Research Article

Cancer Prevention Research

Effect of Bazedoxifene and Conjugated Estrogen (Duavee) on Breast Cancer Risk Biomarkers in High-Risk Women: A Pilot Study

Carol J. Fabian[1], Lauren Nye[1], Kandy R. Powers[1], Jennifer L. Nydegger[1], Amy L. Kreutzjans[1], Teresa A. Phillips[1], Trina Metheny[1], Onalisa Winblad[2], Carola M. Zalles[3], Christy R. Hagan[4], Merit L. Goodman[4], Byron J. Gajewski[5], Devin C. Koestler[5], Prabhakar Chalise[5], and Bruce F. Kimler[6]

Abstract

Interventions that relieve vasomotor symptoms while reducing risk for breast cancer would likely improve uptake of chemoprevention for perimenopausal and postmenopausal women. We conducted a pilot study with 6 months of the tissue selective estrogen complex bazedoxifene (20 mg) and conjugated estrogen (0.45 mg; Duavee) to assess feasibility and effects on risk biomarkers for postmenopausal breast cancer. Risk biomarkers included fully automated mammographic volumetric density (Volpara), benign breast tissue Ki-67 (MIB-1 immunochemistry), and serum levels of progesterone, IGF-1, and IGFBP3, bioavailable estradiol and testosterone. Twenty-eight perimenopausal and postmenopausal women at increased risk for breast cancer were enrolled: 13 in cohort A with baseline Ki-67 < 1% and 15 in cohort B with baseline Ki-67 of 1% to 4%. All completed the study with > 85% drug adherence. Significant changes in biomarkers, uncorrected for multiple comparisons, were a decrease in mammographic fibroglandular volume ($P = 0.043$); decreases in serum progesterone, bioavailable testosterone, and IGF-1 ($P < 0.01$), an increase in serum bioavailable estradiol ($P < 0.001$), and for women from cohort B a reduction in Ki-67 ($P = 0.017$). An improvement in median hot flash score from 15 at baseline to 0 at 6 months, and menopause-specific quality-of-life total, vasomotor, and sexual domain scores were also observed ($P < 0.001$). Given the favorable effects on risk biomarkers and patient reported outcomes, a placebo-controlled phase IIB trial is warranted.

Introduction

In the absence of biopsy evidence of atypical hyperplasia or carcinoma *in situ*, less than 5% of risk eligible women agree to take standard endocrine therapy for breast cancer risk reduction when offered the opportunity (1). The primary reasons for avoidance are fear of side effects coupled with incomplete efficacy, and lack of reliable intermediate marker of response (2–4). Although worry about the rare serious side effect may be a factor, concern about initiating or exacerbating common vasomotor and sexual symptoms is a well-documented barrier to uptake of endocrine agents for breast cancer risk reduction (5). Unfortunately, the peak age interval for interest in primary prevention therapy of 45–60 overlaps with the peak interval for vasomotor symptoms (6, 7). In the Penn Ovarian Aging Study, moderate to severe hot flashes began most commonly between the ages of 45 and 49, averaged 10 years in duration, and afflicted 64% of women (7). Developing an agent for breast cancer prevention that alleviates rather than exacerbates vasomotor and other menopausal symptoms should be an area of high priority.

Tissue selective estrogen complexes (TSEC) combine estrogen for menopause symptoms with a selective estrogen receptor modulator (SERM) to act as an estrogen antagonist in the uterus and breast (8). In preclinical

[1]Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas. [2]Department of Diagnostic Radiology, University of Kansas Medical Center, Kansas City, Kansas. [3]Department of Pathology, Boca Raton Hospital, Boca Raton, Florida [4]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas. [5]Department of Biostatistics and Data Science, University of Kansas Medical Center, Kansas City, Kansas. [6]Department of Radiation Oncology, University of Kansas Medical Center, Kansas City, Kansas.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

Corresponding Author: Bruce F. Kimler, Department of Radiation Oncology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160-7321. Phone: 913-588-4523; Fax: 913-588-3679; E-mail: bkimler@kumc.edu

Cancer Prev Res 2019;12:711–20

doi: 10.1158/1940-6207.CAPR-19-0315

studies, the estrogen antagonist properties of bazedoxifene (BZA) in the uterus were superior to other SERMs such as raloxifene and lasoxifene (8, 9), and its growth inhibitory effects on ER^+ breast cancer cell lines were at least equivalent to active tamoxifen metabolites or the estrogen receptor downregulator fulvestrant (9, 10). Inhibition of cell growth appeared linked to blockade of estrogen upregulation of cyclin D1 (*CCND1*) gene expression as well as accelerated ER alpha protein degradation (10). BZA also inhibited growth of hormone-independent MCF-7 5C cells (10) and tamoxifen-sensitive and -resistant xenografts (11). Conjugated estrogen (CE) rather than estradiol was used to partner with BZA, given its less potent proliferative effect on breast epithelium (12), as well as absence of increase in breast cancer incidence when used without a progestin (13).

Clinical trials demonstrated that BZA combined with CE relieved hot flashes, improved vaginal dryness, and decreased osteoporotic fracture with no increase in uterine hyperplasia or percent dense area on mammogram (14–16). Subsequently BZA 20 mg and CE 0.45 mg marketed as Duavee became the first FDA-approved TSEC for treatment of menopause symptoms and osteoporosis in women with a uterus (17).

Preclinical studies suggested that BZA combined with CE might have a breast cancer preventive rather than simply a breast cancer neutral effect. In benign mammary tissue from ovariectomized nonhuman primates, BZA/CE versus CE alone showed reduced expression of several estrogen early response genes, reduced proliferation-associated *CCND1* as well as decreased Ki-67 immunolabeling (18). BZA/CE also prevented the outgrowth of MCF-7 xenograft tumors in immunosuppressed mice (19).

The preclinical observations along with a favorable clinical side effect profile led to our single-arm study of 6 months of Duavee in women at increased risk for the development of breast cancer. Our objectives were to determine feasibility in terms of accrual and adherence as well as a preliminary assessment of risk biomarker modulation in preparation for a phase IIB placebo-controlled trial. Although we assessed multiple serum risk biomarkers including IGF-1, bioavailable estradiol, bioavailable testosterone, and progesterone (20–22), our focus was on measures of mammographic density and benign tissue proliferation. They are both increased with hormone replacement therapy (23, 24), known to be associated with risk (25, 26), and favorable modulation appears to predict reduced breast cancer incidence in concurrent (27) or subsequent clinical trials (28, 29).

