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DNA methylation modifies the association between obesity and survival after breast cancer diagnosis

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Abstract

Mechanisms underlying the poor breast cancer prognosis among obese women are unresolved. DNA methylation levels are linked to obesity and to breast cancer survival. We hypothesized that obesity may work in conjunction with the epigenome to alter prognosis. Using a population-based sample of women diagnosed with first primary breast cancer, we examined modification of the obesity-mortality association by DNA methylation. In-person interviews were conducted

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Compliance with ethical standards

Ethical standards Institutional Review Board approval was obtained by all participating institutions.

Conflict of Interest The authors declare that they have no conflict of interest.

approximately 3 months after diagnosis. Weight and height were assessed [to estimate body mass index (BMI)], and blood samples collected. Promoter methylation of 13 breast cancer-related genes was assessed in archived tumor by methylation-specific PCR and Methyl Light. Global methylation in white blood cell DNA was assessed by analysis of long interspersed elements-1 (LINE-1) and with the lumino-metric methylation assay (LUMA). Vital status among 1308 patients (with any methylation biomarker and complete BMI assessment) was determined after approximately 15 years of follow-up (N = 194/441 deaths due to breast cancer-specific/all-cause mortality). We used Cox proportional hazards regression to estimate hazard ratios (HRs) and 95 % confidence intervals (CIs) using two-sided p values of 0.05. Breast cancer-specific mortality was higher among obese (BMI ≥ 30) patients with promoter methylation in *APC* (HR = 2.47; 95 % CI = 1.43–4.27) and *TWIST1* (HR = 4.25; 95 % CI = 1.43–12.70) in breast cancer tissue. Estimates were similar, but less pronounced, for all-cause mortality. Increased all-cause (HR =1.81; 95 % CI = 1.19–2.74) and breast cancer-specific (HR = 2.61; 95 % CI = 1.45–4.69) mortality was observed among obese patients with the lowest LUMA levels. The poor breast cancer prognosis associated with obesity may depend on methylation profiles, which warrants further investigation.

Keywords

Body mass index; Epigenetics; Methylation; Breast cancer; Survival

Introduction

Breast cancer (BC) remains the second leading cause of cancer-related death in the United States (US), with an estimated 40,000 deaths occurring in 2015 [1]. Overweight and obesity are associated with poor BC prognosis [2], but the mechanisms underlying this association are unresolved. In the US, one-third of the population is obese [3], and approximately 3.1 million are BC survivors [4]. Thus, understanding how obesity influences BC prognosis could have public health and clinical impact.

Epigenetics is an attractive source of novel biomarkers which exploits the stability of DNA, the reversible nature of epigenetic aberrancies, and can be measured in a range of tissues, including blood [5]. Changes to the epigenome could serve as a useful target for predicting BC prognosis. DNA methylation has been the most studied epigenetic mechanism in human populations and includes both hypermethylation and hypomethylation [6]. Gene-specific methylation in target tissues has been widely investigated, and hypermethylation of tumor suppressor genes has been associated with BC prognosis in several studies, including our own [7, 8]. Global DNA hypomethylation has been evaluated to a lesser extent but is a common phenomenon in carcinogenesis [9] and has similarly been linked to poor BC prognosis [10].

Given BC prognosis is likely influenced by multiple factors, it is plausible that obesity works in conjunction with the epigenome to alter prognosis. Specifically, adiposity may promote tumor progression through the production of excess estrogen [11], which may induce promoter hypermethylation of several important tumor suppressor genes [12]. Despite the strong biologic plausibility, to our knowledge, no epidemiologic study has

examined the interaction between obesity and DNA methylation on BC prognosis. This study examined, in a population-based sample of women with first primary BC, whether the association between obesity and BC mortality was modified by gene-promoter methylation of a panel of 13 BC-related genes measured in tumor tissue (*APC, BRCA1, CCND2, CDH1, DAPK1, ESR1, GSTP1, HIN1, CDKN2A, PGR, RARβ, RASSF1A, and TWIST1*). We also determined whether the obesity-mortality association was modified by global DNA methylation using two methods to assess white blood cell methylation: long interspersed elements-1 (LINE-1) which approximates levels in repetitive elements [13] and the luminometric methylation assay (LUMA) which estimates methylation at CCGG sites [14]. We hypothesized that obesity and aberrant methylation would work synergistically to increase both all-cause and BC-specific mortality following a diagnosis of BC.

Methods

This project draws on the resources of the follow-up component of Long Island. Breast Cancer Study Project (LIBCSP) is a population-based study. Details of the study participants and design for this component have been previously described [15–17]. Written informed consent was obtained for all subjects, and Institutional Review Board approval was obtained from all participating institutions.

