Effects of the Aromatase Inhibitor Letrozole on Normal Breast Epithelial Cell Proliferation and Metabolic Indices in Postmenopausal Women: A Pilot Study for Breast Cancer Prevention

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Abstract

The aromatase enzyme converts androgens to estrogens and is the therapeutic target for aromatase inhibitors in postmenopausal patients with estrogen receptor-positive metastatic breast cancer. Third-generation inhibitors such as letrozole are being considered as potential prophylactic agents for breast cancer. The rationale for their preventive application would be aided by knowledge of their effects on the normal breast and on other estrogen-dependent processes such as bone and lipid metabolism. Thirty-two women without active breast disease were recruited to 3-month treatment with letrozole (2.5 mg/d). Core-cut biopsies from the breast and blood samples were collected before and at the end of treatment. Plasma estradiol levels were markedly suppressed in all but two patients, who were excluded from the efficacy assessment. There was no significant change in the proliferation marker Ki67 (mean change, −23%; 95% confidence interval, −50% to +23%) or estrogen receptor in breast epithelial cells with treatment. Similarly, there were no significant changes in plasma levels of insulin-like growth factor I or lipid profiles. However, there was a significant increase (25%) in the levels of the bone resorption marker C-telopeptide crosslinks (CTxs). We conclude that any prophylactic effect of letrozole is not likely to be dependent on antiproliferative effects on normal breast. Studies in healthy patients will need to recognize the potential for enhanced bone resorption.

Introduction

The need for prevention strategies for breast cancer is highlighted by the fact that the disease constitutes 25% of all female cancers, with approximately 10% of women developing the malignancy during their lives, and 3% dying from it (1). It is estimated that <10% of these cases present as a result of a high-penetrance defect in genes such as BRCA1 and BRCA2 (2). The rest are generally considered as sporadic cancer, although this group may include cancers that develop as a result of genetic predisposition of low penetrance (2). Thus, it is likely that chemopreventives will need to be targeted at populations with only modestly increased risk.

The results of the large randomized National Surgical Adjuvant Breast and Bowel Project P-1 trial have moved the concept of “prevention/retardation” further toward reality and led to the licensing (in the United States) of tamoxifen for this purpose (3). Tamoxifen, however, has a number of side effects, including increased endometrial carcinoma and thromboembolism, which assume a greater significance in the context of preventing breast cancer in healthy women (3). It is thus appropriate to consider other well-tolerated drugs that are effective in the treatment of established breast cancer as possible alternatives to tamoxifen. The justification for their use in cancer prevention pilot strategies will depend on our understanding of the multifactorial etiology of the disease and the ability of these agents to influence this. Accordingly, aromatase inhibitors that inhibit the synthesis of estrogens appear to be good candidates for prophylaxis.

The majority of the factors that are known to be significantly associated with an increased risk of breast cancer can be explained by the mitogenic influence of steroidal hormones, predominantly E_2, on the female breast over a lifetime (4). Enhanced proliferation is accepted as playing a significant role in tumorigenesis by reducing the time for DNA repair subsequent to mutagenic influences (5).

In postmenopausal women, hormone-related risk factors include the use of HRT and a positive association with obesity, which is associated with high plasma E_2 and low SHBG levels, resulting in a higher proportion of biologically active E_2. In postmenopausal women, the highest risk of breast cancer (10, 11). Plasma testos-
terone levels have also been found to be a significant risk factor in some of the above-mentioned studies (12, 13), but in one study, when it was adjusted for E₂ levels, the risk associated with testosterone was completely lost (14). This may be explained by testosterone acting as a precursor for E₂ through the aromatase enzyme and provides a specific rationale for the evaluation of aromatase inhibitors for prevention. In addition to their promotional mitogenic effect, evidence has recently been gathered that estrogens, via their hydroxy metabolites, may have direct genotoxic effects that are independent of the ER status of the cell (15) (16). If this proves to be a significant mechanism, prevention strategies based on estrogen withdrawal may be more effective than those based on antiestrogens.

