

# Constitutional *BRCA1* Methylation and Risk of Incident Triple-Negative Breast Cancer and High-grade Serous Ovarian Cancer

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**IMPORTANCE** About 25% of all triple-negative breast cancers (TNBCs) and 10% to 20% of high-grade serous ovarian cancers (HGSOCs) harbor *BRCA1* promoter methylation. While constitutional *BRCA1* promoter methylation has been observed in normal tissues of some individuals, the potential role of normal tissue methylation as a risk factor for incident TNBC or HGSOC is unknown.

**OBJECTIVE** To assess the potential association between white blood cell *BRCA1* promoter methylation and subsequent risk of incident TNBC and HGSOC.

**DESIGN, SETTING, AND PARTICIPANTS** This case-control study included women who were participating in the Women's Health Initiative study who had not received a diagnosis of either breast or ovarian cancer before study entrance. A total of 637 women developing incident TNBC and 511 women developing incident HGSOC were matched with cancer-free controls (1841 and 2982, respectively) in a nested case-control design. Cancers were confirmed after central medical record review. Blood samples, which were collected at entry, were analyzed for *BRCA1* promoter methylation by massive parallel sequencing. The study was performed in the Mohn Cancer Research Laboratory (Bergen, Norway) between 2019 and 2022.

**MAIN OUTCOMES AND MEASURES** Associations between *BRCA1* methylation and incident TNBC and incident HGSOC were analyzed by Cox proportional hazards regression.

**RESULTS** Of 2478 cases and controls in the TNBC group and 3493 cases and controls in the HGSOC group, respectively, 7 (0.3%) and 3 (0.1%) were American Indian or Alaska Native, 46 (1.9%) and 30 (0.9%) were Asian, 1 (0.04%) and 1 (0.03%) was Native Hawaiian or Pacific Islander, 326 (13.2%) and 125 (3.6%) were Black or African, 56 (2.3%) and 116 (3.3%) were Hispanic, 2046 (82.6%) and 3257 (93.2%) were White, and 35 (1.4%) and 35 (1.0%) were multiracial. Median (range) age at entry was 62 (50-79) years, with a median interval to diagnosis of 9 (TNBC) and 10 (HGSOC) years. Methylated *BRCA1* alleles were present in 194 controls (5.5%). Methylation was associated with risk of incident TNBC (12.4% methylated; HR, 2.35; 95% CI, 1.70-3.23;  $P < .001$ ) and incident HGSOC (9.4% methylated; HR, 1.93; 95% CI, 1.36-2.73;  $P < .001$ ). Restricting analyses to individuals with more than 5 years between sampling and cancer diagnosis yielded similar results (TNBC: HR, 2.52; 95% CI, 1.75-3.63;  $P < .001$ ; HGSOC: HR, 1.82; 95% CI, 1.22-2.72;  $P = .003$ ). Across individuals, methylation was not haplotype-specific, arguing against an underlying cis-acting factor. Within individuals, *BRCA1* methylation was observed on the same allele, indicating clonal expansion from a single methylation event. There was no association found between *BRCA1* methylation and germline pathogenic variant status.

**CONCLUSIONS AND RELEVANCE** The results of this case-control suggest that constitutional normal tissue *BRCA1* promoter methylation is significantly associated with risk of incident TNBC and HGSOC, with potential implications for prediction of these cancers. These findings warrant further research to determine if constitutional methylation of tumor suppressor genes are pancancer risk factors.

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Women with breast cancer type 1 susceptibility gene (*BRCA1*) germline pathogenic variants are at high risk of triple-negative breast cancer (TNBC) and high-grade serous ovarian cancer (HGSOC).<sup>1</sup> *BRCA1*, like *BRCA2* and several other genes, is required for homologous recombination repair of double-stranded DNA breaks.<sup>2</sup> While most TNBCs and HGSOCs have gene expression profiles indicating homologous recombination deficiency (HRD),<sup>3-5</sup> in many cases, no genetic alteration explains the HRD-positive status.

An alternative mechanism to HRD-positive status is *BRCA1* promoter methylation, which causes downregulation of transcription from the *BRCA1* gene.<sup>6,7</sup> *BRCA1* methylation has been detected in tumors of 25% to 30% of TNBCs and 10% to 20% of HGSOCs and has been associated with HRD mutational and gene expression signatures, such as those in cancers in *BRCA1* germline pathogenic variant carriers.<sup>3-5,8</sup> While *BRCA1* methylation is commonly considered to have somatic origin,<sup>9</sup> the HRD mutational signatures indicate that *BRCA1* methylation may occur early in tumor evolution, potentially even as a first triggering event in certain TNBC and HGSOC cases.

