Loss of PTEN Expression, *PIK3CA* Mutations, and Breast Cancer Survival in the Nurses' Health Studies

Tengteng Wang[1,2], Yujing J. Heng[3], Gabrielle M. Baker[3], Vanessa C. Bret-Mounet[3], Liza M. Quintana[3], Lisa Frueh[1], Susan E. Hankinson[4], Michelle D. Holmes[1,2], Wendy Y. Chen[1,5], Walter C. Willett[2,6], Bernard Rosner[1,7], Rulla M. Tamimi[8], and A. Heather Eliassen[1,2,6]

ABSTRACT

Background: The relationships between PTEN loss and/or *PIK3CA* mutation and breast cancer prognosis remain controversial. We aim to examine the associations in large epidemiologic cohorts.

Methods: We followed women with invasive breast cancer from the Nurses' Health Studies with available data on tumor PTEN expression ($n = 4{,}111$) and *PIK3CA* mutation ($n = 2{,}930$). PTEN expression was evaluated by IHC and digitally scored (0%–100%). Pyrosequencing of six hotspot mutations of *PIK3CA* was performed.

Results: We found loss of PTEN expression (≤10%) occurred in 17% of cases, and *PIK3CA* mutations were detected in 11% of cases. After adjusting for clinical and lifestyle factors, PTEN loss was not associated with worse breast cancer-specific mortality among all samples [HR, 0.85; 95% confidence intervals (CI), 0.71–1.03] or among estrogen receptor (ER)-positive tumors (HR, 0.99; 95% CI, 0.79–1.24). However, among ER-negative tumors, PTEN loss was associated with lower breast cancer-specific mortality (HR, 0.68; 95% CI, 0.48–0.95). *PIK3CA* mutation was not strongly associated with breast cancer-specific mortality (HR, 0.89; 95% CI, 0.67–1.17). Compared with tumors without PTEN loss and without *PIK3CA* mutation, those with alterations ($n = 540$) were not at higher risk (HR, 1.07; 95% CI, 0.86–1.34). However, women with both PTEN loss and *PIK3CA* mutation ($n = 38$) were at an increased risk of breast cancer-specific mortality (HR, 1.65; 95% CI, 0.83–3.26).

Conclusions: In this large epidemiologic study, the PTEN-mortality association was more pronounced for ER-negative tumors, and the joint PTEN loss and *PIK3CA* mutation may be associated with worse prognosis.

Impact: Further studies with a larger sample of ER-negative tumors are needed to replicate our findings and elucidate underlying mechanisms.

Introduction

PI3K/Akt signaling cascade is a key regulator of most cancer hallmarks by affecting cell-cycle progression, cell apoptosis, migration, and glucose metabolism (1–3). Regulation of Akt activity is via opposition of PI3K by tumor suppressor PTEN, preventing phosphorylation and Akt activation (4).

PI3K/Akt pathway activation occurs in 50% to 75% of breast cancers (5, 6). The two most common activating alterations of this pathway are the loss of PTEN protein expression and somatic mutations in the PI3K catalytic subunit alpha (*PIK3CA*) gene (1). PTEN loss in breast cancer (with variable definitions across studies) varies from 4% to 82% (7–9), and the *PIK3CA* mutation frequency varies from 7% to 61% (10–12), although the frequency of the coexistence of the two is low (1, 13, 14). However, the true frequency is difficult to interpret given that previous studies generally had small sample sizes (<500), and these studies had considerable differences in breast tumor pathologic characteristics and laboratory measurement methods (1). In addition, the cutoff points for defining PTEN loss and the selection of *PIK3CA* mutation sites varied across studies.

The relationships between tumor PTEN loss and/or *PIK3CA* mutation and breast cancer prognosis remain controversial. In two systematic reviews and meta-analyses, Li and colleagues observed that breast tumors with PTEN loss and/or *PIK3CA* mutation were more aggressive and had worse outcomes (15), whereas Mosele and colleagues suggested that *PIK3CA* mutations are associated with a favorable cancer outcome in women with hormone receptor-positive but HER2-negative (HER2−) breast cancer (16). The frequency of PTEN loss and *PIK3CA* mutation in breast cancers, and their relevance for prognosis, remain unclear.

Herein we aim to comprehensively describe the frequency of PTEN loss of expression and *PIK3CA* mutations and investigate the associations of PTEN loss and *PIK3CA* mutations (individually and jointly) with breast cancer-specific mortality in two large U.S. epidemiological cohort studies, the Nurses' Health Study (NHS) and Nurses' Health Study II (NHSII).

Materials and Methods

Study population

Our study population is identified from two well-characterized cohorts, the NHS and NHSII. The NHS, started in 1976, enrolled 121,700 female registered nurses ages 30 to 55 years from 11 U.S.

[1]Channing Division of Network Medicine, Department of Medicine, Brigham & Women's Hospital, and Harvard Medical School, Boston, Massachusetts. [2]Department of Epidemiology, Harvard T. H. Chan School of Public Health, Boston, Massachusetts. [3]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts. [4]Department of Biostatistics and Epidemiology, University of Massachusetts School of Public Health and Health Sciences, Amherst, Massachusetts. [5]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [6]Department of Nutrition, Harvard T. H. Chan School of Public Health, Boston, Massachusetts. [7]Department of Biostatistics, Harvard T. H. Chan School of Public Health, Boston, Massachusetts. [8]Department of Population Health Sciences, Weill Cornell Medicine, New York, New York.

Rulla M. Tamimi and A. Heather Eliassen contributed equally as the co-senior authors of this article.

