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Alcohol Consumption and Serum Metabolite Concentrations in Young Women

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Abstract

Purpose: Alcohol consumption is an established breast cancer risk factor, though further research is needed to advance our understanding of the mechanism underlying the association. We used global metabolomics profiling to identify serum metabolites and metabolic pathways that could potentially mediate the alcohol-breast cancer association.

Methods: A cross-sectional analysis of reported alcohol consumption and serum metabolite concentrations was conducted among 211 healthy women 25-29 years old who participated in the Dietary Intervention Study in Children 2006 Follow-Up Study (DISC06). Alcohol-metabolite associations were evaluated using multivariable linear mixed effects regression.

Results: Alcohol was significantly (FDR $p < 0.05$) associated with several serum metabolites after adjustment for diet composition and other potential confounders. The amino acid sarcosine, the omega-3 fatty acid eicosapentaenoate (EPA), and the steroid 4-androsten-3beta,17beta-diol monosulfate were positively associated with alcohol intake, while the gamma-tocopherol metabolite gamma-carboxyethyl hydroxychroman (CEHC) was inversely associated. Positive

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associations of alcohol with 2-methylcitrate and 4-androsten-3beta,17beta-diol disulfate were borderline significant (FDR $p < 0.10$). Metabolite set enrichment analysis identified steroids and the glycine pathway as having more members associated with alcohol consumption than expected by chance.

Conclusions: Most of the metabolites associated with alcohol in the current analysis participate in pathways hypothesized to mediate the alcohol-breast cancer association including hormonal, one-carbon metabolism and oxidative stress pathways, but they could also affect risk via alternative pathways. Independent replication of alcohol-metabolite associations and prospective evaluation of confirmed associations with breast cancer risk are needed.

Keywords

Alcohol; serum metabolite; androgen; sarcosine; eicosapentaenoate

Introduction

Approximately 250,000 US women are diagnosed and 40,000 die of breast cancer annually. [5] Yet, the cause of breast cancer remains largely unknown, and of the known risk factors, few are modifiable. Alcohol consumption, an established potentially modifiable breast cancer risk factor, increases risk by approximately 7-10 percent/drink/day across all levels of intake.[3, 13, 15, 73] Risk does not differ by type of alcohol consumed – wine, beer or spirits,[3, 13, 45, 73] suggesting that it is ethanol, per se, and not another constituent that is responsible.

Alcohol-associated increased breast cancer risk is generally thought to be due to altered hormone or one-carbon metabolism, increased oxidative stress, or mutagenesis by acetaldehyde.[27] Alcohol stimulates the hypothalamic-pituitary-adrenal axis leading to increased secretion of androgens that are positively associated with breast cancer risk in both pre- and postmenopausal women.[28, 77] Alcohol also is well-known to inhibit the absorption and metabolism of key nutrients involved in one-carbon metabolism[34, 52, 83] that are critical for maintenance of DNA integrity and epigenetic regulation of gene expression.[6, 88] Ethanol is rapidly metabolized to acetaldehyde, which can lead to redox changes that result in a prooxidant state.[81] Acetaldehyde interacts with many cellular constituents including DNA and proteins and is an established potent mutagen and carcinogen.[71]

Given the plethora of alcohol's adverse effects, metabolomics, which simultaneously quantifies levels of small molecules in multiple metabolic pathways, is ideally suited for advancing our understanding of mechanisms of alcohol's effects on breast cancer risk. Though several studies on metabolic effects of alcohol have been performed,[33, 35, 41, 87, 91] most do not account for dietary composition, which is reported to differ between drinkers and non-drinkers.[14, 55, 65, 72] If in the causal pathway, these dietary differences could potentially mediate alcohol-metabolite associations. Alternatively, diet could confound or have no effect on these associations.

We evaluated associations of reported alcohol intake with serum metabolites in a cross-sectional analysis of women who participated in the Dietary Intervention Study in Children 2006 Follow-Up Study (DISC06). As part of this study women reported usual alcohol intake and diet was ascertained by three non-consecutive 24-hour dietary recalls. We estimated alcohol-metabolite associations both with and without adjustment for diet to obtain unbiased estimates and to assess potential for confounding. For alcohol-metabolite associations that changed following adjustment for diet, we also conducted mediation analysis.