Constitutional epimutations are aberrant normal tissue methylation events occurring in utero.<sup>10</sup> While several studies have assessed white blood cell (WBC) *BRCA1* methylation as a potential risk factor for TNBC or HGSOC,<sup>11-17</sup> apart from 1 study in TNBC<sup>18</sup> and 1 in HGSOC,<sup>19</sup> these studies have provided mixed results because of a limited number of cases and lack of statistical power. However, the main limitation, which was shared by all prior studies, was blood sample collection after cancer diagnosis with potential for disease-related confounding. Consequently, to our knowledge, associations between constitutional *BRCA1* methylation and incident TNBC and HGSOC have not been definitively assessed.

In this article, we assessed the potential association of antecedent WBC *BRCA1* promoter methylation with incident TNBC and HGSOC among Women's Health Initiative (WHI) study participants in a nested case-control study. These tumor forms were selected based on their association with *BRCA1* germline pathogenic variants and previous findings of elevated *BRCA1* methylation among individuals with a diagnosis of HGSOC but not among other ovarian cancers.<sup>19</sup>

## Methods

### Study Design

The WHI study design details were reported previously.<sup>20</sup> Briefly, 161 808 postmenopausal women aged 50 to 79 years with an anticipated survival of longer than 3 years were recruited from 40 US clinical centers between 1993 and 1998 to participate in either an observational study or 1 or more of 4 clinical trials (Supplement 1). At study entry, self-administered questionnaires were used to collect demographic characteristics and medical, reproductive, and family history. Race and ethnicity were determined by participant self-report against fixed categories. Entry blood samples were obtained after at least 12 hours of fasting via a prespecified protocol standardized across all study sites. Samples were shipped on dry ice and stored at -80 °C at Fisher Bioservices (Rockville, Maryland).

### Key Points

**Question** Is mosaic *BRCA1* promoter methylation in normal tissue associated with the risk of incident high-grade serous ovarian cancer (HGSOC) and triple-negative breast cancer (TNBC)?

**Findings** In this nested case-control study including 637 women developing TNBC and 511 developing HGSOC, white blood cell *BRCA1* promoter methylation was associated with a significantly elevated risk of developing both cancer forms (hazard ratio for HGSOC of 1.93 and TNBC of 2.35). The results remained significant in a subgroup analysis of women who received a diagnosis of cancer more than 5 years after blood sampling (hazard ratio of 1.82 and 2.52, respectively).

**Meaning** The study results may serve as proof of concept for early life constitutional methylation as a cancer risk factor.

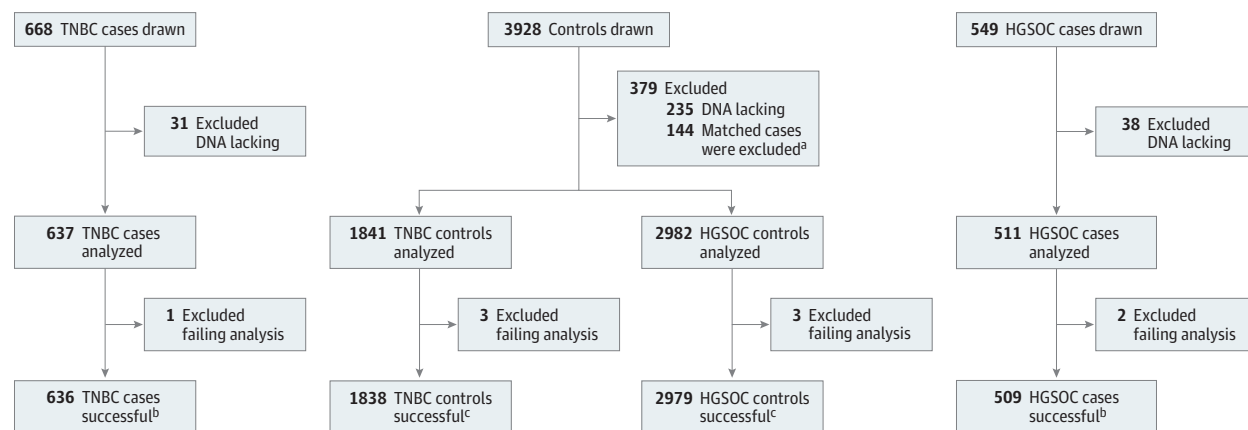
Clinical outcomes were ascertained annually from enrollment in the observational study and every 6 months for clinical trial participants during the 8.5-year intervention period and annually thereafter. Self-reported cancers were initially confirmed with medical record review at the clinical centers by trained physician adjudicators, with final confirmation at the clinical coordinating center.

