Int. J. Cancer: **100,** 678–682 (2002)

Publication of the International Union Against Cancer

ETHNIC VARIATION IN KALLIKREIN EXPRESSION IN NIPPLE ASPIRATE FLUID

Edward R. SAUTER[1]*, Tamara WELCH[1], Angeliki MAGKLARA[2], Gary KLEIN[3] and Eleftherios P. DIAMANDIS[2]

[1]*Department of Surgery, Thomas Jefferson University, Philadelphia, PA, USA*
[2]*Mt. Sinai Hospital, Toronto, Ontario Canada*
[3]*Philadelphia Health Management Corporation, Philadelphia, PA, USA*

Socioeconomic factors cannot entirely explain why black women have an earlier age of breast cancer onset and higher mortality rates, stage for stage, than whites. We and others have shown that prostate-specific antigen [PSA, also known as human kallikrein (hK) 3] is a marker of breast as well as prostate cancer, that hK2 and hK3 are highly homologous at the DNA and protein level and that the level of progesterone, which appears to upregulate hK3, is influenced by ethnicity. We hypothesized that nipple aspiration fluid (NAF) hK2 and hK3 levels are (*i*) lower in black than white women; (*ii*) independently associated with breast cancer; (*iii*) influenced by menopausal status; and (*iv*) in combination are more informative about whether a woman has breast cancer than either marker alone. NAF was assayed for hK2 and hK3, and the results were stratified by ethnicity, presence or absence of cancer and menopausal status. Statistical analysis was then performed. When stratified by ethnicity, hK2 ($p = 0.003$) and hK3 ($p = 0.027$) levels in blacks were lower than in whites. hK2 was lower in premenopausal black than in white subjects, regardless of cancer status. Overall, hK2, hK3 and the ratio hK2/hK3 were lower in subjects with breast cancer than in normal subjects. hK3 was lower in postmenopausal women with breast cancer, regardless of ethnicity. hK2 and hK3 levels were higher in pre- than in postmenopausal whites. Using logistic regression and considering hK2, hK3, hK2/hK3 and ethnicity, hK3 was significantly associated with breast cancer in both pre- ($p < 0.001$) and postmenopausal women ($p = 0.023$). In conclusion, whereas hK2, hK3, hK2/hK3 and ethnicity are each significantly associated with breast cancer bivariately, after entering the strongest predictor, hK3, into a logistic regression model, no other variable accounted for additional variation, although this observation is preliminary due to the limited number of black subjects in the study.

Key words: *kallikreins; breast cancer; prostate-specific antigen; ethnicity*

Over 1,500 Americans die each day from cancer, making it the second leading cause of death (after cardiovascular disease) in the United States.[1] Overall cancer incidence rates are higher in men than women, and black men have the highest incidence of cancer. Among women, whites have the highest incidence.[2] The ethnic variation observed in cancer incidence and survival cannot be ascribed solely to social, cultural, economic and environmental variability.[3] Among individual cancer types, ethnic differences in cancer gene expression are present that may help explain differences in cancer incidence and survival.

Since its discovery more than 20 years ago, prostate-specific antigen (PSA) has been established as the most valuable tool for the early detection, staging and monitoring of prostate cancer.[4] The *KLK2* gene (encoding for hK2) and the *KLK3* gene (encoding for hK3 or PSA) are members of the human kallikrein family,[5] which now comprises 15 structurally similar genes that in humans cluster in a 300 kb region on chromosome 19q13.3-13.4.[6] Each of the kallikreins is a serine protease. All 15 genes in the locus share significant homology at both the DNA and protein level, and all encode for serine proteases with either trypsin- or chymotrypsin-like activity. hK2 exhibits 78% amino acid sequence identity to PSA.[7] At the DNA level, hK2 and PSA share 80% similarity.[8] The secretion of hK2, similar to that of PSA, is stimulated by male sex steroid hormones.[9] hK2 cleaves proPSA to generate enzymatically active PSA.[10] This suggests that hK2 may play a physiologic role in the regulation of PSA activity. PSA and hK2 have been found and measured together in amniotic fluid, breast milk, breast cyst fluid and malignant and nonmalignant breast tissue.[11]

