment is not inconsistent with reality in *in vivo* studies. A variation in result may also relate to differences between the rodent and the primate, however other published experiments have shown consistency with regard to a selection of sex steroids and their analogs and raloxifene would then be the first of these drugs to induce a different response in rabbit and monkey. Finally, the rabbit results are in accordance with an exploratory post-hoc analysis of 4 years of raloxifene therapy versus placebo in osteoporotic women. This evaluation revealed a 40% reduction in cardiovascular clinical endpoints during raloxifene treatment in the subset of women with the highest baseline risk of cardiovascular events (106). A placebo-controlled study in 10,000 postmenopausal women selected to be at cardiovascular risk is currently ongoing in order to determine the effect of raloxifene therapy on the incidence on fatal and non-fatal myocardial infarction (107).

In conclusion, the influence of raloxifene on rabbit aortic athero-

sclerosis is a significant lowering, which is present in both primary and more advanced disease and which is only partially explained by serum lipid and lipoprotein changes. Finally, clinical data have suggested that raloxifene may be cardioprotective (106), but this finding needs confirmation.

A SAFETY EVALUATION OF BREAST AND UTERUS BREAST

Estrogen has a central role in the development of estrogen-dependent breast tumours, but postmenopausal (estrogen deficient) women have a higher risk of being diagnosed with breast cancer than premenopausal women. It has been suggested, that it is the total life-long estrogen exposure which determines risk, but postmenopausal estrogen synthesis in peripheral organs and tissues, such as adipose tissue have also been proposed to play a role. Only very little estrogen may be enough to increase risk, thus even endogenous low estrogen concentration in postmenopausal women have been found to influence the risk of breast cancer (108). Both estrogen receptor α and β are present in breast tissue (9).

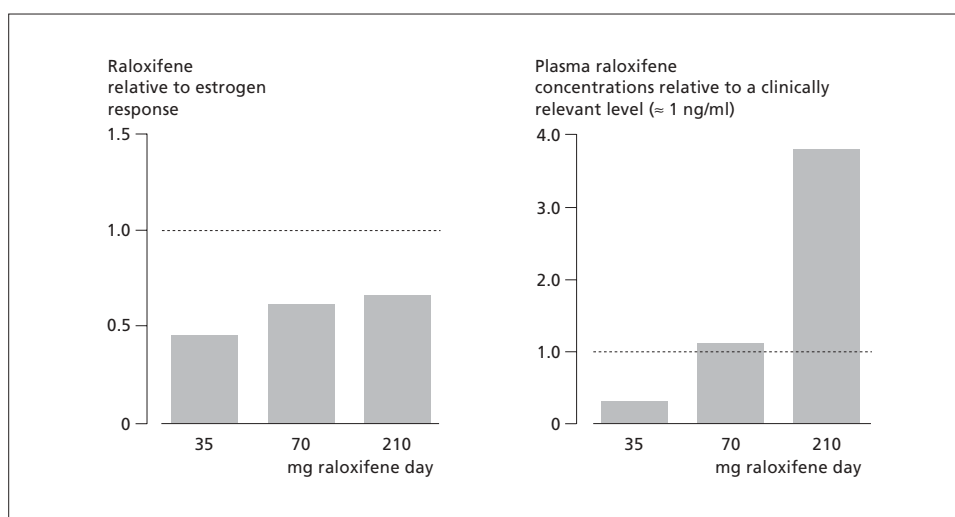
The breast may be monitored by mammography for a qualitative evaluation of tumour risk and recently, breast density measurements – a quantitative assessment based on mammograms – have been introduced (109). Longitudinal data have shown that a high breast density is associated with an increase in the risk of breast cancer (110), but it is not known whether a pharmacologically induced change in breast density influences breast cancer risk.

Breast tenderness is often monitored during adverse event collection in randomised trials in postmenopausal women. Although this symptom is a function of both estrogen and progestogen treatment, it occurs often during progestogen addition in sequential therapies. This association with progestogen therapy is in accordance with the frequent occurrence of breast tenderness in premenstrual premenopausal women. At present, there is no firm evidence for a relation between breast tenderness and mammographic density/breast cancer risk. This would however be a relevant scientific question to ask because combined hormone replacement therapy has been reported to lead to a higher increase in breast density (109) and in breast cancer risk as compared to estrogen therapy alone (111).

Hormone replacement therapy

Breast tenderness occurs significantly more often during hormone replacement therapy regimens (32, 33, 35). By post-hoc evaluation, breast density was examined on the yearly mammograms during a randomised, controlled study of combinations of conjugated equine estrogens and progesterone or medroxyprogesterone acetate (109). There was a significant increase in all active groups, but the increase was larger in the combined groups than in the unopposed group. Based on observational data, it has been reported that hormone re-

Figure 3. Raloxifene and estrogen: anti-atherosclerotic potential. The left hand site shows the response in aortic cholesterol content during raloxifene therapy relative to that of estradiol in the ovariectomized, cholesterol-fed rabbit model. The right hand site shows the corresponding plasma raloxifene concentrations relative to the concentration seen in postmenopausal women during treatment with raloxifene 60 mg (adapted from Bjarnason *et al*, 2000, page 231 [101]).



placement therapy increases the risk of breast cancer with a relative risk of 1.35 (1.21-1.49) for more than 5 years hormone replacement therapy use – an effect which is comparable with that of delaying menopause (112). Recently, this has been confirmed by another observational study, which also demonstrated an increase in the risk of mortality during hormone replacement therapy (111). With regard to the influence of progestogen addition, some data point towards an increase in risk (111) whereas others find no difference from unopposed therapy (113). In the Womens Health Initiative study, a randomised controlled trial comparing conjugated equine estrogens in continuous combination with medroxyprogesterone acetate with placebo, a significant 26% increase in breast cancer was observed during active treatment (43).

Tibolone

Tibolone has not been reported to significantly increase breast tenderness (44, 46, 47). In vitro data have found that tibolone stimulates estrogen sulphotransferase, an effect that theoretically may lead to inhibition of breast cancer development (114). The influence of tibolone on breast density has been investigated in 2 small open studies, one randomised (115) and one uncontrolled, observational (116). In the first, an intermediate increase in density between the small increase seen with unopposed estrogen therapy and the larger increase seen with combined hormone replacement therapy was observed (115), in the other no difference from baseline was found (116). Recently, an open randomised 12-months comparison of tibolone and raloxifene could not demonstrate a significant change in breast density in any group (117). The risk of breast cancer during tibolone therapy has not been studied in a randomised controlled setting, but a large observational study conducted in the UK revealed an increased risk in a similar order of magnitude as seen during unopposed estrogen therapy in the same cohort, but lower than the risk during combined hormone replacement therapy (111).

Raloxifene

Both in early postmenopausal women and in elderly osteoporotic women raloxifene does not induce breast tenderness (49, 52, 53). In vitro and in vivo experiments have demonstrated an inhibitory influence of raloxifene upon breast cancer (118). During 5 year follow-up in hysterectomized women, raloxifene lead to changes in mammographic breast density comparable to placebo (119). In contrast, an increase in breast density was found during unopposed therapy with conjugated equine estrogens. During 3 years, therapy with raloxifene 60 or 120 mg in osteoporotic women reduced the risk of breast cancer by 76% as compared to placebo (120). This effect was caused by a decrease in the risk of estrogen receptor positive cancer with 90%, whereas no effect is observed on estrogen receptor negative cancer. Four-year follow-up revealed comparable results (121). A randomised trial of 22,000 women with breast cancer risk factors is currently ongoing to compare the effect upon breast cancer incidence of raloxifene with tamoxifen.

UTERUS

Estrogen monotherapy in postmenopausal women is associated with increased risk of endometrial hyperplasia and subsequently endometrial carcinoma (122). Addition of a progestogen counteracts this risk and a combined therapy is therefore recommended to ensure endometrial control in women with an intact uterus. Both estrogen receptor α and β have been demonstrated in the endometrium (9).

Hormone replacement therapy

The control of vaginal bleeding during combined hormone replacement therapy depends on the type and dose of the progestogen, the ratio between the estrogen and progestogen component, the regimen, the level of endogenous estradiol and the individual endometrial response (123).

