



Molecular markers in the endometrium at baseline of postmenopausal patients with early breast cancer in the ATAC (Arimidex, tamoxifen, alone, or in combination) trial

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KEY WORDS

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Objective: This study was undertaken to assess baseline endometrial molecular events in the ATAC (Arimidex, tamoxifen, alone, or in combination) trial of breast cancer adjuvant therapy.

Study design: Estrogen receptor (ER) and progesterone receptor (PR) levels and markers of cell proliferation (Ki67) and apoptosis (*Bcl-2*) were assessed in 93 patients at baseline.

Results: An inactive/atrophic endometrium was found in 63 patients, 5 had a proliferative endometrium, and 12 had a secretory endometrium. Thirteen endometrial polyps were analyzed. Inactive endometrium showed high levels of ER in the glandular epithelium, whereas in more than 50% of samples, PR expression was negative or low (+) in the glandular epithelium, and stroma. Ki67 expression was low in both the glandular epithelium and the stroma of the inactive endometrium, whereas *Bcl-2* expression was mostly high or very high (+++/+++++) in the glandular epithelium. *Bcl-2* was strongly expressed (+++/+++++) in the glandular epithelium of polyps.

Conclusion: Although all patients were asymptomatic, some had endometrial pathology.

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The ATAC (Arimidex, tamoxifen, alone, or in combination) trial is a randomized, double-blind trial to assess the efficacy of anastrozole (arimidex; AstraZeneca, Wilmington, Del) and tamoxifen, alone or in combination for 5 years as adjuvant therapy for breast

cancer in postmenopausal women. Tamoxifen has been the mainstay of adjuvant treatment for the past 25 years, but it is associated with the development of endometrial disease, including endometrial adenocarcinoma¹ and possibly uterine sarcoma.² Anastrozole is a nonsteroidal orally active aromatase inhibitor, which is selective, well tolerated, and effective in treating advanced breast cancer.³⁻⁵ The mechanism of action of anastrozole is suppression of estrogen levels via aromatase inhibition. It is devoid of partial agonistic estrogenic activity and would therefore be expected to have no stimulatory effects on the endometrium.

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In the ATAC trial, an endometrial subprotocol was devised to determine whether intrauterine disease development was related to therapy. In this subprotocol, the uterine cavity was examined, and existing uterine disease recorded and removed before treatment.⁶ Endometrial changes occurring during the ATAC trial and their relationship to treatment will be assessed.

To compliment the endometrial subprotocol, estrogen receptor (ER), progesterone receptor (PR), Ki67, and *Bcl-2* levels were assessed at baseline to gain an understanding of the molecular events that may be affected by therapy in the ATAC trial. Estrogens and progestins are known modulators of endometrial proliferation and differentiation via their receptors. As demonstrated in both the normal and malignant endometrium, the ligated ER acts as a potent transcription factor that controls the expression of a number of genes, including genes encoding the PR.⁷ During the proliferative phase of the menstrual cycle, increased levels of estrogen result in the stimulation of epithelial cell proliferation and increased expression of PRs. Cell proliferation is indicated by levels of Ki67, an antigen that is expressed exclusively in the nuclei of proliferating cells. Ki67 levels are highest during the proliferative and early secretory phases of the menstrual cycle and the levels positively correlate with the degree of dedifferentiation of endometrial carcinomas.⁸

The balance between cell proliferation and programmed cell death (apoptosis) is fundamental to the functioning of the menstrual cycle. It is increasingly believed that apoptosis and its control play an important, even pivotal, role in the development of disease. Apoptosis is controlled by a complex network of gene families, including the *Bcl-2* family. *Bcl-2* is an inhibitor of cell death that can extend the survival of cells despite the absence of growth factors. *Bcl-2* expression increases in the proliferative phase and decreases in the secretory phase of the menstrual cycle.⁹⁻¹¹ Studies undertaken by our department have revealed that endometrial polyps demonstrate significantly greater expression of *Bcl-2* compared with proliferative endometrium,¹² suggesting that prolonged overexpression of *Bcl-2* in 1 particular area of the endometrium could lead to a cluster of apoptosis-resistant cells and the formation of a polyp.

Studies have suggested that *Bcl-2* expression is upregulated by estrogen¹³ and downregulated by progesterone¹⁴; thus, *Bcl-2* is implicated in the development of hormone-related disease. It is logical to examine the expression of *Bcl-2* in tamoxifen-exposed endometrial samples to observe whether it is differentially expressed in patients with hormone-related diseases and if this expression is associated with the presence of ER and PR.