Participants provided written informed consent and study protocols were approved at all clinical centers. The current study was additionally approved by the regional ethical committee of the Western Norwegian Health Region.

For the current study, all WHI participants with incident TNBC or HGSOC were included (Supplement 1). Women reporting a history of breast or ovarian cancer at baseline were excluded. For the TNBC study, women with a previous unilateral or bilateral mastectomy for any reason were excluded, while women reporting previous oophorectomy were excluded from the HGSOC study.

The study was conducted as 2 nested case-control studies. Based on up-front statistical power calculations (eMethods in Supplement 2), women with incident TNBC and controls were matched at a 1:3 ratio, whereas women with incident HGSOC and controls were matched at a 1:6 ratio. Controls were matched for age at entry, hormone therapy use, race and ethnicity, and DNA extraction method. In addition, TNBC cases and controls were matched based on age at prior bilateral oophorectomy. Further, controls were required to be alive and disease free at the time of the case diagnosis (eMethods in Supplement 2). The statistical analyses included samples with *BRCA1* promoter methylation determinations from incident TNBC (n = 637), incident HGSOC (n = 511), and matched cancer-free controls (n = 1841 and 2982, respectively), as depicted in Figure 1. To limit the required sample number, 1274 of the controls (35.9%) were included in the TNBC and HGSOC groups. Controls were first drawn for the HGSOC comparison and were then eligible to be selected as controls for the TNBC cases. Five cases had TNBC and HGSOC and were included in the risk assessments for both cancers. The study was conducted according to Strengthening of Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Figure 1. Flowchart Depicting Samples Drawn and Successfully Analyzed From Patients and Controls



<sup>a</sup> A total of 144 controls were excluded because their matched cases had too low DNA concentration.

<sup>b</sup> Five cases had triple-negative breast cancer (TNBC) and high-grade serous ovarian cancer (HGSOc) and were included as cases in the hazard ratio

estimates for TNBC and HGSOc.

<sup>c</sup> A total of 1272 controls were included as controls in the hazard ratio estimates for TNBC and HGSOc.

### *BRCA1* Promoter Methylation Analysis

A detailed description of the methylation analysis is given in eFigure 1 and eTables 1 and 2 in Supplement 2. In brief, WBC genomic DNA was bisulfite converted, and 4 overlapping regions covering the *BRCA1* promoter area were amplified, pooled, indexed, and sequenced to a very high depth (>20 000×) using the Illumina MiSeq System. Methylation status was scored by predefined criteria as variant epiallele frequency (VEF), referring to the frequency of hypermethylated epialleles (eFigures 2 and 3 in Supplement 2).<sup>21</sup>

All samples were analyzed masked to case-control status. The main cutoff value for methylation positivity was computationally defined based on the assay sensitivity and VEF probability density across the entire sample set, which was masked to case-control status (eFigure 4 in Supplement 2).

### Determining Allele Specificity of Methylation

The region covered by sequencing contains a highly prevalent single-nucleotide polymorphism (SNP), rs799905, which is located in the *BRCA1* gene body (eFigure 1 in Supplement 2). By analyzing methylation in sequencing reads covering this SNP, we assessed the potential allele specificity of methylation in individuals heterozygous for the SNP.

### Association Between *BRCA1* Methylation Status and Cancer Risk Genes

We tested for the association of *BRCA1* methylation status with germline pathogenic variants in a subgroup of 234 participants (5.0%) from the study (173 cases and 61 controls) who had previously been tested for germline pathogenic variants in *BRCA1/2* and in 26 additional cancer risk genes (eTable 7 in Supplement 2) as part of another WHI ancillary study.<sup>22</sup>

### Statistical Methods

The potential associations between *BRCA1* methylation and incident TNBC and HGSOc were assessed by estimating hazard ratios

(HRs) and 95% CIs. The HRs were determined using Cox proportional hazards regression in matched case-control groups, including age, race and ethnicity, previous hormone use, DNA extraction method, and (for TNBC) previous oophorectomy as covariates. In addition, we performed hypothesis-generating supportive subgroup analyses.

