Digitized assessment of mammographic breast density in patients who received low-dose intrauterine levonorgestrel in continuous combination with oral estradiol valerate: a pilot study

Eva Lundström, M.D.,[a] *Gunnar Söderqvist, M.D., Ph.D.,*[a] *Gunilla Svane, M.D., Ph.D.,*[b] *Edward Azavedo, M.D., Ph.D.,*[b] *Matts Olovsson, M.D., Ph.D.,*[c] *Lambert Skoog, M.D., Ph.D.,*[d] *Eva von Schoultz, M.D., Ph.D.,*[e] *and Bo von Schoultz, M.D., Ph.D.*[a]

Departments of [a] Obstetrics and Gynecology, [b] Radiology, [d] Pathology and Cytology, and [e] Oncology, Radiumhemmet, Karolinska University Hospital, Stockholm; and [c] Department of Women's and Children's Health and The Centre for Reproductive Biology, Uppsala, Sweden

Objective: To perform a pilot study of the effects on the breast by low-dose intrauterine progestogen combined with estrogen.
Design: A prospective pilot study.
Setting: University hospital.
Patient(s): Twenty postmenopausal women without any previous breast disorder.
Intervention(s): Women were treated with a low-dose intrauterine system releasing 20 μg/24 hours of levonorgestrel in continuous combination with 2 mg of oral E_2 valerate. The effects on mammographic breast density, breast cell proliferation, and hormonal levels were followed for 18 months.
Main Outcome Measure(s): Change in mammographic breast density and breast cell proliferation. Correlations with levels of hormones, growth factors, and binding proteins.
Result(s): Three women showed an apparent increase in density. For the remaining 17 women the changes were only a few percent. Digitized assessment of density showed strong correlations with visual classification scales ($r_s = 0.96–0.97$). There was no increase in proliferation as expressed by the percentage of MIB-1-positive breast cells in fine-needle aspiration biopsies. Increase in breast density displayed a positive correlation with patients age ($r_s = 0.52$) and an inverse relationship with levels of E_2 ($r_s = -0.50$) and free T ($r_s = -0.50$).
Conclusion(s): Low-dose intrauterine administration progestogen may develop into an attractive alternative for hormonal therapy in postmenopausal women as endometrial protection may be achieved at very low systemic levels. (Fertil Steril® 2006;85:989–95. ©2006 by American Society for Reproductive Medicine.)

Key Words: Mammographic breast density, low-dose progestogen, intrauterine system, continuous combined hormone therapy

There is accumulating evidence that progestogens are mitogenic to the human breast when given long term in combination with estrogen to postmenopausal women (1). Combined estrogen (E)/progestogen treatment has been shown to increase the risk of breast cancer but the effect of E alone is much more uncertain (2, 3). In fact, within the large prospective Women's Health Initiative trial E alone for a mean duration of 7 years showed no risk increase but rather a tendency toward a protective effect (4).

Mammographic breast density and breast cell proliferation could be regarded as surrogate markers for the risk of cancer (5, 6). Epidemiological studies have repeatedly shown increased mammographic breast density to be a strong and independent risk factor. Density reveals the effect of the intrinsic hormonal environment and its background genetics on the breast (7, 8). The basis of risk associated with hormonal therapies may lie in the regulation of cell proliferation. Within populations of cells in vitro and in vivo a higher rate of cell proliferation may increase the risk of transformation to the neoplastic phenotype (6).

In animal models as well as in women, breast cell proliferation has been found to increase in short-term studies on hormone therapy (HT) (9, 10). An increase in mammographic breast density has also been reported to occur in a significant number of women during conventional HT (11–13). The increase in both proliferation and density has been shown to be more pronounced during combined E/progestogen treatment than for E only.