The aim of the study presented here is to characterize the baseline expression of ER, PR, Ki67, and *Bcl-2* in all suitable endometrial subprotocol samples taken from the ATAC trial.

Material and methods

Patient selection

Ethical approval for the ATAC trial and the endometrial subprotocol was received from both local and national committees. The main ATAC trial recruited a total of 9366 patients; of these 285 were recruited onto the endometrial subprotocol. The patients were postmenopausal, gynecologically asymptomatic, and had not had a previous hysteroscopy or endometrial ablation. Patients were defined as postmenopausal if they satisfied at least 1 of the following criteria: having had a bilateral oophorectomy; aged 60 years or older; or aged 45 to 59 years with an intact uterus and amenorrheic for 12 months or longer. If a patient had been amenorrheic for less than 12 months (including patients who had undergone a hysterectomy and those who had received hormone replacement therapy or who had been rendered amenorrheic by chemotherapy), follicle-stimulating hormone (FSH) concentrations within the postmenopausal range were required for eligibility. Other inclusion and exclusion criteria of the main ATAC trial have already been described.¹⁵ Any patient who had received neoadjuvant tamoxifen was excluded from the endometrial subprotocol. Thirteen patients were excluded from the endometrial subprotocol without undergoing hysteroscopy (7 withdrew informed consent, 2 had received neoadjuvant tamoxifen, 2 had abnormal ovaries, 1 had cervicitis, and 1 was on warfarin). The remaining 272 patients had a hysteroscopy and a pipelle tissue biopsy attempted before ATAC therapy commenced. Hysteroscopy and biopsy were successful in 264 of the 272 patients. Any endometrial disease present was assessed and removed. Single endometrial samples were formalin fixed, embedded in paraffin, and reviewed by a single designated trial pathologist. The samples were classified as inactive/atrophic, secretory (defined as the presence of epithelial cytoplasmic vacuolation and in the mid-to-late secretory phase, the presence of glandular luminal secretion), or proliferative (epithelial mitoses present). Tissue blocks were identifiable only by a histology code; the endometrial classification was unknown until final data analysis. Of the 264 patients who had a biopsy taken, a histologic classification of the tissue was achieved in 162 cases. Samples were considered suitable for further processing if there was sufficient tissue remaining in the block after routine processing. From the 162 cases, 93 samples were analyzed for this study.

Immunohistochemistry

The tissue was sectioned (5 μ m), dewaxed with xylene, and rehydrated in ethanol. Endogenous peroxidase activity in the tissue was quenched by hydrogen peroxidase solution (30% H₂O₂ in 100% methanol). Heated

Table I Baseline patient demographics

	Anastrozole (n = 39)	Tamoxifen (n = 29)	Combination (n = 25)	Total (n = 93)
Age, y	57.8	59.8	59.6	58.9
Mean (SD)	(7.0)	(7.3)	(8.2)	(7.4)
BMI, kg/m ²	25.9*	26.6*	27.3*	26.4*
Mean (SD)	(4.3)	(3.0)	(5.6)	(4.3)
Hysterectomy (%)				
Yes	0	0	0	0
No	100	100	100	100
HRT				
Yes	48.7	44.8	36.0	44.1
No	51.3	55.2	64.0	55.9

* n = 37 (anastrozole); n = 25 (tamoxifen); n = 22 (combination); n = 84 (total).

antigen retrieval was achieved by microwaving (750 W) the sections in citrate buffer (0.1 mol/L solution, pH 6) on full power for 10 minutes. Sections designated for ER staining were microwaved for 15 minutes. Positive control sections of human tonsil (*Bcl-2* and Ki67) and breast carcinoma (ER and PR) were used throughout. These tissues are known to contain the antigen and always display positive staining. With the use of a Sequensa (Shandon, Runcorn, UK) staining system, the sections were flooded with blocking antibody (150 μ L of 0.15% normal horse serum solution; Vecta, Vector Laboratories, Burlingame, Calif). Primary monoclonal antibodies (Novocastra, Newcastle-upon-Tyne, UK) were diluted as follows: ER (clone 6F11) and PR (clone 1A6) 1 in 60, *Bcl-2* (clone 100/D5) 1 in 80, Ki67 (clone MM1) 1 in 150, and left to incubate on the tissue for 1 hour. Normal horse serum was applied to negative control sections. After a wash (phosphate-buffered saline solution [PBS] buffer, BDH Laboratories, Poole, UK) and a second block with normal horse serum, biotinylated horse antimouse antibody (Vecta) was then applied; the sections were then incubated for 30 minutes. The ABC (avidin-biotin complex; Vecta) was prepared according to the manufacturer's instructions, applied, and incubated for 30 minutes. Antibody binding was visualized with DAB (3, 3' diaminobenzidine tetrahydrochloride) (Sigma, Poole, UK), counterstained with Meyer's hematoxylin (Sigma) and Scott's tap water (20% magnesium sulfate, 7% sodium bicarbonate). Finally, sections were dehydrated, cleared in xylene, and mounted by using DePex (BDH).