Materials and Methods

Design

DISC was a multicenter randomized controlled clinical trial sponsored by the National Heart, Lung, and Blood Institute (NHLBI) to test the safety and efficacy of a dietary intervention to reduce serum low-density lipoprotein cholesterol (LDL-C) in children with elevated LDL-C. The trial's design and results have been described.[19, 21, 23–25, 57, 58, 78] Briefly, between 1988 and 1990, 663 healthy, pre-pubertal, 8-10 year old children, including 301 girls, with elevated LDL-C were recruited into DISC at six clinical centers¹ and randomized to a behavioral dietary intervention or usual care control group. Planned intervention continued until 1997 when the mean age of participants was 16.7 years. In 2006-2008 when participants were 25 to 29 years old, the DISC06 follow-up study was conducted to evaluate the longer-term effects of the diet intervention on biomarkers associated with breast cancer risk in DISC female participants. Assent was obtained from DISC participants and informed consent was obtained from their parents/guardians prior to randomization. Informed consent was obtained from participants prior to the DISC06 follow-up visit. The original DISC protocol was approved by institutional review boards at all participating clinical centers and the data coordinating center.² The DISC06 protocol was also approved by the institutional review board at the Fox Chase Cancer Center.

Participants

Eligibility criteria for girls in the original DISC were: 1) 8 - 10 years old; 2) serum LDL-C in the 80th to 98th percentiles;[82] 3) no major illness or medication that could affect blood lipids or growth; 4) height 5th percentile and weight-for-height in the 5th to 90th percentile; [85] 5) Tanner stage 1 for breast and pubic hair;[76] and 6) normal psychosocial and cognitive development.[1] Girls were excluded if they or family members were following a low-fat diet, a parent had a history of early heart disease or the family planned to move. DISC participants were recruited through schools, health maintenance organizations, and pediatric practices.

All female DISC participants were invited to participate in DISC06 and 260 (86.4 percent) of the 301 females originally randomized took part. Women who were pregnant or breastfeeding at or within 12 weeks before the visit (n=30) or did not have a blood sample

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for metabolomics assays (n=3), were not fasting at blood donation (n=4), did not complete diet recalls (n=11) or had an implausible dietary intake (kcal=13,049/day; n=1) were not eligible for the current analysis, leaving 211 participants.

Data Collection

For the DISC06 follow-up study, each female participant attended a single visit at a DISC clinic between 2006 and 2008. Visits were scheduled to take place within 14 days of onset of next menses whenever possible. All data for a participant were collected on the same day except 24-hour dietary recalls, which were collected over two weeks following the visit. Data were collected by staff masked to treatment assignment. A centralized data collection training session was held before initiation of data collection.

Frequency of usual alcohol intake from wine, beer and spirits was ascertained by questionnaire. Otherwise, dietary data were ascertained via three nonconsecutive 24-hour dietary recalls collected by trained interviewers on two weekdays and one weekend day. Data from the three recalls were averaged to estimate usual nutrient intakes using the University of Minnesota's Nutrition Data System for Research. Participants also completed questionnaires on demographics; medical, reproductive and menstrual histories; medications; smoking; and family history of breast cancer. Leisure physical activity was assessed using the Modifiable Activity Questionnaire.[51] Participants completed menstrual cycle calendars for up to 6 weeks following clinic visits until the start of their next menses.

Blood Sampling

Blood was collected in the morning after an overnight fast by venipuncture using standard procedures. After standing at room temperature for 45 minutes to allow complete clotting, blood was centrifuged and serum was separated and pipetted in 0.5 mL aliquots into cryovials that were stored at -80° C.

Metabolomics Assays

Global metabolomic profiling was performed by Metabolon (Durham, NC). Participants' samples were randomly ordered and 10 percent blind quality control (QC) samples were integrated throughout to monitor laboratory performance. Additionally, a pooled matrix sample served as a technical replicate throughout analyses, extracted water samples served as process blanks, and a cocktail of QC standards spiked into every sample allowed for instrument performance monitoring and aided chromatographic alignment.

Samples were prepared using the automated MicroLab StAR system (Hamilton Co.). Proteins were precipitated with methanol under vigorous shaking for two minutes followed by centrifugation. The resulting extract was divided into five fractions for analysis by four ultra-high performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) based methods with one sample reserved for backup. Samples were placed briefly on a TurboVap (Zymark) to remove organic solvents. The sample extracts were stored overnight under nitrogen before preparation for analysis.

