Int. J. Cancer: **121,** 1079–1085 (2007)

Variation in breast cancer hormone receptor and *HER2* levels by etiologic factors: A population-based analysis

Mark E. Sherman[1]*, David L. Rimm[2], Xiaohong R. Yang[1], Nilanjan Chatterjee[1], Louise A. Brinton[1], Jolanta Lissowska[3], Beata Peplonska[4], Neonila Szeszenia-Dąbrowska[4], Witold Zatonski[3], Richard Cartun[5], Daniza Mandich[5], Grzegorz Rymkiewicz[6], Marcin Ligaj[6], Stanisław Lukaszek[7], Radzislaw Kordek[8], Zynep Kalaylioglu[9], Malini Harigopal[2], Lori Charrette[2], Roni T. Falk[1], Douglas Richesson[1], William F. Anderson[1], Stephen M. Hewitt[10] and Montserrat García-Closas[1]

[1]*Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD*
[2]*Department of Pathology, Yale University School of Medicine, New Haven, CT*
[3]*Department of Cancer Epidemiology and Prevention, Cancer Center and M. Sklodowska-Curie Institute of Oncology, Warsaw, Poland*
[4]*Department of Occupational and Environmental Epidemiology, Nofer Institute of Occupational Medicine, Łódź, Poland*
[5]*Department of Pathology, Hartford Hospital, Hartford, CT*
[6]*Department of Pathology, Cancer Center and M. Sklodowska-Curie Institute of Oncology, Warsaw, Poland*
[7]*Department of Clinical Pathomorphology, Polish Mother's Memorial Hospital-Research Institute, Łódź, Poland*
[8]*Department of Pathology, Medical University of Łódź, Łódź, Poland*
[9]*IMS, Silver Spring, MD*
[10]*Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD*

Evidence suggests that breast cancer hormone receptor status varies by etiologic factors, but studies have been inconsistent. In a population-based case–control study in Poland that included 2,386 cases and 2,502 controls, we assessed ER-α and PR status of tumors based on clinical records according to etiologic exposure data collected *via* interview. For 842 cancers, we evaluated ER-α, ER-β, PR and HER2 levels by semiquantitative microscopic scoring of immunostained tissue microarrays and a quantitative immunofluorescence method, automated quantitative analysis (AQUA™). We related marker levels in tumors to etiologic factors, using standard regression models and novel statistical methods, permitting adjustment for both correlated tumor features and exposures. Results obtained with different assays were generally consistent. Receptor levels varied most significantly with body mass index (BMI), a factor that was inversely related to risk among premenopausal women and directly related to risk among postmenopausal women with larger tumors. After adjustment for correlated markers, exposures and pathologic characteristics, PR and HER2 AQUA levels were inversely related to BMI among premenopausal women (p-trend = 0.01, both comparisons), whereas among postmenopausal women, PR levels were associated directly with BMI (p-trend = 0.002). Among postmenopausal women, analyses demonstrated that BMI was related to an interaction of PR and HER2: odds ratio (OR) = 0.86 (95% CI = 0.69–1.07) for low PR and HER2 expression *vs.* OR = 1.78 (95% CI = 1.25–2.55) for high expression (p-heterogeneity = 0.001). PR and HER2 levels in breast cancer vary by BMI, suggesting a heterogeneous etiology for tumors related to these markers.

Key words: breast; etiology; hormones; epidemiology

Amassing data suggest that breast cancers are characterized by "molecular portraits" that are established at inception, remain stable over time and represent critical determinants of tumor biology.[1] Hormone receptor status is a key parameter in molecular classifications of breast cancer,[2,3] which serves as a marker of hormone-dependent growth and predictor of responsiveness to hormonal treatments. Consequently, researchers have hypothesized that etiologic factors mediated by hormones might be more strongly associated with breast cancers that express hormone receptors when compared with those that are receptor-negative.[4,5]

A recent literature review found evidence that nulliparity, late age at first birth and postmenopausal obesity are associated with greater risk for estrogen receptor-α (ER-α)-positive cancers when compared with ER-α-negative tumors, and that early menarche was more strongly linked to tumors coexpressing ER-α and progesterone receptor (PR).[4] Subsequently, a metaanalysis updating this review affirmed the heterogeneous associations for nulliparity and late age at first birth, but not for age at menarche.[5] However, results of studies have not been entirely consistent, especially when limited by small sample sizes, missing data and nonstandardized receptor assays (summarized[4,5]). Furthermore, analyses have classified receptor status dichotomously, ignoring differences in the percentage of cells expressing receptors, the receptor content per cell and variable criteria for classifying receptor status as positive. Finally, studies have not adjusted for pathologic tumor features, even though risk relationships may vary by tumor size, histologic type, grade and stage.[6]

To assess associations between marker expression and breast cancer risk associated with established etiologic factors, we analyzed data from a population-based case–control study conducted in Poland. To optimize marker assessments, we analyzed tumor marker status using 3 different methods: (*i*) clinical reports of ER-α and PR, mainly determined by immunostaining whole tissue sections; (*ii*) assessment of ER-α, ER-β, PR and HER2 expression by microscopic examination of immunostained tissue microarrays (TMAs); and (*iii*) determination of ER-α, ER-β, PR and HER2 using a novel quantitative immunofluorescent method, automated quantitative analysis (AQUA).[7,8] To account for patterns of marker coexpression, we employed novel statistical models to analyze multiple markers and exposures simultaneously, which permitted the identification of independent associations between marker levels and exposures.[9] These models were also used to adjust for other pathological characteristics (tumor size, histologic type, grade and nodal status), as well as to test *a priori* hypotheses that risk factor associations for cancers characterized by combinations of markers might demonstrate stronger effects than expected,

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat.