Corresponding Author: Tengteng Wang, Brigham and Women's Hospital and Harvard Medical School and Harvard T.H Chan School of Public Health, 181 Longwood Ave, Boston, MA 02115. E-mail: tengteng.wang@channing.harvard.edu

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states (17). The NHSII was initiated in 1989 among 116,429 female registered nurses ages 25 to 42 years from 14 U.S. states (18). At baseline, each participant answered and returned a mailed questionnaire describing characteristics of demographics, reproductive, lifestyle, and medical history (17–19). Updated epidemiologic information is collected through the ongoing biennial follow-up questionnaires (17–19). Written informed consent was implied by the return of the completed questionnaires, and the two studies were conducted in accordance with Declaration of Helsinki guideline (17–19). The study protocols of these two cohorts were approved by the institutional review boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health, and those of participating tumor registries as required (17–19).

Breast cancer diagnoses were self-reported from participants (or next of kin for decedents) on the biennial questionnaires and these diagnoses were further confirmed by study medical personnel via review of medical records (19). For this analysis, the eligible participants included women with confirmed invasive breast cancer between 1978 and 2011 in the NHS and between 1991 and 2011 in the NHSII, for whom tumor tissue were available. PTEN expression was measured in 4,988 eligible women in preassembled tissue microarrays (TMA). *PIK3CA* mutation was measured in 5,318 women eligible for the assay. We further excluded participants who had stage IV tumors or missing information on stage ($n = 361$ for *PIK3CA*; $n = 545$ for PTEN), no PTEN data due to missing or poor-quality tumor cores ($n = 332$), or no *PIK3CA* mutation data due to poor quality of tumor DNA ($n = 2,027$). We excluded stage IV cases primarily because they have much shorter survival than stage I to III tumors, and we focused on long-term survival in this study. After exclusions (**Fig. 1**), 4,111 women were included in the analysis of PTEN, 2,930 women were included in the analysis of *PIK3CA*, and 2,225 women were included in the combined analysis of PTEN and *PIK3CA*.

Assessment of PTEN expression and *PIK3CA* mutations

PTEN expression was evaluated by IHC assay (Rabbit mAb 138G6; Cell Signaling Technology; 1:250 dilution), which was performed on validated tumor TMAs (20). Each participant's tumor was represented by 3 × 0.6 mm formalin-fixed paraffin-embedded (FFPE) cores on the TMA. Up to three cores per individual were scored using Definiens Tissue Studio image analysis software (Munich, Germany), which generated a continuous quantitative estimate of the PTEN expression positivity (0–100%). Positivity was measured as the mean percentage of cells staining positive. It was calculated by dividing the sum of the number of cells staining positive by the sum of the total cell count across cores available (21, 22). Positivity was measured separately for epithelial and stromal cell compartments; we combined measures by compartment to assess total PTEN loss. The loss of PTEN expression was defined as any individual tumor with ≤10% value for the weighted average percentage of cells staining positive. This classification yielded a binary classification for PTEN expression (loss vs. no loss). We also created a four-categories variable using quartile cut-points. One TMA stained for PTEN ($N = 258$) was scored manually by a pathologist and categorized as negative (0%), low positive (1%–10%), or positive (>10%), and expression was evaluated as the maximum across cores.

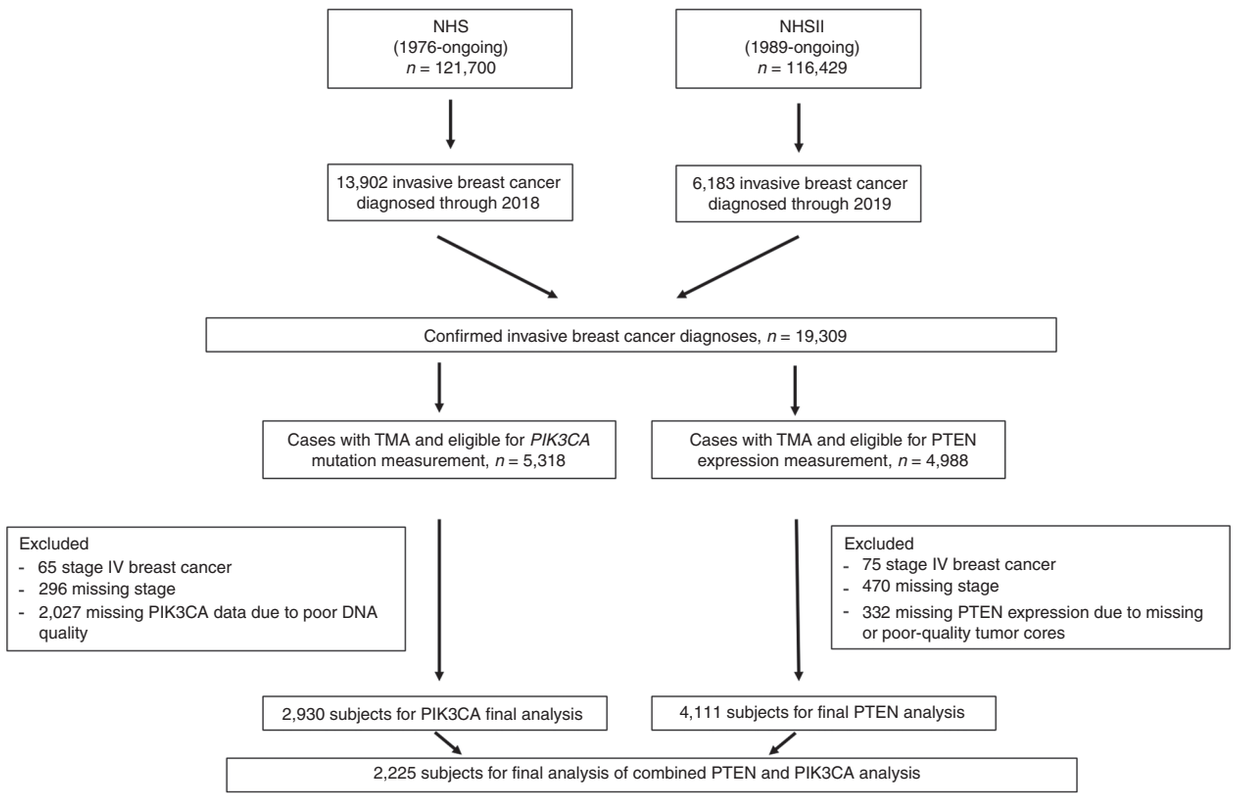

Figure 1.
Flowchart for identification of analytic population for the associations of PTEN loss of expression and *PIK3CA* mutation with breast cancer survival in the NHS and NHSII.