Two researchers independently semiquantitatively scored each section. The researchers were trained by the trial pathologist. Any discrepancies were compared and then reanalyzed by the trial pathologist. With the use of a magnification objective of $\times 40$, the percentage of positive staining throughout the entire tissue section was assessed. Positive cells expressing ER, PR, and Ki67 were identified by a brown precipitate in the nucleus. Brown cytoplasmic staining identified *Bcl-2* expression. In accordance with previously published proto-

Table II Histologic diagnosis of the endometrial samples

Histologic diagnosis	No. of samples (n) analyzed for endometrial subprotocol	No. of samples (n) analyzed for receptor study
Inactive endometrium	117	63
Endometrial polyp	18	12
Secretory endometrium	16	12
Proliferative endometrium	7	5
Atypical hyperplasia	1	—
Polyp with atypia	1	1
Polyp with simple hyperplasia	1	—
Fibroid	1	—
Total	162	93

cols,^{12,16,17} the percentage of positive cells was graded as negative (no expression of antigen), low expression (+) less than 25%, moderate expression (++) 25% to 50%, high expression (+++) 50% to 75%, and very high expression (+++++) 75% to 100%. The glandular epithelium and stromal compartments were scored separately. The intensity of staining in the tissue was not assessed.

Results

Age, body mass index (BMI), hysterectomy status, and previous hormone replacement therapy (HRT) history were similar across the three treatment groups (Table I).

Table II displays the histologic diagnosis of the endometrial samples retrieved and details the endometrial samples used for this study. A total of 162 samples were included in the endometrial subprotocol, and 93 of these had sufficient tissue to be analyzed for this study.

The majority of samples (63) were classed as inactive/atrophic; 12 samples were classified as secretory, 5 samples as proliferative, and 12 samples had endometrial polyps (1 with atypia).

Table III Patient demographics by histologic status

	Inactive (n = 63)	Proliferative (n = 5)	Secretory (n = 12)	Polyps (n = 13)	Total (n = 93)
Age (y)	58.8	58.7	59.5	58.9	58.9
Mean (SD)	(7.4)	(12.2)	(7.9)	(5.0)	(7.4)
BMI (kg/m ²)	26.0*	27.6*	27.4*	27.2*	26.4*
Mean (SD)	(4.5)	(4.8)	(4.3)	(3.6)	(4.3)
Hysterectomy (%)					
Yes	0	0	0	0	0
No	100	100	100	100	100
HRT					
Yes	42.9	60.0	33.3	53.8	44.1
No	57.1	40.0	66.7	46.2	55.9
Estradiol concentration pmol/L geometric mean	22.24	50.12	23.89	23.87	23.54

* n = 56 (inactive); n = 5 (proliferative); n = 11 (secretory); n = 12 (polyps); n = 84 (total).

Table IV Expression of ER, PR, *Bcl-2*, and Ki67 in the glandular epithelium and the stromal compartments of endometrium classified as inactive/atrophic

Expression	ER		PR		Ki67		<i>Bcl-2</i>	
	GE	STR	GE	STR	GE	STR	GE	STR
Inactive/atrophic endometrium (n = 63)								
Negative	3	13	23	44	35	52	9	28
Low (+)	3	12	17	14	24	10	6	27
Moderate (++)	4	12	8	3	3	0	4	6
High (+++)	6	13	6	0	1	1	17	2
Very high (++++)	47	13	9	2	0	0	27	0

Low (+) 0%-25%; moderate (++) 25%-50%; high (+++) 50%-75%; very high (++++) 75%-100%. GE, Glandular epithelium; STR, stroma.

Patient demographics (age, BMI, hysterectomy status, and previous HRT status) were also comparable between the pathologically classified groups, with plasma estradiol levels observed to be similar in the 4 patient groups (inactive, proliferative, secretory, and polyps; Table III).