Grant sponsor: National Cancer Institute.

***Correspondence to:** Mark E. Sherman, M.D., Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD, USA. E-mail: shermanm@mail.nih.gov

Received 15 November 2006; Accepted after revision 22 February 2007
DOI 10.1002/ijc.22812
Published online 8 May 2007 in Wiley InterScience (www.interscience.wiley.com).

based on analyses of single markers (*i.e*. statistical interactions between markers). Specifically, given that coexpression of ER-α and PR predicts a favorable tamoxifen response, whereas coexpression of hormone receptors and HER2 predicts a reduced likelihood of response,[10] we speculated that risk associations for hormonally mediated factors might demonstrate interactions for these combinations of markers.

Material and methods

Study population

As described in detail elsewhere,[6] eligible cases included women between the ages of 20–74 years who were diagnosed with *in situ* or invasive breast cancer in 2000–2003, while residing in 2 cities in Poland, Warsaw and Łódź. Approximately 90% of eligible cases were identified through a rapid ascertainment mechanism in participating hospitals and the remainder was identified through cancer registries. Control women were randomly selected from population lists and frequency-matched to breast cancer cases on city of residence and age (5-year increments). Consent to participate was obtained for 79% (n = 2,386) of eligible cases and 69% (n = 2,502) of eligible controls. Approximately 90% of cancers were invasive at diagnosis. The study was approved by appropriate review boards at the National Cancer Institute and in Poland. In this report, analyses focused on a subset of 842 invasive carcinoma cases whose tumors were prepared as TMAs (see details later).

Exposure assessment

Breast cancer risk factors evaluated in this analysis included: educational level; age at menarche; parity; age at first full-term birth; age at menopause; menopausal status (women who initiated menopausal hormone therapy prior to menopause were classified as unclear status); current body mass index (BMI, kg/m^2, per 5 units) calculated from measured values for ~93% of controls and ~95% of cases (self-reported data were used for the remainder); use of hormone replacement therapy (HRT); family history of breast cancer affecting a first degree relative; history of benign breast disease (benign biopsy performed greater than 1 year prior to diagnosis (cases) or enrollment for controls); and history of mammographic screening. Factors that were relatively uncommon in this population, such as oral contraceptive use (~10%), alcohol consumption (ever ~33%) and long-term breast feeding (mean cumulative time ~1 year), were not considered in this analysis.

Pathology and TMA construction

Tissues were formalin-fixed and paraffin-embedded in Poland, according to local community standards. Surgical pathology reports were abstracted for information related to tumor size, histologic type, grade and stage, and then reclassified in the US to achieve standardized characterizations. Results for ER-α and PR assays performed locally were available for ~80% of invasive cases, of which ~91% were performed using immunohistochemistry and the remainder by biochemical methods. Results were reported as negative or positive.

Using individual paraffin tumor blocks for a subset of 842 unselected invasive breast cancers with available blocks at the time of the study, we constructed 4 TMA blocks with 2-fold representation per tumor using a manual technique (Beecher Scientific, Silver Spring, MD). Duplicate 0.6-mm diameter cores from each tumor were placed in separate TMA blocks to facilitate independent assessment. Sections of TMA blocks were cut at 5 μm thickness and placed on glass slides using a tape transfer method (Instrumedics, Hackensack, NJ). Cut sections were dipped in paraffin and stored at room temperature under gaseous nitrogen to prevent loss of immunoreactivity prior to staining.[11,12]

Immunohistochemical staining and marker assessment

TMA slides were prepared for AQUA analysis as described elsewhere.[7,8] Briefly, slides were deparaffinized, subjected to heat induced antigen retrieval by pressure cooking in citrate buffer (pH 6.0) for ~15 min, and treated with methanol and 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity. Slides were preincubated at 4°C overnight with rabbit anti-pancytokeratin or mouse anti-cytokeratin antibody (1:200) to identify tumor cells, followed by primary antibodies against target markers: ER-α (mouse monoclonal antibody 1D5 at 1:50) for 1 hr; anti-PR (mouse monoclonal PgR 636 at 1:100) for 1 hr; anti-HER2 (rabbit polyclonal diluted at 1:1,000) overnight; or anti-ER-β (rabbit polyclonal prediluted at 10 mg/mL; BioGenix, San Ramon, CA) for 1 hr. Corresponding secondary antibodies were applied using the Envision Double Stain Kit (DAKO) for 1 hr at room temperature: Alexa 488-conjugated goat anti-rabbit or Alexa 488-conjugated goat anti-mouse (1:100, Molecular Probes, Eugene, OR). Secondary antibodies were applied with 4,6-diamidino-2-phenylindole to visualize nuclei. The fluorescent chromogen Cy-5-tyramide (NEN Life Science Products, Boston, MA) was used for target visualization (ER-α, PR, ER-β and HER2).