The effect of progestogens may well differ according to dosage, route of administration, and the estrogenic environment (14, 15). The current discussion about the potential

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Reprint requests: Eva Lundström, M.D., Department of Obstetrics and Gynecology, Karolinska University Hospital, SE-171 76 Stockholm, Sweden (FAX: 46-8-318-114; E-mail: eva.lundstrom@karolinska.se).

adverse effects of progestogen on the breast has stimulated interest in alternative treatment regimens. There are no previous data about the influence on the breast by very low doses of progestogen in combination with E_2.

The levonorgestrel (LNG) releasing intrauterine system (IUS) (Mirena; Leiras Oy/Schering AG, Turku, Finland) has been suggested as an alternative to oral progestogens for postmenopausal hormonal treatment and protection of the endometrium. In combination with E this intrauterine system has been shown to provide bleeding control and effective endometrial suppression (16, 17). In women using the 20 μg/24 hours LNG–IUS the circulating levels of LNG are about 10-fold lower than during oral postmenopausal treatment with, for example, 2 mg of E_2 valerate (E_2V) and 250 μg of LNG (Cycloprogynova; Schering AG, Berlin, Germany) (17).

Therefore, we performed a pilot study on the effects of this treatment on the breast. A group of 20 postmenopausal women were investigated during combined treatment with 2 mg of oral E_2V daily (Progynova) and the 20 μg/24 hours LNG–IUS (Mirena). The effects on mammographic breast density, breast cell proliferation, and hormonal levels were followed for 18 months.

MATERIALS AND METHODS

The study was performed at the Karolinska University Hospital in Sweden and was approved by the independent Ethics Committee (IRB project 96-159). All women gave their written, informed consent before participation in the study.

Subjects

Postmenopausal women aged 50–65 years with the last menstrual bleeding $\geq$12 months before the study and FSH levels $>$35 IU/L were recruited. Women who had used any hormonal treatment in the previous 3 months were excluded. All women were apparently healthy and those with current or a history of thromboembolic disease, breast and endometrial disease, cardiac disorders, liver disease or phorphyria, and those with any uterine malformation were excluded. During the study the use of any other sex steroids, barbiturates, primidone, carbamazepine, rifampicin, griseofulvin, or warfarin was prohibited.

Study Treatments

Twenty women were treated with oral 2 mg of E_2V daily (Progynova; Schering AG) in combination with a low-dose intrauterine system releasing 20 μg/24 hours LNG (Mirena).

Mammographic Breast Density

Mammographic examinations where performed in accordance with the Quality Control Regulations as stipulated by the Swedish National Board of Health and Welfare and the Swedish National Radiation Protection Institute.

Mammograms were obtained at baseline and at 6 and 18 months to determine breast density and to evaluate any abnormalities. Mammographic examinations comprised mediolateral oblique and craniocaudal views of both breasts. Only the mediolateral oblique view of the left breast was used for the visual classifications of breast density. Previous studies have shown very little difference between the left and right breast in the response to hormonal treatment (11, 18). For technical reasons (e.g., to avoid the pectoral muscle when the assessable area was defined) the craniocaudal view was used for the digitized assessment. All mammograms were assessed by two independent radiologists (G.S., E.A.) who were blinded to treatments. Any differences of opinion in the classification of a mammogram were discussed and resolved with a consensus result.

Visual Classification. Mammographic density of all coded films was classified according to the Wolfe classification (19) in four categories: N1, essentially normal breast with a parenchyma composed primarily of fat and with, at most, a few fibrous connective tissue strands; P1, prominent ductal pattern in up to one-fourth of the breast volume; P2, prominent ductal pattern in more than one-fourth of the breast volume; and DY, extremely dense parenchyma, which usually denotes connective tissue hyperplasia. In addition to the Wolfe classification, for each individual woman, all coded films were classified according to a percentage scale (12, 13) with five categories of the amount of dense breast parenchyma in relation to the whole breast volume. The five categories were: 0%–20%, 21%–40%, 41%–60%, 61%–80%, and 81%–100%.

Digitized Breast Density. In addition to visual judgment and classification a computer-based quantitative assessment was performed. The identifying data were removed from the films and the operator (E.L.) was unaware of the patient's identity and duration of treatment.