Power estimates based on previous results of methylation frequency among HGSOc cases and controls<sup>19</sup> are outlined in the eMethods in Supplement 2. In brief, a conservative assumption of 600 TNBC cases and 400 HGSOc cases was made. Matching 600 TNBC with 1800 control samples in a nested design provides a power of 0.88 to detect an HR of 2.0. Similarly, for HGSOc, comparing 400 patients with 2400 controls provides a power of 0.80.

In sensitivity analyses, we analyzed HR associated with *BRCA1* methylation status in different promoter subregions, age groups, cutoff levels for methylation positivity, and methylation positivity assessed by methylation beta values (ratio of methylated to total number of cytosines; eMethods in Supplement 2). As oophorectomy has been associated with a reduced risk of TNBC in *BRCA1* germline pathogenic variant carriers,<sup>23</sup> we also performed subgroup analysis assessing HRs for TNBC among cases and controls who were not undergoing an oophorectomy. Analysis were conducted using R, version 4.0.3 (R Foundation).

## Results

The demographic characteristics and flowchart of TNBC and HGSOc cases and controls are presented in the Table and Figure 1. There was no systemic difference between individuals excluded from analysis because of lack of DNA and final participants. *BRCA1* methylation status was not associated with a self-reported family history of either breast or ovarian cancer (eTable 3 in Supplement 2). Among controls, across all age

Table. Demographic Characteristics of Patients and Controls Included in the Trial

Characteristic	No. (%)		HGSOc	
	TNBC Cases (n = 637)	Controls (n = 1841) <sup>a</sup>	Cases (n = 511)	Controls (n = 2982) <sup>a</sup>
<b>Age, y</b>				
Mean (SD)	62.1 (6.81)	62.1 (6.74)	62.3 (6.42)	62.7 (6.82)
Median (range)	62.0 (50.0-78.0)	62.0 (50.0-79.0)	62.0 (50.0-79.0)	62.0 (50.0-79.0)
IQR (Q1-Q3)	11.0 (57.0-68.0)	10.0 (57.0-67.0)	9.50 (57.5-67.0)	11.0 (57.0-68.0)
<b>Ethnicity</b>				
Not Hispanic or Latino	621 (97.5)	1795 (97.5)	493 (96.5)	2880 (96.6)
Hispanic or Latino	13 (2.0)	43 (2.3)	16 (3.1)	100 (3.4)
Unknown or not reported	3 (0.5)	3 (0.2)	2 (0.4)	2 (0.1)
<b>Race</b>				
American Indian or Alaska Native	1 (0.2)	6 (0.3)	2 (0.4)	1 (0.0)
Asian	13 (2.0)	33 (1.8)	5 (1.0)	25 (0.8)
Native Hawaiian or other Pacific Islander	0	1 (0.1)	0	1 (0.0)
Black or African American	81 (12.7)	245 (13.3)	18 (3.5)	107 (3.6)
White	525 (82.4)	1521 (82.6)	479 (93.7)	2778 (93.2)
Multiracial	12 (1.9)	23 (1.2)	3 (0.6)	32 (1.1)
Unknown or not reported	5 (0.8)	12 (0.7)	4 (0.8)	38 (1.3)
<b>Years from DNA sampling to diagnosis</b>				
Mean (SD)	10.1 (5.23)	NA	10.4 (5.80)	NA
Median (range)	9.00 (0-23.0)	NA	10.0 (0-23.0)	NA
IQR (Q1-Q3)	8.00 (6.00-14.0)	NA	9.00 (6.00-15.0)	NA
Missing	0	1841 (100)	0	2982 (100)
<b>Bilateral oophorectomy</b>				
No	509 (79.9)	1493 (81.1)	511 (100)	2982 (100)
Yes	118 (18.5)	320 (17.4)	0	0
Missing	10 (1.6)	28 (1.5)	0	0
<b>Family history of breast cancer</b>				
No	158 (24.8)	519 (28.2)	137 (26.8)	857 (28.7)
Yes	154 (24.2)	294 (16.0)	94 (18.4)	514 (17.2)
Missing	325 (51.0)	1028 (55.8)	280 (54.8)	1611 (54.0)
<b>Family history of ovarian cancer</b>				
No	289 (45.4)	723 (39.3)	209 (40.9)	1252 (42.0)
Yes	18 (2.8)	42 (2.3)	13 (2.5)	61 (2.0)
Do not know	16 (2.5)	64 (3.5)	17 (3.3)	86 (2.9)
Missing	314 (49.3)	1012 (55.0)	272 (53.2)	1583 (53.1)
<b>High-risk germline variants</b>				
<i>BRCA1</i>	2 (0.3)	0	1 (0.2)	0
<i>BRCA2</i>	1 (0.2)	0	1 (0.2)	0
No variant detected	101 (15.9)	25 (1.4)	4 (0.8)	36 (1.2)
Missing	533 (83.7)	1816 (98.6)	505 (98.8)	2946 (98.8)