For comparison with AQUA data, we performed conventional immunohistochemical stains for ER-α, ER-β, PR and HER2 on slides of TMAs, using standard protocols with antigen retrieval and primary antibody reagents, as listed earlier. One pathologist (M.E.S) scored the stains by examining high-resolution images obtained on a T2 Scanscope (Aperio, Vista, CA) displayed on a computer monitor with microscopic assessment as needed. Tumor representation in cores of immunostained sections was graded as satisfactory, suboptimal or unsatisfactory (<10% of core contained adequate material) for interpretation. Cores in which tumor comprised >10% for the area were scored for percentage of cells stained (0 = no staining; 1 = 0–30%; 2 = 40–70%; and 3 = 80–100%) and staining intensity (0; 1+; 2+; 3+); cores demonstrating unsatisfactory representation of tumor were not scored. Data for suboptimal cores were omitted when the other core from the same tumor was satisfactory.

Statistical analysis

Distributions of exposures among cases and controls were compared by χ^2 tests for categorical variables and by t-tests for continuous variables. To increase comparability of AQUA results for tumor cores contained in different TMA blocks (4 in total) and to normalize the data, we transformed AQUA scores by subtracting the mean and dividing by the standard deviation of results for each block, and then converted the result to the natural logarithm of this "centered" value. For tumors with 2 adequate cores, we determined the maximum and average transformed scores. Results for average and maximum AQUA scores per core showed similar patterns, and we have presented analyses based on maximum values to avoid redundancy. The percent cells stained and intensity scores from conventional microscopic assessment of immunohistochemical stains were combined into a composite, semiquantitative score (0; 1+; 2+; 3+) using *a priori* criteria for classifying off-diagonal results in a 4 × 4 table, in which tumors were crossclassified by percentages of cells stained and intensity. The correlation between transformed AQUA scores for ER-α, ER-β, PR and HER2, and microscopic assessments of the same markers were measured by the Spearman's correlation coefficient.

To assess the relationships between marker levels and exposures among cases, we used multivariate linear regression models with transformed AQUA scores as the outcome and exposures as the explanatory variables. We also performed case–control analyses using polytomous logistic regression to obtain estimates of odds ratios (ORs) and 95% confidence intervals (CIs) for the association between breast tumors classified in quartiles of transformed AQUA scores and exposures. In addition, we employed a novel 2-stage extension of polytomous logistic to evaluate heterogeneity in associations by marker levels and risk factors, adjusted simultaneously for multiple tumor characteristics and other potential confounders.[9] In these models (in which multiple tumor characteristics represented outcome variables and multiple exposures

served as explanatory variables), we evaluated which of the several correlated markers was most important in determining risk factors associations, adjusting for other pathologic features (*i.e.* histology, tumor size, grade and nodal status), and whether exposure associations were related to interactions between markers. Exposure variables in each model included education level; age at menarche; parity; age at first full-term birth; age at menopause; menopausal status; BMI among pre- and postmenopausal women in separate terms; HRT for postmenopausal women; family history of breast cancer; history of benign breast disease; history of mammographic screening; age and study site.

For comparisons with risk factor associations found by AQUA, we performed polytomous regression to assess associations based on microscopically derived semiquantitative marker scores and logistic regression to evaluate relationships according to dichotomous ER-α and PR results reported in the clinical records.

Results

Characteristics of participants

As previously reported,[6] most etiologic factors showed expected associations with overall breast cancer risk in this population, with the exception of BMI among postmenopausal women, which was associated with a significantly increased risk only for tumors larger than 2 cm. The distribution of breast cancer risk factors for the invasive cases included in this analysis ($n = 842$) were similar to those not included ($N = 1{,}302$) (data not shown). Consequently, differences in exposure distributions between cases and controls in this analysis resemble those found for the entire case group (Table I). The tumors included in the TMAs were larger than tumors that were not included ($p < 0.0001$). Tumor histology, grade, axillary node status and hormone receptor status based on hospital reports did not differ significantly for tumors in the TMA and those that were not included after adjusting for tumor size (data not shown).

TABLE I – DISTRIBUTION OF ETIOLOGIC FACTORS FOR BREAST CANCER IN THE STUDY POPULATION (842 CASES AND 2,502 CONTROLS)