Most sequential hormone replacement therapy regimens cause a cyclical vaginal bleeding comparable to premenopausal ovulatory bleeding (123). This is in accordance with our data, but we also found that some women on low dose sequential therapy do not bleed once they are stabilized on the therapy (124). The average number of days with bleeding did not differ according to dose of estradiol or gestodene, but a high estradiol/gestodene ratio was associated with an earlier onset of bleeding (124). Breakthrough bleeding may be evaluated to be the clinically important bleeding pattern in women during hormone replacement therapy. Most women being treated with continuous (124) or interrupted (125) progestogen addition do not bleed, except for a tendency for breakthrough bleeding within the first 6-12 months of intervention. The histology of the endometrium at such bleeding episodes is typically atrophic with weakly developed secretory patterns (123, 124). It is postulated that this bleeding is caused by vascular instability due to abnormalities of vascular growth in combination with an imbalance between tissue degrading matrix metalloproteinases and their inhibitors (123). This is in contrast to breakthrough bleeding during continuous combined oral contraceptives in which inflammatory cell infiltration also is thought to play a role (123). The lowest progestogen dose-regimen, which counteracts histologically verified hyperplasia during concomitant estrogen treatment does not necessarily also lead to an acceptable bleeding control. This usually requires more progestogen.

During unopposed therapy, breakthrough bleeding may occur, but because the endometrium is not transformed in a secretory phase, it is not a regular withdrawal bleeding. Here, the endometrium is characterized by focal severe proliferation and hyperplasia in contrast to the diffuse endometrial changes seen during a regular menstrual bleeding (123).

Tibolone

In elderly women and in early postmenopausal women as well, the incidence of breakthrough bleeding during therapy with 1.25 or 2.5 mg tibolone was 20% (44, 126). Short-term data have shown that treatment with 5 mg tibolone lead to severe bleeding problems (45). Tibolone has not been reported to increase endometrial cancer risk.

In ovariectomized rabbits, the uterus weights on the two lowest dose levels of tibolone were comparable with estrogen and significantly higher than placebo. Interestingly, on the top dose a significant increase over estrogen was seen (100). An increase in uterus weight on tibolone as compared to estradiol in rabbits has been confirmed in another study (102) and similar data have been found in rats (5). Thus, at the doses tested tibolone may be evaluated to have neutral or slightly stimulatory effects with an increasing stimulatory influence when the dose of tibolone is increased.

Raloxifene

Raloxifene therapy induces neither vaginal bleeding nor changes in endometrial thickness as showed in both early postmenopausal women and in elderly women with osteoporosis (49, 52, 53). Moreover, no influence of raloxifene was detected on endometrial cancer incidence in elderly women, although only 10 cancers in 7705 women were diagnosed (120).

Studies of ovariectomized raloxifene-treated rabbits supported both anti-estrogenic and estrogen-like activities on the endometrium as well as a dose-response relationship. In the study of sub-optimum raloxifene doses, the uterine wet weight in the raloxifene group was similar to placebo (63), but when higher raloxifene doses were used, the uterine wet weights in raloxifene groups were significantly lower than placebo (101). Thus, concomitant with increasing raloxifene dose, an increasing inhibition of uterine weight was observed. In all studies, the uterine wet weight in the estradiol groups was significantly higher than both placebo and raloxifene (63, 92, 101). Endometrial receptor analyses revealed that cytosolic estradiol receptor content was down-regulated and nuclear estradiol receptor

content was up-regulated on raloxifene therapy (63, 101). This was different from the endometrial response to estrogen. This effect induced by raloxifene is most likely due to a translocation of estrogen receptors from the cytosol and accumulation in the nucleus, but reasons for this translocation and its effect are unknown. Interestingly, both estradiol and raloxifene up-regulated cytosolic progesterone receptor levels, which is associated with estrogen stimulation (63, 92). Thus, in the uterus, raloxifene exerts both estrogen agonistic and antagonistic effects.

COMMENTS

BONE

Despite the lack of head-to-head comparisons, the comparability in the pattern of responses indicate that the mechanisms of action on bone of estradiol, tibolone and raloxifene are related, and that the effects of tibolone and raloxifene are most likely to be explained by an estrogen agonist effect. For tibolone this is supported by an experiment showing that the inhibitory effect of tibolone on bone loss in ovariectomized rats is abolished by addition of an anti-estrogen but unchanged during addition of an anti-progestogen or an anti-androgen (127). For raloxifene this is supported by mechanistic experiments of the interaction between raloxifene as a ligand and the estrogen receptor (128). Interestingly, raloxifene interacts with non-estrogen response elements to target specific expression of proteins, different from estrogen. The present data indicate that the dose-response relationships of the investigated treatment modalities are different. For example, whereas it is evident that the full anti-resorptive effect on bone is not reached by raloxifene, there seem to be a closer relation with tibolone and estradiol – depending on the dose chosen. This is supported by recent 2-year investigations of combinations of alendronate 10 mg with either conjugated equine estrogen (129) or raloxifene (130). Results from these studies in women with a T-score less than -2, show that the combination of alendronate and hormone replacement therapy increases bone mass slightly (8.3%) as compared to either therapy alone (both 6.0%), whereas the difference between raloxifene alone (2.1%) and the combination of raloxifene and alendronate (5.3%) seems to be larger. This indicates that the full anti-resorptive potential on bone is not reached with either of the maximum doses tested. However, the additional benefit of adding another anti-resorptive drug to a top dose of hormone replacement therapy or tibolone is limited with regard to efficacy on bone mass, non-existing on antifracture efficacy and only sparsely explored with regard to safety. It is not clear if a long-term reduction of bone turnover far below premenopausal levels results in a deleterious bone effect, but recent data have shown that initial therapy for 2 years with alendronate 20 mg per day in healthy, early Danish postmenopausal women lead to a reduction in bone resorption, which was still not normalized 7 years after discontinuation of therapy (131).

Estradiol and tibolone therapy lead to a greater response in an axial site with a content of primarily trabecular bone such as the spine, whereas peripheral sites with a content of primary cortical bone such as the hip and forearm responded with less of an increase or with inhibition of bone loss (33, 44). During raloxifene treatment, however, there was a similar BMD response in axial and peripheral measurement sites (49, 52, 53). One hypothesis accounting for the differential BMD effects of raloxifene versus tibolone and estradiol implies an increase in the amount of mineral per unit of bone during tibolone and estradiol therapy. Animal and human bone micro-radiography studies indicate that, when bone metabolism is markedly reduced by an anti-resorptive treatment, the second phase of mineralisation (which accounts for about 30% of mineral deposited into bone) is significantly prolonged, resulting in an increase in the mean amount of mineral per unit of bone (132). Such an effect is more likely to be observed in sites rich in trabecular bone, such as the spine, as it is characterized by a higher turnover than cortical bone. Thus, the large increase in BMD observed with anti-resorptive

agents that remarkably reduce bone turnover (below the normal postmenopausal range) could be due initially to filling of the remodelling space (greatest in trabecular bone) and later to increase in bone mineral per unit of bone (also greatest in trabecular bone). Such a hypothesis is consistent with the difference in bone turnover marker suppression between raloxifene and estradiol or tibolone. Another aspect of the differential responses in bone mass on raloxifene as compared to traditional regimens of hormone replacement therapy and bisphosphonates lies in the interpretation of fracture risk reduction. Interestingly, the reduction in vertebral fracture risk is in the same order of magnitude on these therapies despite the difference in spinal bone mass response. This is in contrast to fractures of the forearm and the hip, where raloxifene did not demonstrate efficacy. Theoretically, sites with a relatively high content of trabecular bone (such as the vertebrae) may just need a slight reduction in turnover to prevent osteoclast from perforating the trabecular plates and thereby reduce fracture risk (133). Thus, both potent and less potent therapies may reach this low threshold and prove effective here. Conversely, sites with a relatively high content of cortical bone (such as the forearm and the hip) may need a more substantial increase in bone mass, which is only achieved by potent antiresorptive therapy regimens or formation-stimulating agents (133).

It seems from our data that the larger the decrease in bone turnover, the larger the increase in bone mass during hormone replacement therapy (Figure 1). Post-hoc analyses revealed that there was a highly significant association between a short-term change in bone turnover and a long-term response in bone mass for both estradiol (29) and tibolone (134). Interestingly, an association between the short-term decrease in bone turnover and the long-term vertebral fracture risk reduction during raloxifene treatment has been demonstrated (31). This association remained significant after adjustment for age, baseline BMD and fracture status (31). These results may indicate that fracture protection during anti-resorptive therapies can be mechanistically understood from bone turnover decrease over bone mass increase to bone strength increase thereby obtaining fracture prevention. This theory is supported by the demonstration of a positive relationship between the increase in bone mass and reduction in vertebral fracture risk during alendronate therapy (135). We thus conclude, that tibolone and raloxifene have an estrogen-like influence on bone, although with a different dose-response relationship. Whereas tibolone demonstrates comparability with estrogen, the dose-response relationship for raloxifene is broad and with a somewhat weaker maximum effect. The therapies act by decreasing bone resorption, which leads to an increase in bone mass and reduction in fracture risk, the latter has however only been investigated in studies with sufficient power for raloxifene and estrogen, but it remains to be studied for tibolone.

