Published in final edited form as:
Alcohol. 2011 November ; 45(7): 689–699. doi:10.1016/j.alcohol.2010.11.006.

Alcohol consumption in relation to aberrant DNA methylation in breast tumors

Meng Hua Tao[1,2], **Catalin Marian**[3], **Peter G. Shields**[3], **Jing Nie**[1], **Susan E. McCann**[4], **Amy Millen**[1], **Christine Ambrosone**[4], **Alan Hutson**[5], **Stephen B. Edge**[6], **Shiva S. Krishnan**[3], **Bin Xie**[3], **Janet Winston**[7], **Dominica Vito**[1], **Marcia Russell**[8], **Thomas H. Nochajski**[9], **Maurizio Trevisan**[1,10], and **Jo L. Freudenheim**[1]

[1] Department of Social and Preventive Medicine, School of Public Health and Health Professions, University at Buffalo, Buffalo, NY 14214

[2] Department of Hematology and Medical Oncology, Mount Sinai School of Medicine, New York, NY 10029

[5] Lombardi Cancer Center, Georgetown University, Washington, DC 20007

[3] Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Buffalo, NY 14263

[4] Department of Biostatistics, School of Public Health and Health Professions, University at Buffalo, Buffalo, NY 14214

[6] Department of Surgery, Roswell Park Cancer Institute, Buffalo, NY 14263

[7] Potomac Hospital, Woodbridge, VA 22191

[8] Prevention Research Center, Berkeley, CA 94704

[9] School of Social Work, University at Buffalo, Buffalo, NY 14260

[10] University of Nevada Health Sciences System, Las Vegas, NV 89103

Abstract

The mechanism for the observed association of alcohol consumption breast cancer risk is not known; understanding that mechanism could improve understanding of breast carcinogenesis and optimize prevention strategies. Alcohol may impact breast malignancies or tumor progression by altering DNA methylation. We examined promoter methylation of three genes, the *E- cadherin*, *p16*, and *RAR-β_2* genes in archived breast tumor tissues from participants in a population-based case-control study. Real time methylation-specific PCR was performed on 803 paraffin-embedded samples; and lifetime alcohol consumption was queried. Unordered polytomous and unconditional logistic regression were used to derive adjusted odds ratios (OR) and 95% confidence intervals (CI). *RAR-β_2* methylation was not associated with drinking. Among premenopausal women, alcohol consumption was also not associated with promoter methylation for *E- cadherin* and *p16* genes. In case-case comparisons of postmenopausal breast cancer, compared to lifetime never drinkers, promoter methylation likelihood was increased for higher alcohol intake for *E - cadherin* (OR = 2.39, 95% CI, 1.15–4.96), in particular for those with ER-negative tumors (OR = 4.13, 95%

Correspondence to: Dr. Meng Hua Tao, Department of Hematology and Medical Oncology, Mount Sinai School of Medicine, BOX 1128, New York, NY, 10029, Tel: 212-824-7025, Fax: 212-996-0407, menghua.tao@mssm.edu.

CI, 1.16–14.72), and decreased for *p16* (OR = 0.52, 95% CI, 0.29-0.92). There were indications that the association with *p16* was stronger for drinking at younger ages. Methylation was also associated with drinking intensity independent of total consumption for both genes. We found alcohol consumption was associated with DNA methylation in postmenopausal breast tumors, suggesting that the association of alcohol and breast cancer may be related, at least in part, to altered methylation, and may differ by drinking pattern.

Keywords

alcohol consumption; breast cancer; epidemiology; epigenetics; promoter methylation

Introduction

While the risk of breast cancer is associated with alcohol consumption (Hamajima et al., 2002; Key et al., 2006; Petri et al., 2004; Singletary and Gapstur, 2004; Smith-Warner et al., 1998), the mechanism for this association is not well understood. There are several potential hypotheses by which alcohol may contribute to breast malignancies or tumor progression (Castro et al., 2006; Dorgan et al., 2001; Dumitrescu and Shields, 2005; Homann et al., 2005; Rinaldi et al., 2006); one possible mechanism may be the impact of alcohol on one-carbon metabolism and subsequent DNA methylation (Dumitrescu and Shields, 2005; Mason and Choi, 2005). Alcohol affects the bioavailability of the one carbon units, essential to nucleotide synthesis and biological methylation of DNA, RNA and protein, in a number of ways including negative effects on folate absorption, utilization and excretion, and on activity of metabolic enzymes (Choi and Mason, 2002; Dumitrescu and Shields, 2005; Mason and Choi, 2005). Alcohol also can interfere with estrogen pathways and increase the circulation levels of estrogen (Dumitrescu and Shields, 2005); higher estrogens exposure have been suggested to induce aberrant DNA methylation breast carcinogenesis in both *in vivo* and *in vitro* studies (Fernandez and Russo, 2010).

There is increasing evidence that aberrant DNA methylation is significant in carcinogenesis (Szyf et al., 2004; Wajed et al., 2001). These alterations are common in breast cancer and include both global hypomethylation and hypermethylation of promoter regions of specific genes (Szyf et al., 2004). DNA promoter hypermethylation is associated with chromatin condensation, delay of replication, inhibition of transcription initiation, and gene silencing in breast carcinogenesis (Esteller et al., 2001; Szyf et al., 2004; Widschwendter and Jones, 2002). Such silencing is significant in that hypermethylation frequently affects genes with key roles contributing to carcinogenesis. Among those that are frequently found to be hypermethylated in breast tumors are genes functionally important in cell adhesion (Graff et al., 1995), cell cycle regulation (Esteller et al., 2001), and hormone and receptor-mediated cell signaling (e.g., *E-cadherin*, *p16* and *RAR-β_2* (retinoic acid-binding receptor-β_2), respectively) (Widschwendter and Jones, 2002).

The *E-cadherin* gene, a possible tumor suppressor gene, regulates cell adhesion (Graff et al., 1995). Alcohol concentration as low as 0.12% was shown to decrease expression of E-cadherin α, β, and γ, three major catenin proteins important for cell adhesion and tissue integrity (Meng et al., 2000). Loss of expression of *E-cadherin* caused by promoter methylation occurs frequently in breast cancer (Widschwendter and Jones, 2002), suggesting that alcohol consumption might down-regulate the expression of E-cadherin through promoter methylation.

In addition, promoter hypermethylation of other tumor suppressor genes such as *p16* and *RAR-β_2* genes is a common and critical epigenetic event leading to increased proliferation,

genetic instability in human breast cells, and subsequently breast carcinogenesis (Novak et al., 2009). Promoter methylation of *E-cadherin*, *p16* and *RAR-β_2* has also been shown to occur more frequently in breast tumors than benign or adjacent nonmalignant breast tissue (Krassenstein et al., 2004; Parrella et al., 2004) and is associated with poor differentiation, distant metastasis, and estrogen receptor (ER) status in breast tumors (Li et al., 2006; Mehrotra et al., 2004; Shinozaki et al., 2005; Tao et al., 2008; Yan et al., 2000).