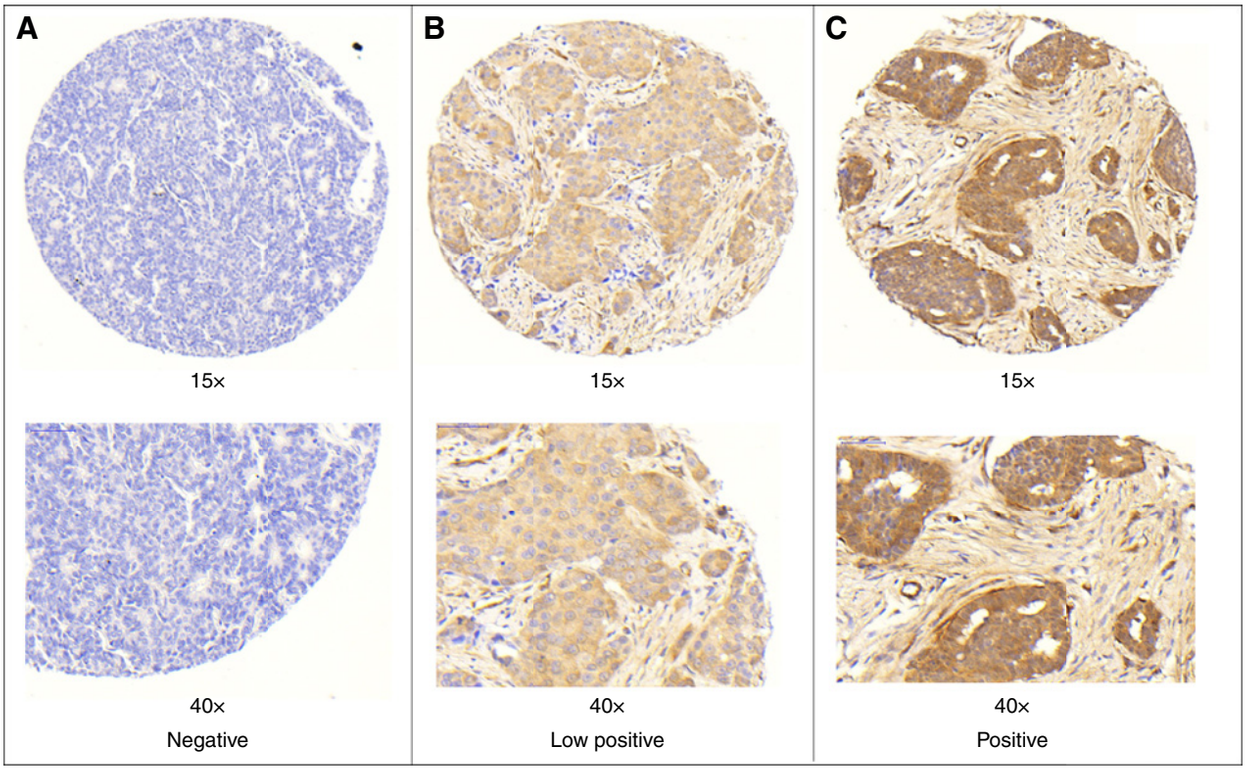

Figure 2.
Manually read PTEN protein expression staining in three tissue microarray cores in the NHS and NHSII. Expression was graded as negative (0%; **A**), low positive (1%–10%; **B**) and positive (>10%; **C**). Images in the top row are at magnification 15×. The bottom row captured the identical image as shown in the top row at magnification 40×.

Representative images on PTEN staining are shown in **Fig. 2**. The Spearman correlation between the Definiens and manual scoring was 0.62. Staining batch variability was corrected by using the average recalibration method (23) with adjustment for age at diagnosis, calendar year of diagnosis, tumor estrogen receptor (ER) status, and HER2 status. In primary analyses, we focused on cytoplasmic PTEN staining. However, in a subset of samples ($n = 3{,}143$), we used similar digital methods to measure nuclear PTEN staining and used that for secondary analyses.

For *PIK3CA* mutation assessment, tumor regions on histopathologic slides were annotated by the pathologists, a 1.5 mm tumor core was taken from the matched FFPE tissue block, DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the manufacturer's instructions. The Qiagen qBiomarker Somatic Mutation PCR Assays and pyrosequencing targeted six most frequently mutated *PIK3CA* hotspots: exon 9 (E542K, E545A, E545K), exon 20 (H1047L, H1047R), and exon 4 (N345K; ref. 5).