Abbreviations: HGSOc, high-grade serous ovarian cancer; NA, not applicable; Q, quartile; TNBC, triple-negative breast cancer.

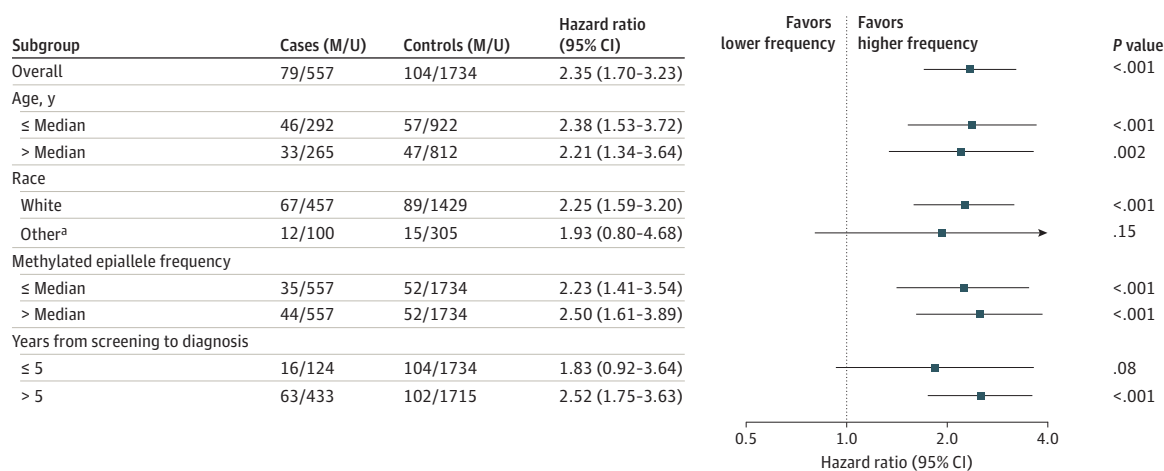
<sup>a</sup> A total of 1274 controls were included as controls for comparison with TNBC and HGSOc. Among these 1274, 2 failed analyses. Thus, 1272 controls were included in the final hazard ratio estimates for TNBC and HGSOc (Figure 1).

groups, 194 (5.5%) had methylated *BRCA1* alleles (eTable 4 in Supplement 2), contrasting with 79 (12.4%) and 48 (9.4%) in TNBC and HGSOc cases, respectively. For cases and controls, most alleles were either fully methylated or unmethylated (eFigures 2 and 3 in Supplement 2).

### Risk of TNBC and HGSOc

For women with incident TNBC, the median (IQR) follow-up from sampling to diagnosis was 9 (8) years. The presence of methylated *BRCA1* alleles in WBCs was significantly associated with incident TNBC risk (HR, 2.35; 95% CI, 1.70-3.23;