In postmenopausal women, third-generation aromatase inhibitors (anastrozole, letrozole, and exemestane) can provide near complete estrogen withdrawal by blocking the aromatase enzyme, which converts androgens to estrogens, directly reducing estrogen levels (17). Letrozole has been found to be more effective than tamoxifen in the treatment of steroid receptor-positive advanced breast cancer and in the neoadjuvant treatment of primary breast cancer (18, 19). The effects of aromatase inhibitors have not been assessed in the normal breast with regard to their potential for prevention, but in Sprague-Dawley rats, fadrozole (another aromatase inhibitor) was found to markedly reduce the incidence of spontaneous mammary tumors (20).

The data above suggest that if proliferation of the target tissue (in this instance, breast tissue) was reduced by an intervention, such as an aromatase inhibitor, this would be compelling evidence for a potentially prophylactic effect related to cancer. The aim of this study was therefore to examine a series of end points that are pertinent to the possible use of letrozole in normal postmenopausal women as a preventive for breast cancer. Analysis of the effect of letrozole on the tissue proliferation marker Ki67 was the primary objective of the study, and the hypothesis was that estrogen withdrawal would lead to a reduction in proliferation. Such decreases have been observed in established breast cancer when using aromatase inhibitors as well as other endocrine agents (21, 22). Secondary objectives included the evaluation of changes in expression of ERs and PgRs, which are both important predictors for response to endocrine therapy in breast cancer (23). Other markers to be assessed included plasma IGF-1 levels, which have been reported to act as both a mitogenic factor and a survival factor in breast cancer (24) and are considered by some to be a possible biomarker of the effectiveness of hormonal therapy (25). Changes in lipid profiles (cholesterol, LDL, and HDL) and markers of bone metabolism were also assessed. Plasma lipids are recognized as markers of risk of cardiovascular disease, and their levels are modified by tamoxifen and HRT. It might be expected that increased osteoporosis could occur as a result of the systemic estrogen deprivation by aromatase inhibitors, and whereas bone mineral density changes take many months to develop, changes in the plasma levels of some bone resorption markers may be measurable in a few weeks and predict eventual bone density changes (26, 27).

**Patients and Methods**

**Patient Selection**

Women who had been treated previously at the Royal Marsden Hospital for benign breast disease or DCIS and LCIS or who had attended the Regional Breast Screening Unit were targeted for recruitment into the study. Women were targeted by searching the hospital data base and then approached via follow-up clinics or mail shots. The latter informed women that a study on breast cancer prevention was occurring and asked for interested volunteers to reply with contact details. Those who replied were then contacted via telephone (if details were given) or letter. During second telephone contacts, eligibility criteria were checked, and, if favorable, full information details were sent. Others were sent information packs directly with details of eligibility. This stepwise method of recruitment was adopted to encourage as many women as possible to receive full information without inducing undue anxiety.

Women were eligible if they: (a) were postmenopausal (≥12 months amenorrhoea; if they were <56 years and had <1 year amenorrhoea after ovariectomy or were hysterectomized before “menopause,” then confirmation with follicle-stimulating hormone and luteinizing hormone was required); (b) had previously treated DCIS/LCIS, had no previous treatment for malignancy to the nondiseased breast (study breast), and last follow-up mammogram was clear; (c) had previously treated benign disease, and last recorded mammogram was clear; and (d) attending screening and last recorded mammogram was clear. Women were ineligible if: (a) there had been previous invasive malignancy to either breast; (b) radiotherapy, as part of DCIS management, had been completed <4 weeks previously; (c) they had taken any prior or were taking concomitant hormonal therapy, e.g., tamoxifen; (d) they had taken HRT <3 months before entry into the study; (e) they were >80 years old and had Eastern Cooperative Oncology Group performance status <2; (f) they had any other medical complaint or were taking medication that may have interfered with the use of letrozole; and (g) they were unable to comply with the study protocol.

Approval was obtained from the relevant ethics committees before commencing recruitment, and the study was conducted in accordance with the Declaration of Helsinki. Participants gave their written informed consent after receiving an ethics committee-approved information sheet.

**Study Design**

Volunteers consented to take 2.5 mg of letrozole p.o. once daily in the evening for 12 weeks, with serum and tissue taken for biomarker assessment before and at the end of letrozole therapy. The primary end point of the study was change of the proliferation biomarker Ki67, and the study was powered accordingly. The power calculations were based on results from our earlier study of the variability of Ki67, assessed in core cuts from the normal breast tissue of mastectomy specimens from postmenopausal women not on tamoxifen or HRT (28). Using the SD of the log (Ki67) score, it was estimated that for an 80% probability of achieving a significant result when the true reduction in Ki67 was ≥50%, 30 volunteers were required (28).