Assessment of covariates

Participants' demographic characteristics, medical history, smoking status, reproductive history, weight, height, and physical activity were self-reported in the biennial follow-up questionnaires (19). Body mass index (BMI, kg/m^2) was calculated and updated using height (m) reported at baseline and weight (kg) reported in the follow-up questionnaires (19). Tumor ER, progesterone receptor (PR), HER2, and androgen receptor (AR) expression were evaluated by IHC when possible or extracted from medical records (except AR). Tumor stage and grade were evaluated via centralized pathologist review or extracted from medical records. Individuals with ≥1% expression in at least one core were defined as positive for ER and PR (19). A 10% cut-point was used to define HER2- and AR-positive expression (22). Finally, information about breast cancer treatment (surgery, chemotherapy, radiation therapy, and endocrine therapy) was obtained from medical records when possible, or self-reported in the breast cancer survivor's supplementary questionnaires.

Outcome ascertainment

Deaths were first reported by participants' family members or by US Postal Service or through the search of the National Death Index (24). Once a death is identified, medical records or death certificate were reviewed to determine the specific causes of death (23). We primarily focused on breast cancer-specific cause of death in this study, and women died from other causes before endpoints were censored at date of death. Study endpoints were defined as death or end of follow-up (June 1, 2016, for the NHS; June 1, 2017, for the NHSII), whichever came first.

Statistical analysis

We combined data from NHS and NHSII and used Cox proportional hazards regression models to estimate HRs and 95% confidence intervals (CI) for the associations of PTEN loss and *PIK3CA* mutation with breast cancer-specific mortality. Person-time of follow-up was calculated from date of diagnosis to death or the

end of the follow-up period. The proportional hazards assumption was tested using the likelihood ratio test by comparing models with versus without interaction terms between binary PTEN or *PIK3CA* status and the follow-up time. Test for trend was performed using the median value for each quintile of the PTEN percentage of positivity as a continuous variable in the regression models.

We fit four models as follows: model 1 was the crude model without any adjustment. Model 2 included age at diagnosis and calendar year of diagnosis. Model 3 was the multivariable-adjusted model and included age and calendar year of diagnosis as well as tumor ER status, stage, grade, self-reported radiation therapy, chemotherapy, and endocrine treatment. HER2 status (and trastuzumab) were not included as main covariates in this model because HER2+ frequency was similar by PTEN/PIK3CA status in our analytical population. Model 4 additionally included the following covariates measured in the questionnaire cycle prior to diagnosis as a proxy for status at diagnosis: menopausal status, BMI, physical activity, cigarette smoking, aspirin use, and menopausal hormone therapy use. All models were stratified by cohort and follow-up period.

We carried out subgroup analyses by time since diagnosis, and by breast tumor ER (for both PTEN and PIK3CA analyses) and AR status (for PTEN analysis only; refs. 25, 26). All statistical analyses were conducted with SAS statistical software version 9.4 (SAS Institute Inc.). *P* values <0.05 were considered significant and all statistical tests were two-sided.

Data availability

The data generated in this study are not publicly available due to participant confidentiality and privacy concerns but are available upon request. Further information including the steps to obtain data from the NHS is described at https://www.nurseshealthstudy.org/researchers.

Results

The loss of PTEN expression (cytoplasmic) occurred in 17.3% of our cases, and the overall mutation of *PIK3CA* (at least one hotspot mutation) presented in 10.8% of cases. Among 2,225 women with both PTEN expression and *PIK3CA* mutation data available, 24.3% had either PTEN loss or *PIK3CA* mutation, or both. In **Table 1**, compared with tumors that had PTEN expression >10%, those with

Table 1. Characteristics of participants at breast cancer diagnosis according to PTEN loss of expression and *PIK3CA* mutation status in the NHS and NHSII.

	PTEN expression ($N = 4,111$)		*PIK3CA* mutation[a] ($N = 2,930$)		PTEN expression and/or *PIK3CA* mutation ($N = 2,225$)	
	Loss ($n = 711, 17.3\%$)	No loss ($n = 3,400, 82.7\%$)	Mutation ($n = 317, 10.8\%$)	No mutation ($n = 2,613, 89.3\%$)	Loss and/or Mutation ($n = 540, 24.3\%$)	No loss and no mutation ($n = 1,685, 75.7\%$)
Mean age of diagnosis, years (SD)	57.7 (10.2)	57.7 (10.6)	58.9 (10.0)	59.2 (10.7)	57.7 (9.9)	57.8 (10.6)
Calendar year of diagnosis, before 2000, %	70.8	66.1	53.3	60.2	65.6	66.4
Postmenopausal, %	66.4	63.9	68.8	67.8	65.0	63.4
Cohort, NHS %	77.8	75.4	75.4	79.5	74.4	75.9
White, %	96.6	96.6	97.5	96.6	96.3	96.9
Mean BMI, kg/m^2 (SD)	26.2 (5.4)	26.1 (5.2)	26.1 (5.0)	26.2 (5.2)	26.1 (5.3)	26.1 (5.2)
Mean physical activity, MET-hours/week (SD)	15.3 (17.6)	17.2 (23.5)	18.4 (24.9)	17.3 (23.1)	15.9 (20.4)	17.4 (24.5)
Current smokers, %	16.7	13.3	17.7	12.4	15.2	12.5
Current aspirin users, %	41.8	41.7	43.9	41.5	43.2	40.4
Ever users, menopausal hormone therapy, %	45.9	44.9	44.5	48.5	45.2	45.0
Estrogen receptor (ER) status, %						
Positive	68.2	81.8	86.1	80.0	75.7	82.3
Negative	31.2	17.9	12.3	19.4	23.7	17.6
Human epidermal growth factor receptor 2 (HER2) status, %						
Positive	13.9	21.3	18.6	16.5	16.9	19.5
Negative	82.3	77.0	77.9	78.5	81.3	79.5
Androgen receptor (AR) status, %						
Positive	54.4	64.4	57.8	53.2	60.4	65.0
Negative	29.3	18.6	13.0	16.5	22.8	18.8
Grade, %						
Grade 1	20.1	19.9	22.4	19.1	19.4	17.6
Grade 2	51.8	52.3	54.3	51.4	53.9	55.0
Grade 3	25.5	23.7	19.2	25.7	24.4	25.6
Stage, %						
Stage I	49.1	53.3	55.2	50.8	50.2	49.7
Stage II	33.2	33.8	32.8	35.1	33.7	35.6
Stage III	17.7	12.9	12.0	14.1	16.1	14.7
Received chemotherapy, %	52.0	48.5	45.4	50.0	52.6	52.1
Received radiation, %	52.6	51.7	57.7	54.6	54.8	52.3
Received endocrine therapy, %	57.7	67.7	73.8	68.7	65.7	69.0