Study participants

Eligible participants for the LIBCSP follow-up study were English-speaking women residing in Nassau and Suffolk counties of Long Island, NY, who were newly diagnosed with a first primary in situ or invasive BC between August 1, 1996 and July 31, 1997. Women were identified using rapid case ascertainment via daily or weekly contact with pathology departments of all 28 hospitals on Long Island and three tertiary care hospitals in New York City. The final LIBCSP follow-up sample consisted of 1508 women with BC, of which 1273 (84 %) had invasive BC as confirmed by review of the medical records. At diagnosis, participants were aged 20–98 years and predominately postmenopausal (67 %) and white (94 %), which was consistent with the underlying racial/ethnic distribution in these counties at the time of data collection.

Data collection

Obesity and other covariates—Self-reported weight and height in the year prior to diagnosis were assessed as part of the baseline interviewer-administered structured 100-min questionnaire, which was completed, on average, within 3 months of diagnosis. These assessments were used to calculate the body mass index (BMI) for each participant [weight (kg)/height (m^2)], as a measure of obesity. Participants were additionally queried on their demographic characteristics (including age, race/ ethnicity, income, and education), medical histories (including family history of BC, exogenous hormone use, and mammography screening), and other potential prognostic factors as previously detailed [15–17]. Medical records were also abstracted for clinically relevant prognostic factors (including treatment and hormone receptor status).

Medical records data—As part of the LIBCSP protocol, medical records were abstracted at baseline and again at the 5-year follow-up to determine tumor characteristics (e.g., ER/PR status, tumor size, and nodal involvement) and treatment regimen of the first primary BC diagnosis.

Gene-specific promoter methylation—Archived FFPE tumor tissue of the first primary BC was obtained, and DNA extraction was performed, as previously described [18]. Thirteen genes known to be involved in breast carcinogenesis, and frequently methylated in promoter regions, were selected for assessing interactions with obesity. Promoter methylation of *ERa, PR*, and *BRCA1* was determined by methylation-specific (MSP)-PCR and was dichotomized (i.e., methylated vs. unmethylated) based on the presence or absence of the PCR band [18, 19]. Methylation status of the 10 remaining genes was assessed by the Methyl Light assay [20, 21]. The percentage of methylation was calculated by the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T = (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{sample}} - (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{full methylated DNA}}$ [22] and multiplying by 100. Using a 4 % cut-off, we dichotomized into methylated or unmethylated cases as previously reported [23].

Global methylation—For 73.1 % of women with BC, trained phlebotomists obtained a non-fasting 40 mL blood sample at the baseline interview, and DNA was isolated as previously described [24]. Details of LUMA and LINE-1 assessment in the LIBCSP have been described previously [14]. Briefly, LUMA followed the modified protocol described by Bjornsson et al. [25] and was expressed as a percentage based on the following equation: methylation methylation(%) = [1 − (HpaII $\Sigma G/\Sigma T$)/(MspI $\Sigma G/\Sigma T$)] * 100 [25]. Four CpG sites in the promoter region of LINE-1 were assessed using a pre-validated pyrosequencing-based methylation assay [20] and were individually analyzed as a T/C single-nucleotide polymorphism using QCpG software (Qiagen). These data were subsequently averaged to provide an overall percentage 5mC status.

Mortality—Vital status through the end of 2011 was determined through the NDI as previously reported [26]. Briefly, after approximately 14.7 (0.2–15.4) years of follow-up, among the 1308 patients with any global or gene-specific methylation assessments and complete BMI data, we identified 441 who died from all causes and 194 whose deaths were related to BC. BC-related deaths were determined using the International Classification of Diseases (codes 174.9 or C-50.9).

Statistical analysis

Among 1308 women with any methylation biomarker and complete BMI assessment, Cox proportional hazards regression [27] was used to estimate hazard ratios (HR) and 95 % confidence intervals (95 % CI) for the association between BMI, methylation status (global and gene-specific), and mortality (all-cause and BC-specific) over the follow-up period of more than 15 years. All statistical test were two-sides (a priori $p = 0.05$). The proportional hazards assumption was assessed using exposure interactions with time [27]. We observed non-proportionality for *CDKN2A*, *PR*, and *RAR*β; as such, exposure-time interactions were included in each of the models for those genes [27]. We observed no violations with remaining genes, global markers, or BMI.

For interaction analyses, we assessed BMI continuously and using the standard World Health Organization classifications (<25.0 kg/m^2; 25.0–29.9 kg/m^2; and ≥30 kg/m^2). Methylation of gene promoters were classified as methylated or unmethylated as described above and global methylation markers (LUMA and LINE-1) were dichotomized at the median. Effect measure modification on the multiplicative scale between BMI and methylation was evaluated using the likelihood ratio test with a 0.05 significance level, comparing proportional hazards regression models with and without the cross-product terms [28].

All models were initially adjusted for age at diagnosis (continuous). We further considered inclusion of other covariates in multivariate models if they were related to either the exposure, modifier, or outcome. These variables included family history of BC (yes/no), history of benign breast disease (yes/no), smoking (ever/never), and race (white, black, and other). Covariates were removed from the multivariate model using backward elimination. Variables remained in the final model if their exclusion changed the effect estimate by > 10 % [31]. None of these covariates met our criteria and thus all models were adjusted for age at diagnosis only.