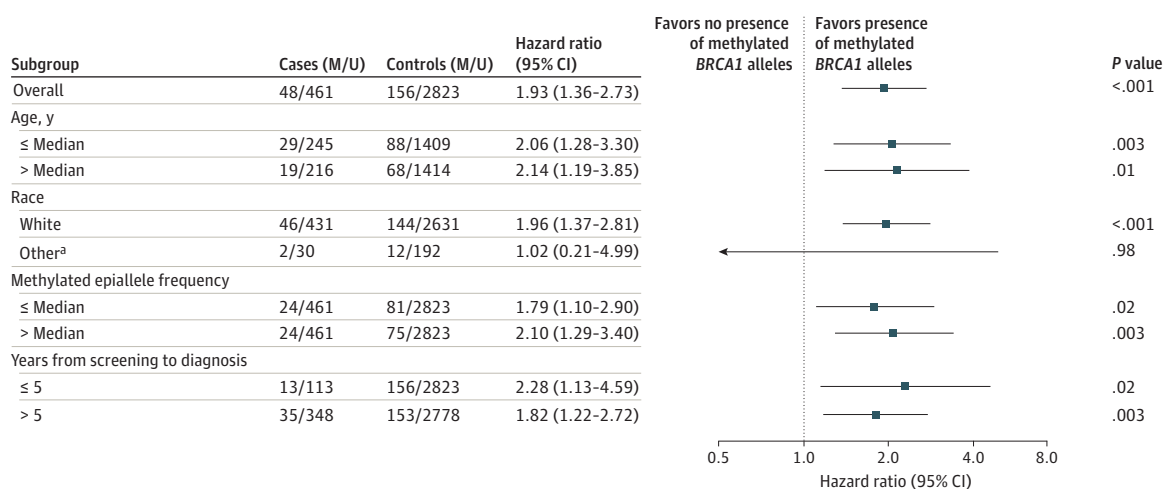
**Figure 2. Hazard Ratios for Triple-Negative Breast Cancer (TNBC) Associated With the Presence of Methylated *BRCA1* Alleles in the Overall Cohort and Selected Subgroups**



M and U represent the number of methylated and unmethylated samples, respectively. Median methylated epiallele frequency equals median variant epiallele frequency for samples classified as methylation-positive.

<sup>a</sup> Other race includes American Indian or Alaska Native, Asian, Native Hawaiian or Pacific Islander, Black or African American, multiracial, unknown, or not reported.

**Figure 3. Hazard Ratios for High-grade Serous Ovarian Cancer (HGSOC) Associated With the Presence of Methylated *BRCA1* Alleles in the Overall Cohort and Selected Subgroups**



M and U represent the number of methylated and unmethylated samples, respectively. Median methylated epiallele frequency here equals median variant epiallele frequency for samples classified as methylation-positive.

<sup>a</sup> Other race includes American Indian or Alaska Native, Asian, Native Hawaiian or Pacific Islander, Black or African American, multiracial, unknown, or not reported.

$P < .001$ ; **Figure 2**). The HR was 1.83 (95% CI, 0.92-3.64;  $P = .08$ ) for TNBC diagnosed 5 years before or less and 2.52 (95% CI, 1.75-3.63;  $P < .001$ ) for those who received a diagnosis more than 5 years after blood sampling. There was no difference between subgroups defined by age at entry or methylation level (Figure 2).

For women with incident HGSOC, the median (IQR) time from sampling to diagnosis was 10 (9) years. Like the findings for TNBC, the presence of methylated *BRCA1* alleles in WBCs was significantly associated with incident HGSOC risk (HR, 1.93;

95% CI, 1.36-2.73;  $P < .001$ ; **Figure 3**). The HR was significant for HGSOC diagnosed 5 years or less (HR, 2.28; 95% CI, 1.13-4.59;  $P = .02$ ) or more than 5 years (HR, 1.82; 95% CI, 1.22-2.72;  $P = .003$ ) after blood sampling. Like for TNBC, the association remained significant in subgroups stratified for age at inclusion and methylation level (Figure 3).

### Sensitivity Analysis

We assessed the potential effect of promoter area selection, analytical cutoff, and different methods for methylation clas-

sification. These analyses revealed results that confirmed the findings from the main analysis (eFigures 5-14 in Supplement 2). Subgroup analysis excluding TNBC cases and controls who underwent oophorectomy or the 5 cases who had TNBC and HGSOV after DNA collection had no significant association with HRs (eFigure 15 in Supplement 2).

### Allele-Specific *BRCA1* Methylation

Assessing methylation in the region harboring *BRCA1* SNP rs799905, we found methylation frequencies to be similar among individuals carrying the different rs799905 alleles. This suggested that *BRCA1* methylation is not associated with a cis-acting factor (factor located on the same allele as the methylation). However, in individuals who were heterozygous for rs799905 for whom allele-specific methylation could be determined, the intraindividual methylation was strongly enriched at a single allele. In more than 90% of individuals, more than 95% of the methylation was associated with one of the rs799905 alleles, indicating that *BRCA1* methylation may have occurred as a single, early event that was followed by clonal expansion of the methylated cell (eFigure 16 in Supplement 2).