Little is known about the etiology of these alterations in methylation. Alcohol drinking may explain the observed changes, at least in part. To our knowledge, there has been just one study of alcohol examining one-carbon metabolism and breast tumor hypermethylation. In that study of African-American women, there was some evidence of an association between higher alcohol intake and hypermethylation of the *ER- α* gene in breast tumors in case control comparisons (Zhu et al., 2003). There is some, although not consistent evidence that alcohol consumption may affect promoter methylation for other cancer sites (de Vogel et al., 2008; Hasegawa et al., 2002; Kraunz et al., 2006; Marsit et al., 2006; Puri et al., 2005; Slattery et al., 2006; van Engeland et al., 2003). It is thought that altered methylation is an early event in carcinogenesis. It may be that drinking earlier in life more than recent drinking is related to methylation. Further, in addition to total intake of alcohol, drinking pattern may also impact the biological consequences of ethanol. Drinking large amounts infrequently may be different from consumption of smaller amounts more frequently even though the absolute intakes are the same. Previous studies have not examined drinking pattern in relation to methylation.

We report here on results of a population-based case control study. We evaluated promoter methylation of *E- cadherin*, *p16*, and *RAR-β_2* in breast tumors in relation to alcohol consumption both over the lifetime and during different periods of the lifetime, examining both absolute intakes as well as intensity of consumption.

Materials and Methods

Study population

A population-based case control study of breast cancer, the Western New York Exposures and Breast Cancer Study (WEB Study) was conducted from 1996–2001. All eligible participants were age 35–79, current residents of Erie or Niagara Counties in New York State, with no previous cancer history other than non-melanoma skin cancer. Cases were identified by trained nurses from medical records of hospitals in those counties. All but two hospitals were included in the study. One of those hospitals did not treat breast cancer patients. In the second, 95% of patients were also seen by one practice of breast surgeons who allowed for case ascertainment through their practice. Eligible cases were women diagnosed with primary, histologically confirmed, incident breast cancer. Among 1,638 eligible cases, 1,170 (72%) participated. Controls were randomly selected from the New York State Department of Motor Vehicles driver's license list (age ≤ 65 years) and the Health Care Finance Administration rolls (age >65 years), and frequency-matched to cases on age and race. Interviews were completed for 2,115 (63%) eligible women. The protocol was approved by the Institutional Review Boards of the University at Buffalo and all participating institutions.

Extensive in-person interviews and self-administered questionnaires were administered to participants including queries regarding demographic factors, medical history, reproductive history and other breast cancer risk factors. The interview focused primarily on lifetime alcohol consumption using the Cognitive Lifetime Drinking History (CLDH) (Russell et al., 1998). Briefly, participants were asked to complete a calendar of important life events (e.g., schooling, marriage, pregnancy). Using that calendar, drinking intervals were identified by

asking participants when they began drinking at least once a month for six months and when their drinking pattern changed. For each interval, participants indicated how often they drank and how many drinks they usually had on weekends, weekdays and days when they had more drinks than usual during a four-week period. Information was collected regarding the proportion of each beverage type. Together with data on beverage-specific drink size, we calculated total consumption and consumption for periods up to two years before diagnosis for cases and two years before interview for controls. Time periods included consumption over the lifetime, for the previous 2–10 years, previous 10–20 years, before age 20 and for each decade of age relevant to that participant (i.e., 20–29, 30–39, 40–49, 50–59). In addition, we examined intensity of consumption, that is, the number of drinks consumed per drinking day for those same time periods. The latter analyses were adjusted for total absolute intake. Those who had never had at least 12 drinks in any 12-month period were classified as lifetime non-drinkers.

For breast cancer cases, information on tumor size, histological grade, and cancer stage (as measured by tumor-node-metastasis (TNM) stage) was abstracted from medical charts by trained research nurses using a standardized protocol. Estrogen receptor (ER) status was determined in the Lombardi Cancer Center by a single pathologist by immunohistochemical analysis as described previously (Tao et al., 2008).

Tumor block promoter methylation determination

Among the 1170 breast cancer cases, we were able to obtain archived tumor blocks for 920 (78.6%). Tumor samples were microdissected from fixed microscope slides in order to minimize the inclusion of normal surrounding tissue. Bisulfite modification was performed on 2μg of tumor DNA isolated from the dissected tissue in accordance with methods described previously (Jeronimo et al., 2001; Tao et al., 2008). For determining promoter methylation of *E- cadherin*, *p16*, and *RAR-β_2*, we used a fluorescence based version of methylation-specific PCR (MSP) using real time PCR amplification of bisulfate converted DNA in an ABI 7900HT real time PCR system as previously described (Eads et al., 2000; Tao et al., 2008). Briefly, each reaction contained 5 μl of Taqman Universal Master Mix (29), 4.5 μl of bisulfite treated DNA and 0.5 μl of a 60X assay by design premix containing the primers and probes that were designed for each respective gene (Applied Biosystems, Carlsbad CA); primers and probes sequences were published elsewhere (Tao et al., 2008). Thermal cycling started with an initial 10 min denaturation at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min, with a final extension of 5 min at 72 °C. As a control to check for modified viable DNA, we used an assay for the *ACTB* gene with primers and probes specifically designed for CpG free sites within the gene sequence, thus amplifying the modified DNA regardless of the methylation status. If the *ACTB* result was negative (i.e. no amplification signal was detected), the DNA was not used in subsequent assays, and re-modification was attempted; the other 3 genes being assayed only if *ACTB* was positive. Each individual DNA sample was assayed in triplicate for each gene for quality control purposes. Additionally, as a positive control, universally methylated DNA (CpGenome; Norcross, GA) was used along with water blanks as a negative control. Finally, we had successful promoter methylation results for 803 cases.

Statistical analysis

Characteristics of participating cases with and without promoter methylation of specific gene and controls were compared using ANOVA for continuous variables and the χ^2 test for categorical variables. For examination of alcohol consumption in relation to gene promoter methylation, we calculated odds ratios (ORs) and 95% confidence intervals (95% CIs), comparing cases with and without promoter methylation to controls using polytomous logistic regression. Additionally, unconditional logistic regression was used for case-case

comparisons of those with and without promoter methylation to estimate ORs and 95% CIs for associations of alcohol consumption with promoter methylation among women with breast cancer. For categorization by alcohol consumption, drinkers were divided into two groups with a cutoff based on the median consumption in controls who were alcohol consumers; non-drinkers were the referent. We also examined tertiles of alcohol consumption among alcohol consumers based on equal distribution of controls. We found similar results for case-control and case-case comparisons and p for trend >0.05. Because of relative low consumption of alcohol in our study population and limited power for stratified analyses, we reported here only those analyses alcohol consumption divided at the median levels. All analyses were adjusted for age, race, education, age at menarche, age at first birth, family history of breast cancer among first degree relatives, history of benign breast disease, body mass index (BMI), smoking status, total caloric intake, folate intake, and age at menopause among the postmenopausal women. Because we had previously found differences in methylation by ER status (Tao et al., 2008), we further adjusted for ER status in the case-case comparisons. Age, age at menarche, age at first birth, BMI, total caloric intake, folate intake, and age at menopause among the postmenopausal women were adjusted in the models as continuous variables, and other factors were adjusted as categorical variables. All statistical tests were based on two-sided probability. Statistical analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC).

Results

Demographic characteristics of cases with and without promoter methylation of *E - cadherin*, *p16*, and *RAR-β_2* gene and of controls are shown in Table 1. The frequency of promoter methylation was 20% (161) for *E - cadherin*, 25.9% (208) for *p16*, and 27.5% (221) for *RAR-β_2*. Mean lifetime alcohol consumption was similar for cases with methylated genes, those without methylation, and for controls. Cases with promoter methylation of *E - cadherin* gene reported younger age at first birth than unmethylated cases and controls. Compared to controls, total energy intake was greater for breast cancer cases with or without promoter methylation of *E - cadherin* or *RAR-β2* gene; while intake of energy was lower for cases with promoter methylation of *p16* gene than unmethylated cases. For cases without hypermethylation of *E - cadherin*, *p16*, or *RAR-β_2* gene, parity was lower than for controls. Cases either with or without promoter methylation of *E - cadherin*, *p16*, or *RAR-β_2* gene were more likely than controls to have a history of benign breast disease and family history of breast cancer.