CARDIOVASCULAR SYSTEM

Whereas we found primarily similarities in the effects of tibolone, raloxifene and estradiol in combination with gestodene on bone (Figure 1), we found differences in the effect of these treatments upon biochemical cardiovascular risk markers (Figure 2) but similarities in the influence on *in vivo* atherogenesis (Figure 3).

The effect on LDL-cholesterol and apolipoprotein B seems to primarily originate from the estradiol stimulation alone, whereas the influence on HDL-cholesterol, apolipoprotein A1 and triglycerides is likely to be caused by the combined effect of estradiol and gestodene in a competitive antagonistic fashion within the dose ranges investigated (Figure 2) (33).

An approach to explain the influence of tibolone on cardiovascular risk markers may be to assume a progestogenic effect of the compound, which is strong relative to its estrogenic effect. Thus, the decreases in HDL-cholesterol and triglycerides may be the result of a progestogenic action, which antagonizes and counteracts the estrogenic action of tibolone. This is in accordance with the influence of

tibolone on fibrinolysis, an effect that seems to be mediated by the estrogen effect alone, comparable to the response seen during combined hormone replacement therapy. It has been suggested that the influence of tibolone on the lipids originates from its androgenic effect. This is however not in accordance with the effect on LDL-cholesterol, which is usually lowered during treatment with androgens. An experiment using adding an anti-progestogen or an anti-androgen to tibolone may further explore these theories. The influence of tibolone treatment on cardiovascular clinical endpoints remains to be studied.

The effect of raloxifene on cardiovascular risk markers may be understood from a theory involving independent, but parallel mechanisms whereby hormone replacement therapy and raloxifene operate to control these parameters. Some of these processes seem to be changeable by raloxifene only, some by estrogen alone, and some by both. Thus, the decrease in LDL-cholesterol is equivalent with that seen during hormone replacement therapy, whereas the effects on HDL-cholesterol, triglycerides and fibrinolysis markers may be induced by estrogen only. Conversely, the effect on coagulation seems to be found for raloxifene alone. Whereas a dose-response relationship was found for traditionally used dose-ranges of estrogens and progestogens (33), this is less pronounced for raloxifene (49) and non-existing for tibolone (44) for the dose levels investigated (Figure 2).

The results of the in vivo experiments may be interpreted in parallel with the results seen on bone, where raloxifene seem to have a moderate, estrogen-like effect. This would be in line with the partial estrogenic lipid effect seen with raloxifene. However, non-lipid mechanisms may be involved in the effect of estradiol as well as raloxifene and tibolone. This is supported by data showing that raloxifene relaxes rabbit and human coronary arteries acutely by an endothelial-dependent mechanism involving nitric oxide (136). Data also indicate that tibolone preserves the endothelium-dependent acetylcholine induced relaxation response in aortic rings from rabbits fed with an atherogenic diet (100). A post-hoc subset analysis from a fracture study of raloxifene indicated that women with cardiovascular risk factors in addition to osteoporosis had benefit from raloxifene intervention in terms of prevention of cardiovascular clinical endpoints (106). This benefit was of comparable size as the reduction in aortic cholesterol content in the rabbit experiments (63, 92, 101).

Almost all observational studies have found a decreased risk of cardiovascular disease during hormone replacement therapy (3, 137, 138). Although cardio-protection has been thought to be plausible, a concern about the epidemiological evidence for a preventive effect of hormone replacement therapy has been the possible bias of comparing women who chose to be compliant with long-term hormone replacement therapy with those who do not take hormone replacement therapy (139). In order to control for known and unknown confounders, blinded, randomised, controlled studies are needed. The Heart and Estrogen/Progestin Replacement Study compared placebo with conjugated equine estrogens in combination with medroxyprogesterone acetate over 4 years on cardiovascular events (42). The surprising null conclusion of this trial in 2,763 women with severe atherosclerosis raised doubts about the exact effect of estrogen and progestogen upon advanced ischemic heart disease (140). The Estrogen Replacement and Atherosclerosis trial which over 3 years compared both unopposed conjugated equine estrogens with a continuous combination of conjugated equine estrogens and medroxyprogesterone acetate with placebo in 309 women with atherosclerosis also found no significant differences between the groups during evaluation of angiographic endpoints (141). Many concerns were raised because of these data: type, dose and regimen of estrogen and progestogen, confounding by concomitant medication, disease severity and age of participants, inadequate power and duration of study and poor compliance. Finally, 5.2 years therapy lead to a 29% increase in the risk of coronary heart disease and a

41% increase in the risk of stroke in the 8506 healthy women, who had been randomised to conjugated equine estrogens in combination with medroxyprogesterone acetate in the Women Health Initiative study (43). Based on these results, it may be concluded that a cardiovascular benefit of combined hormone replacement therapy has not been detected – in fact the influence of this therapy is found to be harmful. The effect of the arm, which compares placebo with conjugated equine estrogens alone in hysterectomized women continues to be studied, which indicates that the risk-benefit ratio on this regimen is at least neutral. This may suggest that combined hormone therapy is more adverse than unopposed estrogen treatment. However, a 2-year randomised study of estradiol alone versus placebo in women with atherosclerosis failed to demonstrate a cardiovascular benefit on clinical endpoints (142). Another randomised 3.3 year study in 226 women with coronary artery disease compared placebo, estradiol, and a combination of estradiol and medroxyprogesterone acetate on change in stenosis on quantitative coronary angiography. Also in this study, there was no difference between either of the groups (143). One theory for the adverse effects of hormone replacement therapy is that a prothrombotic and proinflammatory effect of the therapy outweighs the benefits of the lipid lowering (133). This theory is based on the fact that hormone replacement therapy increases the risk of venous thrombosis (43) and increases C-reactive protein, which is known as a marker of inflammatory activity (144). Changes in haemostasis parameters during hormone replacement therapy and raloxifene treatment have, however, not been linked to the increased risk of venous thrombosis during these therapies.

A possibility to understand Heart and Estrogen/Progestin Replacement Study (42) and the Women Health Initiative Study (43) results lies in consideration of the population characteristics (145). Ideally, one would prefer to include women normally considered for hormone replacement therapy. Unfortunately, this is impossible because women do not accept randomisation to long-term placebo if they suffer from climacteric complaints. Moreover, the women in these US-based studies were relatively heavy. The average body mass index in the Women Health Initiative Study was 29 kg/m² (43) and in Heart and Estrogen/Progestin Replacement Study about 60% of the participants had a body mass index over 27 kg/m² (42). This is important because heavy postmenopausal women have higher endogenous estrogen production than thinner women. This distinction has been shown to be clinically relevant in several estrogen-sensitive organs. For example, postmenopausal women with a high estradiol level in the postmenopausal range have increased risk of breast cancer (108). In addition, we found that a body mass index above 27 kg/m² arrested bone loss in early postmenopausal women (146). In contrast, women with a body mass index below 23 kg/m² had a bone loss, which was twice as high as had those with a body mass index between 23 and 27 kg/m² (146). Thus, fat women without climacteric complaints may be considered to undergo hormone therapy already and it is unknown whether further estrogen supplementation may be harmful.

Additionally, central fat localisation has been found to be a major risk factor for atherosclerosis (147), and the women in Heart and Estrogen/progestin Replacement Study and the Women Health Initiative Study were not characterized with regard to fat distribution. Finally, genetic variability in the response to hormone replacement therapy was suggested during a post-hoc analysis in the Estrogen Replacement and Atherosclerosis Trial, where women with a sequence variant in the estrogen receptor α gene responded with an augmented increase in HDL-cholesterol (148). Thus, questions remain to be answered with regard to characterisation of the population in consideration for therapy and also with regard to progestogen selection.

That hormone replacement therapy, tibolone and raloxifene demonstrate different effects on cardiovascular biochemical markers (Figure 2), comparable effects in rabbit experiments (Figure 3) but

different effects in clinical studies argue against a significant importance of the lipid model. The basis for the lipid-model as an explanation for the effect of estrogen is the observation that a high level of LDL-cholesterol and a low level of HDL-cholesterol are associated with an increase in the risk of cardiovascular disease. However, this does not imply that a pharmacologically induced increase or decrease of these markers will be equivalent with a baseline high or low level of this parameter in untreated individuals. A decrease in LDL-cholesterol during statin therapy has been coupled to the decrease in risk, but the decrease during a regular statin regimen is much larger than the decrease seen during hormone replacement therapy. Interestingly, the failure of a recent pravastatin study was in fact linked to an inadequate reduction in LDL-cholesterol, which was in the same order of magnitude as that seen during hormone replacement therapy (149). But most importantly, the lipid changes seen during hormone replacement therapy do not explain the results on clinically relevant cardiovascular endpoints in randomised, controlled trials of hormone replacement therapy.