Materials and Methods

Medical eligibility

Eligible women were those $\leq$ age 65 in late menopause transition with current vasomotor symptoms, an intact uterus, and at least at moderately increased risk of developing breast cancer. Women with any one or more of the following criteria were risk eligible for the study: first- or second-degree relative with breast cancer < age 60, estimated area of mammographic density of 25% or greater, prior biopsy showing proliferative breast disease, multiple prior biopsies or a 5-year BCRAT (available at https://bcrisktool.cancer.gov) or a 10-year Tyrer-Cuzick IBIS (available at http://www.ems-trials.org/riskevaluator/ evaluator) model risk of at least 2× the average for age group. Late menopause transition was defined as at least 3 months of amenorrhea plus FSH in the postmenopausal range or amenorrhea for 12 or more months (30). Not eligible were women with a known BRCA1/2 mutation, prior LCIS or DCIS, prior deep venous thrombosis, history of hepatic or renal disease, BMI of $\geq$36 kg/m^2, those on tamoxifen, raloxifene, an aromatase inhibitor, or any SERM or prevention trial within 6 months of baseline testing, or systemic hormone replacement within 2 months of baseline testing. Women with a known BRCA1/2 mutation and prior LCIS were excluded at the request of Pfizer, which supplied the drug for the study. Women with BMI of 36 kg/m^2 or greater, considered as class 2 or 3 obesity, were excluded as Duavee had not been tested previously in this group of individuals (15).

Protocols for benign tissue sampling by random periareolar fine-needle aspiration (RPFNA; HSC 4601; NCT00291096) and for the Duavee intervention (Study00002440; NCT02729701) were approved by the University of Kansas Medical Center Human Subjects Committee, the institutional review board that ensures that studies are conducted in accordance with the ethical principles of the Belmont Rule and the U.S. Common Rule. Separate consents were utilized for the screening and interventional protocols with written consent obtained from all subjects. Follow-up post intervention was also performed through HSC 4601.

Baseline testing

Women were required to have a normal mammogram at the University of Kansas Medical Center within 6 months of baseline RPFNA suitable for assessment of volumetric density via Volpara software. Baseline RPFNA (31) yield had to be at least 500 epithelial cells on the slide(s) for assessment of Ki-67. Women with >4% of cells showing Ki-67 immunolabeling were excluded at the request of Pfizer. If less than 12 months since last menstrual period, a postmenopausal FSH was required; and if < age 55, a negative pregnancy test was also required. Dual X-ray absorptiometry (DXA) scan was performed for body composition along with height and weight, history and physical exam, a Menopause Quality-of-Life Questionnaire (MEN-QOL) and hot flash score assessment (32, 33).

Mammographic volumetric density

We chose to use Volpara (Volpara Solutions), a fully automated software system for assessing volumetric density, which is positively associated with risk for breast cancer. Greater relative risks for higher fibroglandular volumes and percent dense volume are similar to those observed for BIRADs patterns and Cumulus percent dense area estimations (25, 34), but with greater intra and interassessment reliability (35). The algorithm used by Volpara assesses thickness of dense tissue at each pixel using the X-ray attenuation of a fatty area as an internal reference. We assessed fibroglandular volume (FGV) as the total sum of both breasts. Likewise, percent dense volume was computed by dividing the total FGV from both breasts by the total volume for both breasts.

RPFNA and cytomorphology

RPFNA was performed (CJF, KRP, LN) on two sites per breast under local anesthesia. The first 2 aspiration passes per site (four sites total) were pooled in a 2-mL cryovial containing 0.25 mL PBS, immediately immersed in liquid nitrogen and transferred to a −80°C freezer within 12 hours for later use in gene-expression assays. Remaining specimens from both breasts were pooled in a single 15 cc tube containing 9 mL of CytoLyt and 1 mL of 10% neutral buffered formalin. Cells were spun, washed, and resuspended in PreservCyt after at least 24 hours in CytoLyt. Aliquots were processed to slides using a ThinPrep (Hologic LP) Non-Gyn standard protocol. Slides for cytomorphology and Ki-67 were Papanicolaou-stained using an RNase-free technique. All slides were assessed by a single cytopathologist (CMZ) who assigned a categorical assessment of nonproliferative, hyperplasia, borderline hyperplasia with atypia, or hyperplasia with atypia as well as a Masood semiquantitative index score (cited in ref. 31).

Ki-67 immunocytochemistry

Immunostaining for Ki-67 with a MIB-1 monoclonal antibody (M7240 Dako Cytomation) was used as an assessment of the proliferative state, as with the exception of G_0, Ki-67 is thought to be expressed in all phases of the cell cycle. Slides having 500 or more epithelial cells visible by Papanicolaou-staining were selected. Following destaining, antigen retrieval was performed with 10 mmol/L citrate buffer (pH 6) in a Biocare decloaking chamber for two minutes at 120°C. The MIB-1 monoclonal antibody was applied at a 1:20 dilution using a Dako autostainer (31). Women with less than 5 positive cells per 500 epithelial cells counted (<1%) were considered cohort A and those with 5 to 20 per 500 epithelial cells counted (1% or greater) as cohort B. If < 500 epithelial cells were observed, a further slide was prepared to bring the total to 500 cells.

Gene expression by RT-qPCR

Total RNA was extracted from frozen RPFNA samples using TRIzol LS (Life Technologies) according to the manufacturer's instructions, with additional RNA purification using an RNeasy MinElute Cleanup Kit (Qiagen). The RNA collected was thus reflective of adipocytes, stroma, and epithelial cells. RNA was amplified using MessageAmpII aRNA amplification kit (Life Technologies) and reverse transcribed to cDNA using SMARTScribe Reverse Transcriptase (Clontech Laboratories, Inc.) and random nonamer primers. Real-time quantitative PCR (qPCR) was performed for selected estrogen and progesterone response genes and others of interest using hydrolysis probes as previously described (31, 36). Baseline and postintervention specimens were assessed together. PCR reactions were run on an Applied Biosystems Prism 7000 Sequence Detection System or a Roche LightCycler 96. Tested transcripts for estrogen response genes included estrogen receptor 1 (*ESR1*) for estrogen receptor alpha (ERα), *ESR2* for estrogen receptor beta (ERβ), trefoil factor 1 (*TFF1*) for pS2, growth regulation by estrogen in breast cancer 1 (*GREB1*), progesterone receptor (*PGR*), amphiregulin (*AREG*), stromal cell derived factor 1 (SDF1α, *CXCL12*) and 2 (SDF1β, *CXCL12*), and lipocalin 2 (*LCN2*). Genes downstream from activated progesterone receptor included cyclin D1 (*CCND1*), fatty acid synthetase (*FASN*), receptor activator of nuclear factor κ B ligand (*RANKL*), serum glucocorticoid kinase 1 (*SGK*), the procoagulant tissue factor (*TF*), pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*), and signal transducer and activator of transcription 5a (*STAT5A*). Other genes impacting metabolism and inflammation that are affected directly or indirectly modulated by estrogen or progesterone include cyclooxygenase 2 (COX2, *PTGS2*), androgen receptor (*AR*), aromatase (*CYP19A1*), steroid sulfatase (*STS*), 3-hydroxybutyrate dehydrogenase, type 2 (*BDH2*), leptin (*LEP*), and adiponectin (*ADIPOQ*). Primer and probe sequences are provided in Supplementary Table S1. The cycle threshold mean value for each transcript (from duplicate assays per specimen) was normalized using two reference transcripts (*PPIA*, peptidylprolyl isomerase A, for cyclophilin A and *CDKN1B* for cyclin-dependent kinase inhibitor 1B) that showed the least change over time. Relative levels of each transcript were calculated using the $\Delta\Delta C_t$ method. The ratio (postintervention: baseline) of final values indicated upregulation (value > 1) or downregulation (value < 1).