	Cases[1]		Controls[1]		p^2
	N	%	*N*	%	
Education level					
Less than high school	238	28	957	38	
High school	305	36	943	38	
Technical training/some college	84	10	208	8	
College degree	209	25	378	15	<0.0001
Number of full-term births					
Nulliparous	135	16	281	11	
1	285	34	746	30	
2	341	40	1,092	44	
3+	81	10	383	15	<0.0001
Hormone replacement therapy					
Never	407	77	1,323	83	
Current/recent use	64	12	93	6	
Past use	27	5	75	5	
Ever used E or P alone	33	6	104	7	<0.0001
Family history of breast cancer					
No	750	89	2,356	94	
Yes	91	11	146	6	<0.0001
History of benign breast disease					
No	731	90	2,323	94	
Yes	84	10	157	6	<0.0001
Ever had a screening mammogram					
No	322	39	1,135	46	
Yes	506	61	1,347	54	0.001
	Mean	SD	Mean	SD	
Age at menarche	13.5	1.7	13.7	1.7	0.01
Age at first full-term birth	24.3	4.4	23.6	4.2	0.0002
Age at menopause	49.8	4.4	49.2	5.0	0.02
Current BMI among premenopausal women	25.3	4.4	26.4	5.1	0.003
Current BMI among postmenopausal women	28.3	5.6	28.6	5.4	0.26

[1]Differences in total numbers of cases and controls and numbers in table are due to missing data.–[2]*p* value from χ^2 test for categorical variables and *t*-test for continuous variables.

Comparison of assays for ER-α, ER-β, PR and HER-2

Transformed AQUA scores for ER-α and PR were significantly higher for tumors classified as positive, as opposed to negative for the corresponding receptor based on clinical testing performed in Poland (both comparisons, $p < 0.0001$). Similarly, AQUA results and microscopic assessment of TMAs were significantly correlated for ER-α ($r^2 = 0.80$); ER-β ($r^2 = 0.58$); PR ($r^2 = 0.86$); and Her 2 ($r^2 = 0.87$), and increased linearly across strata of increasing expression as assessed by microscopic examination of stained TMAs (Fig. 1).

Based on AQUA scores, results were positively correlated for ER-α and ER-β ($r^2 = 0.22$, $p < 0.00001$); ER-α and PR ($r^2 = 0.31$, $p < 0.00001$), and weakly inversely associated for ER-α and HER2 ($r^2 = -0.07$, $p = 0.05$). In addition, AQUA scores for ER-β and PR were positively correlated ($r^2 = 0.11$, $p = 0.003$) and those for PR and HER2 were negatively correlated ($r^2 = -0.20$, $p < 0.00001$).

Associations for breast cancer risk factors and marker levels (AQUA)

Among premenopausal women, we found that risk for tumors with high ER-α levels was lower for women with higher current BMI (OR = 0.47, 95% CI = 0.25–0.87, *p*-trend = 0.004). Similarly, among premenopausal women, we found that risk for tumors with high PR levels was lower for women with higher current BMI (OR = 0.68, 95% CI = 0.49–0.94, *p*-trend = 0.003, Table II). Among postmenopausal women, risk for tumors with high PR levels was elevated for women with increased current BMI (OR = 1.23, 95% CI = 1.05–1.43, *p*-trend = 0.003). We also observed that higher ER-β levels were associated with age at menopause (per 5 year increase) (OR = 1.33, 95% CI = 1.08–1.63, *p*-trend = 0.04), whereas lower PR levels were associated with later menopause (OR = 1.00, 95% CI = 0.84–1.21, *p*-trend = 0.004). Marker levels were not significantly associated with age at menarche, parity, age at first full-term birth and HRT use (Supplementary Table I).

We also assessed relationships between transformed AQUA scores and exposures using an extension of polytomous logistic regression to account for correlation between tumor pathologic characteristics and marker levels (see methods and[9] for details). The associations between PR and BMI remained significant after adjustment for other markers: among premenopausal women, PR levels were inversely related to BMI (adjusted *p*-trend = 0.01), and among postmenopausal women, PR levels were directly related to BMI (adjusted *p*-trend = 0.002). However, associations between ER-β or PR levels and age at menopause were no longer significant (Table II). In addition, among premenopausal women, analyses suggested that HER2 expression and BMI were inversely related in models that considered each marker separately, a result that became statistically significant with simultaneous adjustment for all 4 markers (adjusted *p*-trend = 0.01). Further adjustment for pathologic tumor characteristics (histological type, grade, size and nodal status) did not appreciably change associations between exposures and tumor markers (data not shown).

Breast cancer risk associated with BMI in relation to marker combinations

We used the 2-step extension of the logistic regression model to test for interactions between tumor markers. Tests for interactions between markers and risk associations for BMI among postmenopausal demonstrated significant findings for coexpression of PR and HER2 (*p*-interaction = 0.0003), and for ER-α and HER2 (*p*-interaction = 0.003). Consistent with these analyses, conventional case–control analyses for tumors classified according to PR and