All films were digitized and the dense area of the left craniocaudal view image was measured by using a computer-assisted program (Cumulus, Sierra plus, Vidar Systems Corporation, Medical Imaging, Herndon, VA) (20, 21). In this procedure the operator establishes "thresholds" for the edge of the breast and the edge of dense tissue. A computer then records the number of pixels in the digitized image that fall within the defined areas.

This method of measurement has been shown to give highly reproducible results and details have been given elsewhere (20, 21). In the present study the intra-assay variation was 8%, as calculated from five repeated measurements in five different mammograms (i.e., a total of 25 mammograms). Each value for density was calculated as the mean of three measurements.

Breast Cell Proliferation

Percutanous fine needle aspiration (FNA) biopsies from the upper outer quadrant of the left breast were performed at

baseline and after 2, 6, and 18 months of treatment. As in previous studies (10, 22) this area was chosen due to on average a higher amount of breast epithelium in this location (23). In a previous analysis of 10 different locations in the macaque breast, there were no significant differences with respect to proliferative activity or receptor expression (24). The FNA biopsies were performed after mammography using a needle with an outer diameter of 0.6 mm as described by Franzén and Zajicek (25) and Skoog et al. (26). To produce several identical slides, the aspirated cells were mixed with 0.5 mL of 4% buffered (pH 7.4) formalin in the syringe used to procure cells. Volumes of 110 μL were centrifugated at 700 rpm for 3 minutes and the cells were sedimented onto pretreated glass slides.

Immunocytochemical Analysis

Immunostained cells were quantified using cell counting by two observers unaware of the patient's identity and duration of treatment. Slides were stained for the nuclear antigen Ki-67. The Ki-67/MIB-1 monoclonal antibody reacts with a human nuclear antigen, which is present in proliferating cells but absent in quiescent cells. Cell cycle analysis shows that the antigen is expressed in the phases of G_1, S, G_2, and mitosis (27). The MIB-1 analyses were performed using reagents supplied by Immunotech (Marseilles, France). The staining procedure uses an avidin-biotin peroxidase system, modified for the cytospin technique. We considered samples obtained by FNA to be assessable only if they contained intact cells and no free-lying nuclei. On average 150–200 cells were counted per slide and in all cases a minimum of 40 cells were scored.

Body Composition

Bone mineral area density (in grams per centimeter squared) and total lean and fat mass were determined from the whole body with dual energy X-ray absorptiometry measurements, by using Lunar model DPX-L equipment (Lunar Radiation, Madison, WI) at baseline and after 6 and 18 months. From the whole body dual energy X-ray absorptiometry measurements spinal bone mineral area density was extracted.

Analytical Methods

Venous blood samples were drawn on the day of FNA. Serum concentrations of E_2-17β (E_2) and sex hormone-binding globulin (SHBG) were determined by direct chemiluminescence enzyme immunoassay and T by direct radioimmunoassay using commercial kits (Immulite and Coat-a-Count Testosterone) obtained from Diagnostic Products Corporation, Los Angeles, CA.

Serum levels of PRL and FSH were determined by time-resolved fluorescence immunoassay, using commercial kits from Wallac Oy (Autodelfia; Turku, Finland).

Concentrations were expressed as micrograms per liter of the third PRL IRP 84/500 and as units per liter of the second FSH IRP 78/549.

Insulin-like growth factor I (IGF-I was determined by chemiluminescence enzyme immunoassay using a commercial kit (Advantage) obtained from Nichols Products Corporation. San Juan Capistrano, CA.

Insulin-like growth factor binding protein-3 (IGFBP-3) was analyzed by ELIZA using a commercial kit obtained from Diagnostic Systems Laboratories Inc. (Webster, Texas).