CONCLUSION AND PERSPECTIVES

Table 3 summarizes the differential responses to therapy with raloxifene, tibolone and hormone replacement therapy.

The results on bone parameters are consistent with the effects on clinical fracture endpoints. Thus, a greater response in bone turnover and bone mass within the regimens investigated leads to a greater reduction in fracture risk. This suggests that measurements of bone mass and bone turnover are useful in the identification of regimens to be tested in clinical endpoint studies. Furthermore, the markers may be used in the monitoring of patients undergoing therapy.

The effects on bone are in contrast to the results on cardiovascular risk markers. Whereas a differential effect is seen on lipids and haemostasis assessments, rabbit experiments show comparable results for the therapies studied. These evaluations offer no simple explanation

of the divergent results on clinical endpoints, where hormone replacement therapy seems to be harmful in spite of observational and experimental evidence. Thus, whereas the epidemiological and the randomised controlled studies show consistency with regard to the influence of hormone replacement therapy on bone and breast, they demonstrate inconsistency with regard to cardiovascular disease. Eventually, there is no substitute for the randomised controlled trial in cardiovascular disease.

In the investigation of therapies, which influence several tissues, it is important to consider that the dose response relationship may differ between target organs. Therefore, different doses may be chosen for different target organs depending on the therapeutic window for the condition in need for treatment.

Although tibolone and raloxifene expand the treatment options in postmenopausal women, the ideal alternative to hormone replacement therapy is not yet identified. Tibolone may possess some of the safety problems seen with hormone replacement therapy and raloxifene is less potent on bone and may worsen climacteric symptoms. Knowledge of these therapies may however improve the search for alternatives to hormone replacement therapy. Here, the emerging understanding of the pharmacology of drugs influencing estrogen receptor isoforms is likely to improve a rational drug design. Three interactive mechanisms have been suggested (133): First, the expression of estrogen receptor α and β varies in different target organs. Estrogen receptor α is almost always an activator, whereas estrogen receptor β can inhibit receptor α by forming a heterodimer with it. If a SERM influences both receptor isoforms, the relative availability of the receptors will influence the cellular response. For example, raloxifene has been found to function as an estrogen antagonist when acting through estrogen receptor β on genes containing estrogen response elements but function as a partial agonist when acting on genes through estrogen receptor α (150). Second, receptor-ligand binding results in conformational differences ranging from estrogen at one extreme to an estrogen antagonist (ICI 164, 384) at the other with SERM-bound receptors assuming intermediate shapes (133). Third, depending on the ligand-receptor conformation co-regulatory proteins are activated, which may act either as positive or negative transcriptional regulators. For example, raloxifene and tamoxifen act on mammary cells by recruiting corepressors to estrogen-receptor target promoters. In contrast, tamoxifen acts in endometrial cells by recruiting coactivators, whereas this recruitment does not happen with raloxifene (151). This is in accordance with the clinical effect on the endometrium of both therapies.

The optimum alternative to hormone replacement therapy has the advantages of estrogen but none of its adverse effects and offers protection against breast cancer. Additional strategies could be to develop a drug with exclusive potent effects on bone or on the cardiovascular system. These goals may be searched through knowledge of estrogen receptor isoforms and through studies described in this thesis.

Interestingly, it has been suggested that the differential effects of drugs acting via estrogen receptors may be a general feature among steroid nuclear-receptor families (133). Thus, it is a future possibility that safe analogues of corticosteroids, progestogens and vitamin D may be available.

SUMMARY IN DANISH

Formålet med afhandlingen har været at evaluere effekten af tibolone og raloxifen, som er østrogen-lignende alternativer til hormonsubstitutionsterapi i forebyggelsen af postmenopausalt knogletab samt mht. indflydelse på risikomarkører for hjerte-kar-sygdom. Som sammenligningsgrundlag valgtes hormonsubstitutionsbehandling.

I alt tre randomiserede, placebokontrollerede og dobbeltblinde forsøg af 2-3 års varighed i 970 raske postmenopausale kvinder viste:

Table 3. A qualitative estimation of the influence of tibolone and raloxifene in healthy postmenopausal women with estrogen and progesterone as reference.

	Tibolone	Raloxifene	Estrogen	Progesterone
Bone				
Resorption	↓↓	↓	↓↓	N
Formation	↓↓	↓	↓↓	N or ↑
Bone mass	↑↑	↑	↑↑	N or ↑
Fracture	-	↓	↓↓	-
Cardiovascular system				
<i>Human</i>				
LDL-cholesterol	N	↓	↓	N
HDL-cholesterol	↓↓	N	↑	↓
Triglycerides	↓	N	↑	↓
Fibrinolysis	↑	N	↑	N
Coagulation	N	↓	N	N
<i>In vivo</i>				
Rabbit aortic atherosclerosis	↓↓	↓	↓↓	N, ↑ or ↓
Breast				
Symptoms	N	N	↑	↑
Cancer	↑*	↓↓	↑	N*
Uterus				
<i>Human</i>				
Symptoms	↑	N	↑↑	↑↑
Cancer	-	N	↑*	↓*
<i>In vivo</i>				
Rabbit uterine weight	↑	↓	↑	↓

↑: Increase or stimulatory. ↑↑: Greater increase or stimulatory effect.
 ↓: Decrease or inhibitory. ↓↓: Greater decrease or inhibitory effect.
 -: Data not available. *: Observational evidence only.
 N: Neutral.

1. Tibolonbehandling reducerer knogleomsætningen og øger knoglemineralindholdet. Effekten af de to undersøgte dosisniveauer, 1,25 og 2,5 mg er sammenlignelig og svarer til effekten af behandling med 1-2 mg østrogen.
2. Raloxifenbehandling reducerer ligeledes knogleomsætningen og øger knoglemineralindholdet. Effekten af de tre undersøgte dosisniveauer, 30, 60 og 150 mg er af sammenlignelig størrelsesorden, men synes svagere end effekten af tibolon og standardregimer af hormonsubstitutionsbehandling.
3. Tibolon reducerer HDL-cholesterol og triglycerid, men ændrer ikke LDL-cholesterol. Dette er i overensstemmelse med en overvejende gestagen indflydelse. Tibolon har derimod en østrogenlignende effekt på markører for hæmostase, idet ændring til fordel for fibrinolyse induceres.
4. Raloxifen reducerer LDL-cholesterol, men ændrer ikke HDL-cholesterol eller triglycerid. Dette svarer til en partiel østrogen virkning.

Endvidere viste i alt fire eksperimenter i 284 hun-kaniner i den oophorektomerede kolesterol-fodrede model i varighed af tre uger til halvandet år, at raloxifen reducerer graden af aterosklerosedannelse i aorta, ligeledes med en partiel østrogen virkning.

Vurdering af sikkerhedsprofilen viste, at tibolon medfører en svag stimulation af endometriet, således oplever ca. 20% af kvinderne pletblødning. Raloxifen fører derimod ikke til menstruationslignende blødning og reducerer desuden forekomsten af brystcancer med 76% med en effekt, der udelukkende ses hos østrogen-receptorpositive cancer.

Det konkluderes, at selv om tibolon og raloxifen udvider behandlingsmulighederne hos postmenopausale kvinder, repræsenterer disse lægemidler ikke det ideelle alternativ til hormonbehandling. Tibolon kan medføre i det mindste nogle af de sikkerhedsmæssige problemer forbundet med hormonbehandling, og raloxifen synes mindre effektiv i forebyggelse af osteoporose og afhjælper ikke klimakterielle gener, men kan forværre symptomerne. Imidlertid kan indsigt i de differentierede aspekter af disse lægemidler være med til at øge forståelsen for behandlingseffekter i østrogenfølsomme væv og til at målrette udviklingen af nye lægemidler til postmenopausale kvinder.