Abbreviation: MET, metabolic equivalent task.
[a]Six hotspot mutations were considered: exon 9 (E542K, E545A, E545K), exon 20 (H1047L, H1047R), and exon 4 (N345K).

Table 2. Multivariable analysis of the association between cytoplasmic PTEN expression loss and breast cancer-specific mortality in the NHS and NHSII ($N = 4{,}111$).

	All ($n = 4{,}111$)		ER-positive ($n = 3{,}265$)		ER-negative[e] ($n = 831$)	
Binary (by 10% cutoff of expression)	**No loss ($n = 3{,}400$)**	**Loss ($n = 711$)**	**No loss ($n = 2{,}780$)**	**Loss ($n = 485$)**	**No loss ($n = 609$)**	**Loss ($n = 222$)**
Median (IQR), percent of cells staining positive	38 (23–55)	5 (2–8)	39 (24–56)	5 (1–8)	30 (19–48)	5 (2–8)
No. of events ($n = 774$)	623	151	475	100	147	51
Model 1: HR (95% CI)[a]	1 (referent)	1.16 (0.97–1.38)	1 (referent)	1.20 (0.97–1.49)	1 (referent)	0.96 (0.70–1.31)
Model 2: HR (95% CI)[b]	1 (referent)	1.09 (0.91–1.30)	1 (referent)	1.13 (0.91–1.40)	1 (referent)	0.89 (0.64–1.22)
Model 3: HR (95% CI)[c]	1 (referent)	0.86 (0.72–1.04)	1 (referent)	1.01 (0.81–1.26)	1 (referent)	0.69 (0.49–0.97)
Model 4: HR (95% CI)[d]	1 (referent)	0.85 (0.71–1.03)	1 (referent)	0.99 (0.79–1.24)	1 (referent)	0.68 (0.48–0.95)

[a]Crude model.
[b]Further adjusted for age of diagnosis (categorical) and year of diagnosis (categorical).
[c]Further adjusted for tumor estrogen receptor status (positive, negative, unknown), stage (I, II, III), grade (1, 2, 3, unknown), self-reported radiation therapy (yes, no, or unknown), chemotherapy (yes, no, or unknown), and hormonal treatment (yes, no, or unknown).
[d]Further adjusted for at-diagnosis menopausal status (premenopausal, postmenopausal, and unknown), at-diagnosis BMI (<25, 25–29.9, ≥30 kg/m^2), at-diagnosis physical activity (women: <9, ≥9 MET-hours/week), at-diagnosis cigarette smoking (never, former, current, or unknown), at-diagnosis aspirin use (never, former, current, or unknown), and at-diagnosis menopausal hormone therapy use (current, past, never).
[e]The $P_{interaction}$ for PTEN expression status and ER status is 0.25.

PTEN loss (≤10%) were more likely to be diagnosed before 2000 (71% vs. 66%), be ER− (31% vs. 18%), AR− (29% vs. 19%), and stage III (18% vs. 13%) tumors, and less likely to receive endocrine therapy (58% vs. 68%). Interestingly, tumors with at least one hotspot *PIK3CA* mutation (vs. no hotspot mutation) were more likely to be diagnosed after 2000 (47% vs. 40%), be ER+ (86% vs. 80%), AR+ (58% vs. 53%), and stage I (55% vs. 51%) tumors, and more likely to receive endocrine therapy (74% vs. 69%), and less likely to receive chemotherapy (45% vs. 50%). Combing the two markers, compared with tumors without PTEN loss and *PIK3CA* mutation, those with PTEN loss and/or *PIK3CA* mutation were more likely to be ER− and AR− tumors, and therefore less likely to receive endocrine therapy. Women with PTEN loss and/or *PIK3CA* mutation were also more likely to be current smokers and aspirin users.

Over a median follow-up of 15.8 years, there were 774 breast cancer deaths among 4,111 breast cancer cases. In Model 1, loss of PTEN expression was associated with a slightly, but not significantly, increased risk of breast cancer-specific mortality (HR, 1.16; 95% CI, 0.97–1.38; $P = 0.09$; **Table 2**). In models adjusted for tumor, treatment, and lifestyle factors, PTEN loss was associated with a 15% nonstatistically significant reduction in breast cancer-specific mortality (HR, 0.85; 95% CI, 0.71–1.03; $P = 0.13$). However, in the subgroup analyses by tumor ER status, we observed a significant inverse association among ER− tumors (HR, 0.68; 95% CI, 0.48–0.95; $P = 0.03$), but no association for ER+ breast cancers (HR, 0.99; 95% CI, 0.79–1.24; $P = 0.93$; $P_{interaction} = 0.25$). In sensitivity analyses using quartiles of PTEN staining positivity among all samples, the lowest quartile (vs. highest) was associated with lower breast cancer-specific mortality (HR, 0.80; 95% CI, 0.65–0.99; $P_{trend} = 0.01$; Supplementary Table S1). In a subset of tumors ($n = 2{,}521$) with both cytoplasmic and nuclear PTEN expression data, there was no clear association between cellular localization of PTEN expression and breast cancer mortality (cytoplasmic HR, 1.02; 95% CI, 0.85–1.22; $P = 0.86$; nuclear HR, 0.91; 95% CI, 0.76–1.09; $P = 0.30$; Supplementary Table S2).