Given our baseline BMI measures reflects body size in the year prior to diagnosis, we did not consider tumor characteristics (e.g., tumor stage, grade, size, and nodal involvement)or hormone receptor status as potential confounders of the association between BMI, methylation, and mortality. These covariates are on the causal pathway between BMI and survival and adjustment for them would result in biased parameter estimates [29, 30]. Even upon adding hormone receptor status (any ER/PR positive vs. ER and PR negative) to the multivariate model, we observed no substantial difference in the effect estimates. Further, our findings restricted to women with invasive tumors did not vary substantially from those among all women, likely due to the low proportion of in situ cases (~15 %) in our study population. Our analyses therefore include both invasive and non-invasive cases. All statistical analyses were performed using SAS statistical software version 9.4 (SAS Institute, Cary, NC).

Results

Distribution of clinical characteristics

Table 1 shows the distribution of clinical characteristics among the 1308 women diagnosed with first primary BC with any information on DNA methylation status (gene-specific or global methylation) and BMI. At diagnosis, most patients had a BMI of ≥25, no family history of BC, tumor size <2 cm, and no nodal involvement. The distributions of clinical characteristics by gene-specific methylation marker have been previously described [7, 8].

BMI, gene-promoter methylation, and global methylation: associations with all-cause and BC-specific mortality

In Table 2, we provide effect estimates for obesity and methylation markers, separately, in association with mortality after approximately 15 years of follow-up among our LIBCSP cohort of 1308 women newly diagnosed with first primary BC in 1996–1997. These

LIBCSP-based associations were previously reported for obesity with follow-up through 2002 [32], and for the gene-specific methylation markers with follow-up through 2005 [7, 8], but have now been updated with extended follow-up through 2011. We also newly describe associations between global methylation markers (LUMA and LINE-1) and mortality through 2011. Our updated estimates suggest increased mortality in association with BMI and most methylation markers and are similar to the previously reported estimates in this same cohort based on shorter follow-up time [7, 8, 32] (Table 2).

Associations between BMI, gene-promoter methylation, and mortality

As shown in Table 3, the association between obesity and mortality following a BC diagnosis was modified by promoter methylation status of two genes, *APC* and *TWIST1* ($p < 0.05$ for multiplicative interaction). Among obese patients (defined as a BMI ≥ 30) with an unmethylated *APC* promoter, all-cause mortality was not increased (HR = 0.99; 95 % CI = 0.64–1.53). In contrast, the corresponding effect estimate for methylated APC was increased two-fold (HR = 1.97; 95 % CI = 1.33–2.09). Similar, patterns of association were observed for breast cancer-specific mortality, but the effect sizes were more pronounced (unmethylated *APC* HR = 0.81; 95 % CI = 0.38–1.76 vs. methylated APC HR = 2.47; 95 % CI = 1.43–4.27).

For *TWIST1*, we observed a more than three-fold increased risk of dying at the end of follow-up among obese patients with a methylated *TWIST1* promoter (HR = 3.21; 95 % CI = 1.51–6.83), whereas the corresponding effect estimate for an unmethylated TWIST1 promoter was less pronounced (HR = 1.19; 95 % CI = 0.87–1.63). A similar, but stronger, association between obesity, *TWIST1* methylation and BC-specific mortality was observed (HR = 4.25; 95 % CI = 1.43–12.70), although it was less precise.

CYCLIND2, GSTP1, and *HIN1* promoter methylation also appeared to modify the associations between obesity and BC-specific mortality, but the interaction was of borderline significance ($p < 0.10$).

Associations between BMI, global methylation, and mortality

We observed multiplicative interaction between BMI, LUMA, and all-cause mortality and BC-specific mortality following a BC diagnosis ($p < 0.05$). For example, we observed an 80 % increase in all-cause mortality among obese patients with low LUMA levels (HR = 1.81; 95 % CI = 1.19–2.74) (Table 4). Among obese patients with high LUMA, however, the estimate was less pronounced and imprecise (HR = 1.23; 95 % CI = 0.87–1.73). Similarly, BC-specific mortality was increased more than twofold in obese patients with low LUMA (HR = 2.61; 95 % CI = 1.45–4.69), whereas the corresponding estimates among those with high LUMA were less pronounced (HR = 1.50; 95 % CI = 0.87–2.60).

We found no interaction between BMI, LINE-1, and mortality among women with BC.

Discussion

We are the first to report in a population-based cohort of women with first primary BC, all-cause mortality after 15 years of follow-up was increased two-fold among obese participants

with methylated *APC* or *TWIST1* promoters. Effect estimates were more pronounced for BC-specific mortality. We similarly observed two- and three-fold increases in all-cause and BC-specific mortality, respectively, among obese participants with the lowest levels of global methylation assessed using LUMA. Our findings suggest that the association between BMI and BC mortality may depend upon methylation profiles and warrant further investigation.