REFERENCES

- I Bjarnason NH, Bjarnason K, Haarbo J, Rosenquist C, Christiansen C. Tibolone: prevention of bone loss in late postmenopausal women. *J Clin Endocrinol Metab* 1996; 81: 2419-22.
 - II Bjarnason NH, Bjarnason K, Haarbo J, Coelingh Bennink HJT, Christiansen C. Tibolone: influence on markers of cardiovascular disease. *J Clin Endocrinol Metab* 1997; 82: 1752-6.
 - III Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Christiansen C. Raloxifene inhibits aortic accumulation of cholesterol in ovariectomized, cholesterol-fed rabbits. *Circulation* 1997; 96: 1964-9.
 - IV Delmas PD, Bjarnason NH, Mitlak BH, Ravoux A-C, Shah AS, Huster WJ, Draper MW, Christiansen C. Effects of raloxifene on bone mineral density, serum cholesterol and uterine endometrium in postmenopausal women. *N Engl J Med* 1997; 337: 1641-7.
 - V Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Knadler MP, Christiansen C. Raloxifene reduces atherosclerosis: studies of optimized raloxifene doses in ovariectomized, cholesterol-fed rabbits. *Clin Endocrinol (Oxf)* 2000; 52: 225-33.
 - VI Bjarnason NH, Byrjalsen I, Hassager C, Haarbo J, Christiansen C. Low dose estradiol in combination with gestodene to prevent early postmenopausal bone loss. *Am J Obstet Gynecol* 2000; 183: 550-60.
 - VII Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Christiansen C. Raloxifene and estrogen reduces progression of established atherosclerosis – a study in ovariectomized, cholesterol-fed rabbits. *Atherosclerosis* 2001; 154: 97-102.
1. Consensus Development Statement. Who are candidates for prevention and treatment for osteoporosis? *Osteoporosis Int* 1997; 7: 1-6.
 2. Kupperman HS, Blatt MH, Wiesbader H, Filler W. Comparative clinical evaluation of estrogenic preparations by the menopausal and amenorrheal indices. *J Clin Endocrinol* 1953; 13: 688-703.
 3. Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 1991; 20: 47-63.

4. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsmann AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 2001; 344: 1434-41.
5. van der Vies J. Pharmacological studies with (7 α , 17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one (Org OD 14). *Maturitas* 1987; Suppl. 1: 15-24.
6. Bryant HU, Glasebrook AL, Yang NN, Sato M. A pharmacological review of raloxifene. *J Bone Miner Metab* 1996; 14: 1-9.
7. Eriksen EE, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL. Evidence of estrogen receptors in normal human osteoblast-like cells. *Science* 1988; 241: 84-6.
8. Oursler MJ, Osdoby P, Pyfferoen J, Riggs BL, Spelsberg TC. Avian osteoclasts as estrogen target cells. *Proc Natl Acad Sci USA*. 1991; 88: 6613-7.
9. Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M, Gustafsson JA. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997; 82: 4258-65.
10. Aguirre J, Buttery L, O'Shaughnessy M, Afzal F, Fernandez de Marticorena I, Hukkanen M, Huang P, MacIntyre I, Polak J. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, and defects in osteoblast maturation and activity. *Am J Pathol* 2001; 158: 247-57.
11. Manolagas SC, Jilka RL. Mechanisms of disease: bone marrow, cytokines and bone remodelling: emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 1995; 332: 305-11.
12. Heaney RP, Recker RR, Saville PD. Menopausal changes in calcium balance performance. *J Lab Clin Med* 1978; 92: 953-963.
13. Garnero P, Sornay-Rendu E, Chapuy MC, Delmas PD. Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *J Bone Miner Res* 1996; 11: 337-49.
14. Bjarnason NH, Hassager C, Christiansen C. Postmenopausal bone remodelling and hormone replacement. *Climacteric* 1998; 1: 72-9.
15. Vedi S, Compston JE. The effects of long-term hormone replacement therapy on bone remodelling in postmenopausal women. *Bone* 1996; 19: 535-9.
16. Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN, Hakeda Y, Kumegawa M. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* 1997; 186: 489-95.
17. Melton LJ III. How many women have osteoporosis now? *J Bone Miner Res* 1995; 10: 175-7.
18. Melton LJ III, Atkinson EJ, O'Fallon WJ, Wahner HW, Riggs BL. Long-term fracture prediction of by bone mineral assessed at different skeletal sites. *J Bone Miner Res* 1993; 8: 1227-33.
19. Kanis JA, Melton LJ III, Christiansen C, Johnston CC Jr., Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res* 1994; 9: 1137-41.
20. Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *Br Med J* 1996; 312: 1254-9.
21. Parfitt AM. Trabecular bone architecture in the pathogenesis and prevention of fracture. *Am J Med* 1987; 82: 68-72.
22. Genant HK, Engelke K, Fuerst T, Gluer CC, Grampp S, Harris ST, Jergas M, Lang T, Lu Y, Majumdar S, Mathur A, Takada M. Non-invasive assessment of bone mineral and structure: state of the art. *J Bone Miner Res* 1996; 11: 707-30.
23. Christgau S, Bitsch-Jensen O, Bjarnason NH, Henriksen EEG, Qvist P, Alexandersen P, Henriksen DB. Serum CrossLaps for monitoring the response in individuals undergoing anti-resorptive therapy. *Bone* 2000; 26: 505-11.
24. Wasnich RD, Miller PD. Antifracture efficacy of antiresorptive agents are related to changes in bone density. *J Clin Endocrinol Metab* 2000; 85: 231-6.
25. Baron R. Chapter 1. General principles of bone biology. Pg. 1-8 in "Primer on the metabolic bone diseases and disorders of mineral metabolism" 5th ed. Published by American Society for Bone and Mineral Research, 2003.
26. Schlemmer A, Hassager C, Pedersen BJ, Christiansen C. Posture, age, menopause and osteopenia do not influence the circadian variation in the urinary excretion of pyridinium crosslinks. *J Bone Miner Res* 1994; 9: 1883-8.
27. Bjarnason NH, Henriksen EEG, Alexandersen P, Christgau S, Henriksen DB, Christiansen C. Mechanism of variation in bone resorption. *Bone* 2002; 30: 307-13.
28. Henriksen DB, Alexandersen P, Bjarnason NH, Vilsbøll T, Hartmann B, Henriksen EEG, Byrjalsen I, Krarup T, Holst JJ, Christiansen C. Role of gastrointestinal hormones in postprandial reduction of bone resorption. *J Bone Miner Res* 2003; 18: 2180-2189.
29. Bjarnason NH, Christiansen C. Early response in biochemical markers predicts long-term response in bone mass during HRT in early postmenopausal women. *Bone* 2000; 26: 561-9.