Adjusted ORs and 95% CIs by menopausal status for ever drinking alcohol, for total lifetime alcohol consumption, and for alcohol intake 2–10, and 10–20 years prior to diagnosis/ interview are shown in Tables 2–4. Included are comparisons of: 1) cases without promoter methylation to controls; 2) cases with promoter methylation to controls, and; 3) case-case comparisons of those with methylated gene promoter and those without.

Results for postmenopausal women are shown in Table 2. Compared to abstainers, higher lifetime alcohol consumption was associated with increased gene promoter methylation for *E - cadherin* (OR, 2.39, 95% CI, 1.15–4.96 for the case-case comparison). The association was also similar for those with lower alcohol consumption (OR, 2.06, 95% CI, 1.02–4.16). Associations between alcohol intake and promoter methylation were similar though somewhat stronger point estimates for more recent consumption in the period 2–10 years previous: 2.47 (95% CI, 1.18–5.15) than for intake 10–20 years previous 2.08 (0.99–4.36). Compared to abstainers, there was a decrease in the likelihood of *p16* promoter methylation among postmenopausal women who drank (OR, 0.50, 95% CI, 0.29–0.86 for lifetime lower alcohol intake; OR, 0.52, 95% CI, 0.29–0.92 for higher alcohol intake). ORs were similar for 2–10, and 10–20 years before diagnosis. There was no association between *RAR-β_2*

methylation with alcohol intake among postmenopausal women. Alcohol consumption was not associated with the likelihood of *E - cadherin*, *p16*, or *RAR-β2* promoter methylation among premenopausal breast cancer cases (data not shown).Additionally, we evaluated associations for lifetime intake of specific of alcoholic beverages, *i.e.* beer, liquor or wine. Results were similar to those for total alcohol consumption (data not shown).

Associations between *E-cadherin*, *p16* and *RAR-β2* promoter methylation and drinking intensity (drinks per drinking day) among postmenopausal women are shown in Table 3. Compared to abstainers, both lower and higher intensity of lifetime alcohol consumption were positively associated with methylation of the *E - cadherin*, even after adjusting for total alcohol consumption (OR, 2.21, 95% CI, 1.07–4.57; OR, 2.18, 95% CI, 1.00–4.72, respectively). Additionally, drinking intensity was associated with decreased prevalence of *p16* promoter methylation: 0.48 (95% CI 0.28–0.84) for lifetime low alcohol drinking intensity and 0.43 (95% CI 0.23–0.80) for high alcohol drinking intensity, again after control for total intake. No association was observed for lifetime drinking intensity and *RAR-β2* promoter methylation in postmenopausal women. We further examined the effects of drinking intensity 2–10 years previous and 10–20 years previous on likelihood of promoter methylation. Results were weaker but similar to those for lifetime drinking intensity (data not shown). No associations of drinking intensity with likelihood of promoter methylation with premenopausal breast cancer were observed (data not shown). Additionally, we also conducted analyses on the associations of alcohol consumption and drinking intensity with the likelihood of promoter methylation in at least one of the three genes in tumors among pre- and postmenopausal women. Lifetime alcohol consumption, alcohol intake 2–10, 10–20 years before diagnosis, and drinking intensity were not associated with the likelihood of promoter methylation in at least one gene (data not shown).

In addition, we evaluated associations of alcohol consumption before age 20 and by decade of life (20–29, 30–39, 40–49, 50–59, 60–69) with methylation by menopausal status (Table 4). Among premenopausal women, there was evidence of decreased likelihood of *p16* methylation associated with drinking at age 30–40 but not with the other age intervals and there were no associations for the other genes for the analysis by age interval. Among postmenopausal women, the association with the likelihood of *E - cadherin* methylation was similar for the age intervals. There was some evidence that the association of alcohol consumption with likelihood of *p16* methylation was stronger for intakes before age 40. There were no associations in the likelihood estimates Methylation of *RAR-β_2* again was not associated with alcohol consumption in this analysis by age interval drinking.

We further analyzed associations between alcohol consumption and promoter methylation stratified by ER status (Table 5). We found that the observed increased *E - cadherin* methylation associated with lifetime ever drinking compared to never drinking was limited to women with ER negative tumors (OR, 4.13, 95% CI, 1.16–14.72); there was no association with alcohol consumption for ER positive tumors (OR, 1.33, 95% CI, 0.70–2.53). The reverse association between ever drinking alcohol and *p16* promoter methylation was more significant among ER positive cases (OR, 0.47, 95% CI, 0.27–0.81). Case-case comparisons for alcohol consumption and *RAR-β_2* methylation did not differ by ER status. Because alcohol consumption can affect one-carbon metabolism through its negative impact on folate absorption (Mason JB and Choi SW, 2005), we further examined potential interaction between dietary folate intake and alcohol consumption for each of the specific gene. There was no evidence of greater than multiplicative interaction between dietary folate and alcohol consumption on promoter methylation in breast tumors (data not shown).

Discussion

We found that alcohol drinking was associated with the likelihood of promoter methylation in postmenopausal breast cancer for two of the three genes that we examined, positively for *E - cadherin* and negatively for *p16.* In addition, drinking pattern appeared to affect methylation; associations were similar for drinks per drinking day for both *E - cadherin* methylation and *p16* methylation after adjusting for total intake. The association of alcohol consumption with *E - cadherin* was limited to ER-negative tumors, and there was evidence that associations tended to be stronger for alcohol consumption at younger ages for *p16* in postmenopausal breast cancer. *RAR-β2* methylation did not differ by alcohol consumption.

Frequencies of promoter methylation for *p16* and *RAR-β_2* genes in our sample were similar to previous reports; *E-cadherin* promoter methylation frequency was somewhat lower than has been reported previously (Li et al., 2006; Parrella et al., 2004; Shinozaki et al., 2005). This variation may be related to characteristics of our sample, sample size in this or other studies or it may depend on the sensitivity of the MSP assay and differences in MSP assay design. In our study, we used the same assay conditions for each tumor DNA sample and positive and negative internal controls; our MSP analysis was reliable.

There are few studies examining a possible association between alcohol consumption and promoter methylation in cancers (Kraunz et al., 2006; Marsit et al., 2006; Puri et al., 2005; Slattery et al., 2006; van Engeland et al., 2003; Zhu et al., 2003), and only one of breast cancer (Zhu et al., 2003). In a case control study of 304 African-American cases and 305 controls, there was a trend toward greater risk of breast cancer with hypermethylation of the *ER* α gene associated with alcohol consumption (>0.5 drinks/day), but the confidence interval was wide and included the null (Zhu et al., 2003). In studies of other cancer sites, Marsit et al (Marsit et al., 2006) found increased likelihood of *SRRP1* hypermethylation with any lifetime alcohol exposure for head and neck squamous cell carcinoma; and van Engeland et al (van Engeland et al., 2003) found a marginally positive association between promoter methylation of at least one out of six genes, and low folate/high alcohol intake for colorectal cancer. Other studies did not find such associations (Hasegawa et al., 2002; Kraunz et al., 2006; Puri et al., 2005; Slattery et al., 2006). However, most of these studies did not evaluate drinking patterns or lifetime total alcohol consumption, and none of them examined alcohol intake at different lifetime periods. Our results indicate that alcohol consumption may affect promoter methylation of genes at earlier age periods (before age 40) among postmenopausal breast cancer patients, also providing evidence that DNA methylation alteration is an early event in carcinogenesis, at least for these genes.