The 2,930 breast cancer cases with *PIK3CA* mutation assessment were followed for a median of 15.0 years. Over this period, there were 538 breast cancer-specific deaths. In both crude and multivariable-adjusted models, we did not observe an association between *PIK3CA* mutation and breast cancer-specific mortality (HR, 0.89; 95% CI, 0.67–1.17; $P = 0.40$; **Table 3**). This association was similar when limiting

Table 3. Multivariable analysis of the association between *PIK3CA* mutation status[a] and breast cancer-specific mortality in the NHS and NHSII ($N = 2{,}930$).

	All		ER-positive	
***PIK3CA* mutation status**	**No mutation $n = 2{,}613$**	**With mutation $n = 317$**	**No mutation $n = 2{,}296$**	**With mutation $n = 298$**
No. of events ($n = 538$)	479	59	414	55
Model 1: HR (95% CI)[b]	1 (referent)	1.06 (0.81–1.39)	1 (referent)	1.03 (0.76–1.41)
Model 2: HR (95% CI)[c]	1 (referent)	0.87 (0.67–1.15)	1 (referent)	0.90 (0.66–1.23)
Model 3: HR (95% CI)[d]	1 (referent)	0.94 (0.71–1.24)	1 (referent)	1.11 (0.81–1.53)
Model 4: HR (95% CI)[e]	1 (referent)	0.89 (0.67–1.17)	1 (referent)	1.05 (0.76–1.44)

[a]Six hotspot mutations were considered: exon 9 (E542K, E545A, E545K), exon 20 (H1047L, H1047R), and exon 4 (N345K).
[b]Crude model.
[c]Further adjusted for age of diagnosis (categorical) and year of diagnosis (categorical).
[d]Further adjusted for tumor estrogen receptor status (positive, negative, unknown), stage (I, II, III), grade (1, 2, 3, unknown), self-reported radiation therapy (yes, no, or unknown), chemotherapy (yes, no, or unknown), and hormonal treatment (yes, no, or unknown).
[e]Further adjusted for at-diagnosis menopausal status (premenopausal, postmenopausal, and unknown), at-diagnosis BMI (<25, 25–29.9, ≥30 kg/m^2), at-diagnosis physical activity (women: <9, ≥9 MET-hours/week), at-diagnosis cigarette smoking (never, former, current, or unknown), at-diagnosis aspirin use (never, former, current, or unknown), and at-diagnosis menopausal hormone therapy use (current, past, never).

Table 4. Multivariable analysis of breast cancer-specific survival by combined PTEN loss and/or *PIK3CA* mutation status in the NHS and NHSII ($N = 2,225$).

Four categories	No PTEN loss + *no PIK3CA* mutation ($n = 1,685$)	No PTEN loss + *PIK3CA* mutation ($n = 205$)	PTEN loss + *no PIK3CA* mutation ($n = 297$)	PTEN loss + *PIK3CA* mutation ($n = 38$)
No. of events ($n = 432$)	321	38	61	12
Model 1: HR (95% CI)[a]	1 (referent)	0.99 (0.71–1.38)	1.05 (0.80–1.39)	1.71 (0.96–3.04)
Model 2: HR (95% CI)[b]	1 (referent)	0.98 (0.70–1.37)	1.00 (0.66–1.50)	1.87 (0.97–3.58)
Model 3: HR (95% CI)[c]	1 (referent)	1.10 (0.78–1.55)	0.96 (0.63–1.46)	2.02 (1.03–3.96)
Model 4: HR (95% CI)[d]	1 (referent)	0.99 (0.70–1.41)	0.82 (0.54–1.25)	1.65 (0.83–3.26)

Binary	No PTEN loss + *no PIK3CA* mutation ($n = 1,685$)	PTEN loss and/or *PIK3CA* mutation ($n = 540$)
No. of events ($n = 432$)	321	111
Model 1: HR (95% CI)[a]	1 (referent)	1.07 (0.87–1.33)
Model 2: HR (95% CI)[b]	1 (referent)	0.93 (0.75–1.16)
Model 3: HR (95% CI)[c]	1 (referent)	1.07 (0.85–1.33)
Model 4: HR (95% CI)[d]	1 (referent)	1.07 (0.86–1.34)

[a]Crude model.
[b]Further adjusted for age of diagnosis (categorical) and year of diagnosis (categorical).
[c]Further adjusted for tumor estrogen receptor status (positive, negative, unknown), stage (I, II, III), grade (1, 2, 3, unknown), self-reported radiation therapy (yes, no, or unknown), chemotherapy (yes, no, or unknown), and hormonal treatment (yes, no, or unknown).
[d]Further adjusted for at-diagnosis menopausal status (premenopausal, postmenopausal, and unknown), at-diagnosis BMI (<25, 25–29.9, ≥30 kg/m^2), at-diagnosis physical activity (women: <9, ≥9 MET-hours/week), at-diagnosis cigarette smoking (never, former, current, or unknown), at-diagnosis aspirin use (never, former, current, or unknown), and at-diagnosis menopausal hormone therapy use (current, past, never).

ER+ tumors only (HR, 1.05; 95% CI, 0.76–1.44; $P = 0.79$); analysis among ER− tumors was underpowered.