Both hK2 and hK3 are expressed more in noncancerous than in cancerous tissue, with the degree of downregulation in the prostate being higher for PSA than for hK2.[12] hK2 was shown to be upregulated in the human breast cancer cell line T47D by both androgens and progestins.[13] We previously demonstrated that hK2 was measurable in 53% and PSA in 73% of breast tumor extracts and that their levels of expression were associated.[11] The median PSA/hK2 ratio in male sera is 2.6 (range 0.1–34) based on the assay used in the study.[14] Whereas studies in male specimens such as seminal fluid and prostatic tissue indicated that hK2 was found at levels 1–2% of PSA,[9] we found that median hK2 levels in breast tumor cytosols were approximately 30% of PSA.

hK2 may have a role in differentiating benign prostatic hypertrophy (BPH) from prostate cancer. The hK2/free PSA ratio exhibited the highest significance in discriminating between prostate cancer and BPH,[15] suggesting that hK2 may prove useful as an adjunct to PSA to differentiate BPH from prostate cancer.

Nipple aspiration is a quick and painless technique to obtain breast fluid noninvasively. Breast fluid contains shed ductal epithelial cells and proteins secreted from the ductal epithelium. We were able to collect nipple aspirate fluid (NAF) from essentially all adult women.[16] We have performed nipple aspiration on over 750 women without complications. Both the cells and extracellular fluid obtained hold promise for use in cancer screening. We previously demonstrated that PSA, which is best known for its use in prostate cancer screening, is expressed in NAF and is present at higher levels in pre- than in postmenopausal women. PSA is also found at lower levels in women with cancer than in normal women,[17] and levels in NAF appear to rise in association with the progesterone surge in premenopausal women.[18] Systemic progesterone levels are significantly different in black than in white women,[19] and PSA levels are higher in black than in white men, regardless of whether or not they have prostate cancer.[20] It is therefore reasonable to speculate that hK2 and hK3 expression in NAF vary by ethnicity.

In summary, hK2 and hK3 are expressed in the breast. In breast tissue the levels of these kallikreins have been inversely associated with breast cancer development and progression. The purpose of our study was to investigate whether hK2 and hK3 levels in NAF

Grant sponsor: National Institutes of Health; Grant number: CA-87391.

*Correspondence to: Thomas Jefferson University, 1025 Walnut St., Suite 605, Philadelphia, PA 19107, USA. Fax: +01-215-923-1420.
E-mail: Edward.Sauter@mail.tju.edu

Received 21 March 2002; Revised 27 May 2002; Accepted 28 May 2002

DOI 10.1002/ijc.10556

are (*i*) lower in black than white women; (*ii*) independently associated with breast cancer; (*iii*) influenced by menopausal status; and (*iv*) in combination are more informative about whether a woman has breast cancer than either marker alone. Our findings may help to explain why blacks have a higher risk of death from the disease than whites, even after controlling for potential confounders such as age and disease stage.

MATERIAL AND METHODS

Subject and specimen accrual

Between 1995 and 2000, informed consent was obtained and NAF collected from 285 (244 white and 41 black) women who enrolled in an Institutional Review Board-approved study. The women were between the ages of 24 and 80 years; 86% were white, and 56% were premenopausal. The women were categorized into 1 of 7 risk groups: (1) no risk factors; (2) history of a first-degree relative with breast cancer; (2) history of cancer in the breast contralateral to that being studied; (4) biopsy-proven atypical hyperplasia (AH); (5) biopsy-proven lobular carcinoma *in situ* (LCIS); (6) biopsy-proven ductal carcinoma *in situ*; or (7) biopsy-proven invasive carcinoma. Subjects who fit into 2 risk groups were assigned to the category of greater risk. Because there were very few women in risk categories 2–5, and because we have found that kallikrein expression in subjects with AH and LCIS tend to be similar to subjects without evidence of disease,[17] we collapsed categories 0–5 ("non-cancer") and 6/7 ("cancer") for all analyses.

NAF measures were recorded as coming from either the right or the left breast. Some subjects had measures from 1 side only, whereas others had measures from both sides. For subjects who had measures from both sides, 1 side was randomly selected for the analyses (along with the corresponding side-specific cancer risk assessment). If multiple measures were available from the selected side, they were collapsed into a single value (their median). In essence, only 1 measurement per woman was used in each analysis.