In our study, we found an inverse association between the likelihood of *p16* promoter methylation and lifetime alcohol consumption among postmenopausal women with breast cancer; the associations with alcohol were stronger for drinking before the age of 40. Our results were different from two previous studies examining *p16* methylation of head and neck squamous cell cancers (Kraunz et al., 2006; Puri et al., 2005), which found no difference in prevalence by alcohol intake. A recent animal study also observed that alcohol consumption did not affect *p16* promoter methylation in mice aged either 18 months or 4 months (Sauer et al., 2010). This discrepancy might be partly due to differences in pathways at different cancer sites. It may also be that we were able to detect a difference in our study because of the extensive data regarding alcohol consumption history which we had in our study. However, the biological mechanisms underlying the observed inverse associations are not known.

In an *in vitro* study of MCF-7 cells, ethanol down-regulated the expression of E-cadherin, and increase cell invasion and migration in human breast cancer cell lines (Meng et al.,

2000), and it was proposed that the alcohol consumption may directly decrease the expression of E-cadherin through an epigenetic mechanism. Meanwhile, the ErbB2/Her2/Neu, a member of the epidermal growth factor receptor (EGFR) family, is over-expressed in 20–30% of invasive breast tumors and is associated with poor prognosis. Previous studies reported that the status of ErbB2 expression determine a cellular response to ethanol exposure and high expression of ErbB2 enhances an ethanol-mediated migration and invasion of breast cancer cells *in vitro* (Ke et al., 2006; Ma et al., 2003). DNA methylation of *CDH13*, coding for H-cadherin (a new member of the cadherin superfamily), has been found to be more prevalent in Her2/neu-positive breast tumors (Fiegl et al., 2006). Therefore, it is plausible to postulate that increased EGFR signaling may decrease the expression of E-cadherin through aberrant DNA methylation following the alcohol consumption in breast tumors. No previous studies have evaluated the association of alcohol intake and *E-cadherin* promoter methylation in breast tumors; a study of head and neck squamous cell carcinoma showed a borderline association of *E-cadherin* promoter methylation with increased years of drinking (Hasegawa et al., 2002). We found increased prevalence of *E-cadherin* promoter methylation in breast tumors from postmenopausal drinkers compared with never drinkers. These findings suggest that even the relatively low consumption levels of the study participants may be sufficient to induce aberrant promoter methylation of *E-cadherin*. We found that the association between alcohol consumption and *E-cadherin* promoter methylation was limited to ER-negative breast tumors. However, the biological mechanisms underlying the observed differences by menopausal status and ER status are not known. Further studies would be needed to elucidate a mechanism.

To our knowledge, our study is the first to examine the association between *RAR-β_2* methylation and alcohol consumption in breast tumor, and we found no associations between *RAR-β_2* methylation with alcohol consumption. In addition to the time period of alcohol consumption, the way alcohol is consumed may also affect methylation. Drinks per drinking day was associated with increased likelihood of *E- cadherin* and decreased likelihood of *p16* promoter methylation, even after adjusting for total alcohol intake. Drinking intensity could affect the biological effects of alcohol. There may be important differences between, for example, drinking seven drinks per week as one drink per day, or as seven drinks on a single day each week. In our study, drinking intensity contributed additional information in explaining the difference in likelihood of methylation of these two genes. However, it is important to note that drinking quantity and intensity were highly correlated so that is difficult to separate these different components of drinking behavior.

As in any study of this kind, the strengths and weakness of the study need to be taken into account when considering the findings. Strengths of this study include the population-based study design and relatively large sample size, leading to more stable risk estimates. Nevertheless, the statistical power for examining of subgroups remained limited due to the low frequencies of the promoter methylation, limiting our ability to identify weak associations. Another strength of this study is the detailed information collected regarding lifetime alcohol consumption. The CLDH used in the study has been shown to have high test-retest reliability for estimates of lifetime alcohol consumption (Russell et al., 1997). While recall bias may be a concern for case control study, there is evidence that there is not much bias in recall of alcohol in case control studies of breast cancer (Friedenreich et al., 1991; Giovannucci et al., 1993). Further, it is unlikely that biased recall of alcohol intake would be related to gene promoter methylation, and thus would not differentially affect the case-case comparisons. Among the limitations, the lack of response among cases and controls has the potential for selection bias. However, it is unlikely that there were difference in participation of cases by methylation status; case-case comparisons would not be affected by this bias. A further concern was that we were unable to obtain paraffin-embedded breast tumor tissue for 21.4% of cases. Compared to those for whom we were

unable to obtain tissue, those cases with breast tumor tissue were slightly younger at diagnosis and had a higher TNM stage of breast tumor. They were similar in terms of tumor size, histological grade, nuclear grade, ER and PR status. Further, both age and tumor stage were unrelated to methylation of these genes in this population (Tao et al., 2008); and so selection bias is not a likely explanation for our findings, particularly for the case-case comparisons. Finally, there are concerns with limitations on the outcome measurement of methylation. We examined methylation for three genes that known to be commonly methylated in breast cancer and are known to be significant in three pathways important in breast carcinogenesis. However, clearly we are somewhat limited by the study of a small number of genes. Expansion of our findings to a larger number of genes and genome-wide scale will be important. Further, the methodology used in this study was limited to examination of a single CpG island in the promoter regions. It is assumed that these single regions are sentinels for gene silencing and methylation of other CpG islands, especially in tumors, but it is possible that in some women, these genes are hypermethylated in CpG sequences that we did not study. Finally, in order to assess promoter methylation in this study we used real time MSP that increases the specificity of the MSP by interrogating more than one CpG (Shames et al., 2007). More specifically, we used a fluorescence based version of the MSP technique due to its increased throughput by eliminating the need of gel electrophoresis (Eads et al., 2000). This method has been found to be 10 times more sensitive than the classic MSP method, able to detect methylated sequences from an excess of 10,000-fold unmethylated alleles (Eads et al., 2000). Moreover, due to its increased sensitivity, it can use very small amounts of inferior quality DNA, making it amenable to detect methylation patterns in samples with possible degraded DNA yields and contamination with normal cells, such as archived tumor blocks (Eads et al., 2000). While we are aware of the limitations of this technique given its qualitative nature compared to other quantitative methods such as pyrosequencing, and the fact that it interrogates a limited number of CpG sites, we have followed stringent quality control criteria to ensure confidence in results. Moreover, recent findings show that results from MSP are highly correlated with other methods. By using the highly specific real time MSP, it is likely that our results would be reproduced by other methods (Lee et al., 2008).

In summary, we found an association of alcohol consumption and of drinking pattern with increased promoter methylation of *E- cadherin* and decreased promoter methylation of *p16* genes in postmenopausal breast cancer. The associations were stronger for consumption at younger ages. Thus, our study provides evidence that the observed association of alcohol drinking and breast cancer risk may be related at least in part to alterations in methylation pathways. These findings are important in providing more data to support an etiologic role for alcohol consumption in breast carcinogenesis and for suggesting potential mechanisms for prevention and treatment.

Acknowledgments

This study would not have been possible without the support of all the study participants and the research staff of the WEB Study. This work was supported in part by the National Institute on Alcohol Abuse and Alcoholism (P50-AA09802), the Department of Defense (DAMD 179616202, DAMD 17030446), and the National Cancer Institute (R01CA 092040).