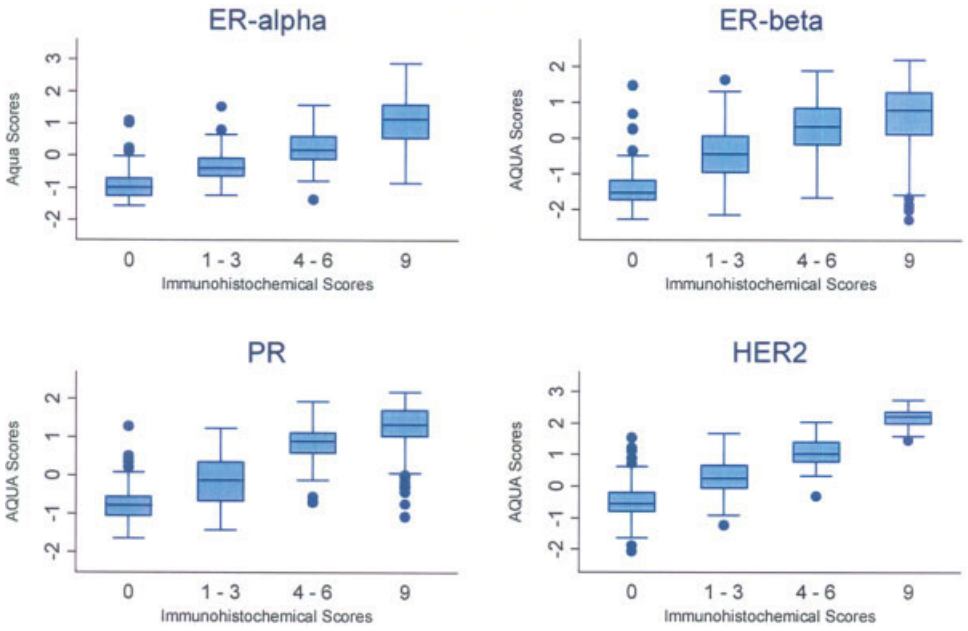

FIGURE 1 – Distribution of transformed AQUA scores by immunohistochemical semiquantitative score categories for 4 tissue markers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II – RELATIONSHIP BETWEEN AGE AT MENOPAUSE AND BMI AND RISK FOR BREAST CANCER SUBTYPES DEFINED BY TUMOR MARKER LEVELS[1] AMONG 842 BREAST CANCER CASES AND 2,502 CONTROLS IN THE POLISH BREAST CANCER STUDY

Tumor marker exposure	Case–control analyses								Case-only analyses	
	1st quartile (low)		2nd quartile		3rd quartile		4th quartile (high)		*p*-trend het.[2]	Adjusted *p*-trend het.[3]
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI		
ER-α										
Age at menopause, per 5 year increase	1.32	1.05–1.65	1.17	0.94–1.47	1.19	0.97–1.46	1.10	0.91–1.33	0.13	0.25
Current BMI among premenopausal, per 5 unit increase	1.08	0.84–1.39	0.77	0.57–1.03	0.64	0.43–0.93	0.47	0.25–0.87	0.004	0.23
Current BMI among postmenopausal, per 5 unit increase	0.87	0.71–1.06	1.11	0.93–1.34	1.00	0.84–1.19	0.98	0.83–1.15	0.84	0.75
ER-β										
Age at menopause, per 5 year increase	1.08	0.89–1.31	1.05	0.87–1.26	1.25	1.01–1.54	1.33	1.08–1.63	0.04	0.23
Current BMI among premenopausal, per 5 unit increase	0.85	0.62–1.17	0.87	0.64–1.20	0.78	0.57–1.07	0.75	0.54–1.04	0.48	0.55
Current BMI among postmenopausal, per 5 unit increase	1.03	0.87–1.21	1.07	0.91–1.25	0.96	0.80–1.14	0.91	0.76–1.08	0.34	0.08
PR										
Age at menopause, per 5 year increase	1.26	1.02–1.55	1.24	1.02–1.49	1.17	0.94–1.45	1.00	0.84–1.21	0.004	0.15
Current BMI among premenopausal, per 5 unit increase	1.03	0.78–1.36	0.98	0.68–1.41	0.68	0.50–0.93	0.68	0.49–0.94	0.003	0.01
Current BMI among postmenopausal, per 5 unit increase	0.83	0.69–1.00	0.95	0.81–1.12	0.98	0.81–1.18	1.23	1.05–1.43	0.003	0.002
HER2										
Age at menopause, per 5 year increase	1.31	1.06–1.61	1.11	0.90–1.36	1.23	1.01–1.50	1.06	0.87–1.28	0.38	0.13
Current BMI among premenopausal, per 5 unit increase	1.00	0.75–1.32	0.81	0.60–1.10	0.77	0.54–1.08	0.70	0.49–0.99	0.17	0.01
Current BMI among postmenopausal, per 5 unit increase	0.95	0.79–1.13	1.03	0.86–1.23	0.96	0.81–1.13	1.02	0.87–1.21	0.54	0.29

[1]Tumors are classified by quartiles of transformed AQUA scores for each of the tumor markers.–[2]*p*-values for heterogeneity (het.) of exposure ORs by tumor subtypes based on case-only analyses assuming a linear relationship between marker levels and exposures. Values are calculated from a linear regression model for each transformed AQUA score and breast cancer etiologic factors as explanatory variables (age, study site, level of education, menopausal status, history of benign breast disease and history of screening mammogram and all exposures in table). See Supplementary Table I for estimates for other exposures included in the model.–[3]*p*-values for heterogeneity (het.) of exposure ORs by tumor subtypes based on case-only analyses assuming a linear relationship between marker levels and exposures, after adjusting for other marker levels. Values are derived from a 2-stage extension of the polytomous logistic regression[9] with all tumor marker levels as the outcome variable and breast cancer etiologic factors as explanatory variables (age, study site, level of education, menopausal status, history of benign breast disease and history of screening mammogram and all exposures in table).