TABLE I – DEMOGRAPHICS OF NAF SAMPLES EVALUATED

	White (%)	Black (%)
Number	244 (86)	41 (14)
Breast cancer status		
Non-cancer	131 (54)	17 (41)
Cancer	113 (46)	24 (59)
Menopausal status		
Pre	141 (58)	19 (46)
Post	103 (42)	22 (54)

Nipple aspiration technique

Nipple aspiration was performed after obtaining informed consent using a modified breast pump. The technique has been described previously.[17] Briefly, the breasts were warmed with moist towels. The subject then massaged her breasts for approximately 2 min. Her nipples were cleansed with a mild soap followed by alcohol. A suction device was then placed first over the right and then over the left breast, if present. Suction was created using a 10 ml syringe and held for 10–15 sec, or until the participant experienced discomfort. Fluid in the form of droplets appeared and was collected into glass capillary tubes. NAF total protein content was determined using a Pierce (Rockford, IL) BCA Protein Assay Reagent Kit. Capillary tubes containing NAF were broken in half and placed in 400 μl of a 0.1 M $NaHCO_3$ solution, pH 7.80. The capillary was then crushed with a steel spatula to release the NAF. Samples for analysis were kept at −80°C and batched for analysis. All analyses were performed under the supervision of a laboratory administrator with over 20 years of experience. Technicians were blinded as to the risk group of each sample.

hK2 and hK3

NAF was analyzed for both hK2 and hK3 whenever possible. Both hK2 and hK3 were measured using time-resolved immunofluorometric assays developed by us.[14,21] hK2 has a detection limit of 6 ng/L and hK3 a detection limit of 1 ng/L. The hK2 assay has less than 0.2% cross-reactivity with hK3. The coefficients of variation for both the hK2 and hK3 assays were $< 10\%$ within the measurement range. All values were recorded as ng hK2 or hK3 per gram total NAF protein.

Statistical analyses

Tables II–IV show the nontransformed mean and median values. As demonstrated by the substantial differences in mean and median values, the data are positively skewed. Performance of a parametric statistical procedure such as the difference of means test (*t*-test) or logistic regression required the transformation of the raw scores to reduce the impact of outliers on the means. Such a

TABLE II – ETHNIC VARIATION IN HK2 AND HK3 (NG/G)[1]

	Whites			Blacks			*p*-value[2]
	No.	Mean (SD)	Median	No.	Mean (SD)	Median	
Overall							
hK2	141	118 (265)	29	34	37 (84)	4	**0.003**
hK3	244	2,948 (8198)	322	41	1,365 (3426)	67	**0.027**
Risk							
Non-cancer							
hK2	82	149 (319)	39	15	75 (116)	23	0.09
hK3	131	4,656 (10,655)	1047	17	2,757 (4832)	519	0.46
Premenopausal							
hK2	59	188 (366)	50	8	60 (76)	25	**0.025**
hK3	93	5,663 (12,210)	1712	8	3,770 (6782)	500	0.35
Postmenopausal							
hK2	23	50 (85.5)	21	7	92 (156)	23	0.38
hK3	38	2,191 (4399)	294	9	1,858 (2127)	519	0.38
Cancer							
hK2	59	75 (154)	18	19	7 (17)	0	**<0.001**
hK3	113	968 (2588)	52	24	378 (1313)	15	0.08
Premenopausal							
hK2	24	68 (99)	20	9	9 (23)	0	**0.01**
hK3	48	1,314 (3319)	97	11	707 (1922)	15	0.59
Postmenopausal							
hK2	35	79 (184)	11	10	6 (9)	0	**0.007**
hK3	65	712 (1864)	38	13	100 (206)	9	**0.05**

[1]Student's *t*-test; all values are per gram total protein. hK, human kallikrein.–[2]Values in bold are significant.

procedure is preferable to conducting nonparametric tests, for these tests do not consider the degree of difference in scores and leave us without a viable multivariate procedure to predict cancer. Log transformations of hK2, hK3 and the ratio of hK2/hK3 were therefore performed that resulted in nonsignificant skewness, enabling bivariate tests of significance utilizing the independent samples *t*-test and the multivariate procedure of logistic regression. Nonetheless, for descriptive clarity, rather than discuss the means of the logarithms used for statistical testing, in the Results section we discuss the actual median values, as they are not influenced by skewed data and are proportionately much closer than the actual mean values to the mean of the logarithmic transformations.