A reduction of at least 50% was chosen because this was exceeded in our previous work with tamoxifen in malignant disease (29).

**Sample Collection**

A 20-ml clotted blood sample was collected before and at the end of the study period at approximately the same time of day on each occasion. The samples were allowed to clot for 2 h at room temperature and then centrifuged for 10 min at 2000 rpm, and the serum was stored at −20°C. Using ultrasound to locate normal glandular breast, core-cut biopsies were taken with a 14-gauge BIP High Speed Core Cut 2 needle before commencing letrozole and on completion

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of 12 weeks of letrozole. Volunteers were requested to refrain from using aspirin or other nonsteroidal inflammatory drugs for 2 weeks before biopsy. Up to seven cores were taken through the same anesthetized (up to 5 ml of 2% lidocaine) entry site, followed by compression and simple dressing. Fewer cores were taken if bleeding occurred that did not cease quickly on compression. One core was immediately frozen in liquid nitrogen and stored at $-70^\circ\text{C}$, and the rest were fixed in 10% neutral buffered formalin and later embedded in a single paraffin wax block. Sections (3 $\mu$m thick) were cut from the embedded cores and mounted on “charged” slides. A section was stained with H&E to assess the presence, if any, of glandular breast tissue and reviewed by a pathologist to ensure that the specimens for investigation contained only normal breast tissue. Volunteers were only commenced on letrozole after this examination had been performed on the pretreatment biopsy sample.

Clinical Data Collection
At the second biopsy, volunteers were asked if significant bruising had occurred after the first procedure and requested to make contact with the clinic if the second biopsy resulted in any significant effects. On completion of letrozole, information on side effects was gathered using a prompted questionnaire listing the main documented side effects of letrozole.

Laboratory Methods

Immunohistochemical Analyses. General reagents were purchased from Sigma (Dorset, United Kingdom). The method for Ki67 staining using MIB-1 antibody (Immunotech) and ER and PgR staining involved microwave antigen retrieval and indirect staining with the avidin-biotin complex technique, largely as described previously (30). Primary antibodies for ER and PgR, however, utilized Novocastra antibodies at 1:40 dilution (monoclonal clone 6F11 and 1A6, respectively; Novocastra, Newcastle-upon-Tyne, United Kingdom). It is known that the baseline proliferative activity is low in postmenopausal women and that errors in scoring of epithelial cells could compromise the ability to detect changes in proliferation (28, 31). The sections were therefore double-stained using a mouse antihuman antibody against smooth muscle actin (SMA Dako Ltd., High Wycombe, United Kingdom) with methods described previously (32) to highlight myoepithelial cells and exclude them from epithelial cell counts (28). Three thousand cells were scored for Ki67 per case using a previously described system, and results were expressed as the percentage of cells stained (30). ER and PgR staining was scored using the “H-score” method, giving a possible range of values from 0 to 300 (33).

Serum Analyses. SHBG and IGF-I were measured using standard kits: Orion Diagnostica (Pharmacia Limited, Milton Keynes, United Kingdom) and DSL 2800 (Diagnostic Systems Laboratories, London, United Kingdom), respectively. E2 was measured using previously described methods from this laboratory (34). Lipid analyses (cholesterol, LDL, and HDL) used an enzymatic method on a Beckman Synchron CX9ALX machine (Beckman, High Wycombe, United Kingdom). Degradation products of type 1 collagen (CTx) were measured using an ELISA (CrossLaps). This test utilizes two highly specific monoclonal antibodies against amino acid sequences in the products from the COOH-terminal telopeptides of type 1 collagen in the serum (Osteometer BioTech A/S, Herlev, Denmark.)

Statistical Methods
The significance of changes in parameter values after the intervention with letrozole were assessed by the Wilcoxon signed-rank test, and CIs for the changes were calculated by assuming that the mean was normally distributed. If there was evidence that a parameter was positively skewed, the values were log-transformed; in this case, the Wilcoxon signed-rank test was used to investigate whether the log of the proportional change was non-zero (which is equivalent to examining whether the proportional change differs significantly from 1). CIs were calculated based on the logs of the proportional changes, and these were back-transformed to express them as proportional changes. Two-sided tests were used in every instance.

