Urinary Metalloproteinases: Noninvasive Biomarkers for Breast Cancer Risk Assessment

Susan E. Pories,[1,8] David Zurakowski,[2,8] Roopali Roy,[2,8] Carolyn C. Lamb,[3,8] Sughra Raza,[4,8] Alexis Exarhopoulos,[2] Rochelle G. Scheib,[5,8] Susan Schumer,[6,8] Corrine Lenahan,[6,8] Virginia Borges,[6] Gwendolyn W. Louis,[2] Ankur Anand,[1] Nina Isakovich,[1] Judi Hirshfield-Bartek,[7] Ulla Wewer,[9] Margaret M. Lotz,[1] and Marsha A. Moses[2,8]

[1]Department of Surgery, Beth Israel Deaconess Medical Center and Mount Auburn Hospital; [2]Vascular Biology Program and Department of Surgery, Children's Hospital of Boston; [3]Department of Radiation Oncology, Mount Auburn Hospital; [4]Department of Radiology, Brigham and Women's Hospital; [5]Department of Medical Oncology, Dana-Farber Harvard Cancer Institute; Departments of [6]Medical Oncology and [7]Nursing, Beth Israel Deaconess Medical Center; [8]Harvard Medical School, Boston, Massachusetts and [9]Institute of Molecular Pathology, University of Copenhagen, Copenhagen Denmark

Abstract

Matrix metalloproteinases (MMP) and a disintegrin and metalloprotease 12 (ADAM 12) can be detected in the urine of breast cancer patients and provide independent prediction of disease status. To evaluate the potential of urinary metalloproteinases as biomarkers to predict breast cancer risk status, urine samples from women with known risk marker lesions, atypical hyperplasia and lobular carcinoma *in situ* (LCIS), were analyzed. Urine samples were obtained from 148 women: 44 women with atypical hyperplasia, 24 women with LCIS, and 80 healthy controls. MMP analysis was done using gelatin zymography and ADAM 12 analysis was done via immunoblotting with monospecific antibodies and subsequent densitometric measurement. Positive urinary MMP-9 levels indicated a 5-fold risk of atypical hyperplasia and >13-fold risk of LCIS compared with normal controls. Urinary ADAM 12 levels were significantly elevated in women with atypical hyperplasia and LCIS from normal controls, with receiver operating characteristic curve analysis showing an area under the curve of 0.914 and 0.950, respectively. To assess clinical applicability, a predictive index was developed using ADAM 12 in conjunction with Gail risk scores for women with atypia. Scores above 2.8 on this ADAM 12-Gail risk prediction index score are predictive of atypical hyperplasia (sensitivity, 0.976; specificity, 0.977). Our data suggest that the noninvasive detection and analysis of urinary ADAM 12 and MMP-9 provide important clinical information for use as biomarkers in the identification of women at increased risk of developing breast cancer. (Cancer Epidemiol Biomarkers Prev 2008;17(5):1034–42)

Introduction

The metalloprotease family of enzymes is integral to the process of tumor progression, from angiogenesis and cell migration to remodeling of the tumor microenvironment, invasion, and metastasis. As part of this process, matrix metalloproteinases (MMPs) enter the circulation and can potentially serve as biomarkers for disease stage and progression. Elevated levels of MMPs and a disintegrin and metalloprotease 12 (ADAM 12) have been shown in breast tumors (1-4) and the serum and plasma of breast cancer patients (5, 6). We were the first to report that urinary MMPs and ADAM 12 could be also be detected in the urine of breast cancer patients and that these urinary MMPs are predictive of disease status (7). This finding confirmed that overproduction of MMPs by a tumor communicating with the vascular and lymphatic systems results in increased levels of MMP activity in urine as well as blood. It has also been reported that levels of other regulatory molecules overproduced by tumors, such as the angiogenic peptide basic fibroblast growth factor, have been measured in body fluids of cancer patients and have been shown to be independent predictors of disease status (8, 9). Since our original report, there are now several studies that support our findings that urinary MMPs predict neoplastic disease status (10-15).

Subsequently, we and others have shown that MMPs are required for the switch to the angiogenic phenotype, an early and critical event in cancer growth and progression (16, 17). We have isolated and identified ADAM 12 as being present in the urine of breast cancer patients (18). ADAM 12 is a member of a glycoprotein family that is related to MMPs. As with MMPs, increased expression of ADAM 12 has been reported previously in breast, colon, and lung carcinoma tissues (4). We have also shown that urinary ADAM 12 levels also significantly increase with disease progression in breast cancer patients and correlate with stage of disease (18).

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Requests for reprints: Marsha A. Moses, Vascular Biology Program and Department of Surgery, Children's Hospital Boston, Karp Family Research Building, 12.214, 300 Longwood Avenue, Boston, MA 02115-5737. Phone: 617-919-2207; Fax: 617-730-0231. E-mail: marsha.moses@childrens.harvard.edu

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The goal of identifying women at high risk of developing breast cancer and providing safe effective risk reduction to this group is compelling. Breast cancer remains the most common cancer among women and the second leading cause of cancer deaths in women today (19, 20). The American Cancer Society estimates that ~182,460 women in the United States will be diagnosed with invasive breast cancer and 40,480 women will die of the disease in 2008 (21). Earlier detection and treatment are thought to improve survival, yet breast cancer can be ''an unpredictable disease'' because even very small lesions at the limit of detection by mammography, magnetic resonance imaging, or palpation can progress to metastatic disease (22-24). Given the current limitations of early detection, identifying women at risk for the disease and providing risk reduction strategies is a critically important goal. As a result, breast cancer risk assessment is becoming an increasingly significant part of the process of counseling women about their health. This has become especially important as genetic testing and risk reduction continue to evolve.