To discern molecular subgroups based on the expression signature of genes of interest, we performed an unsupervised clustering analysis using gene-expression measurements collected at baseline and postintervention. Unsupervised clustering analysis was performed by fitting a Gaussian-distributed recursively partitioned mixture model (37) to the log2-transformed fold change (logFC) values calculated for each gene. The objective of this

analysis was to identify subgroups of participants, where subgroups were defined based on how expression signatures changed from baseline to postintervention across the selected set of genes profiled in this study. Fold-change values were computed by dividing the postintervention expression value by the baseline expression value. As such, genes with a logFC > 0 displayed increase in expression between baseline and postintervention. Conversely, genes with logFC < 0 displayed a decrease in expression between baseline and postintervention. Gaussian-distributed recursively partitioned mixture models were fit using the function glcTree in the R package RPMM. With the exception of the maxlevel argument, the maximum depth to recurse, which was set to one due to the modest sample size of this study, all other parameters in the glcTree function were set to their default values. The logFC values across the panel of selected genes were visualized across the identified clusters by creating a heat map with increasing brightness indicating increased upregulation (red) or downregulation (green) of gene expression.

Hormones, IGF-1, and IGFBP-3

Fasting blood was obtained for assays of estradiol, testosterone, progesterone, sex hormone binding globulin (SHBG), insulin-like growth factor-1 (IGF-1), its binding protein IGFBP-3, and C-reactive protein (CRP). Samples were stored frozen at −80°C until analysis. Baseline and postintervention specimens were run together with pooled serum controls to assess batch variation. Commercial kits from R&D Systems, Inc., were used for enzyme-linked immunosorbent assay (ELISA) of IGF-1 (DG100) and IGFBP-3 (DGB300). Commercial kits from Diagnostics Biochem Canada were used for enzyme immunoassay of estradiol (CAN-E-430), progesterone (CAN-P-305), testosterone (CAN-TE-250), and ELISA of SHBG (CAN-SHBG-4010) and CRP (CAN-CRP-4360, high-sensitivity kit). Bioavailable estradiol and testosterone were calculated according to standard formulae (cited in ref. 31). Limit of detection for estradiol and testosterone were 10 and 22 pg/mL with 8% to 10% intra and interassay coefficient of variation. Limit of detection for progesterone is 0.1 ng/mL with 10% coefficient of variation.

Body composition

Body composition assessment (total mass, lean mass, total fat, and percent body fat) was performed using two GE (GE Healthcare) dual-energy X-ray absorptiometer models: Lunar Prodigy DXA (11 baseline and 1 postintervention) or Lunar iDXA (17 baseline and 27 postintervention). Measurements between the two instruments are well correlated, and the software can detect changes in body composition between 1.6% and 3.8%. The Lunar Prodigy DXA differs from Lunar iDXA primarily in that the software on the latter enables automatic calculation of the amount of visceral fat from the android fat compartment. iDXA estimated visceral fat was available postintervention for 27 women and for both baseline and off study for 17 women.

Patient-reported outcome measurements

The Mayo Clinic Hot Flash Score and the MEN-QOL menopause-specific quality-of-life questionnaire were used to assess vasomotor symptoms and other symptoms related to menopause. The hot flash score was the average number of hot flashes per day × severity (mild = 1, moderate = 2, severe = 3; ref. 32) and was computed at baseline, 4 weeks, 3 months, and 6 months (end of intervention). MEN-QOL consists of 29 items divided into four domains: vasomotor (three questions), psychosocial (seven questions), physical (16 questions), and sexual (three questions). A woman was asked if she had experienced a symptom/issue in the prior month, and if so the extent to which it had affected her. MEN-QOL was administered at baseline and end of intervention. Domain and total scores were computed (33).

Statistical analysis

Because most quantitative measures (especially change in value over time) were not normally distributed, nonparametric statistical approaches were used throughout. For assessment of change in continuous values over the course of the intervention, the Wilcoxon signed-rank test was used. The relationship between variables was assessed using Spearman correlation. A P value of <0.05 was considered an indication of a statistically significant difference, with no correction for multiple comparisons given the large number of secondary, nonpowered endpoints being explored. Thus, caution is advised when interpreting the results.

To gain insight into the potential biological and clinical relevance of the identified clusters, the association between cluster membership and select clinical variables was examined. Wilcoxon rank sum and Fisher exact tests were used to examine the relationship between cluster membership and continuous and categorical variables, respectively.

Results

Accrual and retention

Women were recruited from our high-risk cohort followed in the Breast Cancer Prevention Center. Accrual began May 2016 and concluded August 2018. Forty-two women were considered medically eligible and were screened by RPFNA. Of the 42 screened by RPFNA 14 did not enter the trial. Failure to enter the interventional trial after RPFNA was due to insufficient cell count for immunodetection of Ki-67 (four women); decrease/resolution of hot flashes (four women); decided against trial (two women); and one instance for each of the following reasons:

Table 1. Improvement in quality of life as assessed by the MEN-QOL survey

Measure	Vasomotor	Psychosocial	Physical	Sexual	Total
Baseline score	9 (3–18)	3.5 (0–37)	18 (2–71)	3 (0–18)	42 (9–136)
Postintervention score	0 (0–5)	2 (0–29)	8 (0–39)	0 (0–10)	9.5 (0–67)
Change in score	−8 (−17 to −2)	−1 (−37 to 15)	−9 (−71 to 22)	−3 (−18 to 4)	−23.5 (−136 to 22)
Number improve	28	15	21	20	24
Number worsen	0	6	7	2	4
P value[a]	<0.0001	0.070	0.0077	0.0002	<0.0001

NOTE: Values shown (median and range) are for sums of scores per domain. Larger values represent a greater extent to which individual symptoms or problems were considered bothersome.
[a]Wilcoxon signed-rank test.