Results

Patient Accrual and Characteristics. Using the mail shot method, 335 leaflets were initially sent out. Fig. 1 shows the number of replies and refusals at each stage and indicates that
being able to discuss the study over the phone resulted in the greatest acceptance into the study. Thirty-two women were recruited into the study (23 from the previously treated benign disease [mail-shot] cohort, 7 from the previously treated DCIS/LCIS cohort, and 2 from the Regional Breast Screening Unit). Three withdrew before completing the study: one was not willing to have a repeat biopsy after bruising sustained during the intervention (i.e., estrogen withdrawal) and hence the hypothesis being evaluated were not testable with them.

IGF-I and SHBG levels did not show a significant change between the pre- and posttreatment values (Fig. 3; Table 2). Individual patient changes are shown in Fig. 2 and Table 2. There was a significant and substantial overall fall in E2 over the 12 weeks (P < 0.001; Table 2). Four of the 29 women completing the study had a significant fall in E2, (means); P = 0.02 (Fig. 4a; Table 3). This was a mean increase of 25% (95% CI, 5–49; Fig. 4b). There were no significant associations between pre- or posttreatment serum E2 and CTX or in the differences between the pre and posttreatment values.

**Immunocytochemical Markers.** Two patients required repeat pretreatment biopsy to obtain a cellular tissue sample, and, finally, 26 paired patient tissue samples were suitable for immunocytochemical analysis. Nineteen women reported effects attributed to letrozole (Table 1), with the most common being hot flushes (11), joint discomfort (6), mood alteration (5), and headache (4).

**Serum Markers.** Paired samples were available from all 29 volunteers who completed the study. Assay sensitivity was 3 pmol/liter, and values below that were plotted as 3 pmol/liter. There were no significant associations between pre- or posttreatment serum E2 and CTX or in the differences between the pre and posttreatment values.
munocytochemical analysis. On average, 3 of the 6/7 core-cut biopsies (a sample set) taken contained epithelial cells, and the others contained fat only. Three sample sets contained biopsies (a sample set) taken contained epithelial cells, and the immunocytochemical analysis. On average, 3 of the 6/7 core-cut sections (range, 2–42 core-cut sections) needed to be combined to reach the 3000 cells needed for Ki67 analysis. Overall Ki67 changes were not significant, but Fig. 5a shows the increased variability between pre- and posttreatment samples with marked individual increases and decreases in Ki67 after the study period. There was a mean 23% fall in Ki67 to a mean posttreatment value of 0.99% positive cells (Table 4). The 95% CI indicated that a fall of <50% could not be excluded (Fig. 5b).

There was no significant change overall in ER, with mean pre- and posttreatment values of 126 and 109, respectively (Table 4; Fig. 6). There were no significant associations between pre- or posttreatment values of ER, Ki67, or E2 concentration or differences between these parameters during treatment. PgR staining was conducted, but the number of cells staining was so low (<5%) that this analysis was not pursued because it was considered very unlikely that reduced expression over the study period could have been detected.

Discussion

For aromatase inhibitors such as letrozole to be considered as potential preventive agents in breast cancer, it is helpful to have data on their effects on normal breast, and it is necessary to characterize their potential systemic complications, e.g., on bone and lipid, as mentioned above. This study was therefore conducted to examine the biological effects of letrozole on normal postmenopausal breast and pertinent serum markers.

As mentioned above, the term “prevention” applies in the main to strategies that prevent or retard the development of breast cancer against the background of genetic alteration. A basic molecular event that is accepted as being involved in cancer development and whose change is considered as a possible suitable intermediate marker is cellular proliferation (35). This provided the rationale behind the choice of Ki67 as a primary end point in this study, which was powered to detect a reduction of at least 50% in Ki67 on the basis that such changes have previously been seen in breast carcinomas with endocrine agents and the data excluded this. The CIs of the measured change allowed a change of this degree to be excluded but did not exclude reductions of <50% (CI, 50–123%). It is highly unlikely that other aromatase inhibitors would be effective in reducing Ki67 in normal breast when letrozole is not: we have shown that letrozole essentially completely ablates aromatase activity (36); and in this study, the two patients that did not achieve a near complete suppression of plasma E2 were excluded from the analysis.