Accordingly, atypical hyperplasia and lobular carcinoma *in situ* (LCIS), recognized as markers of an increased risk of breast cancer, are coming under increased scrutiny. Elevated levels of MMPs have been documented in the plasma of women at high risk for breast cancer (5, 6). Additionally, angiogenesis has been documented previously in atypical hyperplasia and LCIS (25). Our group has reported previously that MMPs and ADAM 12 can easily be measured in urine and that elevated levels of these urinary enzymes accompany the angiogenic switch process. Given that MMP activity and angiogenesis are among the earliest events in tumor progression, we hypothesized that the study of these urinary proteins would provide useful information regarding atypical hyperplasia and LCIS, proliferative lesions of the breast that signal increased risk of breast cancer development. Here, in this study, we show that urinary biomarker levels are significantly elevated in women at increased risk for breast cancer and that this information may potentially be used in conjunction with the Gail model as a novel breast cancer risk assessment tool.

Materials and Methods

Study Population. The study population included 148 women: 44 women with biopsy-proven atypical hyperplasia, 24 women with biopsy-proven LCIS, and 80 normal controls. Women undergoing evaluation and treatment for breast complaints were enrolled at Surgery, Radiation Oncology, and Medical Oncology clinics at Beth Israel Deaconess Medical Center, Mount Auburn Hospital, and Dana-Farber Cancer Institute. Normal healthy controls were enrolled from the population of women who came in for routine screening mammograms at Brigham and Women's Hospital and reported no chronic medical problems and no breast complaints, had a normal mammogram reading, and were on no medications.

All participants completed a detailed medical history form at the time of urine donation and gave fully informed consent to join the study. Risk scores for normal controls and women with atypical hyperplasia were calculated using the modified Gail model (26, 27). Gail scores were not calculated for women with LCIS, as this model is not valid for women with this diagnosis. Institutional review board approval for the study was obtained at each institution. Pregnant and breast-feeding women were excluded from the study. Characteristics of the study population are detailed in Table 1.

Urine Sample Collection and Processing. Urine was collected according to the institutional bioethical guidelines pertaining to discarded clinical material. Patients were seen in an ambulatory care setting and provided a voided specimen. Urine samples were kept on ice for no longer than 2 h and then frozen at -20°C, as described previously by us, and then stored at-80°C (7). Protein concentration of urine was determined by the Bradford method using bovine serum albumin as the standard. Before analysis, urine samples were tested for the presence of blood using Ames Multistix 7 reagent strips (Miles), and specimens containing blood were excluded.

MMP Analysis. Urine samples were analyzed by substrate gel electrophoresis (zymography) as reported previously (7). Thirty microliters of each urine sample were subjected to electrophoresis using gelatin as the substrate. Zymograms were processed and evaluated independently without knowledge of the clinical status of the individuals from whom the urine specimens were obtained as reported previously (7). MMP identity was verified by immunoblot analyses using monospecific antibodies (7).

ADAM 12 Analysis. Equal amounts of urinary protein (20 μg) were separated by SDS-PAGE under reducing conditions as described previously (18). Resolved proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) and treated as described previously (28, 29). A polyclonal antibody against human ADAM 12, rb122, was used at a concentration of 1 μg/mL (18, 30). Labeled proteins were visualized with enhanced chemiluminescence (Pierce Chemical). Band intensities were analyzed with UN-SCAN-IT (Silk Scientific) software digitizer technology.

Mammogram Assessment. Mammograms were evaluated using the standard American College of Radiology Breast Imaging Reporting and Data System (31).

Statistical Analysis. Urinary MMPs were compared among atypical hyperplasia, LCIS, and normal controls using χ^2 analysis. ANOVA with Bonferroni-adjusted comparisons was used to evaluate differences in ADAM 12 levels among the three groups (32). Multiple stepwise logistic regression analysis using a backward selection procedure was applied to determine predictors that differentiate atypical hyperplasia and LCIS from controls by considering four MMPs, ADAM 12 as a continuous variable, age, and Gail scores with the likelihood ratio test (LRT) used to assess statistical significance (33). Odds ratios and 95% confidence intervals for significant predictors were determined using exact methods and probability curves for estimating the likelihood of atypical hyperplasia as a function of ADAM 12 levels and Gail 5-year risk scores were derived using regression variables (slope and intercept coefficients) from the final

Table 1. Characteristics of the study groups

Characteristics	Normal controls (n = 80)	Atypical hyperplasia (n = 44)	LCIS (n = 24)
Mean ± SD age (y)	48 ± 8	54 ± 8	55 ± 9
Range	36-76	28-77	40-85
		Percentage of individuals	
Race			
White	77	95	100
Black	11	0	0
Hispanic	10	0	0
Asian	2	5	0
Age at menarche (y)			
7-11	15	16	21
12-13	59	59	46
>13	26	25	33
Age at first live birth (y)			
<20	11	7	0
20-24	17	11	33
25-30	17	27	13
>30	35	27	21
Nulliparous	20	27	33
No. first-degree relatives with breast cancer			
0	78	70	58
1	14	23	29
≥2	1	2	0
Unknown	7	5	3
No. breast biopsies			
0	90	27	8
1	10	41	50
≥2	0	32	42
Atypia on prior biopsy			
No	10	23	17
Yes	0	41	67
Unknown	0	9	8
N/A	90	27	8
Breast Imaging Reporting and Data System code			
Negative	86	9	21
Benign	10	30	42
Probably benign	4	7	0
Suspicious	0	50	33
Highly suggestive of malignancy	0	2*	4
Alcohol usage			
None	15	21	29
<5 drinks/wk	75	68	63
≥5 drinks/wk	10	11	8
Smoking			
Never smoked	61	66	42
Stopped smoking	32	23	42
<1 pack/d	5	9	12
≥1 pack/d	2	2	4
Mean Gail scores, mean ± SD†			
5-year risk	1.0 ± 0.4	3.8 ± 1.9‡	NA
Lifetime risk	10.2 ± 4.1	23.2 ± 10.6‡	NA