References

Castro GD, de Castro CR, Maciel ME, Fanelli SL, de Ferreyra EC, Gómez MI, Castro JA. Ethanol-induced oxidative stress and acetaldehyde formation in rat mammary tissue: potential factors involved in alcohol drinking promotion of breast cancer. Toxicology. 2006; 219:208–219. [PubMed: 16377051]

Choi SW, Mason JB. Folate status: effects on pathways of colorectal carcinogenesis. J Nutr. 2002; 132:2413S–2418S. [PubMed: 12163703]

de Vogel S, Bongaerts BW, Wouters KA, Kester AD, Schouten LJ, de Goeij AF, de Bruïne AP, Goldbohm RA, van den Brandt PA, van Engeland M, et al. Associations of dietary methyl donor intake with MLH1 promoter hypermethylation and related molecular phenotypes in sporadic colorectal cancer. Carcinogenesis. 2008; 29:1765–1773. [PubMed: 18339680]

Dorgan JF, Baer DJ, Albert PS, Judd JT, Brown ED, Corle DK, Campbell WS, Hartman TJ, Tejpar AA, Clevidence BA, et al. Serum hormones and the alcohol-breast cancer association in postmenopausal women. J Natl Cancer Inst. 2001; 93:710–715. [PubMed: 11333294]

Dumitrescu RG, Shields PG. The etiology of alcohol-induced breast cancer. Alcohol. 2005; 35:213–225. [PubMed: 16054983]

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res. 2000; 28:e32. [PubMed: 10734209]

Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res. 2001; 61:3225–3229. [PubMed: 11309270]

Fernandez SV, Russo J. Estrogen and xenoestrogens in breast cancer. Toxicol Pathol. 2010; 38:110–122. [PubMed: 19933552]

Fiegl H, Millinger S, Goebel G, Müller-Holzner E, Marth C, Laird PW, Widschwendter M. Breast cancer DNA methylation profiles in cancer cells and tumor stroma: association with HER-2/neu status in primary breast cancer. Cancer Res. 2006; 66:29–33. [PubMed: 16397211]

Friedenreich CM, Howe GR, Miller AB. An investigation of recall bias in the reporting of past food intake among breast cancer cases and controls. Ann Epidemiol. 1991; 1:439–453. [PubMed: 1669524]

Giovannucci E, Stampfer MJ, Colditz GA, Manson JE, Rosner BA, Longnecker MP, Speizer FE, Willett WC. Recall and selection bias in reporting past alcohol consumption among breast cancer cases. Cancer Causes Control. 1993; 4:441–448. [PubMed: 8218876]

Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB. E-cadherin expression is silence by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res. 1995; 55:5195–5199. [PubMed: 7585573]

Hamajima N, Hirose K, Tajima K, Rohan T, Calle EE, Heath CW Jr, Coates RJ, Liff JM. Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. Br J Cancer. 2002; 87:1234–1245. [PubMed: 12439712]

Hasegawa M, Nelson HH, Peters E, Ringstrom E, Posner M, Kelsey KT. Patterns of gene promoter methylation in squamous cell cancer of the head and neck. Oncogene. 2002; 21:4231–4236. [PubMed: 12082610]

Homann N, Seitz HK, Wang XD, Yokoyama A, Singletary KW, Ishii H. Mechanisms in alcohol-associated carcinogenesis. Alcohol Clin Exp Res. 2005; 29:1317–1320. [PubMed: 16088994]

Jeronimo C, Usadel H, Henrique R, Oliveira J, Lopes C, Nelson WG, Sidransky D. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst. 2001; 93:1747–1752. [PubMed: 11717336]

Ke Z, Lin H, Fan Z, Cai TQ, Kaplan RA, Ma C, Bower KA, Shi X, Luo J. MMP-2 mediates ethanol-induced invasion of mammary epithelial cells over-expressing ErbB2. Int J Cancer. 2006; 119:8–16. [PubMed: 16450376]

Key J, Hodgson S, Omar RZ, Jensen TK, Thompson SG, Boobis AR, Davies DS, Elliott P. Meta-analysis of studies of alcohol and breast cancer with consideration of the methodological issues. Cancer Causes Control. 2006; 17:759–770. [PubMed: 16783604]

Krassenstein R, Sauter E, Dulaimi E, Battagli C, Ehya H, Klein-Szanto A, Cairns P. Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. Clin Cancer Res. 2004; 10:28–32. [PubMed: 14734448]

Kraunz KS, Hsiung D, McClean MD, Osanyingbemi J, Nelson HH, Kelsey KT. Dietary folate is associated with *p16* ^{INK4A} methylation in head and neck squamous cell carcinoma. Int J Cancer. 2006; 119:1553–1557. [PubMed: 16646054]

Lee ES, Issa JP, Roberts DB, Williams MD, Weber RS, Kies MS, El-Naggar AK. Quantitative promoter hypermethylation analysis of cancer-related genes in salivary gland carcinomas: comparison with methylation-specific PCR technique and clinical significance. Clin Cancer Res. 2008; 14:2664–2672. [PubMed: 18451230]

Li SY, Rong M, Iacopetta B. DNA hypermethylation in breast cancer and its association with clinicopathological features. Cancer Lett. 2006; 237:272–280. [PubMed: 16029926]

Ma C, Lin H, Leonard SS, Shi X, Ye J, Luo J. Overexpression of ErbB2 enhances ethanol-stimulated intracellular signaling and invasion of human mammary epithelial and breast cancer cells in vitro. Oncogene. 2003; 22:5281–5290. [PubMed: 12917629]

Marsit CJ, McClean MD, Furniss CS, Kelsey KT. Epigenetic inactivation of the *SFRP* genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. Int J Cancer. 2006; 119:1761–1766. [PubMed: 16708382]

Mason JB, Choi SW. Effects of alcohol on folate metabolism: implications for carcinogenesis. Alcohol. 2005; 35:235–241. [PubMed: 16054985]

Mehrotra J, Vali M, McVeigh M, Kominsky SL, Fackler MJ, Lahti-Domenici J, Polyak K, Sacchi N, Garrett-Mayer E, Argani P, et al. Very high frequency of hypermthylated genes in breast cancer metastasis to the bone, brain, and lung. Clin Cancer Res. 2004; 10:3104–3109. [PubMed: 15131050]

Meng Q, Gao B, Goldberg ID, Rosen EM, Fan S. Stimulation of cell invasion and migration by alcohol in breast cancer cells. Biochem Biophys Res Commun. 2000; 273:448–453. [PubMed: 10873626]

Novak P, Jensen TJ, Garbe JC, Stampfer MR, Futscher BW. Stepwise DNA methylation changes are linked to escape from defined proliferation barriers and mammary epithelial cell immortalization. Cancer Res. 2009; 69:5251–5258. [PubMed: 19509227]

Parrella P, Poeta ML, Gallo AP, Prencipe M, Scintu M, Apicella A, Rossiello R, Liguoro G, Seripa D, Gravina C, et al. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. Clin Cancer Res. 2004; 10:5349–5354. [PubMed: 15328171]

Petri AL, Tjonneland A, Gamborg M, Johansen D, Høidrup S, Sørensen TI, Grønbaek M. Alcohol intake, type of beverage, and risk of breast cancer in pre- and postmenopausal women. Alcohol Clin Exp Res. 2004; 28:1084–1090. [PubMed: 15252295]

Puri SK, Si L, Fan CY, Hanna E. Aberrant promoter hypermethylation of multiple genes in head and neck squamous cell carcinoma. Am J Otolaryngol. 2005; 26:12–17. [PubMed: 15635575]