Due to the highly skewed distribution of hK2 and hK3, as is evident by a comparison of their mean and median values in Tables II–IV, where the means far exceed the medians, we decided to transform the values before conducting significance tests. The logs were taken after adding 1 to each value (to permit inclusion of initial zero values), and this transformation sufficiently removed problematic skewness, thus allowing their inclusion in logistic regression. Statistical tests conducted included independent *t*-tests by ethnicity, presence or absence of cancer, menopausal status and forward stepwise logistic regression, where the simultaneous effects of hK2 , hK3, hK2/hK3 and ethnicity on cancer were ascertained.

RESULTS

The distribution of subjects based on ethnicity, presence or absence of breast cancer and menopausal status is indicated in Table I. The associations of hK2 and hK3 with ethnicity, breast cancer and menopausal status are outlined in Tables II–IV.

Ethnic variation in hK2 and hK3 levels

When looking at all subjects, median values of hK2 were 7-fold higher and those of hK3 were almost 5-fold higher in white than in black women. The expression of both markers was significantly higher in whites (Table II). Among non-cancer subjects, only hK2 expression in premenopausal women differed by ethnicity. Median levels of hK2 for black women with cancer were below the level of detection in more than half of the samples available and were lower than in white women with cancer, whether considering the cancer group overall or groups separated by menopausal status.

TABLE III – ASSOCIATION OF KALLIKREIN EXPRESSION (NG/G) WITH BREAST CANCER[1]

	Non-cancer			Cancer			*p*-value[2]
	No.	Mean (SD)	Median	No.	Mean (SD)	Median	
Overall							
hK2	97	138 (297)	33	78	58 (137)	8	**<0.001**
hK3	148	4,438 (10,164)	975	97	865 (2420)	43	**<0.001**
hK2/hK3	97	2.36 (11.7)	0.029	78	1.89 (10.7)	0.11	**0.003**
Menopausal status							
Premenopausal							
Whites							
hK2	59	188 (366)	50	24	68 (99)	20	0.06
hK3	93	5,663 (12,210)	1712	48	1,314 (3319)	97	**<0.001**
hK2/hK3	59	2.39 (13.7)	0.028	24	4.94 (19.1)	0.15	**0.007**
Blacks							
hK2	8	60 (76)	25	9	9 (23)	0	0.05
hK3	8	3,770 (6782)	500	11	707 (1922)	15	0.15
hK2/hK3	8	4.65 (13.1)	0.018	9	0.41 (0.6)	0.063	0.69
Postmenopausal							
Whites							
hK2	23	50 (85.5)	21	35	79 (184)	11	0.25
hK3	38	2,191 (4399)	294	65	712 (1864)	38	**0.002**
hK2/hK3	23	2.18 (7.0)	0.059	35	0.55 (1.4)	0.086	0.90
Blacks							
hK2	7	92 (156)	23	10	6 (9)	0	0.10
hK3	9	1,858 (2127)	519	13	100 (206)	9	**<0.001**
hK2/hK3	7	0.087 (0.1)	0.046	10	0.56 (0.6)	0.48	0.09

[1]Student's *t*-test; all values are per gram total protein. hK, human kallikrein.–[2]Values in bold are significant.

TABLE IV – ASSOCIATION OF KALLIKREIN EXPRESSION (NG/G) WITH MENOPAUSAL STATUS[1]

	Premenopausal			Postmenopausal			*p*-value[2]
	No.	Mean (SD)	Median	No.	Mean (SD)	Median	
Non-cancer							
Whites							
hK2	59	188 (366)	50	23	50 (85.5)	21	**0.05**
hK3	93	5,663 (12.210)	1712	38	2,191 (4399)	294	**0.003**
Blacks							
hK2	8	60 (76)	25	7	92 (156)	23	0.91
hK3	8	3,770 (6782)	500	9	1,858 (2127)	519	0.76
Cancer							
Whites							
hK2	24	68 (99)	20	35	79 (184)	11	0.31
hK3	48	1,314 (3319)	97	65	712 (1864)	38	0.44
Blacks							
hK2	9	9 (23)	0	10	6 (9)	0	0.93
hK3	11	707 (1922)	15	13	100 (206)	9	0.17

[1]Student's *t*-test; all values are per gram total protein; hK, human kallikrein.–[2]Values in bold are significant.