Several studies, including our own [7, 8, 32], support positive associations between obesity and mortality [33], as well as gene-specific methylation and prognosis [23]. However, to our knowledge, no previous study has considered interaction between obesity, gene methylation, and mortality following BC diagnosis despite strong biologic plausibility. There are several mechanisms thought to influence the adverse role of excess adiposity on BC prognosis. Increased circulating hormones and reduced sex hormone binding globulin are strong possibilities [34, 35]. Excess estrogen is known to promote tumorigenesis [36, 37] and may induce aberrant DNA methylation, altering several genes implicated in breast carcinogenesis [38, 39]. For example, estrogen-induced promoter hypermethylation of *CDH1* and *p16/CDKN2A* has been previously reported [12]. Taken together, these results suggest that the mechanism underlying the obesity-mortality association may be facilitated and/or altered by estrogen-mediated methylation changes.

In our findings reported here, elevated BMI was more strongly associated with mortality among BC patients with methylated *APC* and *TWIST1*. The *APC* tumor suppressor gene gives rise to familial adenomatous polyposis and its role in sporadic colorectal tumors is well documented [40]. Data show that *APC* may similarly be involved in breast carcinogenesis [41] although the frequency of inactivation is unresolved. Our observation of increased mortality among obese women with BC when methylation is present could reflect synergy between adipose-induced estrogen exposure and inactivation of the *APC* tumor suppressor; this is likely facilitated by improper TATA-binding in the promoter and reduced expression [42]. Although adiposity is positively associated with mortality overall in women with BC, we observed a reversal of the association when *APC* methylation was not present. This may suggest that activation of *APC* alleviates the deleterious effect of adipose-induced estrogen on overall and BC-specific mortality. *TWIST1* is an anti-apoptotic and pro-metastatic transcription factor, overexpressed in BC. Methylation of its gene promoter has frequently been observed in malignant breast tissue [42]. While we found substantial increases in mortality following BC diagnosis among obese patients with *TWIST1* methylation, the underlying biology is uncertain. *TWIST1* is thought to function as an oncogene given its role in suppressing apoptosis and promoting metastasis. However, it has been suggested that methylation of the *TWIST1* promoter provides breast epithelial cells with a selective advantage during breast carcinogenesis [43] and may explain the synergy observed with obesity in this study. Further, there appears to be little correlation between *TWIST1* methylation and gene expression [44, 45].

To our knowledge, no previous study has evaluated associations between LUMA and BC prognosis. While LINE-1 hypomethylation has been associated with poor prognosis in epithelial cancers [46, 47], we identified only one investigation of BC where LINE-1 hypomethylation was associated with decreased survival in younger (<55 years) women [5].

In our population-based sample of women with BC, we did not find associations between global methylation and mortality when considering main effects for LUMA or LINE-1, although we did observe interaction between LUMA and BMI in relation to mortality. While typically global DNA hypomethylation increases genomic instability leading to the activation of oncogenes and silencing of tumor suppressors [48], LUMA measures levels of 5-^{m}C in the C^{m}CGG motif which may result in approximation of methylation levels at gene promoters [14]. Thus, low LUMA may associate with better prognosis [49]. Our findings of worse prognosis among obese patients with low LUMA levels may be due to differences in our comparison groups. In the presence of low LUMA, obesity may be particularly deleterious, whereas in presence of high LUMA (and higher genomic instability), the additional risk of death from obesity is minimal. LINE-1 retrotransposon activity may be triggered by stress, including oxidative stress and exposure to DNA damaging agents leading to cancer initiation and progression [50, 51]. Given adiposity is linked to inflammation and oxidative damage, the lack of interaction between BMI and LINE-1 was surprising. However, among older patients LINE-1 hypomethylation is likely a bystander of age-dependent tumor development [5] and may not be predictive of prognosis in the LIBCSP study population, which consists of mostly older women.

Our prospective, population-based study has numerous strengths. We are the first to examine the potential relationship between obesity, methylation (gene-specific and global) and BC survival, and in a comparatively large population-based sample of women diagnosed with a first primary BC with methylation markers and 15 years of follow-up. Our reliance on recalled weight and height is a potential limitation of this study. However, anthropometric data were obtained systematically by trained interviewers [15], and previous studies have found that self-reported anthropometric measures are reasonably accurate when compared with clinical measurements taken at the same time [52]. With regard to estimating gene-specific methylation, we were unable to obtain archived tumor tissue for all LIBCSP cases potentially resulting in selection bias; nonetheless, our population-based sample of BC cases is among the largest with information on methylation status. Our panel of 13 biologically relevant genes limited the number of mechanistic pathways we could evaluate. Employing global methylation markers helps to overcome many of the limitations encountered using gene-specific markers, but it is unknown whether methylation levels in surrogate tissue correlate with levels in target tissue [53]. The LIBSCP study population is primarily comprised white women, which is the largest racial group of BC survivors in the US [54]. While our findings do not apply to African-American women, who are at greatest risk of death from BC, the underlying biologic pathways driving the association between obesity and mortality are unlikely to vary by race and may be relevant for all demographic groups. The racial homogeneity of our study population limits our ability to explore potential variation by intrinsic subtype (luminal A, luminal B, HER2, and triple negative), with known variation in prognostic outcomes. Yet, the largest subtype of BC diagnosed among US women of any race is ER+PR+ [55], which continues to increase with time [56] and is the predominant subtype of BC diagnosed among our study participants. Although we considered hormone receptor status as a potential confounder in the study reported here, we did not find that this tumor characteristic influenced our effects estimates. We did not consider more finely categorized breast cancer subtypes, which may have influenced our