30. Rosenquist C, Qvist P, Bjarnason NH, Christiansen C. Measurement of a more stable region of osteocalcin in human serum using two monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA). *Clin Chem* 1995; 41: 1439-45.
31. Bjarnason NH, Sarkar S, Duong T, Mitlak BH, Delmas PD, Christiansen C. 6 and 12 months changes in bone turnover are related to reduction in vertebral fracture risk during 3 years raloxifene treatment in postmenopausal osteoporosis. *Osteoporosis Int* 2001; 12: 922-30.
32. The writing group for the PEPi trial. Effects of hormone therapy on bone mineral density: results from the postmenopausal estrogen/progestin interventions (PEPi) trial. *JAMA* 1996; 276: 1389-96.
33. Bjarnason NH, Byrjalsen I, Hassager C, Haarbo J, Christiansen C. Low doses of estradiol in combination with gestodene to prevent early postmenopausal bone loss. *Am J Obstet Gynecol* 2000; 183: 550-60.
34. Speroff L, Rowan J, Symons J, Genant H, Wilborn W. The comparative effect on bone density, endometrium, and lipids of continuous hormones as replacement therapy (CHART study). A randomised controlled trial. *JAMA* 1996; 276: 1397-1403.
35. Alexandersen P, Byrjalsen I, Christiansen C. Piperazine oestrone sulphate and interrupted norethisterone in postmenopausal women: effects on bone mass, lipoprotein metabolism, climacteric symptoms, and adverse effects. *Br J Obstet Gynaecol* 2000; 107: 356-64.
36. Ettinger B, Genant HK, Steiger P, Madvig P. Low-dosage micronized 17 β -estradiol prevents bone loss in postmenopausal women. *Am J Obstet Gynecol* 1992; 166: 479-88.
37. Lindsay R, Hart DM, Clark DM. The minimum effective dose of estrogen for prevention of postmenopausal bone loss. *Obstet Gynecol* 1984; 63: 759-63.
38. Christiansen C, Riis BJ. 17 Beta-estradiol and continuous norethisterone: a unique treatment for established osteoporosis in elderly women. *J Clin Endocrinol Metab* 1990; 71: 836-41.
39. Lufkin EG, Wahner HW, O'Fallon WM, Hodgson SF, Kotowicz MA, Lane AW, Judd HL, Caplan RH, Riggs BL. Treatment of postmenopausal osteoporosis with transdermal estrogen. *Ann Intern Med* 1992; 117: 1-9.
40. Lindsay R, Hart DM, Forrest C, Baird C. Prevention of spinal osteoporosis in oophorectomized women. *Lancet* 1980; 2: 1151-3.
41. Mosekilde L, Beck-Nielsen H, Sørensen OH, Pors Nielsen S, Charles P, Vestergaard P, Hermann AP, Gram J, Hansen TB, Abrahamsen B, Ebbesen EN, Stilgren L, Jensen LB, Brot C, Hansen B, Tofteng CL, Eiken P, Kolthoff N. Hormonal replacement therapy reduces forearm fracture incidence in recent postmenopausal women – results of the Danish Osteoporosis Prevention Study. *Maturitas* 2000; 36: 181-93.
42. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA* 1998; 280: 605-13.
43. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J; Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomised controlled trial. *JAMA* 2002; 288: 321-33.
44. Bjarnason NH, Bjarnason K, Haarbo J, Rosenquist C, Christiansen C. Tibolone: prevention of bone loss in late postmenopausal women. *J Clin Endocrinol Metab* 1996; 81: 2419-22.
45. Lindsay R, Hart DM, Kraszewski A. Prospective double-blind trial of synthetic steroid (Org OD 14) for preventing postmenopausal osteoporosis. *Br Med J* 1980; 280: 1207-9.
46. Berning B, Kuijk CV, Kuiper JW, Bennink HJ, Kicovic PM, Fauser BC. Effects of two doses of tibolone on trabecular and cortical bone loss in early postmenopausal women: a two-year randomized, placebo-controlled study. *Bone* 1996; 19: 395-9.
47. Gallagher JC, Baylink DJ, Freeman R, McClung MR. Prevention of bone loss with tibolone in postmenopausal women: results of two randomized, double-blind, placebo-controlled dose-finding studies. *J Clin Endocrinol Metab* 2001; 86: 4717-26.
48. Draper MW, Flowers DE, Huster WJ, Neild JA, Harper KD, Arnaud C. A controlled trial of raloxifene (LY139481) HCl: impact on bone turnover and serum lipid profile in healthy postmenopausal women. *J Bone Miner Res* 1996; 11: 835-842.
49. Delmas PD, Bjarnason NH, Mitlak BH, Ravoux A-C, Shah AS, Huster WJ, Draper MW, Christiansen C. Effects of raloxifene on bone mineral density, serum cholesterol and uterine endometrium in postmenopausal women. *N Engl J Med* 1997; 337: 1641-7.
50. Johnston CC Jr, Bjarnason NH, Cohen FJ, Shah AS, Lindsay R, Mitlak BH, Huster W, Draper MW, Harper KD, Heath, H 3rd, Gennari C, Christiansen C, Arnaud C, Delmas PD. Long-term effects of raloxifene on bone mineral density, bone turnover, and serum lipid levels in early postmenopausal women: three-year data from 2 double-blind, randomized, placebo-controlled trials. *Arch Intern Med* 2000; 160: 3444-50.
51. Jolly EE, Bjarnason NH, Neven P, Plouffe L Jr., Johnston CC Jr., Watts SD, Arnaud CD, Mason TM, Crans G, Akers R, Draper MW. Prevention of osteoporosis and uterine effects in postmenopausal women taking raloxifene for 5 years. *Menopause* 2003; 10: 337-44.
52. Meunier PJ, Vignot E, Garnero P, Confavreux E, Paris E, Liu-Leage S, Sarkar S, Liu T, Wong M, Draper MW. Treatment of postmenopausal women with osteoporosis or low bone density with raloxifene. Raloxifene Study Group. *Osteoporosis Int* 1999; 10: 330-6.
53. Ettinger B, Black DM, Mitlak BH, Knickerbocker RK, Nickelsen T, Genant HK, Christiansen C, Delmas PD, Zanchetta JR, Stakkestad J, Glüer CG, Krueger K, Cohen FJ, Eckert S, Ensrud KE, Avioli LV, Lips P, Cummings SR for the MORE Investigators. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene. *JAMA* 1999; 282: 637-645.
54. Delmas PD, Ensrud KE, Adachi JD, Harper KD, Sarkar S, Gennari C, Reginster JY, Pols HA, Recker RR, Harris ST, Wu W, Genant HK, Black DM, Eastell R. Multiple outcomes of raloxifene evaluation investigators. Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial. *Clin Endocrinol Metab* 2002; 87: 3609-17.
55. Barrett-Connor E, Stuenkel C. Hormones and heart disease in women: Heart and estrogen/progestin replacement study in perspective. *J Clin Endocrinol Metab* 1999; 84: 1848-53.
56. Tunstall-Pedoe H. Myth and paradox of coronary risk and the menopause. *Lancet* 1998; 351: 1425-1427.
57. Jensen J, Nilas L, Christiansen C. Influence of menopause on serum lipids and lipoproteins. *Maturitas* 1990; 12: 321-31.
58. Mosca L. The role of hormone replacement therapy in the prevention of postmenopausal heart disease. *Arch Int Med* 2000; 160: 2263-72.
59. Do KA, Green A, Guthrie JR, Dudley EC, Burger HG, Dennerstein L. Longitudinal study of risk factors for coronary heart disease across the menopausal transition. *Am J Epidemiol* 2000; 151: 584-93.
60. Gebara OCE, Mittlemann MA, Sutherland P, Lipinska I, Matheny T, Xu P, Welty FK, Wilson PWF, Levy D, Muller JE, Tofler GH. Association between increased estrogen status and increased fibrinolytic potential in the Framingham offspring study. *Circulation* 1995; 91: 1952-8.
61. Kannel WB, Wolf PA, Castelli WP, D'Agostino RB. Fibrinogen and the risk of cardiovascular disease: the Framingham study. *JAMA* 1987; 258: 1183-6.
62. Obasanjo IO, Clarkson TB, Weaver DS. Effects of the anabolic steroid nandrolone decanoate on plasma lipids and coronary arteries of female cynomolgus macaques. *Metabolism* 1996; 45: 463-8.
63. Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Christiansen C. Raloxifene inhibits aortic accumulation of cholesterol in ovariectomized, cholesterol-fed rabbits. *Circulation* 1997; 96: 1964-9.
64. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999; 340: 1801-11.
65. White RE, Darkow DJ, Lang JLF. Estrogen relaxes coronary arteries by BK_{Ca} channels through a cGMP-dependent mechanism. *Circ Res* 1995; 77: 936-42.
66. Guetta V, Quyyumi AA, Prasad A, Panza JA, Waclawiw M, Cannon RO 3rd. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. *Circulation* 1997; 96: 2795-2801.
67. Nabulsi AA, Folsom AR, White A, Patsch W, Heiss G, Wu KK, Szklo M for the Atherosclerosis Risk in Communities Study Investigators. Association of hormone replacement with various cardiovascular risk factors in postmenopausal women. *N Engl J Med* 1993; 328: 1069-75.
68. Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999; 340: 115-25.
69. Zaman AG, Helft G, Worthley SG, Badimon JJ. The role of plaque rupture and thrombosis in coronary artery disease. *Atherosclerosis* 2000; 149: 251-66.
70. Levine GN, Keaney JF Jr., Vita JA. Cholesterol reduction in cardiovascular disease: clinical benefits and possible mechanisms. *N Engl J Med* 1995; 332: 512-21.
71. LaRosa JC, He J, Vupputuri S. Effect of statins on risk of coronary disease: a meta-analysis of randomized controlled trials. *JAMA* 1999; 282: 2340-6.
72. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; 18: 499-502.
73. Thompson SG, Kienast J, Pyke SDM, Haverkate F, van de Loo JCW for the European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. *N Engl J Med* 1995; 332: 635-41.
74. Becker U, Bartl K, Wahlefeld AW. A functional photometric assay for plasma fibrinogen. *Thromb Res* 1984; 35: 475-84.
75. Hesse R, Tritschler W, Castelfranchi G, Bablok W. Antithrombin III: Referenzwerte mit einem chromogenen substrat (Chromozym TH). *Blut* 1981; 42: 227-34.
76. Hamsten A. Hemostatic function and coronary heart disease. *N Engl J Med* 1995; 332: 677-8.
77. Bergsdorf N, Nilsson T, Wallén P. An enzyme linked immunosorbent assay for determination of tissue plasminogen activator applied to