Inactive/atrophic endometrium

A total of 63 (68%) patients sampled in this study had an inactive endometrium. Three quarters of these samples demonstrated very high (++++) expression of ER in the glandular epithelium (Table IV). There was an even distribution of ER expression in the stroma ranging from negative to very high (++++). More than half of the endometrial samples had negative or low (+) expression of PR in the glandular epithelium with stromal expression of PR being assessed as mostly negative. Ki67 expression in the glandular epithelium and stroma was mainly negative or low (+). *Bcl-2* expression was for the most part high to very high (++++/++++) in the glandular epithelium and negative or low (+) in the stroma.

Table V Expression of ER, PR, *Bcl-2*, and Ki67 in the glandular epithelium and the stromal compartments of endometrium classified as proliferative

Expression	ER		PR		Ki67		<i>Bcl-2</i>	
	GE	STR	GE	STR	GE	STR	GE	STR
Proliferative endometrium (n = 5)								
Negative	0	0	1	3	1	2	0	2
Low (+)	0	1	0	0	0	3	0	0
Moderate (++)	0	0	0	2	2	0	1	2
High (+++)	0	3	0	0	2	0	1	1
Very high (++++)	5	1	4	0	0	0	3	0

Low (+) 0%-25%; moderate (++) 25%-50%; high (+++) 50%-75%; very high (++++) 75%-100%.

Proliferative endometrium

All 5 samples from patients with proliferative endometrium demonstrated very high (++++) levels of expression of ER in the glandular epithelium, 4 of the 5 samples had very high (++++) levels of expression of PR in the glandular epithelium, and 3 of the 5 samples had very high (++++) levels of expression of *Bcl-2* in the glandular epithelium (Table V). Of the 5 samples, 4 had moderate (++) or high (+++) levels of expression of Ki67 in the glandular epithelium. Expression of the markers in the stroma was generally lower than in the glandular epithelium.

Secretory endometrium

For the 12 samples of endometrium classified as secretory, ER expression was mostly very high (++++) in the glandular epithelium. Stromal expression of ER was lower compared with the glandular epithelium, with 8 of the 12 samples demonstrating low (+) expression. The expression of PR in the glandular

Table VI Expression of ER, PR, *Bcl-2*, and Ki67 in the glandular epithelium and the stromal compartments of endometrium classified as secretory

Expression	ER		PR		Ki67		<i>Bcl-2</i>	
	GE	STR	GE	STR	GE	STR	GE	STR
Secretory endometrium (n = 12)								
Negative	0	0	4	9	5	6	2	7
Low (+)	0	8	4	3	4	6	1	4
Moderate (++)	1	1	0	0	3	0	7	1
High (+++)	2	1	3	0	0	0	1	0
Very high (++++)	9	2	1	0	0	0	1	0

Low (+) 0%-25%; moderate (++) 25%-50%; high (+++) 50%-75%; very high (++++) 75%-100%.

epithelium ranged from negative to very high (++++), although the majority of samples showing negative to low (+) expression. Stromal expression of PR was confined to negative or very low (+). Ki67 expression ranged from negative to moderate (++) in both the stroma and the glandular epithelium, with 9 of the 12 samples demonstrating negative to low (+) expression in the glandular epithelium (Table VI). *Bcl-2* expression ranged from negative to very high (++++) in the glandular epithelium, although 7 of the 12 samples had moderate (++) expression levels. Stromal expression of *Bcl-2* was less positive with 11 of the 12 samples having negative or low (+) expression levels.

Endometrial polyps

A total of 13 endometrial polyps were analyzed separately from the surrounding endometrium. ER expression in the glandular epithelium was very high (++++) in all but 1 of the endometrial polyps. The pattern of stromal expression was similar to the glandular epithelium with 12 of the 13 polyps demonstrating high (+++) or very high (++++) expression of ER. PR expression in the glandular epithelium and the stroma ranged from negative to very high (++++). Ki67 expression ranged from negative to moderate (++) with 8 of the 13 polyps being low (+) in the glandular epithelium. Stromal expression of Ki67 was mostly negative (10 of the 13 polyps) with 1 polyp demonstrating moderate (++) expression levels. *Bcl-2* expression demonstrated a range of expression in the glandular epithelium; however, 11 of the 13 polyps had high (+++) or very high (++++) expression levels (Table VII). Stromal expression of *Bcl-2* was lower with all the polyps demonstrating negative to moderate (++) levels.