Abbreviation: NA, not applicable.
*Patient in this group did not have a mammogram, although magnetic resonance imaging was negative.
†Includes only individuals over age 35 years.
‡$P < 0.001$, compared with normal controls.

multivariate model (34). Receiver operating characteristic curve analysis was applied to assess diagnostic accuracy of ADAM 12, Gail scores, and the combination for differentiating atypical hyperplasia from normal (35). Statistical analysis was done using the SPSS software package (version 15.0; SPSS). Two-tailed values of $P < 0.05$ were considered statistically significant. Power analysis was conducted *a priori* and indicated that a minimum sample size of 24 patients in each of the atypical hyperplasia and LCIS groups and 80 controls would provide 90% power ($\alpha = 0.05$; $\beta = 0.20$) to detect a significant difference of 20% in the positive expression of each MMP between patients and controls and using a binomial Z-test for independent proportions (36) and a 30% difference in mean ADAM 12 levels between the study groups using ANOVA (version 6.0, nQuery Advisor; Statistical Solutions).

Results

Study Population. The study population consisted of 148 women: 44 women with atypical hyperplasia, 24 women with LCIS, and 80 normal controls. All diagnoses were biopsy proven. Gail score calculations were consistent with an average risk of breast cancer in

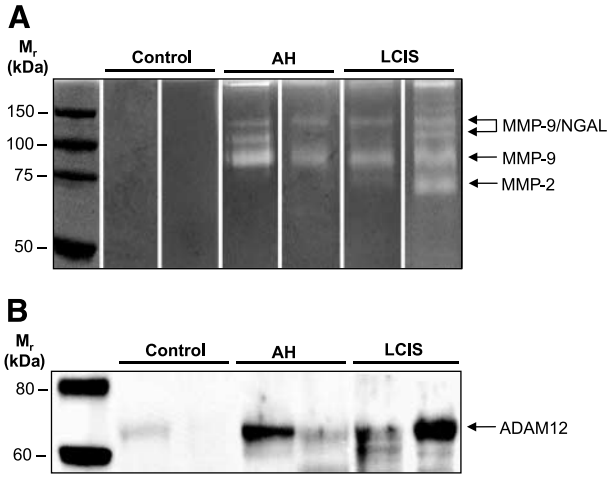

Figure 1. A, urinary MMPs are present in the urine of women at risk to develop breast cancer: MMP-2 (gelatinase A), MMP-9 (gelatinase B), MMP-9/NGAL complex, and the high-molecular-weight MMP species are the predominant MMP species detectable in human urine by gelatin zymography. **B,** ADAM 12 is present in the urine of women diagnosed with atypical hyperplasia or LCIS and at increased risk of developing breast cancer at significantly higher levels than normal controls. The 68-kDa active form of ADAM 12 is the predominant species detected in human urine by immunoblot analysis using an ADAM 12–specific antibody. Band intensities were analyzed and converted to DU using UN-SCAN-IT (Silk Scientific) software digitizer technology (18).

the normal controls having a mean 5-year risk of 1.0 and were elevated in the patients with atypia with a mean risk of 3.8. There was no significant difference in smoking or drinking habits between the groups. Mammograms were read as normal with Breast Imaging Reporting and Data System scores 1 or 2 in 96% of the normal controls, 60% of the women with LCIS, and 36% of the women with atypia. Fifty-two percent of the women with atypia and 36% of the women with LCIS had mammograms classified as Breast Imaging Reporting and Data System 4 or 5, which are scores suspicious for, or highly suggestive of, malignancy. The study group was slightly older than the control group; appropriate adjustments were made in the statistical analyses (Table 1).

Urinary MMP and ADAM 12 Expression. MMP-9, MMP-2, the MMP-9/NGAL complex, and ADAM 12 were consistently detected in the urine of the majority of the patients studied. Representative zymograms for MMP-9 and Western blots for ADAM 12 are shown in Fig. 1.

Statistical Analysis. With respect to ADAM 12, univariate analysis indicated that women with atypical hyperplasia and LCIS had mean levels of 20.7 ± 16.8 and 14.7 ± 6.9 densitometric units (DU), respectively, which were significantly higher than normal controls (2.1 ± 2.8 DU) as determined by ANOVA with Bonferroni adjustment (both $P < 0.001$). The median ADAM 12 level for normal controls was 0. Atypical hyperplasia and LCIS groups did not differ significantly from each other in mean or median ADAM 12 level ($P > 0.20$; Table 2*A*). There were also significant differences in the percentage of individuals with positive MMP-9 between normal controls and women diagnosed with atypical hyperplasia or LCIS (Pearson $\chi^2 = 6.17$ on 2 *df*; $P < 0.05$).

Stepwise multiple logistic regression analysis revealed that continuous ADAM 12 level ($P < 0.0001$), positive MMP-9 ($P = 0.02$), and age ($P = 0.04$) were independently predictive in differentiating women diagnosed with atypical hyperplasia from controls (Table 2*B*). The adjusted odds ratio for ADAM 12 in differentiating

Table 2.