findings. However, hormone receptor-positive tumors (ER+ or PR+) strongly correlate with the Luminal subtypes, which are associated with better prognosis. Similarly, the hormone receptor-negative tumors strongly correlate with both the HER2+ and triple-negative subtypes, which have been linked to poorer outcomes. Finally, although invasive cases have worst prognosis overall compared to in situ cases, both groups were included in our analysis. We calculated the frequency of methylation in the two groups independently (data not shown) and found similar prevalence (average difference across all genes was 5 %). These data support the hypothesis that in DNA methylation occurs prior to disease onset and are unlikely to be influenced by tumor aggressiveness. We have included in Supplemental Table 1 associations for *APC*, *TWIST1*, and LUMA among invasive cases only.

In summary, we are the first to show that promoter methylation of *APC* and *TWIST1*, as well as levels of global methylation assessed using LUMA, may modify the well-established association between obesity and mortality following a BC diagnosis. Pending additional replication, our findings could help to identify women with BC who would most greatly benefit from increased surveillance. Our results may also provide clues to mechanistic pathways by which obesity influences BC prognosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Distribution of clinical characteristics among the 1308 participants with any information on methylation (gene-specific and/ or global) and body mass index in a population-based cohort of women diagnosed with first primary breast cancer, Long Island Breast Cancer Study Project

Covariate	*N* (%)
Age at diagnosis	
<50 years	373 (28.5)
≥50 years	935 (71.5)
Menopausal status	
Premenopausal	401 (31.3)
Postmenopausal	880 (68.7)
Family history of breast cancer	
No	1025 (80.8)
Yes	243 (19.2)
Body mass index (BMI)	
BMI < 25 kg/m^2	584 (44.7)
BMI 25–29.9 kg/m^2	423 (32.3)
BMI ≥ 30 kg/m^2	301 (23.0)
Cancer type	
In situ	203 (15.5)
Invasive	1105 (84.5)
Estrogen receptor status	
Positive	653 (74.5)
Negative	223 (25.5)
Progesterone receptor status	
Positive	564 (64.4)
Negative	312 (35.6)
Tumor size	
<2 cm	473 (65.9)
≥2 cm	245 (34.1)
Nodal involvement	
0	548 (75.9)
1	174 (24.1)
Treatment type	
No chemotherapy	538 (60.1)
Chemotherapy	357 (39.9)
No radiation	356 (39.6)
Radiation	542 (60.4)
No hormone therapy	335 (38.0)
Hormone therapy	547 (62.0)

Table 2

Age-adjusted hazard ratios (HRs) and 95 % confidence intervals (CIs) for the association between gene methylation status, global methylation status, and body mass index (BMI) and 15-year all-cause and breast cancer-specific mortality among a population-based sample of 1308 women with a first primary breast cancer, Long Island Breast Cancer Study Project

	All-cause mortality			Breast cancer-specific mortality		
	No. deaths/cases	**HR**	**95 % CI**	**No. deaths/cases**	**HR**	**95 % CI**
Gene methylation[a,b]						
APC						
Unmethylated	138/413	1.00	Reference	52/413	1.00	Reference
Methylated	148/387	1.17	(0.93, 1.48)	72/387	1.53	(1.07, 2.20)
BRCA1						
Unmethylated	113/347	1.00	Reference	37/347	1.00	Reference
Methylated	190/504	1.30	(1.03, 1.64)	92/504	1.78	(1.22, 2.62)
CDH1						
Unmethylated	255/721	1.00	Reference	107/721	1.00	Reference
Methylated	19/44	1.35	(0.85, 2.15)	7/44	1.22	(0.57, 2.63)
CYCLIND2						
Unmethylated	207/615	1.00	Reference	89/615	1.00	Reference
Methylated	67/150	1.19	(0.90, 1.57)	25/150	1.27	(0.81, 1.99)
DAPK						
Unmethylated	231/657	1.00	Reference	94/657	1.00	Reference
Methylated	43/108	0.99	(0.71, 1.38)	20/108	1.25	(0.77, 2.04)
ESR1						
Unmethylated	163/460	1.00	Reference	67/460	1.00	Reference
Methylated	139/383	1.06	(0.84, 1.33)	62/383	1.13	(0.80, 1.60)
GSTP1						
Unmethylated	177/552	1.00	Reference	71/552	1.00	Reference
Methylated	97/213	1.56	(1.22, 2.00)	43/213	1.85	(1.27, 2.71)
HIN1						
Unmethylated	97/284	1.00	Reference	38/284	1.00	Reference
Methylated	177/481	1.09	(0.85, 1.40)	76/481	1.18	(0.80, 1.74)