The only other clinical study reported on normal breast of postmenopausal women that assessed change in Ki67 after an antiestrogenic therapy involved tamoxifen and also showed no significant change in the marker after a range of 4–21 days (37). In contrast, using similar analytical procedures, we found that the aromatase inhibitors vorozole and tamoxifen reduced Ki67 in ER-positive primary breast carcinomas by a mean 73% and 49%, respectively, after 3 months. It would therefore appear that ER-positive breast tumors may have a greater sensitivity to the prevailing estrogen environment and thus to these interventions. Such concepts are consistent with hormonal prevention in postmenopausal women being more likely to have effects on existing subclinical tumors or possibly preinvasive disease than on normal epithelium. It should be noted, however, that this study did not target women at increased risk, for example, as a result of high plasma or estradiol levels (10, 11), and we cannot exclude a substantial effect on normal breast epithelium of such a group. Similarly, this study cannot exclude potential preventive effects that letrozole may exert on subclinical disease or via its action on possible estrogen-derived mutagenic DNA adducts (see “Introduction”).

The lack of a significant change in ER after estrogen withdrawal is of interest because tamoxifen treatment was found to lead to a significant increase in ER expression in normal breast (37). This was explained as up-regulation resulting from the efficient reduction of E2 to the cells by tamoxifen, analogous to the up-regulation seen in the estrogenic transition period between premenopausal and postmenopausal status (38). From this, it might be expected that perhaps up-regulation would occur with further estrogen withdrawal as implemented in our study, but this does not appear to be the case, and again

![Fig. 3](image_url) Effect of 3-month treatment with letrozole on changes in serum IGF-I on 27 volunteers.

**Table 3** Effects of 3 months of treatment with letrozole on mean serum lipids and bone resorption marker-CTx levels in 27 volunteers

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Time</th>
<th>Mean (95% CI)</th>
<th>P (w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/liter)</td>
<td>Pretreatment</td>
<td>5.6 (5.3–5.9)</td>
<td>0.6</td>
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<tr>
<td></td>
<td>12 wks</td>
<td>5.7 (5.3–6.0)</td>
<td></td>
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<tr>
<td>LDL (mmol/liter)</td>
<td>Pretreatment</td>
<td>3.8 (3.5–4.1)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>12 wks</td>
<td>3.8 (3.5–4.1)</td>
<td></td>
</tr>
<tr>
<td>HDL (mmol/liter)</td>
<td>Pretreatment</td>
<td>1.3 (1.2–1.4)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>12 wks</td>
<td>1.4 (1.2–1.5)</td>
<td></td>
</tr>
<tr>
<td>CTx (pmol/liter)</td>
<td>Pretreatment</td>
<td>1900.7 (1500.3–2388.4)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>12 wks</td>
<td>2368.5 (1986.5–2833.5)</td>
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*Bold indicates significant P.*
the explanation may be that there is no increased sensitivity at these very low levels of estrogen. The contrasting finding with tamoxifen might be due to the different mechanism of action of tamoxifen through its binding to the ER or possibly effects on other pathways such as IGF-I suppression.

IGF-I is mitogenic to breast cancer cells (24). Clinical studies in breast cancer have consistently shown a reduction in plasma levels of IGF-I with tamoxifen treatment but increases or no change with aromatase inhibitor therapy (39–41). Our study is the first to report the effect on serum IGF-I using an aromatase inhibitor in normal postmenopausal women, and we found no significant change, consistent with some of the reports in breast cancer patients. Little is known regarding the role that IGF-I and its family members play in normal breast. Associations of increased risk of breast cancer development with high serum IGF-I levels have been reported in premenopausal but not postmenopausal women (42). Based on this and on the