>4% of cells staining positive for Ki-67, decided on standard hormone replacement instead, desired TAH/BSO, and worried about estrogen in drug. Of the 28 high-risk women entering the 6-month intervention with Duavee, 13 were enrolled in cohort A (Ki-67 < 1%) and 15 were enrolled in cohort B (Ki-67 = 1%–4%). The accrual rate was less than expected primarily due to ineligibility of women with a BRCA1/2 mutation, or prior hysterectomy with a prior biopsy showing LCIS. All 28 women completed the 6-month intervention and are evaluable with no serious adverse events and > 85% adherence.

Patient-reported outcomes

Hot flash frequency and intensity dramatically improved ($P < 0.001$) as median hot flash score (average daily frequency and intensity) dropped from 15 at baseline to 0 after the intervention). In general, hot flashes were reported as relieved within 2 weeks of starting Duavee. MEN-QOL total score, as well as vasomotor, physical, and sexual domains significantly improved ($P < 0.01$; Table 1).

Baseline characteristics by cohort

Median age was similar for cohorts A (54) and B (53). The proportion of women who were nulliparous was higher for cohort B (33%) than for cohort A (8%). The proportion women who had previously used a SERM was small and similar for both cohorts (8% cohort A and 7% cohort B).

Both cohorts had a median baseline cytomorphology index score of 13 consistent with low-level hyperplasia. More women in cohort B (baseline Ki-67 immunolabeling 1%–4% of epithelial cells) had previously used HRT (6/15 B vs. 3/13 A), had a prior diagnostic biopsy indicating atypical hyperplasia (6/15 B vs. 2/13 A), and were considered obese by BMI (5/15 B vs. 2/13 A). Despite meeting eligibility criteria, two women in cohort B and one in cohort A had baseline progesterone levels considered in the premenopausal range at >1 ng/mL.

Change in blood biomarkers

Twenty-four of 27 women had increases in bioavailable estradiol and 25 of 27 had reductions in bioavailable testosterone ($P < 0.001$). There were significant decreases in IGF-1 ($P < 0.001$), IGFBP3 ($P = 0.024$), and the molar ratio of IGF-1: IGFBP3 ($P < 0.001$). Nineteen of 27 women had reductions in serum progesterone ($P = 0.006$). There were no trends for CRP (Table 2).

Change in mammographic density

Median baseline fibroglandular volume (FGV) was 133 cm^3 for both breasts (range, 36–336 cm^3), and median percent dense volume was 9.7% (range, 3.5%–34%) for the 26 women with evaluable baseline and 6-month Volpara processed mammograms (Table 3). Fibroglandular volume decreased by a median of 12 cm^3 (6 cm^3/breast) or a median relative decrease of 11% (range, −61% to +43%; $P = 0.043$). Baseline and change in FGV varied

Table 2. Baseline and postintervention assessment of serum total and bioavailable estradiol and testosterone, SHBG, progesterone, IGF-1, IGFBP3, and CRP

Variable or biomarker	Baseline	Postintervention	Difference	*P* value (Wilcoxon)
SHBG, nmol/L	68 (26–241)	106 (38–274)	31 (−5 to 65)	0.0001
Estradiol, pg/mL	54 (24–458)	107 (43–914)	56 (−5 to 752)	0.0001
Estradiol, nmol/L	0.2 (0.1–1.7)	0.4 (0.2–3.4)	0.2 (0.0–2.8)	
Bioavailable estradiol, pmol/L	2.7 (0.7–23.2)	3.6 (1.5–19.7)	1.3 (−8.8 to 12.3)	0.0001
Testosterone, ng/mL	1.29 (0.50–12.11)	1.07 (0.42–7.39)	−0.12 (−5.78 to 2.27)	0.029
Testosterone, nmol/L	4.5 (1.7–42)	3.7 (1.5–25.7)	−0.4 (−20 to 7.9)	
Bioavailable testosterone, pmol/L	43 (13–802)	27 (10–213)	−17 (−588 to 25)	0.0001
Progesterone, ng/mL	0.33 (0.17–11.81)	0.26 (0.07–5.92)	−0.09 (−7.09 to 1.17)	0.0058
IGF-I, ng/mL	109 (60–184)	87 (50–128)	−17 (−101 to 25)	0.0001
IGF-I, nmol/L	14 (8–24)	11 (7–17)	−2 (−13 to 3)	
IGFBP-3, ng/mL	2,153 (1,582–2,944)	2,052 (1,402–2,937)	−48 (−597 to 276)	0.024
IGFBP-3, nmol/L	75 (55–103)	72 (49–103)	−1.5 (−20 to 10)	
IGF-I:IGFBP-3 molar ratio	0.19 (0.13–0.32)	0.16 (0.10–0.26)	−0.03 (−0.1 to 0.05)	0.0002
CRP, μg/mL	1.5 (0.1–7.7)	1.4 (0.1–8.4)	−0.04 (−4.1 to 4.8)	0.79

NOTE: Median (range) values are provided for 27 subjects with paired specimens.

Table 3. Volpara assessment of mammographic features

Variable or biomarker	Baseline	Postintervention	Difference	Relative difference, %	*P* value (Wilcoxon)
Total breast volume, cm^3	1,364 (326–4,419)	1,224 (374–5,160)	18 (−904 to 1,673)	2 (−37 to 48)	0.45
Fibroglandular volume (total), cm^3	133 (36–336)	111 (37–265)	−12 (−204 to 62)	−11 (−61 to 43)	0.043
Percent dense volume, total FGV/total breast volume	9.7 (3.5–34.0)	8.3 (2.5–34.2)	−1.2 (−11.0 to 8.9)	−16 (−68 to 61)	0.11

NOTE: Median and range values for 26 women with baseline and postintervention digital mammograms.

positively with BMI, but correction for change in BMI did not alter the finding of significant change with 6 months of Duavee. Percent dense volume decreased by a median of 1.2% from a baseline of 9.7% but this was not statistically significant ($P = 0.11$). In this small sample, there was no evidence of differential change in FGV by baseline dense volume above or below 10%. However, women with baseline fibroglandular volume above the median of 133 cm^3 had over twice the change in FGV as women with FGV < 133 cm^3 (medians of −26.9 cm^3 vs. −10.3 cm^3). Change in fibroglandular volume or percent dense volume was not significantly correlated with change in systemic hormone levels or percentage of benign breast epithelial cells expressing Ki-67.