- patients with thromboembolic disease. *Thromb Haemostat* 1983; 50: 740-4.
78. Declerck PJ, Alessi M-C, Verstreken M, Kruithof EKO, Juhan-Vague I, Collen D. Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood* 1988; 71: 220-5.
 79. Eriksson E, Rånby M, Gyzander E, Risberg B. Determination of plasminogen activator inhibitor in plasma using t-PA and a chromogenic single-point poly-D-lysine stimulated assay. *Thromb Res* 1988; 50: 91-101.
 80. Haarbo J, Leth-Espensen P, Stender S, Christiansen C. Estrogen monotherapy and combined estrogen-progestogen replacement therapy attenuate aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *J Clin Invest* 1991; 87: 1274-9.
 81. Haarbo J, Svendsen OL, Christiansen C. Progestogens do not affect aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. *Circ Res* 1992; 70: 1198-1202.
 82. Kushawa RS, Lewis DS, Carey KD, McGill HC. Effects of estrogen and progesterone on plasma lipoproteins and experimental atherosclerosis in the baboon (*Papio Sp.*). *Arterioscler Thromb* 1991; 11: 23-31.
 83. Clarkson TB, Adams MR, Kaplan JR, Shively CA, Koritnik DR. From menarche to menopause: coronary artery atherosclerosis and protection in cynomolgus monkeys. *Am J Obstet Gynecol* 1989; 160: 1280-5.
 84. Alexandersen P, Haarbo J, Breinholt V, Christiansen C. Dietary phytoestrogens and estrogen inhibit experimental atherosclerosis. *Climacteric* 2001; 4:151-9.
 85. Arad Y, Badimon JJ, Badimon L, Hembree WC, Ginsberg HN. Dehydroepiandrosterone feeding prevents aortic fatty streak formation and cholesterol accumulation in cholesterol-fed rabbits. *Arteriosclerosis* 1989; 9: 159-66.
 86. Thorpe SM. Steroid receptors in breast cancer: sources of inter-laboratory variation in dextran-charcoal assays. *Breast Cancer Res Treat* 1987; 9: 175-89.
 87. Haarbo J, Hassager C, Jensen SB, Riis BJ, Christiansen C. Serum lipids, lipoproteins and apolipoproteins during postmenopausal estrogen replacement therapy combined with either 19-nortestosterone derivatives or 17-hydroxyprogesterone derivatives. *Am J Med* 1991; 90: 584-9.
 88. Haarbo J, Christiansen C. Treatment-induced cyclic variation in serum lipids, lipoproteins, and apolipoproteins after 2 years of combined hormone replacement therapy: exaggerated cyclic variations in smokers. *Obstet Gynecol* 1992; 80: 639-44.
 89. Walsh BW, Kuller LH, Wild RA, Paul S, Farmer M, Lawrence JB, Shah AS, Anderson PW. The effect of raloxifene on serum lipids and coagulation factors in healthy, postmenopausal women. *JAMA* 1998; 279: 1445-51.
 90. Koh KK, Mincemoyer R, Bui MN, Csako G, Pucino F, Guetta V, Wacławiw M, Cannon RO III. Effects of hormone-replacement therapy on fibrinolysis in postmenopausal women. *N Engl J Med* 1997; 336: 683-90.
 91. Haarbo J, Christiansen C. The impact of female sex hormones on secondary prevention of atherosclerosis in ovariectomized, cholesterol-fed rabbits. *Atherosclerosis* 1996; 123: 39-44.
 92. Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Christiansen C. Raloxifene and estrogen reduces progression of established atherosclerosis – a study in ovariectomized, cholesterol-fed rabbits. *Atherosclerosis* 2001; 154: 97-102.
 93. Holm P, Andersen HL, Andersen MR, Erhardtson E, Stender S. The direct antiatherogenic effect of estrogen is present, absent, or reversed depending on the state of the arterial endothelium. A time course study in cholesterol-clamped rabbits. *Circulation* 1999; 100: 1727-33.
 94. Hanke H, Hanke S, Bruck B, Brehme U, Gugel N, Finking G, Muck AO, Schmahl FW, Hombach V, Haasis R. Inhibition of the protective effect of estrogen by progesterone in experimental atherosclerosis. *Atherosclerosis* 1996; 121: 129-38.
 95. Alexandersen P, Haarbo J, Sandholdt I, Shalmi M, Lawaetz H, Christiansen C. Norethindrone acetate enhances the antiatherogenic effect of 17 β estradiol. *Arterioscler Thromb Vasc Biol* 1998; 18: 902-7.
 96. Bjarnason NH, Bjarnason K, Haarbo J, Coelingh Bennink HJT, Christiansen C. Tibolone: Influence on markers of cardiovascular disease. *J Clin Endocrinol Metab* 1997; 82: 1752-6.
 97. Farish E, Barnes JF, Rolton HA, Spowart K, Fletcher CD, Hart DM. Effect of tibolone on lipoprotein(a) and HDL subfractions. *Maturitas* 1995; 20: 215-9.
 98. Rymer J, Crook D, Sidhu M, Chapman M, Stevenson JC. Effects of tibolone on serum concentration of lipoprotein(a) in postmenopausal women. *Acta Endocrinol (Copenh.)* 1993; 128: 259-62.
 99. Haenggi W, Riesen W, Birkhaeuser MH. Postmenopausal hormone replacement therapy with tibolone decreases serum lipoprotein(a). *Eur J Clin Chem Biochem* 1993; 31: 645-50.
 100. Zandberg P, Peters JLM, Demacker PNM, Smit MJ, de Reeder EG, Meuleman DG. Tibolone prevents atherosclerotic lesion formation in cholesterol-fed, ovariectomized rabbits. *Arterioscler Thromb Vasc Biol* 1998; 18: 1844-1854.
 101. Bjarnason NH, Haarbo J, Byrjalsen I, Kauffman RF, Knadler MP, Christiansen C. Raloxifene reduces atherosclerosis: studies of optimized raloxifene doses in ovariectomized, cholesterol-fed rabbits. *Clin Endocrinol (Oxf)* 2000; 52: 225-33.
 102. Zandberg P, Peters JLM, Demacker PN, de Reeder EG, Smit MJ, Meuleman DG. Comparison of the antiatherosclerotic effect of tibolone with that of estradiol and ethinyl estradiol in cholesterol-fed, ovariectomized rabbits. *Menopause* 2001; 8: 96-105.
 103. Clarkson TB, Anthony MS, Wagner JD. A comparison of tibolone and conjugated equine estrogens effects on coronary artery atherosclerosis and bone density of postmenopausal monkeys. *J Clin Endocrinol Metab* 2001; 86: 5396-404.
 104. Clarkson TB, Anthony MS, Jerome CP. Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys. *J Clin Endocrinol Metab* 1998; 83: 721-726.
 105. Bryant HU, Kauffman RF, Iversen P, Cox DA, Mitlak BH, Heath H 3rd. Comment on: Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys. Authors' response by Clarkson TB, Anthony MS. *J Clin Endocrinol Metab* 1998; 83: 3001-3004.
 106. Barrett-Connor E, Grady D, Sashegyi A, Anderson PW, Cox DA, Hozowski K, Rautaharju P, Harper KD; MORE Investigators (Multiple Outcomes of Raloxifene Evaluation). Raloxifene and cardiovascular events in osteoporotic postmenopausal women: four-year results from the MORE (Multiple Outcomes of Raloxifene Evaluation) randomized trial. *JAMA* 2002; 287: 847-857.
 107. Wenger NK, Barrett-Connor E, Collins P, Grady D, Kornitzer M, Mosca L, Sashegyi A, Baygani SK, Anderson PW, Moscarelli E for the RUTH study investigators. Baseline characteristics of participants in the raloxifene use for the heart (RUTH) trial. *Am J Cardiol* 2002; 90: 1204-10.
 108. Cauley JA, Lucas FL, Kuller LH, Stone K, Browner W, Cummings SR. Elevated serum estradiol and testosterone concentrations are associated with a high risk of breast cancer. *Ann Intern Med* 1999; 130: 270-277.
 109. Greendale GA, Reboussin BA, Sie A, Singh HR, Olson LK, Gatewood O, Bassett LW, Wasilaukas C, Bush T, Barrett-Connor E. Effects of estrogen and estrogen-progestin on mammographic parenchymal density. Postmenopausal estrogen/progestin interventions (PEPI) investigators. *Ann Intern Med* 1999; 130: 262-269.
 110. Brisson J, Morrison AS, Khalid N. Mammographic parenchymal features and breast cancer in the breast cancer detection demonstration project. *J Natl Cancer Inst* 1988; 80: 1534-40.
 111. Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003; 362: 419-27.
 112. Collaborative group on hormonal factors in breast cancer. Breast cancer and hormone replacement therapy: collaborative reanalyses of data from 51 epidemiological studies of 52705 women with breast cancer and 108411 women without breast cancer. *Lancet* 1997; 350: 1047-1059.
 113. Colditz GA, Hankinson SE, Hunter DJ, Willett WC, Manson JE, Stampfer MJ, Hennekens C, Rosner B, Speizer FE. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. *N Engl J Med* 1995; 332: 1589-1593.
 114. Chetrite GS, Kloosterboer HJ, Philippe J-C, Pasqualini JR. Effect of Org OD14 (LIVIAL) and its metabolites on human estrogen sulphotransferase activity in the hormone-dependent MCF-7 and T-47D, and the hormone independent MDA-MB-231, breast cancer cell lines. *Anti-cancer Research* 1999; 19: 269-276.
 115. Colacurci N, Mele D, De Franciscis P, Costa V, Fortunato N, De Seta L. Effects of tibolone on the breast. *Eur J Obstet Gynecol Reprod Biol* 1998; 80: 235-238.
 116. Erel CT, Elter K, Akman C, Ersavasti G, Altug A, Seyisoglu H, Ertungalp E. Mammographic changes in women receiving tibolone therapy. *Fertil Steril* 1998; 69: 870-875.
 117. Christodoulakos GE, Lambrinoudaki IV, Vourtsi AD, Panoulis KP, Kelekis DA, Creatas GC. Mammographic changes associated with raloxifene and tibolone therapy in postmenopausal women: a prospective study. *Menopause*. 2002; 9: 110-6
 118. Gottardis MM, Jordan VC. Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Res* 1987; 47: 4020-4.
 119. Freedman M, San Martin J, O'Gorman J, Eckert S, Lippman ME, Lo SC, Walls EL, Zeng J. Digitized mammography: a clinical trial of postmenopausal women randomly assigned to receive raloxifene, estrogen, or placebo. *J Natl Cancer Inst* 2001; 93: 51-6.
 120. Cummings SR, Eckert S, Krueger K, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glasman JE, Costa A, Jordan VC. The effect of raloxifene on risk of breast cancer in postmenopausal women. Results from the MORE randomized trial. *JAMA* 1999; 281: 2189-97.
 121. Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, Farrerons J, Karasik A, Mellstrom D, Ng KW, Stepan JJ, Powles TJ, Morrow M, Costa A, Silfen SL, Walls EL, Schmitt H, Muchmore DB, Jordan