The detection limits and within and between-assay coefficients of variation were for E_2: 73 pmol/L, 8%, and 9%; SHBG: 0.05 nmol/L, 4%, and 8%; T: 0.1 nmol/L, 6%, and 10%; PRL: 0.04 μg/L, 2%, and 4%; FSH: 0.05 IU/L, 2%, and 3%; IGF- I, 6 μg/L, 4.8%, and 6.7%; and for IGFBP-3: 0.04 ng/mL, 9%, and 10%.

Apparent concentrations of free T were calculated from values for total T; SHBG and a fixed albumin concentration of 40 g/L by successive approximation using a computer program based on an equation system derived from the law of mass action (28).

Plasma levels of LNG were determined by radioimmunoassay after extraction with diethyl ether according to Weiner and Johansson (29) with slight modifications according to Olsson (30). Anti-LNG 11α-hemisuccinate-bovine serum albumin (rabbit) and tracer (15, 16 [^{3}H]-*d*-norgestrel, specific activity 30–50 Ci/mmol) were obtained from Schering AG, Berlin, Germany. Because of a small plasma blank, the practical detection limit was 0.08 nmol/L. No corrections were made for procedural losses, nor were the plasma blanks subtracted. The extraction recovery was 89%–95%. With the extraction volume of 200 μL, the detection limit was 0.16 nmol/L. For random samples the within-assay coefficient of variation was 9% for samples $<$1.0 nmol/L and 6% for those $>$1.0 nmol/L. The between-assay coefficient of variation was 11% for samples $<$1.0 nmol/L and 8% for those $>$1.0 nmol/L. The antibody did not crossreact with any naturally occurring steroids.

Other Assessments

Physical examination including a pelvic examination was carried out before entering the study and after 6 and 18 months of treatment. Endometrial thickness was measured by vaginal ultrasound and endometrial biopsies (Endorette; Cooper Surgical Inc., Trumbull, CT) were performed at each visit. A cervical smear was carried out at baseline. The women were questioned about any untoward medical events and breast symptoms at baseline and at visits after 6 and 18 months.

Statistical Analyses

Differences were analyzed by the Wilcoxon signed rank test. Correlations were assessed by the Spearman's rank correlation test. A P value of $<.05$ was considered as significant.

TABLE 1

Serum levels (mean ± SEM) of hormones, growth factors, and binding proteins at baseline and after 2, 6, and 18 months in 20 postmenopausal women during treatment with oral E_2V (2 mg)/LNG-IUS (20 μg/24 hours).

	Baseline (n = 20)	2 mo (n = 20)	6 mo (n = 20)	18 mo (n = 18)
FSH (IU/L)	66.3 ± 4.4	32.9 ± 4.2	34.3 ± 4.8	35.6 ± 4.9[c]
E_2 (pmol/L)	43.4 ± 4.6	195.5 ± 25.6	214.6 ± 28.0	199.7 ± 21.4[c]
T (nmol/L)	0.6 ± 0.05	0.5 ± 0.05	0.5 ± 0.05	0.7 ± 0.02 ns
Free T (pmol/L)	9 ± 1	7 ± 1	7 ± 1	7 ± 3[a]
SHBG (nmol/L)	54.4 ± 4.1	66.3 ± 3.1	69.1 ± 4.5	75.7 ± 5.3[b]
PRL (μg/L)	5.4 ± 0.4	6.1 ± 0.6	5.4 ± 0.4	5.3 ± 0.5 ns
IGF-1 (μg/L)	129.5 ± 12.5	118.8 ± 9.0	117.4 ± 7.8	113.5 ± 8.1 ns
IGFBP-3 (ng/mL)	4.3 ± 0.2	6.5 ± 2.3	4.4 ± 0.2	4.3 ± 0.2 ns
LNG (nmol/L)	—	1.05 ± 0.07	1.00 ± 0.06	1.12 ± 0.06

Note: Values are means ± SEM Changes from baseline [a] $P<.05$, [b] $P<.01$, [c] $P<.001$; ns = not significant.

Lundström. Breast density and low-dose intrauterine progestogen. Fertil Steril 2006.