(A) Univariate analysis of urinary ADAM 12 levels for normal controls and patient study groups

	Normal controls (n = 80)	Atypical hyperplasia (n = 44)	LCIS (n = 24)	Atypical hyperplasia/ LCIS (n = 68)
ADAM 12 (DU), mean ± SD	2.1 ± 2.9	20.7 ± 16.8*	14.7 ± 6.9*	18.5 ± 14.7*
Median (interquartile range)	0 (0-3)	3 (9-27)*	15 (12-18)*	13 (11-24)*
Full range	0-11	0-80	0-27	0-80

(B) Multivariable logistic regression analysis of variables predicting atypical hyperplasia and LCIS

Variable	β coefficient	Odds ratio (95% confidence interval)	P
		Predictors of Atypical hyperplasia	
ADAM 12 (DU)	0.33	1.4 (1.2-1.6)	<0.0001
MMP-9	1.62	5.1 (1.4-17.9)	0.02
Age (y)	0.08	1.1 (1.0-1.2)	0.04
		Predictors of LCIS	
ADAM 12 (DU)	0.47	1.6 (1.3-2.0)	<0.0001
MMP-9	2.61	13.8 (1.7-110.7)	0.01
Age (y)	0.09	1.1 (1.0-1.2)	0.05

NOTE: ADAM 12 levels were compared using ANOVA with Bonferroni adjustment for means and the Mann-Whitney *U* test for medians. MMP-2, MMP-9/NGAL, and MMP > 150 were not statistically significant ($P > 0.05$).
*$P < 0.001$ for the comparison with normal controls.

atypical hyperplasia from control is 1.4, implying that each 10-unit increase is associated with an increased odds of 28 times (1.4^{10}) that the individual has atypical hyperplasia rather than being a normal healthy control. This is equivalent to an increased probability of 97%, where probability = odds / (1 + odds) = 28 / 29. For binary MMP-9 analysis, an individual who is positive has an estimated risk five times higher to have atypical hyperplasia compared with testing negative for MMP-9 (odds ratio, 5.1; 95% confidence interval, 1.4-17.9). Other variables tested, including MMP-2, MMP-9/NGAL, and MMP > 150 kDa, were not predictive of atypical hyperplasia (all $P > 0.05$).

When evaluating the predictors of LCIS, logistic regression indicated significant multivariate predictors identical to those for atypical hyperplasia, including ADAM 12 ($P < 0.0001$), MMP-9 ($P = 0.014$), and age ($P = 0.05$; Table 2*B*). These variables provide independent information in differentiating women with LCIS from normal controls, where the adjusted odds ratio for ADAM 12 is 1.6, implying that each 10-unit increase is associated with an increased odds 110 times (1.6^{10}) that the individual has LCIS rather than being a normal control. This is equivalent to an increased probability of >99%. A woman who is positive for MMP-9 has a risk of LCIS over 13 times higher compared with an individual testing negative for MMP-9 (odds ratio, 13.8; 95% confidence interval, 1.7-110.7). Other variables, including MMP-2, MMP-9/NGAL, and MMP > 150 kDa, were not significant predictors of LCIS (all $P > 0.05$).

By means of logistic regression analysis, nonlinear equations were derived to estimate the probability of atypical hyperplasia and LCIS based on different intervals ADAM 12 and were highly significant for atypical hyperplasia (LRT, 58.4; $P < 0.0001$) and for LCIS (LRT, 53.3; $P < 0.0001$). Empirical data for controls and patients with a diagnosis of atypical hyperplasia (Fig. 2*A*) or LCIS (Fig. 2*B*) are represented by bars for