	All-cause mortality			Breast cancer-specific mortality		
	No. deaths/cases	**HR**	**95 % CI**	**No. deaths/cases**	**HR**	**95 % CI**
CDKN2A						
Unmethylated	267/747	1.00	Reference	111/747	1.00	Reference
Methylated	12/30	5.30[d]	(2.03, 13.81)	10/30	2.28	(1.19, 4.35)
PR						
Unmethylated	260/749	1.00	Reference	103/749	1.00	Reference
Methylated	43/102	1.36	(0.98, 1.88)	26/102	0.70[d]	(0.30, 1.63)
RARB						
Unmethylated	193/554	1.00	Reference	73/554	1.00	Reference
Methylated	81/211	1.89[d]	(1.14, 3.14)	41/211	1.50	(1.02, 2.20)
RASSF1A						
Unmethylated	34/113	1.00	Reference	13/113	1.00	Reference
Methylated	240/652	1.21	(0.84, 1.74)	101/652	1.42	(0.80, 2.53)
TWIST1						
Unmethylated	223/649	1.00	Reference	91/649	1.00	Reference
Methylated	51/116	1.25	(0.91, 1.70)	23/116	1.58	(1.00, 2.50)
Global methylation						
LUMA						
<Median (0.556)	124/366	1.00	Reference	58/366	1.00	Reference
≥Median	216/689	0.94	(0.75, 1.18)	90/689	0.81	(0.58, 1.13)
LINE1						
≥Median	160/517	1.00	Reference	66/517	1.00	Reference
<Median (78.735)	183/547	1.06	(0.85, 1.31)	83/574	1.18	(0.86, 1.63)
BMI[c]						
All women						
BMI < 25 kg/m^2	170/584	1.00	Reference	77/584	1.00	Reference
BMI 25–29.9 kg/m^2	142/423	0.98	(0.79, 1.23)	59/423	1.05	(0.75 1.49)
BMI ≥ 30 kg/m^2	129/301	1.36	(1.08, 1.71)	58/301	1.63	(1.15, 2.30)

[a] Xu et al. [18] previously reported age-adjusted associations for *APC*, *BRCA1*, and *CDKN2A*, with follow-up through 2005 [7]

[b] Cho et al. 2010 previously reported age-adjusted associations for *CYCLIND2*, *DAPK*, *GSTP1*, *HIN*, *RAR*β, *RASSF1A, and TWIST1, with* follow-up through 2005 [8]

[c] Cleveland et al. 2007 previously reported age- and hypertension-adjusted associations for pre- and postmenopausal pre-diagnostic BMI, with follow-up though 2002 [32]

[d] Proportional hazard assumption violated. Exposure*time interactions ($p < 0.05$) included in model

Table 3

Age-adjusted hazard ratios (HRs) and 95 % confidence intervals (CIs) for the association between BMI and 15-year all-cause and breast cancer-specific mortality stratified by gene methylation status (methylated vs. unmethylated tumors) among 1308 women diagnosed with a first primary breast cancer, Long Island Breast Cancer Study Project

	All-cause mortality						**Breast cancer-specific mortality**					
	Unmethylated			**Methylated**			**Unmethylated**			**Methylated**		
Gene promoter												
Body Mass Index (BMI) categories	No. deaths/cases	HR	95 % CI	No. deaths/cases	HR	95 % CI	No. deaths/cases	HR	95 % CI		HR	95 % CI
APC												
BMI < 25 kg/m^2	55/195	1.00	Reference	52/158	1.00	Reference	25/195	1.00	Reference	23/158	1.00	Reference
BMI 25–29.9 kg/m^2	49/119	1.26	(0.86, 1.87)	44/131	0.99	(0.66, 1.47)	17/119	1.28	(0.69, 2.40)	20/131	1.03	(0.57, 1.88)
BMI ≥ 30 kg/m^2	32/94	0.99	(0.64, 1.53)	51/96	1.97	(1.33, 2.09)	9/94	0.81	(0.38, 1.76)	29/96	2.47	(1.43, 4.27)
p interaction	0.001						0.003					
BRCA1												
BMI < 25 kg/m^2	45/153	1.00	Reference	71/221	1.00	Reference	18/153	1.00	Reference	33/221	1.00	Reference
BMI 25–29.9 kg/m^2	34/104	0.96	(0.61, 1.49)	63/163	1.11	(0.79, 1.57)	9/104	0.78	(0.35, 1.75)	29/163	1.24	(0.75, 2.05)
BMI ≥ 30 kg/m^2	33/88	1.20	(0.77, 1.88)	54/114	1.45	(1.02, 2.06)	10/88	1.09	(0.50, 2.39)	29/114	1.89	(1.14, 3.12)
p interaction	0.489						0.151					
CDH1												
BMI < 25 kg/m^2	98/319	1.00	Reference	7/19	1.00	Reference	44/319	1.00	Reference	<5/19	Not estimated[a]	
BMI 25–29.9 kg/m^2	78/218	1.03	(0.77, 1.39)	7/16	1.38	(0.47, 4.01)	26/218	0.92	(0.56, 1.49)	<5/16	not estimated	
BMI > 30 kg/m^2	76/177	1.34	(1.00, 1.81)	5/9	2.15	(0.67, 6.95)	36/177	1.64	(1.05, 2.56)	<5/9	Not estimated	
p interaction	0.400						-					
CYCLIND2												
BMI < 25 kg/m^2	83/276	1.00	Reference	22/62	1.00	Reference	40/276	1.00	Reference	6/62	1.00	Reference
BMI 25–29.9 kg/m^2	63/184	1.04	(0.75, 1.45)	22/50	1.07	(0.59, 1.94)	20/184	0.80	(0.47, 1.36)	9/50	2.01	(0.70, 5.72)
BMI > 30 kg/m^2	59/149	1.30	(0.93, 1.81)	22/37	1.64	(0.91, 2.96)	28/149	1.42	(0.87, 2.31)	10/37	3.41	(1.21, 9.59)
p interaction	0.480						0.084					
DAPK												
BMI < 25 kg/m^2	92/297	1.00	Reference	13/41	1.00	Reference	39/297	1.00	Reference	7/41	1.00	Reference