Rinaldi S, Peeters PH, Bezemer ID, Dossus L, Biessy C, Sacerdote C, Berrino F, Panico S. Relationship of alcohol intake and sex steroid concentrations in blood in pre- and post-menopausal women: the European Prospective Investigation into Cancer and Nutrition. Cancer Causes Control. 2006; 17:1033–1043. [PubMed: 16933054]

Russell M, Marshall JR, Trevisan M, Freudenheim JL, Chan AW, Markovic N, Vána JE, Priore RL. Test-retest reliability of the cognitive lifetime drinking history. Am J Epidemiol. 1997; 146:975–981. [PubMed: 9400340]

Russell M, Peirce RS, Vána JE, Nochajski TH, Carosella AM, Muti P, Freudenheim JL, Trevisan M. Relations among alcohol consumption measures derived from the Cognitive Lifetime Drinking History. Drug Alcohol Rev. 1998; 17:377–387. [PubMed: 16203505]

Sauer J, Jang H, Zimmerly E, Kim KC, Liu Z, Chanson A, Smith DE, Mason JB, Friso S, Choi SW. Ageing, chronic alcohol consumption and folate are determinants of genomic DNA methylation, p16 promoter methylation and the expression of p16 in the mouse colon. Br J Nutr. 2010; 104:24–30. [PubMed: 20205967]

Shames DS, Minna JD, Gazdar AF. Methods for detecting DNA methylation in tumors: from bench to bedside. Cancer Lett. 2007; 251:187–198. [PubMed: 17166656]

Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, Taback B. Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. Clin Cancer Res. 2005; 11:2156–2162. [PubMed: 15788661]

Singletary KW, Gapstur SM. Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. JAMA. 2004; 286:2143–2151. [PubMed: 11694156]

Slattery ML, Curtin K, Sweeney C, Levin TR, Potter J, Wolff RK, Albertsen H, Samowitz W. Diet and lifestyle factor associations with CpG island methylator phenotype and BRAF mutations in colon cancer. Int J Cancer. 2006; 120:656–663. [PubMed: 17096326]

Smith-Warner SA, Spiegelman D, Yaun SS, van den Brandt PA, Folsom AR, Goldbohm RA, Grahm S, Howe GR. Alcohol and breast cancer in women: a pooled analysis of cohort studies. JAMA. 1998; 279:535–540. [PubMed: 9480365]

Szyf M, Pakneshan P, Rabbani SA. DNA methylation and breast cancer. Biochem Parmacol. 2004; 68:1187–1197.

Tao MH, Shields PG, Nie J, Millen A, Ambrosone CB, Edge SB, Krishnan SS, Marian C, Xie B, Winston J, et al. DNA hypermethylation and clinicopathological features in breast cancer: the Western New York Exposures and Breast Cancer (WEB) Study. Breast Cancer Res Treat. 2008; 114:559–568. [PubMed: 18463976]

van Engeland M, Weijenberg MP, Roemaen GM, Brink M, de Bruine AP, Goldbohm RA, van den Brandt PA, Baylin SB, de Goeij AF, Herman JG. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. Cancer Res. 2003; 63:3133–3137. [PubMed: 12810640]

Wajed SA, Laird PW, DeMeester TR. DNA methylation: an alternative pathway to cancer. Ann Surg. 2001; 234:10–20. [PubMed: 11420478]

Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene. 2002; 21:5462–5482. [PubMed: 12154408]

Yan PS, Perry MR, Laux DE, Asare AL, Caldwell CW, Huang TH. CpG island arrays: an application toward deciphering epigenetic signatures of breast cancer. Clin Cancer Res. 2000; 6:1432–1438. [PubMed: 10778974]

Zhu K, Davidson NE, Hunter S, Yang X, Payne-Wilks K, Roland CL, Phillips D, Bentley C, Dai M, Williams SM. Methyl-group dietary intake and risk of breast cancer among African-American women: a case-control study by methylation status of the estrogen receptor alpha genes. Cancer Causes Control. 2003; 14:827–836. [PubMed: 14682440]

Table 1

Descriptive characteristics of breast cancer cases and controls by promoter methylation status of *E-cadherin*, *p16*, and *RAR-β_2*, WEB Study 1996–2001

	E-cadherin		***p16***		***RAR-β_2***		
	M (n = 161)[§]	**UM (n = 642)[§]**	**M (n = 208)[§]**	**UM (n = 595)[§]**	**M (n = 221)[§]**	**UM (n = 582)[§]**	**Controls**
Age, y (mean ± SD)	58.0 ± 11.8	57.4 ± 11.2	58.0 ± 11.2	57.4 ± 11.3	57.4 ± 11.3	57.6 ± 11.3	57.8 ± 11.8
Race/ethnicity							
White	148 (91.9%)	594 (92.5%)	192 (92.3%)	550 (92.4%)	210 (95.0%) *a*	532 (91.4%)	1910 (90.3%)
Non-white	13 (8.1%)	48 (7.5%)	16 (7.7%)	45 (7.6%)	11 (5.0%)	50 (8.6%)	205 (9.7%)
Age at menarche, y (mean ± SD)	12.5 ± 1.5	12.6 ± 1.6	12.6 ± 1.6	12.6 ± 1.6	12.6 ± 1.5	12.5 ± 1.6	12.7 ± 1.6
Parity (mean ± SD) [†]	2.9 ± 1.5	2.8 ± 1.4 *2*	3.0 ± 1.5	2.8 ± 1.4 *2*	2.9 ± 1.5	2.8 ± 1.4 *2*	3.1 ± 1.6
Age at first birth, y (mean ± SD) [†]	23.4 ± 4.9 *a, 3*	24.5 ± 4.8	24.0 ± 4.7	24.3 ± 4.9	24.0 ± 4.5	24.3 ± 5.0	24.1 ± 4.5
BMI, kg/m^2 (mean ± SD)	28.1 ± 5.6	28.5 ± 6.5	28.5 ± 6.2	28.4 ± 6.3	28.6 ± 6.1	28.4 ± 6.4	28.2 ± 6.3
Family history of breast cancer [*]							
Yes	33 (22.0%) *1*	124 (20.8%) *2*	47 (24.5%) *1*	110 (19.8%) *2*	41 (19.7%) *a*	116 (21.5%) *2*	252 (12.8%)
No	117 (78.0%)	473 (79.2%)	145 (75.5%)	445 (80.2%)	167 (80.3%)	423 (78.5%)	1712 (87.2%)
History of benign breast disease [*]							
Yes	61 (38.6%) *1*	208 (33.1%) *2*	79 (38.7%) *1*	190 (32.6%) *2*	70 (31.8%) *1*	199 (35.1%) *2*	457 (21.8%)
No	98 (61.4%)	420 (66.9%)	125 (61.3%)	393 (67.4%)	150 (68.2%)	368 (64.9%)	1636 (78.2%)
Daily total energy, kcal (mean ± SD)	1538.3 ± 630.5 *1*	1506.3 ± 566.2 *b*	1415.5 ± 471.6 *3*	1546.8 ± 609.3 *2*	1549.8 ± 657.6 *1*	1498.7 ± 546.7	1452.0 ± 558.2
Smoking							
Never	75 (46.9%)	290 (45.2%)	100 (48.1%)	265 (44.7%)	102 (46.2%)	263 (45.3%)	1015 (48.3%)
Ever	85 (53.1%)	351 (54.8%)	108 (51.9%)	328 (55.3%)	119 (53.8%)	317 (54.7%)	1087 (51.7%)
Lifetime alcohol (oz)	3509.3 ± 6278.0	2901.8 ± 4766.5	3049.5 ± 5194.3	3023.3 ± 5104.7	2626.4 ± 3703.2	3177.4 ± 5548.6	3545.6 ± 12546.9
Postmenopausal	111 (68.9%)	455 (70.9%)	147 (70.7%)	419 (70.4%)	157 (71.0%)	409 (70.3%)	1503 (71.1%)
Hormone replacement therapy [**]							
Never	60 (54.1%)	209 (46.2%)	72 (49.0%)	197 (47.4%)	73 (47.1%)	196 (48.0%)	720 (50.0%)
Ever	51 (45.9%)	243 (53.8%)	75 (51.0%)	219 (52.6%)	82 (52.9%)	212 (52.0%)	721 (50.0%)

[§] M – methylated; UM – un-methylated;

[†] Among parous women.