Comment

Although several articles have investigated the expression of ER, PR, Ki67, and *Bcl-2* in the postmenopausal

Table VII Expression of ER, PR, *Bcl-2*, and Ki67 in the glandular epithelium and the stromal compartments of endometrial polyps

Expression	ER		PR		Ki67		<i>Bcl-2</i>	
	GE	STR	GE	STR	GE	STR	GE	STR
Endometrial polyps (n = 13)								
Negative	0	0	2	4	2	10	1	6
Low (+)	1	1	1	4	8	2	1	3
Moderate (++)	0	0	3	1	3	1	0	4
High (+++)	0	6	1	2	0	0	3	0
Very high (++++)	12	6	6	2	0	0	8	0

Low (+) 0%-25%; moderate (++) 25%-50%; high (+++) 50%-75%; very high (++++) 75%-100%.

endometrium (PMEM),¹⁸⁻²³ the ATAC endometrial subprotocol receptor study is, to our knowledge, the first multicentered longitudinal study to investigate molecular markers in samples from asymptomatic women with breast carcinoma before adjuvant treatment to establish a “baseline” mark of endometrial status. In this study, the PMEM was classified as proliferative secretory or inactive/atrophic, reflecting the heterogeneity of the PMEM. This heterogeneity may explain the complexity of the partial agonist activity of tamoxifen and its variable effects on the endometrium.

The majority of patients in this study had an inactive endometrium, which demonstrated high levels of ER expression in the glandular epithelium and a range of expression in the stroma. Expression of PR was generally negative or low (+) in the glandular epithelium and negative in the stroma. Compared with a previous study,¹⁹ which investigated PMEM from symptomatic women, the samples from the ATAC trial that were classified as inactive demonstrated greater expression of ER in both the glandular epithelium and the stroma. However, both studies found similar patterns of PR expression. It is known that the PMEM retains ERs that can be stimulated by estrogen after the menopause, and it is generally accepted that PR expression is an indicator of estrogenic activity in the endometrium.^{24,25} It is interesting that even though the samples from the ATAC trial demonstrated more ER, there was no corresponding increase in PR expression. This would suggest that there is no increase in estrogenic activity in these samples. Overall, the expression of ER and PR in the endometrial samples from the ATAC trial are comparable with the results of other studies of PMEM of asymptomatic women.^{22,23}

The inactive endometrium samples demonstrated low Ki67 expression in both the glandular epithelium and the stroma. This is to be expected as it indicates low mitotic levels and a low cell division. This is a well-characterized feature of the inactive PMEM.²⁶ *Bcl-2*

expression in the inactive endometrial samples was mostly high or very high (+++/++++) in the glandular epithelium. The high levels of *Bcl-2* are likely to indicate low levels of apoptosis in the inactive endometrium samples and coupled with the low expression of Ki67, it would appear that the endometrium is quiescent with little cellular turnover.

It has been assumed that after the menopause, chronic estrogen deficiency prevents endometrial stimulation through the normal cyclical changes. Lack of estrogen causes atrophy of the vagina, cervix, and uterus, including the endometrium. It is evident from this study that even after menopause, there is still a low, yet sufficient, level of estrogen to stimulate receptor expression and trigger the endometrium through its cyclical functional adaptations in some women. This could mean that certain individuals have a unique low-estrogen stimulation threshold, which once reached, stimulates the receptors and triggers estrogenic effects in the tissues. The group of patients with proliferative or secretory endometrium could experience an adverse response to therapy in the ATAC trial and be more susceptible to developing disease. Conversely, their endometrium may give them a form of protection from endometrial side effects.

The expression of ER and PR in the endometrial polyps of the ATAC study group demonstrated similar patterns to that observed in our previous study of premenopausal endometrial polyps.¹² *Bcl-2* was highly expressed (+++/++++) in the glandular epithelium of the polyps, which may be indicative of decreased levels of apoptosis. This is a noted characteristic of premenopausal endometrial polyps and is thought to play a pivotal role in their pathogenesis. Overall, Ki67 expression was low in the samples from the ATAC trial. There is, as yet, no indication that these endometrial polyps have an increased potential for malignant transformation. They do not appear to have any unusual physiologic characteristics that make them unique from other symptomatic postmenopausal endometrial polyps. Data from year 1 will indicate whether having had a previous polyp at baseline predisposes an individual to having further endometrial disease develop.

Although all the patients in this study were asymptomatic, some (~15%) had endometrial disease. It will be interesting to observe whether this baseline disease correlates with response to treatment.

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