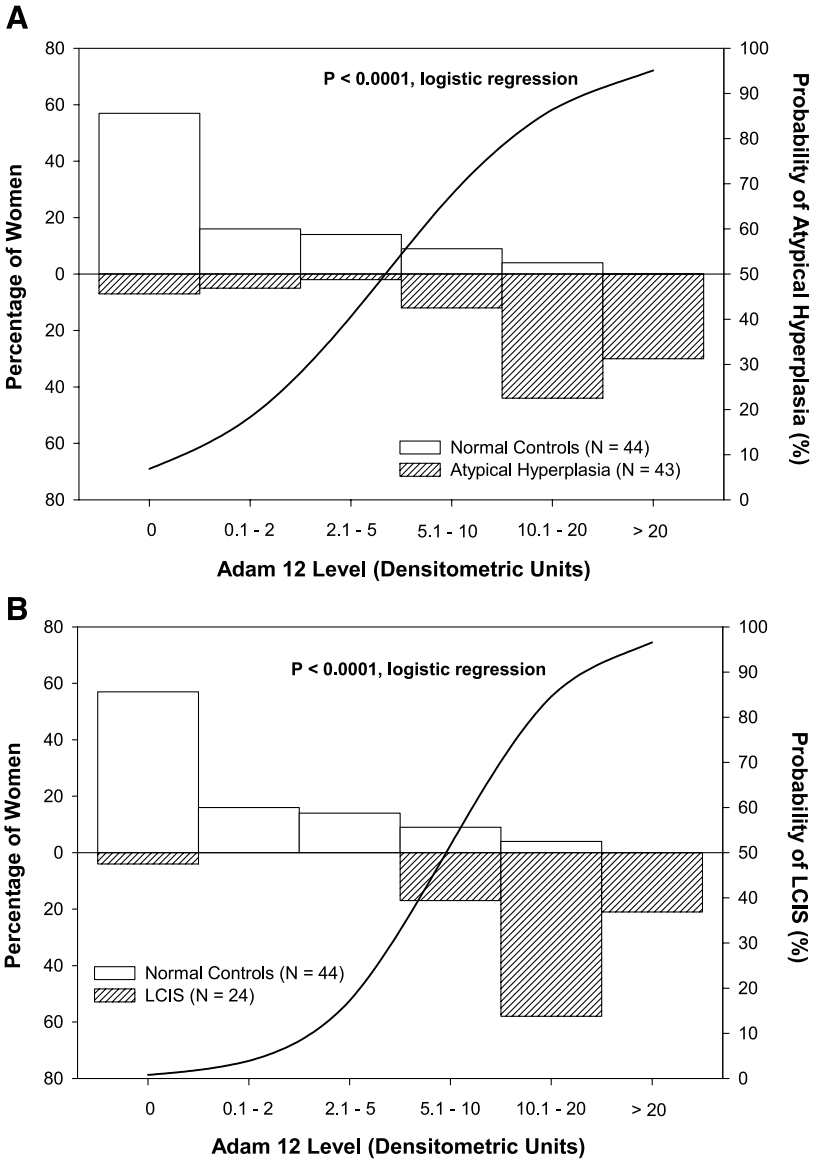

Figure 2. Theoretical curve illustrating the probability of atypical hyperplasia (**A**) and LCIS (**B**) compared with the normal controls on ADAM 12 level. Empirical data are shown as histograms representing the percentage of women in each group with ADAM 12 levels within each of the intervals on the *X axis*. Logistic regression analysis indicated a highly significant nonlinear relationship between increasing ADAM 12 level and the increasing probability of atypical hyperplasia (LRT, 58.4 on 1 *df*; $P < 0.0001$) and LCIS (LRT, 53.3 on 1 *df*; $P < 0.0001$). Nearly 60% of controls had ADAM 12 levels of 0, whereas 75% of women diagnosed with atypical hyperplasia and 80% with LCIS had ADAM 12 levels greater than 10 DU.

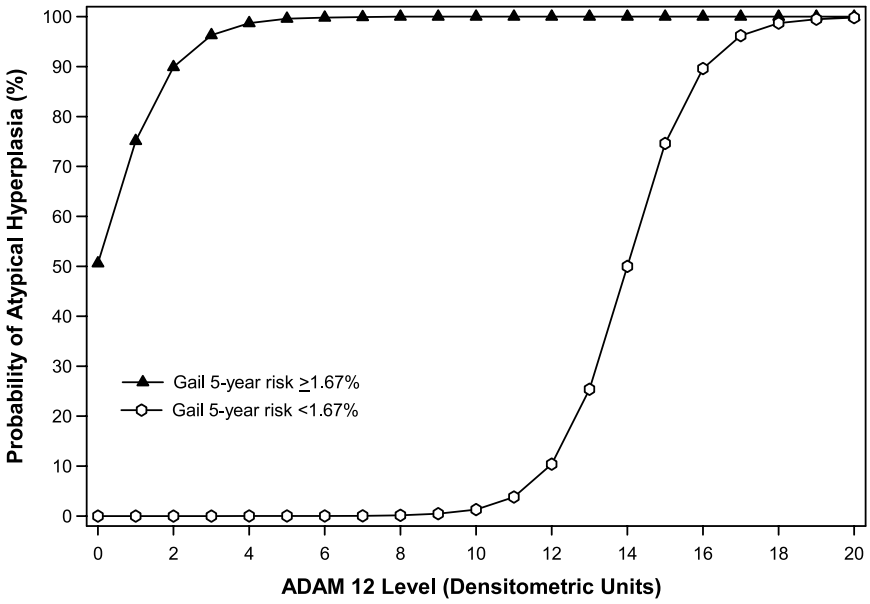

Figure 3. Probability curves showing the combined value of using ADAM 12 level with Gail scores to predict the likelihood of atypical hyperplasia. Curves for Gail risk subgroups were derived by multiple logistic regression, which confirmed that ADAM 12 level and Gail scores were each independently predictive of an abnormal diagnosis of atypical hyperplasia. A woman with a low-risk Gail 5-year score of <1.67% has a low predicted probability of atypical hyperplasia if she also has an ADAM 12 level <12 DU.

each group, reflecting the percentage of women with ADAM 12 levels in each interval. Theoretical curves illustrating the probability of atypical hyperplasia or LCIS diagnosis compared with normal are shown according to ADAM 12 interval in each figure and clearly show the separation between the patients and controls.

As shown in Fig. 2*A*, 57% of controls and only 7% of patients with atypical hyperplasia had ADAM 12 levels of 0 DU, whereas 75% of patients with atypical hyperplasia and only 4% of controls had levels over 10 DU. The predicted probability of atypical hyperplasia is 7% for individuals who have ADAM 12 levels of 0, 40% for those with levels between 5 and 10 DU, and 85% for women with ADAM 12 levels 10 to 20 DU and 95% for levels over 20 DU. Comparatively, as depicted in Fig. 2*B*, 57% of controls and only 4% of patients with LCIS had ADAM 12 levels of 0, whereas almost 80% of patients with LCIS and only 4% of controls had levels over 10 DU. The probability of LCIS is <5% for individuals with positive ADAM 12 levels <2 DU, 52% for those with levels 5 to 10 DU, 85% for women with ADAM 12 levels of 10 to 20 DU, and 97% for women with levels >20 DU.