Association of kallikrein expression with breast cancer

Median levels were 4-fold higher for hK2 and 20-fold higher for hK3 in women without than in women with breast cancer (Table III, Fig. 1). The median ratio of hK2/hK3 was almost 4-fold lower in normal women than in women with breast cancer. The expression of the markers and the ratio of the 2 were significantly associated with breast cancer. hK3 levels were significantly lower in both pre- and postmenopausal white women with cancer than normal women and in postmenopausal black women with breast cancer than normal black women. The hK2/hK3 ratio was significantly lower in normal premenopausal women than in premenopausal women with breast cancer. Median differences in hK3 were more pronounced in both pre- (33.3- *vs.* 17.6-fold lower) and in postmenopausal (57.7- *vs.* 7.7-fold lower) black than in white women with breast cancer.

Association of kallikrein expression with menopausal status

Median expression levels of hK2 and hK3 were 2.4- and 5.8-fold higher in pre- compared with postmenopausal white women without breast cancer. The differences in expression of both markers were significant (Table IV). The differences were not observed in black women without breast cancer, nor in white or black women with breast cancer.

Association of hK2, hK3, hK2/hK3 and ethnicity with breast cancer

Whereas bivariate analyses were performed to determine all of the previously presented results, stepwise logistic regression was conducted to determine which markers contributed additional information to predict whether a subject had breast cancer. Since we had previously observed that that hK3 expression was significantly higher in pre- than in postmenopausal women,[17] separate logistic regressions were conducted for pre- and postmenopausal subjects to see whether these markers had the same impact for each group. For both pre- and postmenopausal subjects, hK3 had the strongest independent impact, and once hK3 was entered into the forward conditional model, knowledge of hK2, hK2/hK3 and ethnicity did not improve the model to predict which subjects had breast cancer. hK3 was a better predictor of cancer for pre- ($p < 0.001$, Nagelkerke $R^2 = 0.266$) than for postmenopausal women ($p = 0.023$, Nagelkerke $R^2 = 0.099$).

DISCUSSION

A major goal of our study was to determine whether kallikrein levels in NAF are different in white and black women with and without breast cancer. Socioeconomic causes do not entirely explain differences in breast cancer incidence and survival between blacks and whites. Our belief was that levels of NAF hK2 and hK3, which are known to be lower in the tissue of women with breast cancer than in normal women, may be different based on ethnicity. If this is true, physiologic differences in kallikrein expression may contribute to disparities in the breast cancer survival rates of white and black women.

The distribution of ethnicity (Table I) represents the patient population to which we have access. Blacks without breast cancer were less likely to participate than whites, as reflected in the fact that the majority of samples from blacks were from women with cancer as opposed to samples from whites, in whom most samples came from women without the disease.

There were substantial differences in hK2 and hK3 expression based on ethnicity (Table II). These differences were most notable in women with breast cancer and were more dramatic for hK2 than hK3. Indeed, most samples from black women with breast cancer contained levels of hK2 too low to detect, whereas median levels for samples from white women ranged from 11 to 20 ng/g.

Both hK2 and hK3 were significantly higher in women without than in women with breast cancer (Table III). The difference in hK3 expression was significant in subset analyses for postmenopausal white and black women, and for premenopausal white women, whereas it was observed in subset analyses of hK2 only in premenopausal black women. This more powerful association of hK3 with breast cancer explains why it was the most important variable in our logistic regression model.