HER2 levels showed that elevated BMI among postmenopausal women was not associated with risk for tumors with low PR and low HER2 levels (OR = 0.86, 95% CI = 0.69–1.07), whereas heavier women were at significantly increased risk for tumors with high levels of both markers (OR = 1.78, 95% CI = 1.25–2.55, *p*-heterogeneity = 0.001, Table III). Although the test for interaction between ER-α and HER2 was also significant in the 2-step models including other tumor markers, relative risks stratified by ER and HER2 categories did not show a consistent pattern of heterogeneity, *e.g.*, relative risk for tumors with low ER and HER2 levels was not significantly different from the relative risk for tumors with high ER and HER2 levels.

TABLE III – ASSOCIATION BETWEEN INCREASING BMI AMONG POSTMENOPAUSAL WOMEN AND BREAST CANCER RISK FOR TUMORS WITH DIFFERENT LEVELS OF PR AND HER2 PROTEINS IN TUMOR TISSUES

PR level	HER2	*N*	Median BMI (kg/m^2)	OR[1] (95% CI) per 5 unit increase	p^1	p^2 het.
Cases[3]						
Low	Negative	92	27.4	0.86 (0.69–1.07)	0.17	reference
Low	Positive	45	26.3	0.84 (0.61–1.15)	0.28	0.76
Intermediate	Negative	189	27.8	0.94 (0.81–1.09)	0.43	0.63
Intermediate	Positive	72	27.4	0.97 (0.77–1.22)	0.78	0.63
High	Negative	114	28.7	1.15 (0.97–1.37)	0.12	0.05
High	Positive	17	29.9	1.78 (1.25–2.55)	0.002	0.001
Controls		1,674	28.0			

[1]OR (95%CI) and corresponding *p* values from polytomous logistic regression models (case–control analyses) evaluating the relationship between increasing BMI and risk for developing different tumor subtypes. Estimates are adjusted for age, study site, level of education, age at menarche, parity, age at first full-term pregnancy, HRT use, age at menopause, menopausal status, history of benign breast disease and history of a screening mammogram.–[2]*p* value from case-only analyses of the association between BMI and tumor subtypes, using tumors with low PR levels and HER2 negative as the reference group.–[3]PR: low, intermediate and high levels are defined as AQUA score for PR <25th, 25–<75th and ≥75th percentile, respectively. HER2: negative and positive are defined as AQUA scores for HER2 <75th and ≥75th, respectively.

Associations for breast cancer risk factors and marker levels by standard immunohistochemistry

Analysis of breast cancer risk by marker levels determined by microscopic review of immunostained TMAs yielded associations similar to those found with AQUA (data not shown). We obtained information on hormone receptor status from medical records for about 80% of invasive cases (1,742 cases had ER-α information and 1,716 cases had PR information), including the 842 cases in the TMAs. Analyses based on ER-α and PR status from medical records were also consistent with results obtained by other methods (Supplementary Table II). Analyses by hormone receptor status, including all cases with this information, showed significant modification of BMI associations with breast cancer risk by PR status, as in the more detailed analyses using AQUA scores in the subset of 842 cases. Risk associations for parity (protective factor) and late age at first full-term (risk factor) were stronger for ER-α-positive when compared with ER-α-negative tumors; however, tests for heterogeneity of ORs by ER status were not statistically significant. Furthermore, analyses of AQUA scores among the 842 cases in TMAs showed nonlinear associations of increasing AQUA levels of ER-α and reproductive exposures.

Discussion

This analysis supports the view that hormone receptor expression is linearly related to breast cancer risk associations for BMI, and that coexpression of HER2 may modify these relationships.

In addition to analyzing data for ER-α and PR status based on medical records, we attempted to optimize marker assessments by employing 2 additional techniques, which provided semiquantitative and quantitative levels. Results for different assay methods were highly correlated and demonstrated expected patterns of marker coexpression, providing reassurances that misclassification was minimized. Specifically, we found that ER-α and PR expression were correlated and that both markers were positively associated with ER-β and inversely related to HER2, as previously reported in several studies.[10,13,14]

In all analyses, whether based on AQUA, manual interpretation of TMAs or hospital data, we found that PR levels were inversely related to current BMI among premenopausal women and that PR expression was directly associated with BMI among postmenopausal women. In analyses considering each marker separately, ER-α levels were inversely related to BMI among premenopausal women, but the association became weaker and nonsignificant in models that adjusted for both markers. The inverse association between receptor expression and BMI among premenopausal women, and the direct relationship for BMI in postmenopausal women are consistent with most previous reports.[4] In our analyses, PR was the strongest determinant of etiologic heterogeneity. PR expression may represent a better marker of hormone-dependent growth than ER-α levels, because transcription of the PR gene requires formation of estradiol–ER-α complexes, implying both the availability of ligand and formation of functional ligand–receptor complexes.[15]