Urinary ADAM 12 levels were then multiplexed with Gail 5-year risk scores, which reflect clinical information (27). Gail scores less than 1.67% are considered low risk, whereas scores equal to or over 1.67% are high risk for the development of breast cancer. Multiple logistic regression analysis identified ADAM 12 level (LRT, 19.92; $P < 0.0001$) as a significant predictor for differentiating atypical hyperplasia from controls. The results of this modeling approach can be seen as the increasing probability of atypical hyperplasia with increasing ADAM 12 levels separately according to high or low Gail 5-year risk (Fig. 3). For example, the probability of atypical hyperplasia for an ADAM 12 level of 2 DU is 90% for women who have Gail scores ≥1.67% and essentially 0% for those with low-risk Gail scores <1.67%. On the other hand, the probability of atypical hyperplasia in women with low-risk Gail scores <1.67% (bottom curve) starts to increase with moderately high ADAM 12 levels (e.g., levels of ≥12 DU). For example, ADAM 12 levels of 14 and 15 DU are associated with probabilities of 50% and 75%, respectively, in this subgroup of women with low-risk Gail 5-year scores. There is an estimated ≥90% probability of atypical hyperplasia in individuals with Gail 5-year risk of <1.67% and ADAM 12 levels of ≥16 DU. As Gail scores are not appropriate for use in women with LCIS, this model applies only to women with atypical hyperplasia.

Similarly, receiver operating characteristic analysis of continuous ADAM 12 levels alone shows excellent discrimination in differentiating women with atypical hyperplasia from normal controls, with area under the receiver operating characteristic curve of 0.914. Urinary ADAM 12 levels also provide exceptional discrimination in differentiating women with LCIS from normal controls, with area under the curve of 0.950. As the maximum area under the curve is 1.0 for any test, these scores show the enormous predictive accuracy of urinary ADAM 12 levels.

When urinary ADAM 12 levels are multiplexed with the clinical information that Gail 5-year risk scores provide, further receiver operating characteristic analysis indicates that the optimal combination is Gail + 0.15 × ADAM 12 (area under the curve = 0.996). Therefore, the best performance is obtained when both ADAM 12 and the Gail risk score are used together. The optimal cutoff for this combination index is 2.8. Using the cutoff of 2.8, the sensitivity for the combination is 0.976 (41 of 42 atypical hyperplasia cases were classified correctly) and specificity is 0.977 (43 of 44 controls were classified correctly). Consequently, use of this ADAM 12-Gail combination index in our population yields only one false positive (one control has a combination index of 2.95, scoring above 2.8) and one false negative (one woman with atypical hyperplasia has a combination of 2.3, scoring below the 2.8 cutoff).

Potential Confounders. Because the atypical hyperplasia and LCIS study groups were older than the

controls and had a higher percentage of menopausal women, we examined five potential confounders to assess their association with ADAM 12 and MMP-9. Age, timing of urine collection, menopausal status, alcohol usage, and smoking were each examined to determine whether any of them correlated with these biomarkers and thus could be confounding variables in this study. None of these five variables were significantly associated with urinary ADAM 12 or MMP-9 results.

Age. There were no significant correlations between age and ADAM 12 levels in normal controls (Pearson r = 0.09; P = 0.54), atypical hyperplasia (Pearson r = -0.03; P = 0.83), or LCIS (Pearson r = -0.04; P = 0.83). Comparing MMP-9 positive versus MMP-9 negative, there were no significant differences in age based on the Student's t test: normal controls (48.1 $\pm$ 8.5 versus 47.6 $\pm$ 7.9; P = 0.80), atypical hyperplasia (54.6 $\pm$ 7.4 versus 53.4 $\pm$ 8.9; P = 0.60), and LCIS (56.2 $\pm$ 9.8 versus 52.3 $\pm$ 7.2; P = 0.39).

Timing of Urine Collection. ADAM 12 levels (DU) collected before or after diagnosis were not significantly different according to timing of urine collection based on Kruskal-Wallis test (atypical hyperplasia, P = 0.32; LCIS, P = 0.07). With respect to MMP-9, χ^2 analysis revealed no relationship between the timing of urine collection and the percentage of MMP-9 positive expression for atypical hyperplasia (P = 0.83) or LCIS (P = 0.48).

Menopausal Status. Menopausal women were compared with premenopausal women with regard to urinary ADAM 12 and MMP levels. ADAM 12 levels (DU) were not significantly different according to menopausal status based on the Mann-Whitney U test (controls, P = 0.81; atypical hyperplasia, P = 0.37; LCIS, P = 0.08). MMP-9-positive rates according to menopausal status indicated no differences based on Fisher's exact tests (controls, P = 0.47; atypical hyperplasia, P = 0.11; LCIS, P = 0.99).

Alcohol Usage. Women who did not drink alcohol were compared with those who consumed less than five alcoholic beverages a week and those who drank five or more alcoholic beverages a week. ADAM 12 levels (DU) were not significantly different according to alcohol usage based on the Kruskal-Wallis test (controls, P = 0.28; atypical hyperplasia, P = 0.15; LCIS, P = 0.50). The MMP-9-positive rates also showed no differences in alcohol usage with χ^2 analysis (controls, P = 0.57; atypical hyperplasia, P = 0.61; LCIS, P = 0.63).

Smoking. Women who never smoked were compared with those who smoked in the past but stopped smoking, those who continue to smoke less than a pack a week, and those who smoked a pack or more a week. ADAM 12 levels (DU) were not significantly different according to smoking status based on the Kruskal-Wallis test (controls, P = 0.82; atypical hyperplasia, P = 0.62; LCIS, P = 0.55). There was also no difference in MMP-9-positive rates according to smoking status based on χ^2 analysis (controls, P = 0.47; atypical hyperplasia, P = 0.78; LCIS, P = 0.09).

Discussion

As the options for breast cancer risk reduction improve, the identification of women with an elevated risk for developing breast cancer is becoming increasingly important (37). Current approaches to risk assessment include a complete clinical evaluation with careful physical exam, radiologic studies, family history, and risk profiling using mathematical models, such as BRCAPRO and Gail, to aid in decision making (38).