Change in Ki-67 and gene expression in benign breast tissue sampled by RPFNA

No change was observed in the cytomorphology index scores from baseline to postintervention specimens. The median score of 13 both pre- and postintervention was consistent with low-grade hyperplasia without atypia over the 6-month period (Supplementary Table S2). Women in cohort B had a median baseline Ki-67 of 1.6% (range, 1%–3.6%) and a median postintervention Ki-67 of 0.4% (range, 0%–3.2%). This was a significant reduction from baseline ($P = 0.017$; Fig. 1). There was no overall change in Ki-67 in breast tissue samples from cohort A women with a median baseline and postintervention value of 0.2% (range, 0%–0.8% baseline and 0%–5.4% postintervention; $P = 0.44$). One individual each from cohort A and B (2/28 total) exhibited a protocol-defined significant increase in proliferation (>2% Ki-67-positive cells for cohort A or doubling of initial Ki-67 frequency for cohort B). Both women had low baseline BMI (19.5 and 20.5 kg/m^2, respectively), low baseline estradiol and bioavailable levels (<40 pg/mL total, 1.1 pmol or lower), and exhibited increases in serum progesterone in addition to increases in bioavailable estradiol.

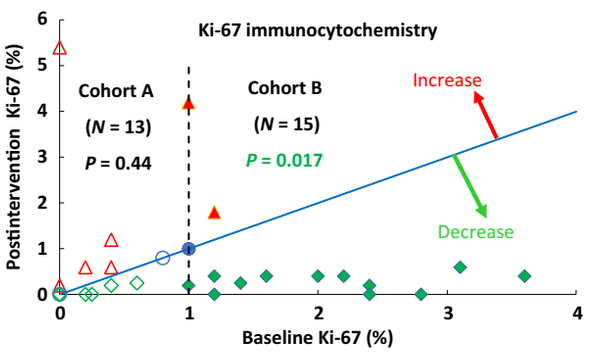

Figure 1.
Percentage of breast epithelial cells acquired by RPFNA that stained immunocytochemically positive for Ki-67 at baseline and after a 6-month intervention with Duavee. Comparison of change by the nonparametric Wilcoxon test.

We assessed a number of genes known to be responsive to increases in systemic estrogen and/or downstream from activated estrogen and progesterone receptors. An unsupervised cluster analysis (Fig. 2) identified two clusters (cluster 1 with 10 women and cluster 2 with 17 women) with differences in change in early estrogen response genes. Women in cluster 1 tended to have increased expression of 2 or more of the early estrogen response genes *ESR1* (ER alpha), *TFF1* (pS2), *GREB1a*, *PGR*, and *AREG* (Amphiregulin), but did not exhibit increased expression of *CCND1* (cyclin D1) or other genes downstream of activated progesterone receptor such as *STAT5a*, *PdK4*, and *STK*. A trend toward decreased expression was observed of several genes thought to play a role in breast cancer development with predominant expression in stroma such as *LEP* (leptin), *FASN* (fatty acid synthetase), *CXCL12* (two splice variants of stromal derived growth factor, SDF1α and SDF1β), and *CYP19A1* (aromatase). Women in cluster 2 exhibited predominately decreased expression of both the early estrogen response genes and the stromal genes listed above. Several clinical factors were examined to explain clustering membership including age, body mass index and body composition variables baseline and change in bioavailable estradiol, baseline and change in progesterone, and baseline and change in Ki-67 immunolabeling and fibroglandular volume. Five of the 10 women in the early estrogen response gene cluster (cluster 1) had a postintervention visceral adipose tissue (VAT) > 1.2 kg, which is consistent with increased risk for metabolic syndrome (38), compared with only 1 of 16 women in cluster 2 ($P = 0.018$, Fisher exact test).

Body composition

Detailed body composition is given in Supplementary Table S3. A small increase was observed in fat mass (median 1 kg) and percent body fat (median 0.9%) but not in lean mass or VAT. For the latter, there is the caveat that change in VAT could only be assessed for 17 women.

Off-study follow-up

Twenty-one of 28 women elected to continue Duavee off study following trial completion. The remaining seven did

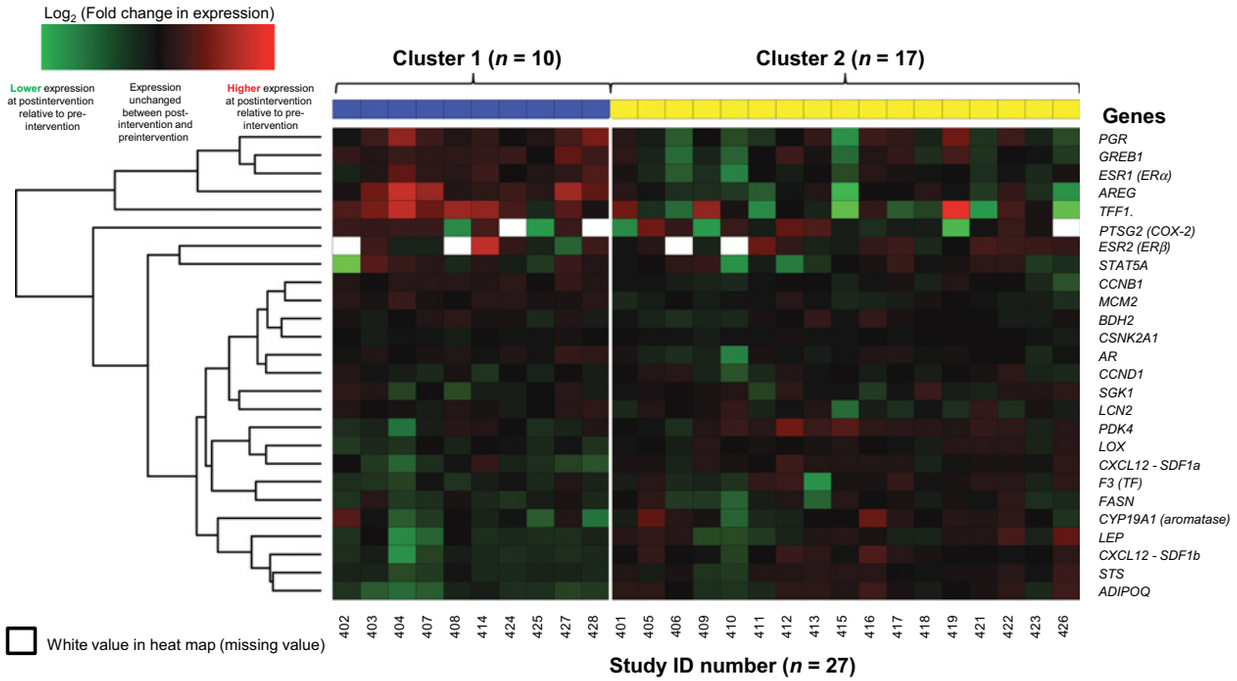

Figure 2.