The LC/MS platform was based on a Water ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with an electrospray ionization source and Orbitrap mass analyzer operated at 35,000 mass resolution. After drying sample extracts were reconstituted in solvents compatible with each of the four analytical methods. The first two methods used reverse phase UPLC-MS/MS with positive ion mode electrospray ionization. One chromatographically optimized for more hydrophilic compounds by gradient eluting the extract from a C18 column using water and methanol with 0.05% perfluoropentanoic acid and 0.1% formic acid. Whereas the other chromatographically optimized for more hydrophobic compounds by gradient eluting the extract from the same C18 column using methanol, acetonitrile, and water with 0.05% perfluoropentanoic acid and 0.01% formic acid. The third method used reverse phase UPLC-MS/MS with negative ion mode electrospray ionization. The basic extracts were gradient eluted from a separate dedicated C18 column using methanol and water with 6.5mM ammonium bicarbonate at pH 8. The fourth method also used negative ionization following gradient elution from a HILIC column using water and acetonitrile with 10mM ammonium formate at pH 10.8. The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Biochemical identifications were based on three criteria: retention index, accurate mass match to the library, and the MS/MS forward and reverse scores between the experimental data and authentic standards. At the time DISC06 samples were assayed, more than 3300 commercially available purified standard compounds had been acquired and characterized.

Proprietary visualization and interpretation software were used to confirm the consistency of peak identification among samples. Peaks were quantified using area-under-the-curve.

Statistical Analysis

A total of 705 named metabolites were semi-quantified as relative peak intensity. Metabolites with 30 percent of values less than the limit of detection or with coefficients of variation 25 percent calculated from masked quality control samples were dropped, leaving 449 metabolites for analysis. For metabolites with <30 percent of values below the limit of detection, undetected values were imputed at the lowest observed value multiplied by 0.75. Metabolites were transformed to the natural log scale and extreme values were winsorized using the median absolute deviation.[54]

The association of alcohol consumption with diet composition was assessed by the R² from a linear regression model including alcohol intake (drinks/day) as the dependent variable and energy (kcal/day), fat (percent kcal/day) and carbohydrate (percent kcal/day) intakes as independent variables. Analyses also were performed replacing total fat in models with fat subtypes – saturated, polyunsaturated and monounsaturated fatty acids. Because the adjusted R² from this model was slightly smaller than the adjusted R² from the more parsimonious model and because associations of serum metabolites with alcohol were similar when total

fat or fat subtypes were included in models, only results from simpler models including total fat are reported.

Univariate associations of individual metabolites with reported alcohol intake were evaluated using simple linear regression including each metabolite as a dependent variable and alcohol consumption as an independent continuous variable (drinks/week). Multivariable linear mixed effects regression was then used to adjust for a set of potential confounders identified a priori. Initial multivariable models included clinic as a random effect while body mass index (BMI; kg/m²), race, education, smoking, hormonal contraceptive use, parity, physical activity, menstrual cycle phase, time of blood collection and DISC treatment group were included as fixed effects. Multivariable models were then refit also including energy, fat and carbohydrate intakes to adjust for diet composition. Model details are included as a footnote to Table 2. Percentage differences in serum metabolite concentrations associated with a one drink/week increase in reported alcohol consumption were estimated from models as $\% = (\exp(\beta) - 1) \times 100$. We applied the false discovery rate (FDR) to control for multiple comparisons.[9]

To evaluate the potential influence of extreme values on observed associations, analyses were repeated after winsorizing reported alcohol intake using the median absolute deviation. [54]. We also conducted additional analyses restricted to non-smokers.

To further explore associations of metabolites associated with alcohol (FDR p<0.10) in diet adjusted models, analyses were repeated including alcohol as a factor with five levels.

For metabolites significantly associated with alcohol in continuous multivariable models (FDR p<0.05), we conducted mediation analysis if after adjustment for diet, the alcohol effect size decreased by at least 20 percent and was no longer significant (FDR p 0.10). Analyses were performed using the model-based approach as implemented in R package *mediation*. [80] Mediation was evaluated separately for dietary fat and carbohydrates using models similar to those described above but including only fixed effects and using bootstrap variances.

Metabolite Set Enrichment Analysis (MSEA) with a one-sided Fisher's exact test was used to identify over-represented classes of metabolites associated with reported alcohol intake. [67] Metabolites associated with alcohol at p<0.10 in diet adjusted models were considered related. Metabolite classes were defined a priori by Metabolon.

To quantify the variance of participants' metabolite levels relative to assay variance, we divided the variance of participants' analytical samples by the total variance, estimated as the sum of the variances of analytical samples plus 23 masked, replicate quality control aliquots from a single pool.

All tests of statistical significance were two-sided except where otherwise stated. All analyses were conducted using SAS 9.4 and R 3.5 statistical software.