RESULTS

Mean values for age, years since menopause, and body mass index (BMI) at baseline for the 20 women included in the study were 57 years, 6.3 years, and 24.8, respectively. One woman discontinued after 12 months due to irregular bleeding. Vaginal ultrasound and endometrial biopsy were compatible with atrophic endometrium.

Values for serum parameters at baseline and during treatment are given in Table 1. Compliance was confirmed by the increased serum levels of E_2 and LNG. There was also an increase in SHBG and a simultaneous decline in free T. Levels of PRL, IGF-I and IGFBP-3 were unchanged throughout the observation period.

During 18 months of treatment there was a small increase in bone mineral area density spine (mean: +4%; $P<.01$). The BMI was unchanged but there was a slight decrease in total fat mass (mean: −7%; $P<.01$) and an increase in lean body mass (mean: +3.7%; $P<.01$).

Endometrial thickness and endometrial biopsies were unchanged throughout the study. At the end of the 18-month study 16 of the 19 women expressed that they were very satisfied with their treatment and they continued with the same regimen.

Mammographic Breast Density

Visual Classification. No clinical or mammographic breast abnormalities were recorded during the study period. When breast density among the 20 women was classified at baseline the distribution according to Wolfe was N1 = 2, P1 = 8, and P2 = 10. The corresponding figures for the percentage scale were 0%–20% = 7, 20%–40% = 6, 40%–60% = 2, and 60%–80% = 5.

During treatment three women (patient nos. 5, 9, and 12) were judged to fulfill the criteria for an upgrading of one class by both of the two visual classification scales. The individual density increase in patient 9 is shown in Figure 1. In all of the other women density was unchanged compared to baseline.

Digitized Density Assessment. Individual values for the amount of dense breast tissue according to the digitized assessment are shown in Table 2. The baseline mean value for the percentage area of dense breast (35.4%) showed a slight increase (38.6%) after 6 months ($P<.01$). After 18 months no further significant increase was recorded.

Digitized assessment at all points showed strong and highly significant correlations with the visual percentage classification (r_s range, 0.96–0.97; $P<.0001$). As illustrated in Table 2 the same three women (patient nos. 5, 9, and 12), who were upgraded during treatment according to the visual classifications, showed a marked increase in density also at digitized assessment.

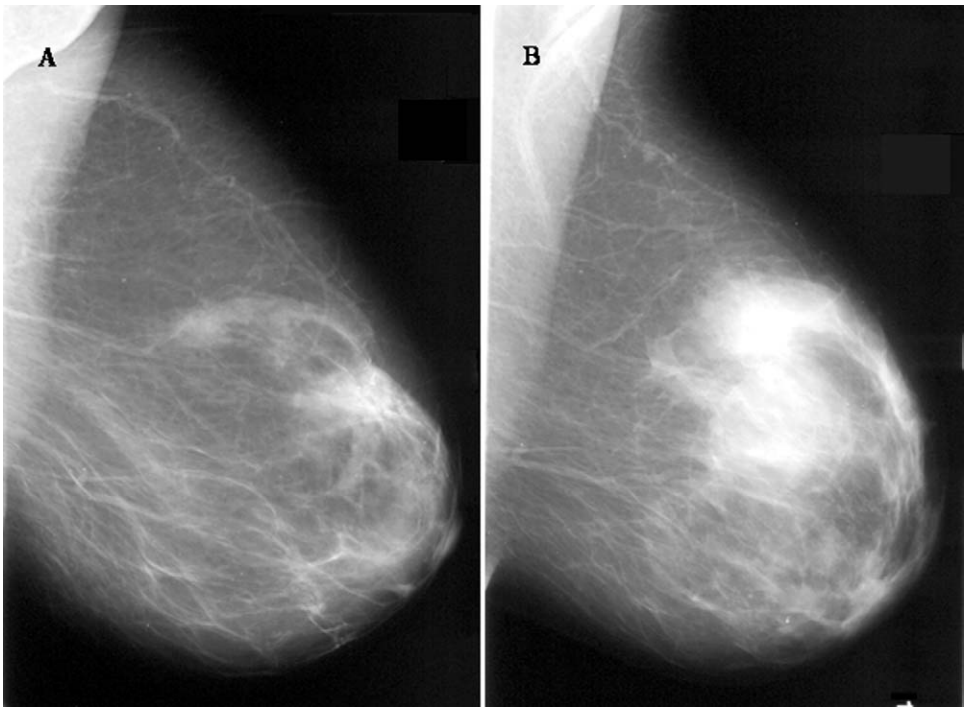
The age of these women at baseline were in the upper range of the study group (i.e., 60, 62, and 62 years, respectively) but hormonal levels including LNG and other characteristics showed no apparent difference. For the remaining 17 women changes in density (increase/decrease) were only in the order of a few percent. However, even for those women a very small but still significant increase in mean density from 38.2% to 39.4% was recorded after 6 months ($P<.05$).