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Effects of 3 months of treatment with letrozole on mean immunocytochemical levels in 24 volunteers</th>
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</thead>
<tbody>
<tr>
<td>Geometric means were used for Ki67, and arithmetic means were used for ER. <em>Ps</em> are as described in Table 1.</td>
<td></td>
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<tr>
<td>Analyte</td>
<td>Time</td>
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<td>---------</td>
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</tr>
<tr>
<td>Ki67%</td>
<td>Pretreatment</td>
</tr>
<tr>
<td></td>
<td>12 wks</td>
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<tr>
<td>ER (H-score)</td>
<td>Pretreatment</td>
</tr>
<tr>
<td></td>
<td>12 wks</td>
</tr>
</tbody>
</table>

reductions in IGF-I seen with tamoxifen and retinoids in normal women, it has been postulated that IGF-I may be an appropriate surrogate marker in breast cancer prevention (25). If correct, this may be agent specific and may not apply to the effects of estrogen deprivation with aromatase inhibitors.
IGF-I is also considered a potent mitogen of osteoblasts, and reduced IGF-I levels in normal postmenopausal women have been associated with osteoporosis (43, 44). Given the lack of suppression of IGF-I seen with letrozole, this mechanism cannot explain the change seen here in the bone resorption marker CTx.

The preservative effects of tamoxifen on bone mineral density in postmenopausal women are advantageous and contribute to the argument for its use in the prevention setting (3, 45, 46). In contrast, there have been theoretical concerns over the effect that the profound estrogen deprivation achieved with third-generation aromatase inhibitors might have on bone turnover (47). There are no published data on the effects of aromatase inhibition on bone density or intermediate markers of bone loss such as CTx in normal women. Studies in women with advanced breast cancer are confounded by the propensity of the disease to metastasize to bone and cause osteolysis. The CTx assay is specific for mature bone products of resorption from the degradation of the COOH-terminal telopeptide of type I collagen (48). The advantages to its use are the ease of storage, accurate collection and reduction in variability compared with urine analysis (27, 48). In two randomized trials using HRT, it has been shown to correlate significantly with bone mineral density and to predict for change in the latter at a future point (26, 27). In a recent study in primary breast cancer, we found a nonsignificant rise in serum CTx with the aromatase inhibitor vorozole compared with a significant fall in the tamoxifen group (21). In the current study, we found a significant increase in CTx, which is consistent with an increase in bone resorption.

In unfasted samples, CTx shows circadian changes with a nadir plateau extending from 10.00 to 16.00 h (27). Our patients had samples taken within this nadir, and thus results are not likely to have been significantly affected by circadian variation. Although we found no correlation between serum E2 and CTx, others using larger numbers have shown a significant inverse correlation between E2 and CTx (49). The degree of bone mineral density change resulting from the increase in CTx observed here is not predictable from currently available data. Whereas the current data do not allow any consideration as to how any effect might be confined to particular subgroups of women, it is possible that those women with the highest plasma estrogen levels might be the most sensitive to the effects of estrogen deprivation. Because this group has higher bone mineral density, any resorptive effects may have less clinical significance. This hypothesis is being be examined in ongoing studies. Additionally, relatively straightforward strategies may be possible to avoid any increased bone resorption being clinically detrimental.

Our study showed no substantial effects on plasma lipids, indicating that aromatase inhibitors are unlikely to contribute to a negative cardiovascular risk in postmenopausal women. These findings are consistent with data on other aromatase inhibitors in breast cancer (21, 50, 51) but contrast with those recently reported with letrozole (52), where increases in total cholesterol \( (P = 0.05) \) and LDL cholesterol \( (P < 0.01) \) were observed. In contrast to our data and most published data on aromatase inhibitors, these data were derived from fasting patients and are therefore probably more sensitive to detecting any change. However, they were from an advanced breast cancer population in which many other drugs are generally in use with potential confounding effects on metabolic indices.

Accrual into the study was difficult even when conducted in our specialist cancer center. However, with sufficient time for explanation, the target number of volunteers was recruited. The relatively invasive procedures used in the study were well tolerated by the volunteers, and the acceptability of the approach was demonstrated by only one volunteer refusing a second set of biopsies. This experience was in a relatively normal-risk population, and in a well-motivated high-risk group, accrual might be considerably easier. Thus, although our experience in this study has led us to question the usefulness of Ki67 as an intermediate marker in the normal breast of postmenopausal women, the approach of obtaining normal breast tissue by core biopsy for the assessment of biological end points in prevention studies would appear to be viable.

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