Results

Participant characteristics at the DISC06 clinic visit are shown in Table 1. Ninety percent of women were white with a mean age of 27.2 ± 1.1 years. Their mean BMI was 25.5 ± 5.5 kg/m²; 24 percent were overweight and 19 percent were obese.[10] At the time of the visit, 26 percent of participants smoked cigarettes and 55 percent used hormonal contraceptives. By design, most (82 percent) were in the luteal phase of their menstrual cycles. All participants fasted overnight and 91 percent of blood collections took place before 11am. Reported alcohol intake ranged from 0 to 40 drinks per week with a median of 3.0 (IQR (interquartile range) = 0.9 – 6.0) drinks per week. Participants' mean energy intake was 1735 ± 487 kcal/day, with 17 percent of energy provided by protein, 50 percent by carbohydrates and 31 percent by fat. The distribution of participant characteristics across categories of alcohol intake is included as Supplemental Table 1.

Diet macronutrient composition was strongly and significantly associated with alcohol intake. The adjusted R² from a linear model that regressed reported alcohol intake (drinks/wk) on energy (kcal/day), fat and carbohydrate (percent kcal/day) intakes was 0.152 ($p < 0.0001$). Though alcohol was not significantly associated with energy intake ($\beta = 0.0002$, $p = 0.08$), fat ($\beta = -0.5014$, $p < 0.0001$) and carbohydrate ($\beta = -0.4374$, $p < 0.0001$) intakes were independently significantly inversely associated with alcohol.

Table 2 presents associations of reported alcohol intake with metabolites that were associated in multivariable adjusted analysis with or without inclusion of diet. Associations of alcohol with all metabolites evaluated along with an estimate of the contribution of participant variance to total metabolite variance are included as Supplemental Table 2. Adjustment for diet composition generally attenuated alcohol-metabolite associations. Prior to adjustment for diet, 14 metabolites were significantly associated with alcohol consumption after adjustment for multiple comparisons (FDR $p < 0.05$), while after adjustment for diet four metabolites were significantly associated with alcohol and two were borderline significant (FDR $p < 0.10$).

Figure 1 graphically summarizes diet adjusted alcohol-metabolite associations by metabolite superclass. After adjusting for multiple comparisons, the amino acid sarcosine increased significantly by 3.89 percent/drink/wk (FDR $p = 0.045$), whereas the tricarboxylic acid (TCA) cycle metabolite 2-methylcitrate increased slightly by 1.16 percent/drink/wk and was borderline significant (FDR $p = 0.067$). Adjusted for multiple comparisons several lipids also increased in association with alcohol; the omega-3 fatty acid eicosapentaenoate (EPA) increased significantly by 2.58 percent/drink/wk (FDR $p = 0.045$) and the steroids 4-androsten-3beta,17beta-diol mono- and disulfate increased by 3.78 percent /drink/wk (FDR $p = 0.045$) and 3.36 percent/drink/wk (FDR $p = 0.054$), respectively. In contrast, the gamma-tocopherol metabolite gamma-carboxyethyl hydroxychroman (gamma-CEHC) decreased significantly by 2.40 percent/drink/wk (FDR $p = 0.045$). The association of alcohol with serum EPA was not materially changed by further adjustment for dietary EPA ($\beta = 2.36$ percent/drink/wk; $p = 6.08e^{-4}$), and its association with serum gamma-CEHC was unchanged by further adjustment for dietary gamma tocopherol from food and supplements ($\beta = -2.39$ percent/drink/wk; $p = 4.35e^{-4}$). Results of the alcohol - gamma-CEHC association also were

unchanged by adjustment for alpha-tocopherol from food and/or supplements or total vitamin E from food and/or supplements (data not shown).

Winsorizing alcohol intake recoded 23 to 21.8 drinks per week. Effect sizes tended to be larger after winsorizing because of the truncated distribution of alcohol intakes. However, p-values generally were similar to those shown in Table 2, indicating results were not due to influential outliers (data not shown). Restricting analyses to non-smokers also did not change our main results materially (data not shown).

Associations of alcohol intake modeled as a factor with 5 levels and metabolites associated in continuous diet adjusted models at FDR $p < 0.10$ are shown in Table 3. Women who consumed 10 drinks/week had serum sarcosine and 4-androsten-3beta,17beta-diol disulfate levels that were more than twice as high as those who consumed < 1 drink/week. Their 4-androsten-3beta,17beta-diol monosulfate levels also were almost twice as high. EPA and 2-methylcitrate also were 60 and 20 percent higher, respectively, among the heaviest compared to lightest drinkers, whereas gamma-CEHC was 41 percent lower. Among drinkers serum metabolite levels changed monotonically with increasing alcohol intake except for 2-methylcitrate, which was associated only at 10 drinks/week.