Combining PTEN and *PIK3CA* status, 432 breast cancer deaths occurred among 2,225 women with the two markers data available. Compared with tumors without PTEN loss and without *PIK3CA* mutation, those with PTEN loss and/or *PIK3CA* mutation were not at higher risk (HR, 1.07; 95% CI, 0.86–1.34; $P = 0.58$). However, women with both PTEN loss and *PIK3CA* mutation ($n = 38$, breast cancer-specific deaths = 12) had a higher risk of breast cancer mortality (HR, 2.02; 95% CI, 1.03–3.96; $P = 0.04$), but the association became less pronounced after further adjusting for lifestyle factors (HR, 1.65; 95% CI, 0.83–3.26; $P = 0.15$; **Table 4**).

Sensitivity analyses evaluating whether association differed by time since diagnosis, using the median survival time of 7 years, yielded similar associations with breast cancer-specific mortality to those found in primary analyses. We also explored the PTEN-mortality association by AR expression status and similar results were found for AR+ (HR, 0.88; 95% CI, 0.69–1.12; $P = 0.30$) and AR− tumors (HR, 0.70; 5% CI, 0.48–1.01; $P = 0.06$; Supplementary Table S3). Results changed only minimally after including women with stage 4 breast tumors (Supplementary Table S4). In addition, adjusting for HER2 status also did not alter the results substantially (with HER2: HR, 0.87; 95% CI, 0.72–1.06; $P = 0.17$; without HER2: HR, 0.85; 95% CI, 0.71–1.03; $P = 0.13$).

Discussion

In this analysis within two large epidemiologic cohorts, we observed that loss of PTEN expression was not associated with worse breast cancer-specific mortality after fully adjusting for tumor, treatment, and lifestyle characteristics. However, the PTEN loss was associated with a significantly decreased risk of mortality for women with ER− tumors but not those with ER+ tumors. *PIK3CA* mutation was not strongly associated with breast cancer-specific mortality. However, women with tumors that have jointly loss of PTEN expression and *PIK3CA* mutation status were at elevated risk of breast cancer-specific mortality, although the proportion of coexistence status was low (2%).

Approximately 17% of the breast cancers in our study demonstrated cytoplasmic PTEN loss. The frequency of PTEN loss reported in previous studies and systematic review varies from 4% to 82% (7–9). These discrepant frequencies could be explained by the considerably different scoring methods and definitions of PTEN loss used across studies. Specifically, there were four measurements used to assess PTEN expression: percent of cells staining positive, staining intensity, *H* score, and other immunoreactive scores. In each method, different cut-points were used to define PTEN loss. For example, most studies have used 0% and 10% of cells staining positive as the cut-points to define PTEN loss (8, 27–31), whereas others used 5%, 15%, 25%, and 50% (1, 4, 32–34). Moreover, different PTEN antibodies may also result in different rates of PTEN loss. The frequency of loss among studies that used similar methods as ours (percent of cells staining positive and 10% cut-point) ranged from 19% to 70% (3, 27–29, 35, 36). The higher frequencies in those studies compared with ours is likely because most of them combined cytoplasmic and nuclear distribution together. Although the frequency of cytoplasmic loss was 17%, our combined frequency for cytoplasmic and nuclear PTEN loss in subsamples is 46%: consistent with the literature (3, 27–29, 35, 36). Our findings are also generally consistent with prior knowledge that the frequency of PTEN loss was more frequent in ER− breast cancer (9).

Tumor suppressor gene *PTEN* is located on the 10q23 chromosome, and plays an essential role to control cell cycle, growth, and survival (37, 38). The prognostic value of PTEN loss in human cancers has been heavily investigated. A comprehensive meta-analysis that included 32 small studies (total $n = 4,393$) published before 2013 found that the PTEN loss was significantly associated with unfavorable overall survival and disease-free survival in breast cancer patients, but they

also observed considerable publication bias (9). In contrast, our finding among 4,111 nurses with breast cancer suggested that PTEN loss was not independently associated with worse survival, after full adjustment for confounding factors. This finding was consistent with several large randomized clinical trials, although they focused on specific tumor types. For example, in patients with early HER2+ disease who received adjuvant chemotherapy and trastuzumab, PTEN loss had no clear prognostic significance in the BCIRG-006 trial (39) and the NCCTG N9831 trial (30).

Although we did not observe a significant association between PTEN loss and breast cancer mortality overall, we found a strong inverse association of PTEN loss with mortality among women with ER− tumors. To our best knowledge, this study is the first to examine whether the association of PTEN loss with breast cancer prognosis differs by ER status. Previous studies on PTEN loss and breast cancer prognosis were limited by small sample size or focused on ER+ or HER2+ tumors only (35, 40, 41). One triple-negative breast cancer study conducted among Middle Eastern ethnic women ($N = 149$) observed poorer survival for those with PTEN loss (41). Recently, evidence from animal studies suggests that AR may upregulate PTEN transcription in breast cancer because there is an AR-binding motif located in the *PTEN* promoter (25, 26). Although we did not observe an interaction between PTEN and AR expression in our analysis, interestingly, studies reported that ERβ also plays a role in controlling tumor growth by regulating PTEN expression in AR + TNBC (25, 42). Additional research is needed to clarify how the interplay between PTEN, ER, and AR expression affects progression.

PIK3CA mutation is one of the most frequently described mutations in breast cancer (10–12). We focused on six most frequent hotspot mutations identified from previous studies and the cancer somatic mutation database (5, 12). The *PIK3CA* overall mutation frequency in our breast tumors was 10% and the three most frequent of the six sites were H1047R, E542K, and E545A (Supplementary Table S5). Our frequencies (overall and specific hotspot) were lower than in previous reports. The most recent and largest study using the data from the cBio Cancer Genomics Portal reported a frequency of 36% for *PIK3CA* somatic mutations in breast cancer (12). Potential reasons for the lower frequency may be due differences in the study population in that NHS has more ER+ tumor and stage I tumors than public databases, and we targeted six hotspots whereas others performed whole exome/genome sequencing which captured more mutation sites. However, the pattern of mutation frequency in our subgroups was consistent with the literature, which shows these mutation frequencies are much higher in ER+ than ER− breast cancers.