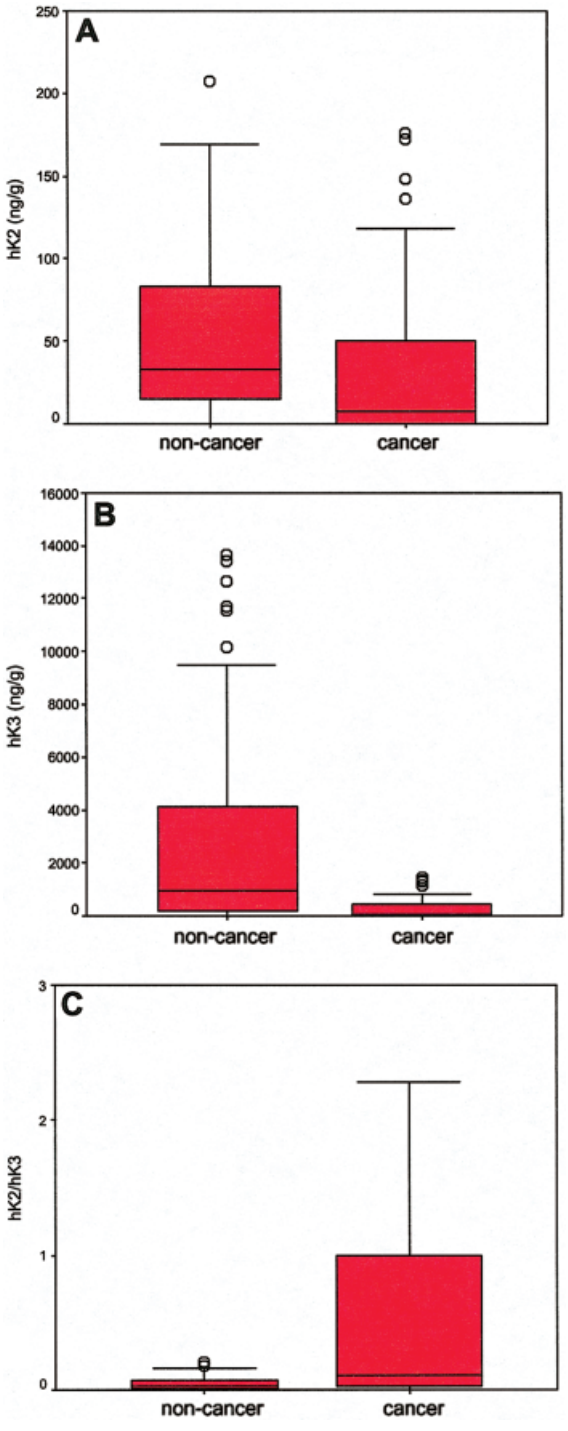

FIGURE 1 – Boxplots demonstating median values and interquartile ranges for (*a*) hK2, (*b*) hK3 (PSA) and (*c*) hK2/hK3. For both hK2 and hK3, subjects without cancer (non-cancer) tended to have higher expression than did subjects with cancer. This was reversed for hK2/hK3, with higher values in the cancer than the non-cancer group. There were 19 extreme values for hK2 (12 non-cancer and 7 cancer), 25 for hK3 (9 non-cancer and 16 cancer) and 21 for hK2/hK3 (17 non-cancer and 4 cancer). Extreme values are not shown.

As we stated earlier, it has been reported that knowing both hK2 and hK3 may be better than knowing one or the other.[15] The ratio of hK2/hK3 appears to better differentiate a benign from a cancerous prostate condition than either marker alone. We assessed the ability of hK2/hK3 levels in NAF to identify women with breast cancer. Although in bivariate analysis the ratio differentiated women with from women without breast cancer, it did not provide additional predictive information once hK3 was known.

Menopausal status appeared to influence hK2 and hK3 expression significantly in white but not in black women without breast cancer. Although it is not entirely clear why this might be, perhaps this reflects the fact that kallikreins, which have been reported to be under the influence of ovarian hormones[21] and may be upregulated by progesterone,[18] may not be under similar regulation in black and white women. On the other hand, neither white nor black women with breast cancer had differences in hK2 or hK3 expression based on menopausal status, suggesting that PSA regulation by ovarian steroids is lost with the development of breast cancer.

Despite significant associations of hK2 and hK3 with ethnicity, breast cancer and menopausal status, when the effects of these markers were looked at simultaneously in a stepwise logistic regression model, hK3 had the strongest influence on predicting breast cancer, and after hK3 was known, the other markers added little to predict breast cancer. It is not altogether surprising that hK2 did not add information, given its high homology with hK3. It is less clear why ethnicity did not independently contribute to the prediction of breast cancer, although in bivariate analyses the major contribution of ethnicity was to predict differences in hK2, not hK3, which may explain why it did little to improve the ability of hK3 to predict breast cancer. In addition, the limited number of black women in our study may have minimized our ability to detect a unique contribution of ethnicity to cancer prediction.

The results of our study support previous findings in a smaller sample size that PSA levels are lower in women with breast cancer[17] and suggest that PSA may have a protective role in breast cancer. The finding of lower hK2 levels in the NAF of black women, although not significant in predicting breast cancer after knowing hK3, warrants further study to determine whether physiologic differences in kallikrein expression exist based on ethnicity and how these differences may be associated with adverse survival.

ACKNOWLEDGMENTS

Our study was not sponsored by or affiliated with any commercial interest. None of the authors have stock/equity interests or patent licenses related to this work.

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