Of the 14 metabolites significantly associated with alcohol at FDR $p < 0.05$ in multivariable models before adjustment for diet, eight had an FDR $p > 0.10$ after adjustment, and alcohol effect sizes decreased by 20 percent or more for three of these. Results of mediation analysis shown in Table 4 suggest that dietary fat significantly ($p = 0.002$) mediated 33 percent of the association of alcohol with the phospholipid 1-palmitoyl-2-oleoyl-GPC, whereas both fat and carbohydrate contributed to mediation of the association of alcohol with the lipid myo-inositol. However, small absolute values of the sensitivity parameter ρ when the average causal mediation effect equals 0 indicate results are sensitive to possible uncontrolled confounding.[40]

Metabolite Set Enrichment Analysis (MSEA) identified two classes of metabolites, steroids and glycine pathway metabolites as having more members associated with alcohol consumption in diet adjusted analysis than expected by chance. Seven of 19 steroid metabolites including 4-androsten-3beta,17beta-diol mono- and disulfate, 5-alpha-androstan-3beta,17beta-diol disulfate, etiocholanolone glucuronide, pregnenolone disulfate and pregnenolone-3-glucuronide were associated with alcohol ($p = 0.007$). Similarly, 4 of 10 glycine pathway metabolites including sarcosine, N-acetylglycine, dimethylglycine and threonine were associated ($p = 0.03$).

Discussion

Alcohol consumption was strongly associated with serum metabolites across a broad range of metabolite classes in this cross-sectional study of young adult women. Accounting for multiple comparisons, 14 metabolites were significantly associated with alcohol before diet adjustment, while after adjustment four remained significant and two were borderline significant. Results are compatible with alcohol-induced alterations in hormonal, one-carbon

metabolism and oxidative stress pathways, all of which are implicated in breast cancer etiology.

Diets of drinkers and non-drinkers differ.[14, 55, 65, 72] In an analysis from NHANESIII, alcohol consumption was significantly positively associated with total energy intake but inversely associated with energy derived from protein, fat and particularly carbohydrate, suggesting that energy from alcohol replaces that from other macronutrients.[55] Even so, most studies of metabolomic profiles associated with alcohol do not adjust for diet or adjust only for energy intake. In our analysis eight metabolites that were significantly associated with alcohol before adjustment for diet composition were no longer associated after adjustment. With adjustment, alcohol effect sizes decreased by 20 percent or more for three of these metabolites, which appeared to be due to a combination of confounding and mediation.

Steroids as a class were significantly positively upregulated by alcohol. In particular, the mono- and disulfates of the testosterone precursor 4-androsten-3beta,17beta-diol were strongly and significantly positively associated with alcohol after adjustment for multiple comparisons. Two additional testosterone metabolites, 5-alpha-androstan-3beta,17beta-diol disulfate and etiocholanolone glucuronide, as well as several progesterone metabolites also were significantly positively associated with alcohol before, but not after, adjustment for multiple comparisons. Alcohol stimulates the hypothalamic-pituitary-adrenal axis leading to increased adrenal androgen production, and 4-androsten-3beta,17beta-diol was previously reported to be increased in association with alcohol.[33, 60, 91] Associations of testosterone with breast cancer risk are well established in both pre- and postmenopausal women.[28, 77] 4-Androsten-3beta,17beta-diol monosulfate also was significantly positively associated with postmenopausal breast cancer in a prospective study.[60] Thus, alcohol could potentially increase breast cancer risk by increasing circulating androgens.

To our knowledge this is the first report of significant alterations of the glycine pathway by alcohol. Sarcosine (methylglycine), an intermediate in this pathway, was strongly and significantly positively associated with alcohol intake in diet-adjusted models after accounting for multiple comparisons. N-acetylglycine and dimethylglycine also were positively associated while threonine was inversely associated with alcohol before, though not after, adjustment for multiple comparisons. We are not aware of any prior reports of an association of alcohol with serum sarcosine levels, but alcohol has been reported to increase sarcosine's precursors choline and dimethylglycine under some conditions.[35, 62] The glycine pathway fuels one-carbon metabolism,[4] and approximately 60 percent of methyl groups required for methylation of homocysteine to methionine in one-carbon metabolism are contributed by betaine,[59] a precursor of dimethylglycine. Thus, in addition to established mechanisms,[83] alcohol could potentially inhibit one-carbon metabolism by interfering with the glycine pathway. Sarcosine is metabolized to glycine, an integral component of glutathione, which is the primary cellular antioxidant,[4] and elevated sarcosine in association with alcohol could alternatively be related to oxidative stress. We are not aware of any reports of an association of sarcosine with breast cancer risk, but breast tumor expression of sarcosine-related proteins has been reported.[11, 49, 89]