- VC, Ste-Marie LG. Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. *Breast Cancer Res Treat* 2001; 65: 125-34.
122. Grady D, Gebretsadik T, Kerlikowske K, Ernster V, Petitti D. Hormone replacement therapy and endometrial cancer risk: a meta-analysis. *Obstet Gynecol* 1995; 85: 304-13.
 123. Ferenczy A. Patophysiology of endometrial bleeding. *Maturitas* 2003; 45: 1-14.
 124. Byrjalsen I, Bjarnason NH, Christiansen C. Progestational effects of combinations of gestodene on the postmenopausal endometrium during hormone replacement therapy. *Am J Obstet Gynecol* 1999; 180: 539-49.
 125. Byrjalsen I, Alexandersen P, Christiansen C. Piperazine oestrone sulphate and interrupted norethisterone: effects of the postmenopausal endometrium. *Br J Obstet Gynaecol* 2000; 107: 356-64.
 126. Rymer J, Fogelman I, Chapman MG. The incidence of vaginal bleeding with tibolone treatment. *Br J Obstet Gynaecol* 1994; 101: 53-6.
 127. Ederveen AG, Kloosterboer HJ. Tibolone exerts its protective effect on trabecular bone loss through the estrogen receptor. *J Bone Miner Res* 2001; 16: 1651-7.
 128. Yang NN, Venugopalan M, Hardikar S, Glasebrook A. Identification of an estrogen response element activated by metabolites of 17 β -estradiol and raloxifene. *Science* 1996; 273: 1222-5.
 129. Bone HG, Greenspan SL, McKeever C, Bell N, Davidson M, Downs RW, Emkey R, Meunier PJ, Miller SS, Mulloy AL, Recker RR, Weiss SR, Heyden N, Musliner T, Suryawanshi S, Yates AJ, Lombardi A. Alendronate and estrogen effects in postmenopausal women with low bone mineral density. Alendronate/Estrogen Study Group. *J Clin Endocrinol Metab* 2000; 85: 720-6.
 130. Johnell O, Scheele WH, Lu Y, Reginster JY, Need AG, Seeman E. Additive effects of raloxifene and alendronate on bone density and biochemical markers of bone remodeling in postmenopausal women with osteoporosis. *J Clin Endocrinol Metab* 2002; 87: 985-92.
 131. Bagger YZ, Tanko LB, Alexandersen P, Ravn P, Christiansen C. Alendronate has a residual effect on bone mass in postmenopausal Danish women up to 7 years after treatment withdrawal. *Bone* 2003; 33: 301-7.
 132. Meunier PJ, Boivin G. Bone mineral density reflects bone mass but also the degree of mineralization of bone: therapeutic implications. *Bone* 1997; 21: 373-7.
 133. Riggs BL, Hartmann LC. Selective estrogen-receptor modulators – mechanisms of action and application to clinical practice. *N Engl J Med* 2003; 348: 618-29.
 134. Bjarnason NH, Bjarnason K, Hassager C, Christiansen C. The response in spinal bone mass to tibolone treatment is related to bone turnover in elderly women. *Bone* 1997; 20: 151-5.
 135. Hochberg MC, Ross PD, Black D, Cummings SR, Genant HK, Nevitt MC et al. Larger increases in bone mineral density during alendronate therapy are associated with a lower risk of new vertebral fracture in women with postmenopausal osteoporosis. *Arthritis & Rheumatism* 1999; 42: 1246-54.
 136. Figtree GA, Lu Y, Webb CM, Collins P. Raloxifene acutely relaxes rabbit coronary arteries in vitro by an estrogen receptor-dependent and nitric oxide-dependent mechanism. *Circulation* 1999; 100: 1095-1101.
 137. Grodstein F, Stampfer MJ, Manson JE, Colditz GA, Willett WC, Rosner B, Speizer FE, Hennekens CH. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. *N Engl J Med* 1996; 335: 453-61.
 138. Grodstein F, Stampfer M, Colditz GA, Willett WC, Manson JE, Joffe M, Rosner B, Fuchs C, Hankinson SE, Hunter DJ, Hennekens CH, Speizer FE. Postmenopausal hormone therapy and mortality. *N Engl J Med* 1997; 336: 1769-75.
 139. Barrett-Connor E. Hormone replacement therapy. *Br J Med* 1998; 317: 457-61.
 140. Herrington DM. The HERS trial results: paradigms lost? *Ann Intern Med* 1999; 131: 463-466.
 141. Herrington DM, Reboussin DM, Brosnihan KB, Sharp PC, Shumaker SA, Snyder TE, Furberg CD, Kowalchuk GJ, Stuckey TD, Rogers WJ, Givens DH, Waters D. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med* 2000; 343: 522-529.
 142. Cherry N, Gilmour K, Hannaford P, Heagerty A, Khan MA, Kitchener H, McNamee R, Elstein M, Kay C, Seif M, Buckley H; ESPRIT team. Oestrogen therapy for prevention of reinfarction in postmenopausal women: a randomised placebo controlled trial. *Lancet* 2002; 360: 2001-08.
 143. Hodis HN, Mack WJ, Azen SP, Lobo RA, Shoupe D, Mahrer PR, Faxon DP, Cashin-Hemphill L, Sanmarco ME, French WJ, Shook TL, Gaarder TD, Mehra AO, Rabbani R, Sevianian A, Shil AB, Torres M, Vogelbach KH, Selzer RH; Women's Estrogen-Progestin Lipid-Lowering Hormone Atherosclerosis Regression Trial Research Group. Hormone therapy and the progression of coronary artery atherosclerosis in postmenopausal women. *N Engl J Med* 2003; 349: 535-45.
 144. Walsh BW, Paul S, Wild RA, Dean RA, Tracy RP, Cox DA, Anderson PW. The effects of hormone replacement therapy and raloxifene on C-reactive protein and homocysteine in healthy postmenopausal women: a randomized, controlled trial. *J Clin Endocrinol Metab* 2000; 85: 214-8.
 145. Gambacciani M, Rosano GM, Monteleone P, Fini M, Genazzani AR. Clinical relevance of the HERS trial. *Lancet* 2002; 360: 641.
 146. Bjarnason NH, Christiansen C. The influence of thinness and smoking on bone loss and response to HRT in early postmenopausal women. *J Clin Endocrinol Metab* 2000; 85: 590-6.
 147. Tanko LB, Bagger YZ, Alexandersen P, Larsen PJ, Christiansen C. Peripheral adiposity exhibits an independent dominant antiatherogenic effect in elderly women. *Circulation* 2003; 107: 1626-31.
 148. Herrington DM, Howard TD, Hawkins GA, Reboussin DM, Xu J, Zheng SL, Brosnihan KB, Meyers DA, Blecker ER. Estrogen-receptor polymorphisms and effects on high density lipoprotein cholesterol in women with coronary disease. *N Engl J Med* 2002; 346: 967-74.
 149. ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial. Major outcomes in moderately hypercholesterolemic, hypertensive patients randomized to pravastatin vs usual care: The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT-LLT). *JAMA* 2002; 288: 2998-3007.
 150. Hall JM, McDonnell DP. The estrogen receptor β -isoform (ER β) of the human estrogen receptor modulates ER α transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* 1999; 140: 5566-78.
 151. Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. *Science* 2002; 295: 2465-8.