Heat map demonstrating the results of unsupervised clustering analysis of PCR data. For individual subjects, an increase in gene expression is indicated by red and a decrease in gene expression is indicated by green. The 27 subjects with paired specimens are separated into two clusters (cluster 1, $n = 10$ and cluster 2, $n = 17$), primarily on the basis of alterations in the estrogen early response genes *PGR, GREB1, ESR1, AREG*, and *TFF1*.

not continue primarily because of high co-pays or their insurance would not approve the drug unless they had tried two other nonhormonal medications for vasomotor symptoms. One who initially did not take off-study Duavee initiated it a year after she completed trial because of return of vasomotor symptoms and regain of FGV. Fibroglandular volume decreased again after drug was restarted.

Of the 21 who continued Duavee by clinical prescription after completion of the trial, four have discontinued, three because of insurance coverage and one due to thyroid problems. Eighteen of the 26 women who entered with Volpara evaluable baseline mammograms have had further follow-up mammograms with Volpara software 12 to 24 months following interventional trial completion. Eleven of 18 women continue to have reduction in mammographic density from baseline. The remainder have not yet had their clinical 12-month post study completion mammogram.

Discussion

We demonstrated excellent retention and favorable change in multiple risk biomarkers for breast cancer with 6 months of Duavee in perimenopausal and postmenopausal women with vasomotor symptoms. Hot flashes decreased dramatically, which was similar to results observed in large placebo-controlled trials (14).

The increases in SHBG and estradiol, as well as decreases in bioavailable testosterone and IGF-1, are consistent with the effects of the oral estrogen component of Duavee (39). As the SERMs tamoxifen and raloxifene also reduce systemic IGF-1 in postmenopausal women (40), it is possible that both the BZA and CE components of Duavee are responsible for the reduction in systemic IGF-1. Systemic progesterone levels in postmenopausal women have recently been reported to be positively associated with breast cancer risk (22), but it is not clear whether our observed reduction in serum progesterone was the result of Duavee or progressive luteal failure with late menopause transition (30).

Although a small increase was noted in total and percent body fat, this is unlikely to be of clinical significance. Further, for over half of the women the baseline and postintervention percent and total fat measures were performed with two different types of DXA units. Although measurements between the Lunar Prodigy DXA and Lunar iDXA are highly correlated, the differences are within the margin of error (41).

We found that FGV, a demonstrated risk biomarker (34), was significantly reduced after 6 months of Duavee in the 26 women with Volpara software assessed mammograms. The FGV decrease after Duavee contrasts with the lack of change in age-matched controls assessed during the same time frame as the Duavee trial (Fig. 3). These results

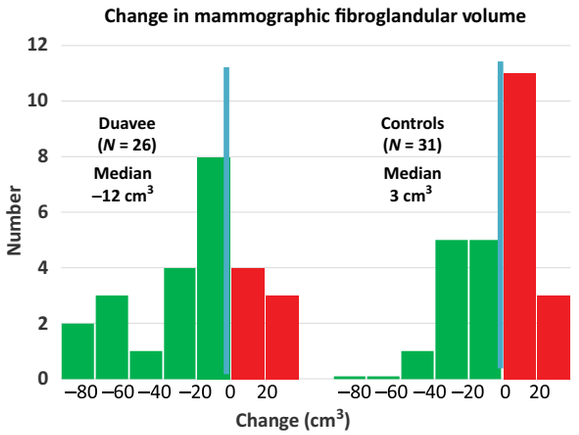

Figure 3.
Distribution of change in mammographic total fibroglandular volume assessed by Volpara software for subjects on the Duavee pilot study and age-matched controls with two mammograms separated by 6 to 12 months.

differed from those of BZA/CE versus placebo in the SMART trials where the Cumulus computer-assisted method was used to assess percent dense area (14). Small changes in density in the SMART trials might have been missed with the Cumulus semiautomated method if baseline density is low, as is often the case in postmenopausal women (25). Change in FGV volume after 6 months of Duavee (median total for both breasts of -12 cm^3 or -6 cm^3/per breast) in our pilot is in the range of the annual change in FGV of -8 cm^3 over an average of 3 years in contralateral breasts for premenopausal women with breast cancer taking tamoxifen (42). In addition to breast cancer risk, endogenous levels of progesterone in postmenopausal women have been reported as positively correlated with percent mammographically dense area (43). We did not observe a correlation between change in serum progesterone and change in FGV and/or percent density possibly due to our sample size. We did not measure change in serum osteoprotegerin, a decoy receptor for receptor activator of nuclear factor κB (RANK) ligand, which has been reported to be inversely associated with breast density among postmenopausal women (44).

Similar to findings reported for cynomolgus monkeys (18) we observed a reduction in benign breast tissue Ki-67 in women from cohort B who had measurable proliferation (1% or higher Ki-67 immunolabeling) at baseline. Reduction in systemic progesterone levels due to physiologic progression of menopause transition (45); and/or disruption of estrogen and progesterone receptor signaling by Duavee are potential explanations for the observed decreases in proliferation (8, 15). Progesterone increases proliferation-associated gene expression by increasing transcription factors including *STAT5a, PDK4*, and *RANKL* (46, 47). We found no strong evidence of change in overall gene expression including the above transcription factors. The limited sample size ($n = 27$) and potential for variation in the proportion of epithelial and stromal content between baseline and postintervention sampling results in suboptimal statistical power for detecting associations between clinical features and cluster membership in the exploratory studies of benign tissue gene expression. However, the higher proportion of women in cluster 1 with elevated visceral adipose as well as increases in estrogen response gene expression is intriguing in view of the hyperinsulinemia and elevated levels of proinflammatory cytokines often found in women with increased visceral adipose and cross-talk between the Insulin/IGF-1 system and estrogen (48). Change in mammographic breast density which is composed predominately of stroma in postmenopausal women, was independent of change in epithelial cell proliferation in line with our prior findings and that of others (49, 50).