### Association of *BRCA1* Methylation With Germline Pathogenic Variant Status

*BRCA1* methylation was not associated with germline mutation status for either *BRCA1* or *BRCA2*. Similarly, methylation was not associated with the germline status of any of the 26 other cancer risk genes analyzed (eTable 7 in Supplement 2).

## Discussion

In a nested case-control design in postmenopausal WHI participants, including women with incident TNBC (n = 637), incident HGSOV (n = 511), and matched cancer-free controls (n = 1841 and 2982, respectively), WBC *BRCA1* promoter methylation was significantly associated with higher risk of incident TNBC and incident HGSOV. The association was also significant in analyses restricted to cancers diagnosed more than 5 years after sampling. While an association between *BRCA1* methylation in normal tissue and TNBC and HGSOV was established previously,<sup>11,18,19</sup> these studies were performed on normal tissue obtained after the patients received their cancer diagnoses. Thus, this study's findings represent a potential conceptual breakthrough, the results suggesting that *BRCA1* normal tissue methylation in association with TNBC and HGSOV occurs before, and not because of, cancer development. The association of *BRCA1* normal tissue methylation with higher risk for TNBC and HGSOV, the 2 major cancers associated with germline *BRCA1* pathogenic variants, supports this conclusion. Given the frequency of *BRCA1* methylation in TNBC and HGSOV, *BRCA1* normal tissue methylation may be an underlying cause of a substantial fraction of TNBC and HGSOV cases.

Constitutional epimutations may be classified as primary epimutations (methylation in absence of any genetic aberration) or secondary epimutations that are associated with rare

germline genetic variants.<sup>10,24</sup> Secondary epimutations have been detected in only a few cases associated with the Lynch syndrome-associated *MLH1* gene, but also *BRCA1*.<sup>24,25</sup> In such cases, the fraction of methylated alleles is typically around 50% (ie, 100% of cells carrying the epigenetic variant in a heterozygous individual), and cancer penetrance is high. In contrast, primary *BRCA1* normal tissue methylation assessed in WBCs is not a rare event; it occurs as a low-mosaic phenomenon in 4% to 10% of adult women and newborn girls without cancer.<sup>19,26</sup> A potential pathogenic role of mosaic methylations may be paralleled with an elevated cancer risk associated with mosaic germline pathogenic variants in *BRCA1* as well as other tumor suppressor genes.<sup>27-31</sup> The finding that *BRCA1* methylation was not associated with a family history of breast/ovarian cancer was expected, considering the magnitude of the HRs reported.

Recent trials have provided preliminary evidence suggesting that *BRCA1* methylation may be associated with a more favorable response to chemotherapy, as well as polyadenosine diphosphate-ribose polymerase inhibition, in breast cancer.<sup>8,32</sup> Neither trial distinguished somatic from constitutional methylation. Thus, it remains to be determined if constitutionally methylated tumors have the same phenotypical characteristics as those methylated somatically.

One may assume that the effect of *BRCA1* methylation and *BRCA1* pathogenic variants is similar in individual cells. In this article, we found the odds ratios for TNBC as well as HGSOV associated with WBC *BRCA1* methylation to be modest as compared with the odds ratio of more than 50 for TNBC in *BRCA1* germline pathogenic variant carriers.<sup>33</sup> This likely reflects the fact that germline pathogenic variants affect all cells in the body, whereas only a small fraction, 0.1% to 10% of the cells, carry methylated alleles. The present study's finding that *BRCA1* methylation occurs independent of rs799905 genotype across individuals is consistent with previous findings,<sup>19</sup> arguing against a cis-acting genetic factor, subject to mendelian inheritance, as the underlying cause of methylation.

An important question is to what extent WBC *BRCA1* methylation represents *BRCA1* methylation in normal tissue across other organs, like the ovaries, fallopian tubes, breasts, and others. Global DNA methylation pattern may vary across different tissue compartments and even between WBC subfractions.<sup>34,35</sup> However, we previously found WBC *BRCA1* promoter methylation to be strongly associated with methylation status in other benign tissues.<sup>19</sup> This finding, in concert with the observation that *BRCA1* mosaic promoter methylation occurs across all age groups, including newborns, supports the hypothesis that this methylation is constitutive,<sup>10</sup> thus affecting different tissue compartments derived from all the embryonic germ layers.