High-risk patients are encouraged to comply with a regimen of close surveillance, including mammograms and breast exams. If the lifetime risk is 25% to 30%, more intensive screening with breast magnetic resonance imaging alternating with mammograms as well as genetic counseling and testing can also be considered (39, 40). Although mammography remains the ''gold standard'' for breast cancer detection, mammogram screening yields a false-negative rate of 10% to 30% and is compromised in women with high breast density (41). False positives are also a substantial problem given that 10% of all screens are read as abnormal and almost all of these are false positives (42, 43). Lifestyle changes, such as decreasing fat and alcohol intake and exercise benefits, can also be encouraged (44). Finally, medical risk reduction can be approached by avoidance of exogenous estrogens and consideration of Tamoxifen, Raloxifene, or an aromatase inhibitor while acknowledging that the side effects of these medications preclude use in many women (45). Ultimately, more reliable, less invasive, and less expensive approaches for breast cancer risk assessment and early detection, as well as better alternatives for medical intervention, are needed.

In this study, we have analyzed the urinary expression of MMP-9 and ADAM 12 in patients with biopsy-proven atypical hyperplasia and LCIS, indicators of an increased risk of developing breast cancer. We have also carefully examined potential confounding variables, including age, timing of urine collection, menopausal status, alcohol usage, and smoking, to determine whether any of them were correlated with these biomarkers. We found that none of these five variables were significantly associated with the urinary ADAM 12 or MMP-9 results. However, in keeping with current guidelines for careful analysis of new biomarkers (46), our results require further validation in a much larger population before clinical application.

It is important to note that MMPs are involved in the modulation of modulate normal cellular behavior and cell-cell communication as well as tumor angiogenesis and progression (47). For this reason, MMPs might be elevated in conditions that are not specifically breast cancer related (48-50) and would never be used as a sole determinant of risk assessment or diagnosis. Therefore, in clinical context, and in conjunction with the Gail model and a full assessment of the patient, these noninvasive biomarkers could be a useful adjunct to currently available risk assessment tools.

Our data show that urinary ADAM 12 and MMP-9 are highly significant predictors of breast cancer risk markers, atypical hyperplasia and LCIS. ADAM 12 levels in particular were found to provide excellent discrimination in differentiating women with atypical hyperplasia or LCIS from normal controls. Moreover, when ADAM 12 levels are multiplexed with the Gail risk score, the resultant index appears to provide an accurate tool to distinguish normal controls from women with atypical hyperplasia (sensitivity, 0.976; specificity, 0.977). Presently, the Gail model is not appropriate for use in