	All-cause mortality						Breast cancer-specific mortality					
	Unmethylated			Methylated			Unmethylated			Methylated		
BMI 25–29.9 kg/m^2	70/198	0.99	(0.73, 1.35)	15/36	1.45	(0.69, 3.05)	22/198	0.88	(0.52, 1.48)	7/36	1.35	(0.47, 3.86)
BMI ≥ 30 kg/m^2	67/156	1.33	(0.97, 1.82)	14/30	1.63	(0.77, 3.47)	32/156	1.69	(1.05, 2.70)	6/30	1.50	(0.50, 4.50)
p interaction	0.353						0.347					
ESR1												
BMI < 25 kg/m^2	57/195	1.00	Reference	58/175	1.00	Reference	30/195	1.00	Reference	21/175	1.00	Reference
BMI 25–29.9 kg/m^2	55/153	1.04	(0.72, 1.51)	42/110	1.13	(0.76, 1.69)	17/153	0.78	(0.43, 1.43)	21/110	1.61	(0.88, 2.95)
BMI ≥ 30 kg/m^2	49/108	1.53	(1.04, 2.24)	38/94	1.15	(0.77, 1.74)	20/108	1.48	(0.84, 2.62)	19/94	1.67	(0.89, 3.12)
p interaction	0.202						0.082					
GSTP1												
BMI < 25 kg/m^2	65/247	1.00	Reference	40/91	1.00	Reference	25/247	1.00	Reference	21/91	1.00	Reference
BMI 25–29.9 kg/m^2	56/169	1.14	(0.80, 1.63)	29/65	0.89	(0.55, 1.44)	19/169	1.16	(0.64, 2.12)	20/65	0.70	(0.3, 1.48)
BMI ≥ 30 kg/m^2	54/130	1.54	(1.08, 2.21)	27/56	1.07	(0.65, 1.74)	26/130	2.12	(1.22, 3.68)	12/56	1.05	(0.52, 2.14)
p interaction	0.192						0.080					
HIN1												
BMI < 25 kg/m^2	47/140	1.00	Reference	58/198	1.00	Reference	17/140	1.00	Reference	29/198	1.00	Reference
BMI 25–29.9 kg/m^2	28/78	1.00	(0.63, 1.60)	57/156	1.10	(0.76, 1.59)	12/78	1.25	(0.59, 2.62)	17/156	0.82	(0.45, 1.49)
BMI ≥ 30 kg/m^2	21/63	0.95	(0.57, 1.59)	60/123	1.65	(1.15, 2.36)	9/63	1.12	(0.50, 2.53)	29/123	1.95	(1.16, 3.28)
p interaction	0.077						0.071					
CDKN2A												
BMI < 25 kg/m^2	102/334	1.00	Reference	<5/8	Not estimated		45/334	1.00	Reference	<5/8	Not estimated	
BMI 25–29.9 kg/m^2	86/226	1.11	(0.83, 1.48)	6/16	Not estimated		31/226	1.09	(0.69, 1.73)	6/16	Not estimated	
BMI ≥ 30 kg/m^2	76/180	1.37	(1.02, 1.84)	<5/6	Not estimated		34/180	1.56	(1.00, 2.44)	<5/6	nOt estimated	
p interaction	-						-					
PR												
BMI < 25 kg/m^2	94/321	1.00	Reference	22/53	1.00	Reference	37/321	1.00	Reference	14/53	1.00	Reference
BMI 25–29.9 kg/m^2	85/238	1.07	(0.79, 1.43)	12/29	1.33	(0.65, 2.71)	31/238	1.23	(0.76, 2.00)	7/29	1.05	(0.42, 2.61)
BMI ≥ 30 kg/m^2	78/183	1.40	(1.04, 1.89)	9/19	1.15	(0.53, 2.50)	34/183	1.91	(1.19, 3.07)	5/19	1.09	(0.39, 3.05)
p interaction	0.400						0.286					
RARB												