* Subjects with missing values were excluded from the analysis.

‡ Daily folate intake from diet and supplements.

** Among postmenopausal women.

[a] Comparison of methylated cases to controls, $p \leq 0.05$;

[b] Comparison of unmethylated cases to controls, $p \leq 0.05$;

[c] Comparison of methylated to unmethylated cases, $p \leq 0.05$.

[1] Comparison of methylated cases to controls, $p \leq 0.01$;

[2] Comparison of unmethylated cases to controls, $p \leq 0.01$;

[3] Comparison of methylated to unmethylated cases, $p \leq 0.01$.

Table 2

Alcohol consumption and promoter methylation of *E-cadherin*, *p16*, and *RAR-β_2* genes in postmenopausal breast tumors: case-control and case-case comparisons

				U vs Controls	M vs Controls	M vs U
	Controls	**M**	**U**	**OR1 (95% CI)** [1]	**OR2 (95% CI)** [1]	**OR3 (95% CI)** [2]
E-cadherin						
Lifetime alcohol intake [a]						
Lifetime abstainer	260	13	89	1.0	1.0	1.0
Low alcohol intake	590	48	182	0.96 (0.70–1.30)	1.93 (1.01–3.71)	2.06 (1.02–4.16)
High alcohol intake	589	49	176	0.92 (0.67–1.26)	2.06 (1.06–4.03)	2.39 (1.15–4.96)
Alcohol consumption previous 2–10 years [a]						
Lifetime abstainer	260	13	89	1.0	1.0	1.0
Low alcohol intake	603	44	174	0.87 (0.64–1.19)	1.69 (0.88–3.27)	1.96 (0.97–3.98)
High alcohol intake	595	53	187	0.92 (0.67–1.26)	2.07 (1.07–4.03)	2.47 (1.18–5.15)
Alcohol consumption previous 10–20 years [a]						
Lifetime abstainer	260	13	89	1.0	1.0	1.0
Low alcohol intake	600	49	171	0.86 (0.63–1.18)	1.89 (0.99–3.62)	2.20 (1.09–4.43)
High alcohol intake	595	48	190	0.95 (0.69–1.30)	1.85 (0.94–3.63)	2.08 (0.99–4.36)
p16						
Lifetime alcohol intake [a]						
Lifetime abstainer	260	36	66	1.0	1.0	1.0
Low alcohol intake	590	54	176	1.29 (0.93–1.80)	0.69 (0.43–1.09)	0.50 (0.29–0.86)
High alcohol intake	589	54	171	1.26 (0.89–1.78)	0.69 (0.42–1.12)	0.52 (0.29–0.92)
Alcohol consumption previous 2–10 years [a]						
Lifetime abstainer	260	36	66	1.0	1.0	1.0
Low alcohol intake	603	54	164	1.12 (0.82–1.62)	0.64 (0.40–1.03)	0.53 (0.30–0.91)
High alcohol intake	595	56	184	1.27 (0.90–1.79)	0.68 (0.42–1.11)	0.47 (0.27–0.85)
Alcohol consumption previous 10–20 years [a]						
Lifetime abstainer	260	36	66	1.0	1.0	1.0
Low alcohol intake	600	58	162	1.16 (0.83–1.62)	0.69 (0.44–1.10)	0.56 (0.32–0.97)

				U vs Controls	M vs Controls	M vs U
	Controls	**M**	**U**	**OR1 (95% CI)** [1]	**OR2 (95% CI)** [1]	**OR3 (95% CI)** [2]
High alcohol intake	595	52	186	1.29 (0.92–1.83)	0.64 (0.39–1.04)	0.44 (0.24–0.78)
RAR-β₂						
Lifetime alcohol intake [a]						
Lifetime abstainer	260	31	71	1.0	1.0	1.0
Low alcohol intake	590	65	165	1.11 (0.80–1.54)	1.00 (0.62–1.59)	0.87 (0.51–1.48)
High alcohol intake	589	58	166	1.13 (0.80–1.58)	0.89 (0.54–1.46)	0.79 (0.45–1.39)
Alcohol consumption previous 2–10 years [a]						
Lifetime abstainer	260	31	71	1.0	1.0	1.0
Low alcohol intake	603	62	156	1.01 (0.72–1.42)	0.88 (0.55–1.42)	0.85 (0.49–1.47)
High alcohol intake	595	63	177	1.15 (0.82–1.62)	0.86 (0.53–1.41)	0.74 (0.42–1.32)
Alcohol consumption previous 10–20 years [a]						
Lifetime abstainer	260	31	71	1.0	1.0	1.0
Low alcohol intake	600	60	160	1.05 (0.76–1.47)	0.86 (0.53–1.38)	0.79 (0.46–1.36)
High alcohol intake	595	65	173	1.12 (0.80–1.58)	0.91 (0.56–1.49)	0.82 (0.47–1.45)

[1]Odds ratios and 95% confidence intervals adjusted for age, race, education, age at menarche, age at first birth, age at menopause, BMI, family history of breast cancer among first degree relatives, history of benign breast disease, energy, folate, smoking status.

[2]Odds ratios and 95% confidence intervals also adjusted for ER status.

[a]Cutoff point (oz): 1162 for lifetime alcohol until 2 years prior to diagnosis/interview; 106 for alcohol consumption between 2–10 years previous; 184 for alcohol consumption 10–20 years previous.

Table 3

Intensity of alcohol (drinks per drinking day) intake and *E-cadherin*, *p16*, and *RAR-β_2* promoter methylation in postmenopausal breast tumors: case-control and case-case comparisons

				U vs Controls	M vs Controls	M vs U
	Controls	**M**	**U**	**OR1 (95% CI)** [1]	**OR2 (95% CI)** [1]	**OR3 (95% CI)** [2]
E-cadherin						
Lifetime No. of drinks per usual drinking day [a, 3]						
Lifetime abstainer	260	13	89	1.0	1.0	1.0
Low alcohol intake	588	49	179	0.94 (0.69–1.27)	2.02 (1.06–3.86)	2.21 (1.07–4.57)
High alcohol intake	587	48	178	0.95 (0.70–1.30)	2.03 (1.04–3.96)	2.18 (1.00–4.72)
p16						
Lifetime No. of drinks per usual drinking day [a, 3]						
Lifetime abstainer	260	36	66	1.0	1.0	1.0
Low alcohol intake	588	54	174	1.29 (0.92–1.80)	0.69 (0.44–1.10)	0.48 (0.28–0.84)
High alcohol intake	587	53	173	1.33 (0.94–1.87)	0.65 (0.40–1.05)	0.43 (0.23–0.80)
RAR-β_2						
Lifetime No. of drinks per usual drinking day [a, 3]						
Lifetime abstainer	260	31	71	1.0	1.0	1.0
Low alcohol intake	588	66	162	1.10 (0.79–1.52)	1.03 (0.63–1.61)	0.92 (0.54–1.58)
High alcohol intake	587	58	168	1.15 (0.82–1.61)	0.92 (0.56–1.50)	0.76 (0.44–1.34)

[1] Odds ratios and 95% confidence intervals adjusted for age, race, education, age at menarche, age at first birth, age at menopause, BMI, family history of breast cancer among first degree relatives, history of benign breast disease, energy, folate, smoking status.