The observed differences in associations for hormone receptor levels and BMI among pre- and postmenopausal women are biologically plausible. Obesity among premenopausal women has been associated with lower serum hormone levels, which could reduce risks for developing receptor-positive, hormone-dependent cancers.[16] In contrast, obesity among postmenopausal women is associated with increased hormone levels, which may enhance growth of receptor-positive tumors.[17] We speculate that among premenopausal obese women, low-serum hormone levels may lead to upregulated ER-α and PR levels in benign ("normal") epithelium, creating conditions conducive to exaggerated hormone responses after menopause as cumulative estrogen exposure rises. This view is supported by limited data linking elevated serum hormone levels and weight gain, specifically to risk for receptor-positive tumors.[18–20]

Our analyses also suggested that risk relationships for postmenopausal obesity vary for tumors coexpressing either ER-α or PR with HER2, a pattern that has been designated as molecular subtype "Luminal B."[2,3] In particular, obesity among postmenopausal women was associated with an ~80% higher risk for strong PR and HER2 coexpressing tumors when compared with tumors with low levels. HER2 overexpression may contribute to carcinogenesis by upregulating aromatase and by activating growth factor pathways, suggesting crosstalk between hormonal and nonhormonal mechanisms of carcinogenesis.[21] Furthermore, obesity in postmenopausal women is associated with elevated estrogen levels, which may lead to activation of HER1 and HER2 kinases. Consistent with this view, Arpino *et al*. suggested that tamoxifen, acting as a weak estrogen, activates HER1 and HER2 kinases, thereby inducing tamoxifen resistance in receptor-positive tumors with HER2 amplification.[22] Investigation of other genes included within the "molecular signature" of ER-α-positive breast cancer may reveal additional unrecognized intersections between hormonal and nonhormonal pathways.[23] Finally, data suggesting that normal epithelium associated with breast cancer shows inappropriately high hormone receptor expression with respect to serum hormone levels and abnormal coexpression of ER-α and proliferation markers point to exaggerated responses to hormones as a mechanism that may augment effects of excess hormone exposures.[24,25]

In analyses considering each tumor marker separately, we found that ER-β was directly related and PR expression was indirectly

associated with age at menopause. Late age at menopause has been linked to lower serum estrogen levels,[26] which would lead to reduced ER-α-mediated transcription of PR. Furthermore, ER-β may also reduce ER-α-mediated transcription of target genes such as PR.[27,28] However, the associations between marker levels and age at menopause were not significant in modified polytomous regression models that simultaneously adjusted for all markers. Similarly, in some analyses, we reproduced previously reported findings suggesting stronger effects for parity (protective factor) and late age at first full-term birth (risk factor) among ER-α-positive tumors.[4] However, these associations were weak and were not found consistently with all assays techniques. Although these associations may reflect chance observations, our ability to detect these relationships with categorical rather continuous classifications of receptor status (*i.e.* AQUA) may reflect the nonlinear nature of the associations.

Research has shed light on etiologic differences between receptor-positive and -negative tumors, but the genesis of the latter remains poorly understood.[29] ER-α-negative tumors may arise from hormonally stimulated precursors that lose receptor expression prior to diagnosis. Alternatively, ER-α-negative and -positive tumors may develop from different progenitor cells at the outset. Furthermore, these proposals are not mutually exclusive; different receptor-negative tumors may develop through different pathways. Focused studies to reveal the etiology of ER-α-negative tumors may be warranted, because these tumors are often clinically aggressive and difficult to treat effectively.

Strengths of the Polish study include its large size, population-based sample, use of standardized and quantitative assays and novel statistical modeling. Although we observed the expected inverse relationship between BMI and cancer risk among premenopausal women, the direct association with risk among postmenopausal women found in most studies was limited to larger tumors in Poland. This may reflect the age truncation of our population at 74 years and the fact that 40% of women were never screened. However, we minimized potential confounding by pathologic factors (size, grade, nodal status) by employing novel statistical methods, enabling us to adjust for these parameters in models that included risk factors and markers. In addition, some etiologic exposures, such as use of birth control pills, HRT and alcohol, were uncommon and therefore not examined. Our study included a high percentage of large, high-grade, and hormone receptor-negative tumors. In fact, small tumors were underrepresented in the case subset prepared as TMAs when compared with the full study, but we attempted to reduce the impact of this concern by statistical adjustment. Finally, some misclassification of markers secondary to sampling, variable fixation, interpretive errors and other factors is unavoidable.

In summary, our data suggest that molecular characteristics of breast cancers vary by BMI. Accordingly, it may be possible in the future to link etiologic exposures to abnormalities in molecular pathways, permitting the identification of molecular targets for risk assessment, screening and prevention.

Acknowledgements

We thank Ms. Anita Soni and Ms. Elena Adrianza, (Westat, Rockville, MD) for their work on study management; Pei Chao (IMS, Silver Spring, MD) for her work on data and sample management; physicians, pathologists and nurses from participating centers in Poland as well as interviewers and study participants for their efforts during field-work. Funding for this work was provided by the Intramural Research Program of the National Cancer Institute, including a Breast Cancer Think Tank award to M.E.S. Dr. Rimm is a founder, stock holder and consultant to HistoRx, a private corporation to which Yale University has given exclusive rights to produce and distribute the software and technologies embedded in AQUA; Yale University retains patent rights for the AQUA technology.