This was a single-arm pilot study and was not designed to definitively conclude that BZA 20 mg combined with CE 0.45 mg (Duavee) was associated with favorable modulation of any specific serum, tissue or imaging biomarkers. It is entirely possible that many of these changes could be the result of advancing menopause transition; plus, no adjustments were made for multiple comparisons. The observations are also limited to symptomatic women undergoing menopause transition. However, the pilot results are encouraging and provide the basis for further investigation of this agent for primary prevention in high-risk perimenopausal and postmenopausal women in a phase IIB placebo-controlled trial.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.J. Fabian, B.F. Kimler
Development of methodology: C.J. Fabian, L. Nye, T.A. Phillips
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Fabian, L. Nye, K.R. Powers, J.L. Nydegger, A.L. Kreutzjans, T.A. Phillips, T. Metheny, O. Winblad, C.M. Zalles, C.R. Hagan,
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Nydegger, T.A. Phillips, C.R. Hagan, B.J. Gajewski, D.C. Koestler, P. Chalise, B.F. Kimler
Writing, review, and/or revision of the manuscript: C.J. Fabian, L. Nye, T.A. Phillips, T. Metheny, O. Winblad, C.R. Hagan, B.J. Gajewski, D.C. Koestler, P. Chalise, B.F. Kimler
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.J. Fabian, J.L. Nydegger, T.A. Phillips, O. Winblad, M.L. Goodman, B.F. Kimler
Study supervision: C.J. Fabian, L. Nye

Acknowledgments

The authors thank Pfizer, Inc. for providing the study agent. This study was supported in part by grants from the Breast Cancer Research Foundation (BCRF-16-049, BCRF-17-049, and BCRF-18-049) an NIH Clinical and Translational Science Award grant (UL1 TR000001, formerly UL1RR033179) awarded to the University of Kansas Medical Center, and an internal clinical pilot grant program of the KUMC

Research Institute; and used the Biostatistics & Informatics Shared Resource supported by National Cancer Institute Cancer Center Support Grant P30 CA168524.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 20, 2019; revised July 29, 2019; accepted August 9, 2019; published first August 16, 2019.

References

1. Kaplan CP, Haas JS, Perez-Stable EJ, Gregorich SE, Somkin C, Des Jarlais G, et al. Breast cancer risk reduction options: awareness, discussion, and use among women from four ethnic groups. Cancer Epidemiol Biomarkers Prev 2006;15:162–6.
2. Bambhroliya A, Chavez-MacGregor M, Brewster AM. Barriers to the use of breast cancer risk reduction therapies. J Natl Compr Canc Netw 2015;13:927–35.
3. Ropka ME, Keim J, Philbrick JT. Patient decisions about breast cancer chemoprevention: a systematic review and meta-analysis. J Clin Oncol 2010;28:3090–5.
4. Crew KD. What factors influence decision-making about breast cancer chemoprevention among high-risk women? Cancer Prev Res (Phila) 2017;10:609–11.
5. Smith SG, Sestak I, Forster A, Partridge A, Side L, Wolf MS, et al. Factors affecting uptake and adherence to breast cancer chemoprevention: a systematic review and meta-analysis. Ann Oncol 2016;27:575–90.
6. Bastian LA, Lipkus IM, Kuchibhatla MN, Weng HH, Halabi S, Ryan PD, et al. Women's interest in chemoprevention for breast cancer. Arch Intern Med 2001;161:1639–44.
7. Freeman EW, Sammel MD, Lin H, Liu Z, Gracia CR. Duration of menopausal hot flushes and associated risk factors. Obstet Gynecol 2011;117:1095–1104.
8. Pickar JH, Boucher M, Morgenstern D. Tissue selective estrogen complex (TSEC): a review. Menopause 2018;25:1033–45.
9. Chang KC, Wang Y, Bodine PV, Nagpal S, Komm BS. Gene expression profiling studies of three SERMs and their conjugated estrogen combinations in human breast cancer cells: insights into the unique antagonistic effects of bazedoxifene on conjugated estrogens. J Steroid Biochem Mol Biol 2010;118:117–24.
10. Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. Mol Pharmacol 2011;80:610–20.
11. Wardell E, Nelson ER, Chao CA, McDonnell DP. Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen-resistant breast cancer: implications for treatment of advanced disease. Clin Cancer Res 2013;19:2420–31.
12. Yue W, Wang J, Atkins KA, Bottalico L, Mesaros C, Blair IA, et al. Effect of a tissue selective estrogen complex on breast cancer: role of unique properties of conjugated equine estrogen. Int J Cancer 2018;143:1259–68.
13. Anderson GL, Chlebowski RT, Aragaki AK, Kuller LH, Manson JE, Gass M, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomised placebo-controlled trial. Lancet Oncol 2012;13:476–86.
14. Kagan R, Komm BS, Ryan KA, Lavenberg J, Yu CR, Pinkerton JV. Timing and persistence of effect of conjugated estrogens/bazedoxifene in postmenopausal women. Menopause 2016;23:1204–13.
15. Pinkerton JV. Tissue-selective estrogen complex for menopausal hormone therapy. Clin Obstet Gynecol 2018;61:463–9.
16. Harvey JA, Pinkerton JV, Baracat EC, Shi H, Chines AA, Mirkin S. Breast density changes in a randomized controlled trial evaluating bazedoxifene/conjugated estrogens. Menopause 2013;20:138–45.
17. Komm BS, Mirkin S, Jenkins SN. Development of conjugated estrogens/bazedoxifene, the first tissue selective estrogen complex (TSEC) for management of menopausal hot flashes and postmenopausal bone loss. Steroids 2014;90:71–81.
18. Ethun KF, Wood CE, Register TC, Cline JM, Appt SE, Clarkson TB. Effects of bazedoxifene acetate with and without conjugated equine estrogens on the breast of postmenopausal monkeys. Menopause 2012;19:1242–52.
19. Santen RJ, Song Y, Wang JP, Yue W. Preclinical breast effects of a tissue selective estrogen complex (TSEC) including conjugated estrogen with bazedoxifene. J Steroid Biochem Mol Biol 2017;170:61–4.
20. Horne HN, Sherman ME, Pfeiffer RM, Figueroa JD, Khodr ZG, Falk RT, et al. Circulating insulin-like growth factor-I, insulin-like growth factor binding protein-3 and terminal duct lobular unit involution of the breast: a cross-sectional study of women with benign breast disease. Breast Cancer Res 2016;18:24.
21. Farhat GN, Cummings SR, Chlebowski RT, Parimi N, Cauley JA, Rohan TE, et al. Sex hormone levels and risks of estrogen receptor-negative and estrogen receptor-positive breast cancers. J Natl Cancer Inst 2011;103:562–70.
22. Trabert B, Bauer DC, Brinton LA, Buist DSM, Cauley JA, Dallal CM, et al. Circulating progesterone is associated with increased postmenopausal breast cancer risk: B-Fit cohort. In: Proceedings of the 110th Annual Meeting of the American Association for Cancer Research; 2019 Mar 29 - Apr 3; Atlanta, GA. Philadelphia (PA): AACR; 2019. Abstract nr 589.
23. Byrne C, Ursin G, Martin CF, Peck JD, Cole EB, Zeng D, et al. Mammographic density change with estrogen and progestin therapy and breast cancer risk. J Natl Cancer Inst 2017;109.
24. Hofseth LJ, Raafat AM, Osuch JR, Pathak DR, Slomski CA, Haslam SZ. Hormone replacement therapy with estrogen or estrogen plus medroxyprogesterone acetate is associated with increased epithelial proliferation in the normal postmenopausal breast. J Clin Endocrinol Metab 1999;84:4559–65.
25. Eng A, Gallant Z, Shepherd J, McCormack V, Li J, Dowsett M, et al. Digital mammographic density and breast cancer risk: a case-control study of six alternative density assessment methods. Breast Cancer Res 2014;16:439.
26. Huh SJ, Oh H, Peterson MA, Almendro V, Hu R, Bowden M, et al. The proliferative activity of mammary epithelial cells in normal tissue predicts breast cancer risk in premenopausal women. Cancer Res 2016;76:1926–34.
27. Cuzick J, Warwick J, Pinney E, Duffy SW, Cawthorn S, Howell A, et al. Tamoxifen-induced reduction in mammographic density and breast cancer risk reduction: a nested case-control study. J Natl Cancer Inst 2011;103:744–52.