[2] Odds ratios and 95% confidence intervals also adjusted for ER status.

[3] Odds ratios and 95% confidence intervals also adjusted for total amount of alcohol consumption.

[a] Lifetime no of drinks per usual drinking day, cutoff point: 2; No of drinks per usual drinking day 2–10 years previous, cutoff point: 1; No of drinks per usual drinking day 10–20 years previous, cutoff point: 1.

Table 4

Alcohol consumptions by decades of age and likelihood of *E-cadherin*, *p16* and *RAR-β_2* promoter methylation in breast tumors stratified by menopausal status, case-case comparisons

	E-cadherin			*p16*			*RAR-β_2*		
	N	**N**	**OR (95% CI)** [1]	**N**	**N**	**OR (95% CI)** [1]	**N**	**N**	**OR (95% CI)** [1]
	M	**U**		**M**	**U**		**M**	**U**	
Premenopausal									
Alcohol intake before age 20 [a]									
Abstainer	7	19	1.0	10	18	1.0	10	18	1.0
Low	19	83	0.66 (0.23–1.94)	21	81	0.47 (0.17–1.32)	25	77	0.64 (0.23–1.78)
High	22	82	0.71 (0.23–2.21)	30	74	0.85 (0.28–2.54)	29	75	0.76 (0.26–2.23)
Alcohol intake age 20–30 [b]									
Abstainer	7	21	1.0	10	18	1.0	10	18	1.0
Low	18	72	0.76 (0.25–2.35)	20	70	0.49 (0.18–1.41)	22	68	0.59 (0.21–1.70)
High	24	94	0.70 (0.22–2.21)	31	87	0.61 (0.21–1.78)	32	86	0.55 (0.19–1.62)
Alcohol intake age 30–40 [c]									
Abstainer	7	19	1.0	9	17	1.0	9	17	1.0
Low	13	61	0.87 (0.31–2.39)	17	57	0.41 (0.16–1.05)	23	51	1.45 (0.58–3.62)
High	23	73	1.16 (0.46–2.95)	21	75	0.39 (0.16–0.95)	25	71	1.01 (0.42–2.44)
Postmenopausal									
Alcohol intake before age 20 [a]									
Abstainer	13	89	1.0	36	66	1.0	31	71	1.0
Low	54	196	2.16 (1.08–4.32)	67	183	0.58 (0.34–0.98)	65	185	0.82 (0.48–1.40)
High	43	162	2.22 (1.07–4.63)	41	164	0.42 (0.23–0.75)	59	146	0.85 (0.48–1.50)
Alcohol intake age 20–30 [b]									
Abstainer	13	89	1.0	36	66	1.0	31	71	1.0
Low	52	185	2.31 (1.13–4.72)	62	175	0.53 (0.31–0.91)	62	175	0.75 (0.43–1.29)
High	46	176	2.41 (1.13–5.16)	47	175	0.42 (0.23–0.76)	62	160	0.81 (0.46–1.43)
Alcohol intake age 30–40 [c]									
Abstainer	13	89	1.0	36	66	1.0	31	71	1.0

	E-cadherin			*p16*			*RAR-β₂*		
	N	**N**	**OR (95% CI)** [1]	**N**	**N**	**OR (95% CI)** [1]	**N**	**N**	**OR (95% CI)** [1]
	M	**U**		**M**	**U**		**M**	**U**	
Low	50	186	2.25 (1.10–4.61)	63	173	0.54 (0.31–0.92)	62	174	0.74 (0.43–1.28)
High	48	176	2.52 (1.18–5.37)	47	177	0.42 (0.23–0.75)	63	161	0.81 (0.46–1.44)
Alcohol intake age 40–50 [d]									
Abstainer	12	85	1.0	35	62	1.0	31	66	1.0
Low	45	168	1.69 (0.90–3.17)	55	158	0.76 (0.45–1.27)	56	157	0.63 (0.38–1.04)
High	46	168	1.78 (0.92–3.44)	52	162	0.67 (0.39–1.17)	55	159	0.62 (0.37–1.06)
Alcohol intake age 50–60 [e]									
Abstainer	9	64	1.0	26	47	1.0	27	46	1.0
Low	27	102	1.35 (0.72–2.52)	31	98	0.70 (0.40–1.24)	29	100	0.47 (0.26–0.83)
High	35	107	1.65 (0.89–3.06)	37	105	0.84 (0.48–1.49)	37	105	0.67 (0.38–1.18)

[1] Odds ratios and 95% confidence intervals adjusted for age, race, education, age at menarche, age at first birth, menopausal status, BMI, family history of breast cancer among first degree relatives, history of benign breast disease, energy, folate, smoking status, and ER status.

[a] Alcohol intake before age 20 years, cutoff point (oz): 136 premenopausal women, 0 postmenopausal women.

[b] Alcohol consumption between age 20–30 years, cutoff point (oz): 400 premenopausal women, 189 postmenopausal women.

[c] Alcohol consumption between age 30–40 years, cutoff point (oz): 196 premenopausal women, 177 postmenopausal women.

[d] Alcohol consumption between age 40–50 years, cutoff point (oz): 256 premenopausal women, 187 postmenopausal women.

[e] Alcohol consumption between age 50–60 years, cutoff point (oz): 147 postmenopausal women.

Table 5

Relationship between alcohol consumptions and promoter methylation of *E-cadherin*, *p16* and *RAR-β_2* genes stratified by ER status: case-case comparisons

	Ecad		OR (95% CI) [1]	*p16*		OR (95% CI) [1]	*RAR-β_2*		OR (95% CI) [1]
	N	N		N	N		N	N	
	M	U		M	U		M	U	
ER positive									
Alcohol drinking									
Never	16	69	1.0	29	56	1.0	28	57	1.0
Ever	91	350	1.33 (0.70–2.53)	98	344	0.47 (0.27–0.81)	126	315	0.69 (0.41–1.18)
Lifetime alcohol intake [a]									
Abstainer	16	69	1.0	29	56	1.0	28	57	1.0
Low	46	158	1.29 (0.66–2.50)	40	164	0.45 (0.25–0.83)	58	146	0.73 (0.41–1.29)
High	45	191	1.09 (0.54–2.19)	58	178	0.59 (0.32–1.09)	67	169	0.69 (0.38–1.25)
ER negative									
Alcohol drinking									
Never	4	33	1.0	15	22	1.0	10	27	1.0
Ever	41	154	4.13 (1.16–14.72)	55	140	0.59 (0.25–1.39)	44	151	0.81 (0.32–2.07)

[1] Odds ratios and 95% confidence intervals adjusted for age, race, education, age at menarche, age at first birth, BMI, family history of breast cancer among first degree relatives, history of benign breast disease, energy, smoking status, and menopausal status.

[a] Lifetime alcohol until 2 years prior to diagnosis/interview, cutoff point (oz): 1002 premenopausal women, 1162 postmenopausal women.