References

1. Lacroix M, Toillon RA, Leclercq G. Stable 'portrait' of breast tumors during progression: data from biology, pathology and genetics. Endocr Relat Cancer 2004;11:497–522.
2. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–752.
3. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006;295:2492–2502.
4. Althuis MD, Fergenbaum JH, Garcia-Closas M, Brinton LA, Madigan MP, Sherman ME. Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. Cancer Epidemiol Biomarkers Prev 2004;13:1558–1568.
5. Ma H, Bernstein L, Pike MC, Ursin G. Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. Breast Cancer Res 2006;8:R43.
6. Garcia-Closas M, Brinton LA, Lissowska J, Chatterjee N, Peplonska B, Anderson WF, Szeszenia-Dabrowska N, Bardin-Mikolajczak A, Zatonski W, Blair A, Kalaylioglu Z, Rymkiewicz G, et al. Established breast cancer risk factors by clinically important tumour characteristics. Br J Cancer 2006;95:123–9.
7. McCabe A, Dolled-Filhart M, Camp RL, Rimm DL. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. J Natl Cancer Inst 2005;97:1808–15.
8. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med 2002;8:1323–7.
9. Chatterjee N. A two-stage regression model for epidemiological studies with multivariate disease classification data. J Am Stat Assoc 2004;99:127–38.
10. Kaptain S, Tan LK, Chen B. Her-2/neu and breast cancer. Diagn Mol Pathol 2001;10:139–52.
11. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer Epidemiol Biomarkers Prev 2004;13:667–72.
12. DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. Lab Invest 2004;84:1071–8.
13. Lal P, Tan LK, Chen B. Correlation of HER-2 status with estrogen and progesterone receptors and histologic features in 3,655 invasive breast carcinomas. Am J Clin Pathol 2005;123:541–6.
14. Saunders PT, Millar MR, Williams K, Macpherson S, Bayne C, O'Sullivan C, Anderson TJ, Groome NP, Miller WR. Expression of oestrogen receptor β (ERβ1) protein in human breast cancer biopsies. Br J Cancer 2002;86:250–6.
15. Miyoshi Y, Akazawa K, Kamigaki S, Ueda S, Yanagisawa T, Inoue T, Yamamura J, Taguchi T, Tamaki Y, Noguchi S. Prognostic significance of intra-tumoral estradiol level in breast cancer patients. Cancer Lett 2004;216:115–21.
16. Potischman N, Swanson CA, Siiteri P, Hoover RN. Reversal of relation between body mass and endogenous estrogen concentrations with menopausal status. J Natl Cancer Inst 1996;88:756–8.
17. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 2004;4:579–91.
18. Eliassen AH, Colditz GA, Rosner B, Willett WC, Hankinson SE. Adult weight change and risk of postmenopausal breast cancer. JAMA 2006;296:193–201.
19. Fentiman IS, Hanby A, Allen DS, Key T, Meilahn EN. Hormone dependency of breast tumours developing in the Guernsey Cohort Study. Breast Cancer Res Treat 2006;97:205–8.
20. Missmer SA, Eliassen AH, Barbieri RL, Hankinson SE. Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. J Natl Cancer Inst 2004;96:1856–65.
21. Howe LR, Chang SH, Tolle KC, Dillon R, Young LJ, Cardiff RD, Newman RA, Yang P, Thaler HT, Muller WJ, Hudis C, Brown AM, et al. HER-2/neu-induced mammary tumorigenesis and angiogenesis are reduced incyclooxygenase-2 knockout mice. Cancer Res 2005;65:10113–9.
22. Arpino G, Weiss H, Lee AV, Schiff R, De Placido S, Osborne CK, Elledge RM. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 2005;97:1254–61.

23. Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 2001;61:5979–84.
24. Khan SA, Sachdeva A, Naim S, Meguid MM, Marx W, Simon H, Halverson JD, Numann PJ. The normal breast epithelium of women with breast cancer displays an aberrant response to estradiol. Cancer Epidemiol Biomarkers Prev 1999;8:867–72.
25. Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, Sibson DR, Sloane JP. Estrogen receptor-positive proliferating cells in the normal and precancerous breast. Am J Pathol 1999;155: 1811–15.
26. Ness RB, Buhari A, Gutai J, Kuller LH. Reproductive history in relation to plasma hormone levels in healthy post-menopausal women. Maturitas 2000;35:149–57.
27. Matthews J, Wihlen B, Tujague M, Wan J, Strom A, Gustafsson JA. Estrogen receptor (ER) β modulates ERα-mediated transcriptional activation by altering the recruitment of c-Fos and c-Jun to estrogen-responsive promoters. Mol Endocrinol 2006;20:534–43.
28. Saji S, Hirose M, Toi M. Clinical significance of estrogen receptor β in breast cancer. Cancer Chemother Pharmacol 2005;56(Suppl 1):21–6.
29. Allred DC, Brown P, Medina D. The origins of estrogen receptor α-positive and estrogen receptor α-negative human breast cancer. Breast Cancer Res 2004;6:240–5.