28. de Lima GR, Facina G, Shida JY, Chein MB, Tanaka P, Dardes RC, et al. Effects of low dose tamoxifen on normal breast tissue from premenopausal women. Eur J Cancer 2003;39:891–8.
29. DeCensi A, Puntoni M, Guerrieri-Gonzaga A, Caviglia S, Avino F, Cortesi L, et al. Randomized placebo controlled trial of low-dose tamoxifen to prevent local and contralateral recurrence in breast intraepithelial neoplasia. J Clin Oncol 2019;37:1629–37.
30. Burger HG, Hale GE, Robertson DM, Dennerstein L. A review of hormonal changes during the menopausal transition: focus on findings from the Melbourne Women's Midlife Health Project. Hum Reprod Update 2007;13:559–65.
31. Fabian CJ, Kimler BF, Zalles CM, Phillips TA, Metheny T, Petroff BK, et al. Clinical trial of acolbifene in premenopausal women at high risk for breast cancer. Cancer Prev Res (Phila) 2015;8: 1146–55.
32. Loprinzi L, Barton DL, Sloan JA, Zahasky KM, Smith DA, Pruthi S, et al. Pilot evaluation of gabapentin for treating hot flashes. Mayo Clin Proc 2002;77:1159–63.
33. Lewis JE, Hilditch JR, Wong CJ. Further psychometric property development of the Menopause-Specific Quality of Life questionnaire and development of a modified version, MENQOL-Intervention questionnaire. Maturitas 2005;50: 209–21.
34. Brand JS, Czene K, Shepherd JA, Leifland K, Heddson B, Sundbom A, et al. Automated measurement of volumetric mammographic density: a tool for widespread breast cancer risk assessment. Cancer Epidemiol Biomarkers Prev 2014;23:1764–72.
35. Alonzo-Proulx O, Mawdsley GE, Patrie JT, Yaffe MJ, Harvey JA. Reliability of automated breast density measurements. Radiology 2015;275:366–76.
36. Walter KR, Goodman ML, Singhal H, Hall JA, Li T, Holloran SM, et al. Interferon-stimulated genes are transcriptionally repressed by PR in breast cancer. Mol Cancer Res 2017;15:1331–40.
37. Houseman EA. Model-based clustering of DNA methylation array data: a recursive-partitioning algorithm for high-dimensional data arising as a mixture of beta distributions. BMC Bioinformatics 2008;9:365.
38. Neeland IJ, Grundy SM, Li X, Adams-Huet B, Vega GL. Comparison of visceral fat mass measurement by dual-X-ray absorptiometry and magnetic resonance imaging in a multiethnic cohort: the Dallas Heart Study. Nutr Diabetes 2016;6:e221.
39. Southmayd EA, De Souza MJ. A summary of the influence of exogenous estrogen administration across the lifespan on the GH/IGF-1 axis and implications for bone health. Growth Horm IGF Res 2017;32:2–13.
40. Torrisi R, Baglietto L, Johansson H, Veronesi G, Bonanni B, Guerrieri-Gonzaga A, et al. Effect of raloxifene on IGF-I and IGFBP-3 in postmenopausal women with breast cancer. Br J Cancer 2001;85:1838–41.
41. Morrison SA, Petri RM, Hunter HL, Raju D, Gower B. Comparison of the Lunar Prodigy and iDXA dual-energy x-ray absorptiometers for assessing total and regional body composition. J Clin Densitom 2016;19:290–7.
42. Engmann NJ, Scott CG, Jensen MR, Ma L, Brandt KR, Mahmoudzadeh AP, et al. Longitudinal changes in volumetric breast density with tamoxifen and aromatase inhibitors. Cancer Epidemiol Biomarkers Prev 2017;26:930–7.
43. Hada M, Oh H, Fan S, Falk RT, Geller B, Vacek P, et al. Relationship of serum progesterone and progesterone metabolites with mammographic density. In: Proceedings of the 110th Annual Meeting of the American Association for Cancer Research; 2019 Mar 29–Apr 3; Atlanta, GA. Philadelphia (PA): AACR; 2019. Abstract nr 588.
44. Moran O, Zaman T, Eisen A, Demsky R, Blackmore K, Knight JA, et al. Serum osteoprotegerin levels and mammographic density among high-risk women. Cancer Causes Control 2018;29: 507–17.
45. Wright DJ, Earnhardt JN, Perry R, Bailey S, Komm B, Minck DR, et al. Carcinogenicity and hormone studies with the tissue-selective estrogen receptor modulator bazadoxifene. J Cell Physiol 2013;228:724–33.
46. Hagan CR, Knutson TP, Lange CA. A common docking domain in progesterone receptor-B links DUSP6 and CK2 signaling to proliferative transcriptional programs in breast cancer cells. Nucleic Acids Res 2013;41:8926–42.
47. Obr AE, Grimm SL, Bishop KA, Pike JW, Lydon JP, Edwards DP. Progesterone receptor and Stat5 signaling cross talk through RANKL in mammary epithelial cells. Mol Endocrinol 2013;7: 1808–24.
48. De Marco P, Cirillo F, Vivacqua A, Malaguarnera R, Belfiore A, Maggiolini M. Novel aspects concerning the functional cross-talk between the insulin/IGF-i system and estrogen signaling in cancer cells. Front Endocrinol (Lausanne) 2015;6:30.
49. Khan QJ, Kimler BF, O'Dea AP, Zalles CM, Sharma P, Fabian CJ. Mammographic density does not correlate with Ki-67 expression or cytomorphology in benign breast cells obtained by random periareolar fine needle aspiration from women at high risk for breast cancer. Breast Cancer Res 2007;9:R35.
50. Verheus M, Maskarinec G, Erber E, Steude JS, Killeen J, Hernandez BY, et al. Mammographic density and epithelial histopathologic markers. BMC Cancer 2009;9:182.