women with LCIS. The potential of an accurate, noninvasive urine test for assessing breast cancer risk with is enormously appealing. Urine tests would be less invasive, less costly than current screening modalities, and easily tolerated and would encourage higher compliance with screening and therapeutic monitoring. Once validated in larger studies, such a test could potentially provide a useful adjunct for breast cancer risk assessment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Brown PD, Bloxidge RE, Anderson E, Howell A. Expression of activated gelatinase in human invasive breast carcinoma. Clin Exp Metastasis 1993;11:183–9.
2. Poulsom R, Hanby AM, Pignatelli M, et al. Expression of gelatinase A and TIMP-2 mRNAs in desmoplastic fibroblasts in both mammary carcinomas and basal cell carcinomas of the skin. J Clin Pathol 1993; 46:429–36.
3. Zucker S, Lysik RM, DiMassimo BI, et al. Plasma assay of gelatinase B: tissue inhibitor of metalloproteinase complexes in cancer. Cancer 1995;76:700–8.
4. Iba K, Albrechtsen R, Gilpin BJ, Loechel F, Wewer UM. Cysteine-rich domain of human ADAM 12 (meltrin α) supports tumor cell adhesion. Am J Pathol 1999;154:1489–501.
5. Somiari SB, Shriver CD, Heckman C, et al. Plasma concentration and activity of matrix metalloproteinase 2 and 9 in patients with breast disease, breast cancer and at risk of developing breast cancer. Cancer Lett 2006;233:98–107.
6. Somiari SB, Somiari RI, Heckman CM, et al. Circulating MMP2 and MMP9 in breast cancer-potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories. Int J Cancer 2006;119:1403–11.
7. Moses MA, Wiederschain D, Loughlin KR, Zurakowski D, Lamb CC, Freeman MR. Increased incidence of matrix metalloproteinases in urine of cancer patients. Cancer Res 1998;58:1395–9.
8. Nguyen M, Watanabe H, Budson AE, Richie JP, Folkman J. Elevated levels of the angiogenic peptide basic fibroblast growth factor in urine of bladder cancer patients. J Natl Cancer Inst 1993;85: 241–2.
9. Nguyen M, Watanabe H, Budson AE, Richie JP, Hayes DF, Folkman J. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. J Natl Cancer Inst 1994;86:356–61.
10. Durkan GC, Nutt JE, Rajjayabun PH, Neal DE, Lunec J, Mellon JK. Prognostic significance of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 in voided urine samples from patients with transitional cell carcinoma of the bladder. Clin Cancer Res 2001;7:3450–6.
11. Gerhards S, Jung K, Koenig F, et al. Excretion of matrix metalloproteinases 2 and 9 in urine is associated with a high stage and grade of bladder carcinoma. Urology 2001;57:675–9.
12. Hanemaaijer R, Sier CF, Visser H, et al. MMP-9 activity in urine from patients with various tumors, as measured by a novel MMP activity assay using modified urokinase as a substrate. Ann N Y Acad Sci 1999;878:141–9.
13. Nutt JE, Durkan GC, Mellon JK, Lunec J. Matrix metalloproteinases (MMPs) in bladder cancer: the induction of MMP9 by epidermal growth factor and its detection in urine. BJU Int 2003;91:99–104.
14. Sherief MH, Low SH, Miura M, Kudo N, Novick AC, Weimbs T. Matrix metalloproteinase activity in urine of patients with renal cell carcinoma leads to degradation of extracellular matrix proteins: possible use as a screening assay. J Urol 2003;169:1530–4.
15. Sier CF, Casetta G, Verheijen JH, et al. Enhanced urinary gelatinase activities (matrix metalloproteinases 2 and 9) are associated with early-stage bladder carcinoma: a comparison with clinically used tumor markers. Clin Cancer Res 2000;6:2333–40.
16. Fang J, Shing Y, Wiederschain D, et al. Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci U S A 2000;97:3884–9.
17. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000;2:737–44.
18. Roy R, Wewer UM, Zurakowski D, Pories SE, Moses MA. ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem 2004;279:51323–30.
19. Parkin DM, Fernandez LM. Use of statistics to assess the global burden of breast cancer. Breast J 2006;12 Suppl 1:S70–80.
20. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
21. Jemal A, Siegel R, Ward E, et al. Cancer Statistics, 2008. CA Cancer J Clin 2008;58:71–96.
22. Strategies for managing the breast cancer research program: a report to the U.S. Army Medical Research and Development Command. Institute of Medicine National Academy Press; 1993.
23. Kronqvist P, Kuopio T, Nykanen M, Helenius H, Anttinen J, Klemi P. Predicting aggressive outcome in $T_1N_0M_0$ breast cancer. Br J Cancer 2004;91:277–81.
24. Verschraegen C, Vinh-Hung V, Cserni G, et al. Modeling the effect of tumor size in early breast cancer. Ann Surg 2005;241:309–18.
25. Viacava P, Naccarato AG, Bocci G, et al. Angiogenesis and VEGF expression in pre-invasive lesions of the human breast. J Pathol 2004; 204:140–6.
26. Costantino JP, Gail MH, Pee D, et al. Validation studies for models projecting the risk of invasive and total breast cancer incidence. J Natl Cancer Inst 1999;91:1541–8.
27. Gail MH, Brinton LA, Byar DP, et al. Projecting individualized probabilities of developing breast cancer for white females who are being examined annually. J Natl Cancer Inst 1989;81:1879–86.
28. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem 2001;276:37258–65.
29. Towbin H, Staehelin T, Gordon J. Immunoblotting in the clinical laboratory. J Clin Chem Clin Biochem 1989;27:495–501.
30. Gilpin BJ, Loechel F, Mattei MG, Engvall E, Albrechtsen R, Wewer UM. A novel, secreted form of human ADAM 12 (meltrin α) provokes myogenesis *in vivo*. J Biol Chem 1998;273:157–66.
31. Lacquement MA, Mitchell D, Hollingsworth AB. Positive predictive value of the Breast Imaging Reporting and Data System. J Am Coll Surg 1999;189:34–40.
32. Glantz SA. Primer of biostatistics. 5th ed. New York: McGraw-Hill; 2005.
33. Hosmer DW, Lemeshow S. Applied logistic regression. 2nd ed. New York: John Wiley; 2000.
34. Breslow NE. Day NE Statistical methods in cancer research. Lyon (France): IARC; 1980.
35. Zhou Z-H, Obuchowski NA, McClish DK. Statistical methods in diagnostic medicine. New York: Wiley-Interscience; 2002.
36. Rosner B. Fundamentals of biostatistics. Belmont (CA): Duxbury; 2006.
37. Hollingsworth AB, Singletary SE, Morrow M, et al. Current comprehensive assessment and management of women at increased risk for breast cancer. Am J Surg 2004;187:349–62.
38. Ozanne EM, Klemp JR, Esserman LJ. Breast cancer risk assessment and prevention: a framework for shared decision-making consultations. Breast J 2006;12:103–13.
39. James PA, Doherty R, Harris M, et al. Optimal selection of individuals for BRCA mutation testing: a comparison of available methods. J Clin Oncol 2006;24:707–15.
40. Saslow D, Boetes C, Burke W, et al. American Cancer Society guidelines for breast screening with MRI as an adjunct to mammography. CA Cancer J Clin 2007;57:75–89.
41. Barlow WE, Lehman CD, Zheng Y, et al. Performance of diagnostic mammography for women with signs or symptoms of breast cancer. J Natl Cancer Inst 2002;94:1151–9.

42. Brown ML, Houn F, Sickles EA, Kessler LG. Screening mammography in community practice: positive predictive value of abnormal findings and yield of follow-up diagnostic procedures. AJR Am J Roentgenol 1995;165:1373–7.
43. Fletcher SW, Elmore JG. False-positive mammograms—can the USA learn from Europe? Lancet 2005;365:7–8.
44. Linos E, Holmes MD, Willett WC. Diet and breast cancer. Curr Oncol Rep 2007;9:31–41.
45. Vogel VG, Constantino JP, Wickerham DL, et al. Effects of Tamoxifen vs Raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial. JAMA 2006;295:2727–41.
46. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
47. McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 2001;13:534–40.
48. Smith ER, Manfredi M, Scott RM, Black PM, Moses MA. A recurrent craniopharyngioma illustrates the potential usefulness of urinary matrix metalloproteinases as noninvasive biomarkers: case report. Neurosurgery 2007;60:E1148–9; discussion E1149.
49. Moses MA, Harper J, Folkman J. Doxycycline treatment for lymphangioleiomyomatosis with urinary monitoring for MMPs. N Engl J Med 2006;354:2621–2.
50. Fitzsimmons PJ, Forough R, Lawrence ME, et al. Urinary levels of matrix metalloproteinase 9 and 2 and tissue inhibitor of matrix metalloproteinase in patients with coronary artery disease. Atherosclerosis 2007;194:196–203.