General methylation patterns may change with age.<sup>36</sup> However, in a previous study,<sup>19</sup> we found WBC *BRCA1* methylation among females across all age groups, with a slight drop in frequency during lifetime. In the present study, we found *BRCA1* methylation to be predominantly monoallelic, nearly completely restricted to the same allele across affected cells within the same individual, as previously indicated by Hansman and colleagues<sup>13</sup> in a small group of patients. While this

finding does not fully exclude the possibility of dynamic modulations of *BRCA1* methylation through life, it supports the hypothesis that normal tissue *BRCA1* methylation has a clonal origin, arising in a single cell at an early embryonic stage.

An important question is whether *BRCA1* methylation could be a secondary event to germline pathogenic variants in *BRCA1* or other tumor suppressor genes. Our subgroup analysis revealed no evidence for such a covariance. Regarding *BRCA1* pathogenic variants, in a previous study analyzing more than 250 individuals harboring *BRCA1* germline pathogenic variants who had received a diagnosis of HGSOC, we found constitutional methylation frequency to be lower compared with individuals harboring either germline *BRCA2* variants or being wild type for both genes.<sup>19</sup>

### Limitations

All WHI participants were postmenopausal, with a median age of 62 years, with 813 (17%) being 70 years or older at entry. In general, TNBC is more common in younger women. Thus, while TNBC constitutes about 15% of all US breast cancers, the percentage of TNBC in WHI is 7%.<sup>37</sup> Moreover, the prevalence of tumor *BRCA1* methylation is higher in younger compared with older women with TNBC.<sup>4</sup> Thus, in our previous study assessing *BRCA1* WBC methylation status in women at all ages who already received a diagnosis of HGSOC<sup>19</sup> the odds ratio associated with HGSOC varied between 2.2 to 2.9 and was higher among younger compared with older individuals. This indicates that the risk of TNBC and HGSOC associated with WBC *BRCA1* methylation may be underestimated in the present study. While information on *BRCA1* germline pathogenic variant status was not available for all patients in the current study, in the subgroup from whom *BRCA1* methylation could be compared with *BRCA1/2* germline pathogenic variant status, we detected no association between germline variant status and *BRCA1* methylation. Notably, *BRCA1* promoter methylation and *BRCA1* germline pathogenic variants have been reported to be mutually exclusive in breast cancer tissue<sup>4</sup> as well as in WBC collected from patients with a diagnosis of HGSOC.<sup>19</sup> The present study was not powered to assess different racial and

ethnic groups, precluding conclusions for risk in African American individuals, for whom the incidence of TNBC is known to be elevated.

For cancer risk studies, the findings should be confirmed in independent cohorts. To our knowledge, there are few population-based cohorts that are enrolling sufficient numbers of participants, including follow-ups with regular health assessment, that are adequate to confirm our findings. However, our study contains an indirect independent validation because it suggests that there is an association between *BRCA1* methylation and TNBC and HGSOC, the 2 cancer forms most strongly associated with germline *BRCA1* pathogenic variants.

### Conclusions

In this case-control study, we found normal tissue (WBC) *BRCA1* promoter methylation to be associated with an elevated HR for incident TNBC and HGSOC (also when restricting analysis to cancers developing more than 5 years after WBC sampling). The study findings have 2 major implications. First, WBC *BRCA1* methylation may help identify women at elevated risk of TNBC and HGSOC. This raises the question whether methylation carriers should be offered breast cancer screening at a younger age compared with the general population. While multimodal screening for HGSOC has not been recommended for women without deleterious germline *BRCA1/BRCA2* pathogenic variants,<sup>38</sup> the study findings add evidence to future discussion of potential screening strategies for this serious cancer diagnosis. Second, the current study findings, in concert with previous data revealing WBC *BRCA1* methylation to occur in women across all age groups, even newborns,<sup>19</sup> point to *BRCA1* methylation as an early embryonic event that is followed by clonal expansion. Prenatal events have been associated with risk of breast cancer, as well as other cancers.<sup>39-41</sup> Thus, this study's findings should trigger further research assessing the potential association of constitutional methylation of other genes with the risk of other cancer types and research into the cause(s) of constitutional normal tissue methylation of tumor suppressor genes.

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