Mutations in any of these hotspots have been shown to be functional (43–45), and are associated with hyper-activation of the PI3K signaling pathway, resulting in increased cell growth and survival (2, 46). However, conflicting data suggest that *PIK3CA* mutations may be associated with either a favorable or a poor outcome, compared with the wild type (47–49). In a prior systematic review, that included several retrospective studies of 2,587 patients, gain-of-function mutations in *PIK3CA* were associated with superior clinical outcomes in patients with breast cancer, in particular for women with ER+ tumors (50). However, we did not observe a significant prognostic value of *PIK3CA* in our study population after fully adjusting for potential confounders, which was consistent with the findings from fully adjusted models of two more recent pooled studies (51, 52). The potential reasons are likely due to variations in study population, tumor characteristics, range of sequencing, treatment regimen (ER+/HER2− tumors may eligible to receive PIK3 inhibitor), and sample sizes across different studies.

Both PTEN loss and *PIK3CA* mutations lead to dysregulation of the PI3K/Akt pathway in breast cancer. However, loss of PTEN expression is rarely correlated with *PIK3CA* mutation. Only 2% of our study participants with PTEN loss also had a *PIK3CA* mutation, which was consistent with a prior study (1). As previously discussed, loss of PTEN is more frequent in ER− tumors. However, mutations in *PIK3CA* are more frequently observed in ER+ tumors. These results indicate crosstalk between the PI3K/Akt pathway and the hormonal pathways, and PTEN loss and *PIK3CA* mutations may have opposite prognostic impacts on breast cancer. However, women with simultaneous PTEN loss and *PIK3CA* mutation had a significantly increased risk of dying from breast cancer than those who had no alterations of the two markers. We cannot rule out, however, that this finding was due to chance, given the small number of events ($n = 12$) among the subgroup of women with joint PTEN loss and *PIK3CA* mutation ($n = 38$).

This study represents the largest to date examining PTEN loss and *PIK3CA* mutation status and breast cancer survival. Our findings do not support worse prognosis for tumors with PTEN loss, and in fact showed potential differential associations by ER status, with better prognosis for ER− tumors with PTEN loss. Moreover, using automated imaging methods allowed us to examine PTEN expression status in a large study population, which correlates well with manual reading methods. The comprehensive information of lifestyle, clinical, and tumor molecular characteristics allowed us to rigorously adjust for confounding factors and investigate the potential heterogeneity of PTEN/PIK3CA-mortality associations by tumor subtypes.

We acknowledge several limitations in this study. First, currently there is no standardized methodology for testing, scoring, and defining PTEN loss, therefore our findings based on the automated imaging quantification measures and arbitrary cut-points (e.g., 10% and quartile cutoffs) should be interpreted with caution. This also highlights the need for a global method for the evaluation of PTEN loss to facilitate future research and clinical practice. Second, our assessment of markers in archived FFPE tumor tissue likely includes some measurement error. However, such errors would likely be randomly distributed and drive our results towards the null. Third, we were only able to measure a subset of the full NHS study population with invasive breast cancer for PTEN loss and *PIK3CA* mutation and we had approximately 40% missing PIK3CA data due to poor tissue DNA quality or quantity. However, the subsets with PTEN and/or PIK3CA measurements are in general comparable to our full cohort in terms of sociodemographic and breast tumor characteristics (Supplementary Table S6). Moreover, the results after applying the inverse probability weighting method were very similar to the complete case analysis. Therefore, our findings were not likely substantially influenced by selection bias from tissue selection and missing data of PTEN loss and/or *PIK3CA* mutation. Finally, our study is still limited by small sample size of ER− tumors. Further experimental and epidemiologic studies are needed to replicate our findings and elucidate the mechanisms underlying PTEN/PIK3CA-mortality associations by ER status.

In sum, in two large prospective U.S. cohort studies, the loss of PTEN expression was not associated with worse breast cancer survival. However, reduced breast cancer-specific mortality for PTEN loss was observed among ER− tumors. Although limited by small numbers, we observed that joint PTEN loss of expression and *PIK3CA* mutation was associated with worse breast cancer survival. Future studies with larger numbers of ER− breast cancer should examine more closely the biology of the PI3K/Akt pathway to develop a deep understanding of the underlying mechanisms.

Authors' Disclosures

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Authors' Contributions

T. Wang: Formal analysis, investigation, writing–original draft, writing–review and editing. **Y.J. Heng:** Data curation, investigation, methodology, writing–review and editing. **G.M. Baker:** Data curation, methodology, writing–review and editing. **V.C. Bret-Mounet:** Data curation, investigation, writing–review and editing. **L.M. Quintana:** Data curation, investigation. **L. Frueh:** Investigation, writing–review and editing. **S.E. Hankinson:** Conceptualization, resources, funding acquisition, investigation, writing–review and editing. **M.D. Holmes:** Data curation, investigation, writing–review and editing. **W.Y. Chen:** Data curation, investigation, writing–review and editing. **W.C. Willett:** Conceptualization, funding acquisition, investigation, methodology, writing–review and editing. **B. Rosner:** Investigation, methodology, writing–review and editing. **R.M. Tamimi:** Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, project administration, writing–review and editing. **A.H. Eliassen:** Conceptualization, resources, data curation, supervision, funding acquisition, investigation, methodology, project administration, writing–review and editing.

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Note

Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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