Subjective symptoms of breast tenderness during treatment were reported by patients 5 and 9 (most pronounced) but also by patients 3, 6, and 8, where no marked increase in density was recorded.

Mammographic breast density at baseline showed a significant positive correlation to SHBG levels (r_s = 0.61; $P<.01$) and a negative association with total fat mass (r_s =

FIGURE 1

Change in mammographic breast density between baseline (**A**) and 18 months (**B**) in one woman during treatment with oral E_2V (2 mg)/LNG-IUS (20 μg/24 hours).



Lundström. Breast density and low-dose intrauterine progestogen. Fertil Steril 2006.

−0.48; $P<.05$). The increase in breast density after 6 months of treatment also displayed a positive correlation with age of patient ($r_s = 0.52$; $P<.05$) and an inverse relationship with circulating levels of E_2 ($r_s = -0.5$; $P<.05$). At 18 months there was also an association with PRL ($r_s = 0.51$; $P<.05$) and with free T ($r_s = -0.50$; $P<.05$).

FNA Biopsy

From 20 women a total of 79 FNA biopsies were obtained. Fifty-three of these aspirates (67%) were evaluable for MIB-1 content. Twenty-six biopsies were nonevaluable because of too few cells in the aspirate. The mean percentages of MIB-1-positive breast cells were 0.9%, 2.1%, 2.1%, and 1.5% at baseline, 2, 6, and 18 months, respectively, with no significant differences between visits.

The individual change in proliferation (MIB-1-positive cells) between baseline and 6 months of treatment displayed a strong association with digitized breast density assessment at 6 months ($r_s = 0.7$; $P<.05$). Otherwise there were no apparent correlations with hormonal levels, age of patient, and body composition.

DISCUSSION

During the past years serious concerns have been raised about the long-term safety of combined HT and in particular about the effects on the breast. There is a need to define treatment regimens for postmenopausal women that have a minimum of effects on the breast but still maintain the many advantages of such treatment.

In the present small pilot study 2 mg of E_2V and the 20 μg/24 hours LNG–IUS appeared as an accepted alternative for continuous combined E/progestogen treatment. In agreement with previous and larger studies there was good bleeding control and effective endometrial suppression (16, 17). Beneficial effects on bone density and body composition were also recorded after 18 months of treatment.

When mammographic breast density was assessed by traditional visual scoring systems three women (15%) responded with an increase fulfilling the criteria for an upgrading of the Wolfe and percentage scales.

Clearly the population sample was much too small to allow any conclusive comparisons but in previous studies of combined E/progestogen treatment by the same investigators, using the same methods, around 30% to 50% of women showed a corresponding increase in density (11–13).

Also in previous studies on 2 mg of E_2/1 mg of norethisterone acetate and 2 mg of E_2V/2 mg of dienogest there was a three- to fourfold increase in breast cell proliferation after 6 months of treatment (10, 22). Here the cell yield from FNA

TABLE 2

Individual values for the amount of dense breast tissue (digitized assessment, %) in 20 postmenopausal women at baseline and after 6 and 18 months of treatment with oral E_2V (2 mg)/LNG-IUS (20 μg/24 hours).