	All-cause mortality						Breast cancer-specific mortality					
	Unmethylated			Methylated			Unmethylated			Methylated		
BMI < 25 kg/m^2	75/242	1.00	Reference	30/96	1.00	Reference	30/242	1.00	Reference	16/96	1.00	Reference
BMI 25–29.9 kg/m^2	57/166	1.01	(0.72, 1.43)	28/68	1.18	(0.70, 1.97)	15/166	0.77	(0.41, 1.44)	14/68	1.35	(0.65, 2.79)
BMI ≥ 30 kg/m^2	58/139	1.28	(0.91, 1.81)	23/47	1.66	(0.96, 2.85)	27/139	1.64	(0.97, 2.77)	11/47	1.81	(0.84, 3.90)
p interaction	0.434						0.284					
RASSF1A												
BMI < 25 kg/m^2	18/51	1.00	Reference	87/287	1.00	Reference	6/51	1.00	Reference	40/287	1.00	Reference
BMI 25–29.9 kg/m^2	7/29	0.61	(0.25, 1.49)	78/205	1.15	(0.84, 1.55)	2/29	0.57	(0.11, 2.91)	27/205	1.02	(0.63, 1.67)
BMI ≥ 30 kg/m^2	9/32	0.76	(0.34, 1.70)	72/154	1.53	(1.12, 2.09)	5/32	1.28	(0.38, 4.36)	33/154	1.77	(1.12, 2.82)
p interaction	0.050						0.355					
TWIST1												
BMI < 25 kg/m^2	93/291	1.00	Reference	12/47	1.00	Reference	40/291	1.00	Reference	6/47	1.00	Reference
BMI 25–29.9 kg/m^2	63/191	0.91	(0.66, 1.25)	22/43	2.12	(1.04, 4.31)	19/191	0.70	(0.41, 1.22)	10/43	2.49	(0.90, 6.86)
BMI ≥ 30 kg/m^2	65/162	1.19	(0.87, 1.63)	16/24	3.21	(1.51, 6.83)	31/162	1.39	(0.87, 2.24)	7/24	4.25	(1.43, 12.70)
p interaction	0.010						0.015					

[a]Point estimate was not calculated because cell sizes less than five

Table 4

Age-adjusted hazard ratios (HRs) and 95 % confidence intervals (CIs) for the association between body mass index (BMI) and 15-year all-cause and breast cancer-specific mortality among a population-based sample of 1308 women with a first primary breast cancer, stratified by global methylation status (measured by LUMA and LINE-1), Long Island Breast Cancer Study Project

Global marker	**All-cause mortality**						**Breast cancer-specific mortality**					
BMI categories	**No. deaths/cases**	**HR**	**95 % CI**	**No. deaths/cases**	**HR**	**95 % CI**	**No. deaths/cases**	**HR**	**95 % CI**	**No. deaths/cases**	**HR**	**95 % CI**
LUMA methylation[a]	<Median (0.556)			>Median			<Median (0.556)			>Median		
BMI < 25 kg/m^2	44/164	1.00	Reference	80/307	1.00	Reference	21/164	1.00	Reference	33/307	1.00	Reference
BMI 25–29.9 kg/m^2	33/111	0.91	(0.58, 1.44)	78/226	1.11	(0.81, 1.52)	12/111	0.82	(0.40, 1.67)	35/226	1.50	(0.92, 2.43)
BMI ≥ 30 kg/m^2	46/88	1.81	(1.19, 2.74)	56/150	1.23	(0.87, 1.73)	25/88	2.61	(1.45, 4.69)	22/150	1.50	(0.87, 2.60)
p interaction	0.035						0.007					
LINE-1 methylation[b]	>Median(78.735)			<Median			>Median (78.735)			<Median		
BMI < 25 kg/m^2	49/183	1.00	Reference	62/202	1.00	Reference	20/183	1.00	Reference	32/202	1.00	Reference
BMI 25–29.9 kg/m^2	49/133	1.12	(0.76, 1.65)	54/151	1.01	(0.71, 1.43)	21/133	1.52	(0.82, 2.81)	23/151	1.05	(0.62, 1.77)
BMI ≥ 30 kg/m^2	50/98	1.55	(1.06, 2.28)	50/106	1.40	(0.97, 2.01)	23/98	2.46	(1.36, 4.46)	25/106	1.75	(1.04, 2.97)
p interaction	0.621						0.313					

[a] LUMA methylation median value 0.556, high levels of LUMA hypothesized to be deleterious

[b] LINE-1 methylation median value 78.735, low levels of LINE-1 hypothesized to be deleterious