Patient no	Baseline (%)	6 mo (%)	18 mo (%)
1	50.4	51.2	53.7
2	36.5	37.8	36.7
3	16.5	18.5	18.4
4	18.3	18.8	19.2
5	16.6	25	25.2
6	63.8	65	69.3
7	63.4	64.7	65
8	62	65.1	65.4
9	18.4	33.2	33.1
10	63.6	67.9	66
11	31.6	35.2	36.3
12	23.5	44.6	42.9
13	32.5	36.5	35.9
14	9.3	8.4	8.7
15	32.5	32.9	32.8
16	11.1	10	11.2
17	33.8	33.4	31.2
18	62.5	64.4	64.3
19	54.9	53.9	53.4
20	6.1	5.8	—
Mean ± SEM	35.4 ± 4.6	38.6 ± 4.6	40.5 ± 4.5
Range	6.1–63.8	5.8–67.9	8.7–69.3

Lundström. Breast density and low-dose intrauterine progestogen. Fertil Steril 2006.

biopsies and the percentage of assessable samples was quite similar but we observed no significant increase in proliferative activity during 18 months of follow-up. However, mean values showed a numerical increase and there was also a strong association between individual density and change in proliferative activity.

Previously, in women taking oral contraceptives containing 150 μg of LNG, we found a positive correlation between breast cell proliferation and serum levels of the progestogen (31). In that study serum levels of LNG were around 11 nmol/L as compared to the average values of 1 nmol/L in the present material. Thus, LNG, like other progestogens, when given together with E, may stimulate breast cell proliferation. The results suggest that this effect can be reduced or perhaps even avoided by lowering of the systemic levels of progestogen during treatment.

For the first time we applied a highly sensitive computer-assisted digitized technique for the quantification of breast density during clinical treatment. We found a very strong correlation between this method and the traditional visual classifications that represent a rather crude measurement where an increase in density of about 20% to 25% could be required for an upgrading of one class. Because mammographic breast density should be regarded as a continuous variable the digitized method allows a more sensitive measurement. With this approach we found a very small but still significant increase in density also when the three women with a marked increase in density were excluded.

Mammographic breast density has emerged as a risk factor for breast cancer. However, this radiological feature of the breast probably reflects the cumulative exposure to hormones, reproductive events, and other factors that may occur during women's life span and influence breast cancer incidence (32). Although any increase in density during HT could be regarded as an unwanted side effect it seems unlikely that a change of a few percent that occurs during a limited period in life would have a significant influence on the risk for breast cancer in an individual woman.

Constitutional and hormonal factors are important predictors of mammographic density (33). We have previously shown that women react very differently to the same type and dose of hormonal treatment. (10–13, 18, 22). Overall lean nulliparous women with a short duration of menopause have higher density at baseline, whereas older women with a low BMI respond stronger to treatment. Also in the present small material breast density was associated with SHBG and PRL and displayed a negative correlation with fat mass and levels of E_2 and free T. Further efforts should be made to identify and characterize those women with a marked response to treatment (e.g., in terms of genetic predisposition and differences in local metabolism in the breast).

The results of our small study should be interpreted with much caution. Data suggest, however, that low-dose intrauterine administration of progestogen may develop into an attractive alternative for postmenopausal hormone therapy. The local administration allows endometrial protection at lower systemic levels of progestogen than many other treatment options.

The 20 μg/24 hours LNG–IUS was developed for contraception and treatment of menorrhagia in fertile women. When this device was given in combination with E_2 to postmenopausal women we found no increase in breast cell proliferation. Still some women reacted with an apparent increase in breast density. A smaller version of the LNG–IUS, the menopausal LNG system releasing only 10 μg/24 hours is currently evaluated in postmenopausal women (34). A prospective randomized controlled clinical trial to clarify the effects of the menopausal LNG system on breast cell proliferation and density is highly warranted.

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