We observed higher levels of 2-methylcitrate associated with alcohol consumption. Though circulating citrate was reduced in association with alcohol consumption in two metabolomics studies,[35, 87] to our knowledge there have been no prior reports of associations with 2-methylcitrate. 2-Methylcitrate is formed by the condensation of propionyl CoA with oxaloacetate in a reaction that is catalyzed by citrate synthase and is more common to bacteria and fungi than humans.[2] In humans propionyl CoA is primarily metabolized via the vitamin B12 dependent methylmalonyl pathway,[20] though under certain circumstances, such as vitamin B12 deficiency, it can be metabolized via citrate synthase to 2-methylcitrate.[2] Alcohol decreases circulating vitamin B12,[53] and 2-methylcitrate levels are reportedly elevated in vitamin B12 deficiency.[37, 43] Even so, the magnitude of the association of alcohol with 2-methylcitrate in our study was small and should be interpreted cautiously.

In the current analysis gamma-CEHC, a gamma-tocopherol metabolite, was significantly inversely associated with alcohol intake, and the association was unchanged by adjustment for dietary gamma-tocopherol. Alcohol is rapidly oxidized to acetaldehyde, which leads to oxidative stress as a consequence of increased free radical generation and depletion of antioxidant defenses.[16] Tocopherols are one of the primary defense systems acting by preventing the generation of free radicals.[44, 84] Gamma-tocopherol also is anti-inflammatory, and its CEHC derivative acts as an antioxidant and anti-inflammatory as well.[38, 42] Plasma tocopherols generally are not associated[30, 31, 60] or are positively associated with alcohol consumption in cross sectional studies.[86, 92] In a controlled feeding study, consumption of two alcoholic beverages per day increased serum alpha-tocopherol but gamma-tocopherol was unchanged.[36] Gamma-tocopherol has been shown to inhibit development of estrogen dependent mammary tumors in animal models.[74] However, in most prospective epidemiologic studies gamma-tocopherol is not significantly associated with breast cancer,[7, 26, 29, 68, 75] with the exception of one that observed a positive association.[46] In a recent prospective metabolomics study,[60] serum gamma-CEHC glucuronide also was significantly positively associated with postmenopausal breast cancer risk. Thus, even though the biology of gamma-CEHC suggests our observed lower serum levels with alcohol consumption could potentially increase breast cancer risk, this is not supported by empirical evidence. In a small reproducibility study, the intraclass correlation coefficient for serum gamma-CEHC measured in samples collected twice, approximately one year apart, from 15 premenopausal women was 0.27 (unpublished data), suggesting poor reproducibility over time, which could contribute to inconsistent findings.

EPA was significantly positively associated with alcohol consumption in the current analysis, and the association was not altered by adjustment for EPA intake from food and supplements. EPA was previously positively associated with moderate alcohol consumption.[17, 18] Though these studies suggest the association may be specific to wine,[17, 18] we only ascertained usual total alcohol intake. EPA is an omega-3 fatty acid plentiful in fish, a component of the Mediterranean diet associated with decreased cancer risk and mortality at several sites including the breast.[69] Though plasma EPA levels generally are not associated with breast cancer risk,[8, 12, 61, 66] most prospective studies support a protective effect of dietary EPA or marine omega-3 fatty acids for breast cancer.[8, 50, 70,

90] Increased serum EPA may counter some of alcohol's adverse health effects while contributing to some of its beneficial effects when consumed in moderation.

Our study had several strengths. The sample size was reasonably large and participants, who were all women, reported a wide range of usual alcohol intake. Data on potential confounders were ascertained concurrently with alcohol intake. Usual diet was assessed by three nonconsecutive 24-hour recalls, the gold standard for population research.[79] Participants fasted overnight prior to donating blood, which was processed following a standardized protocol, and metabolomics assays were performed by Metabolon, a leader in the field. There also were some limitations. All participants had elevated serum LDL-C at baseline[82] and met several additional eligibility criteria, which could limit generalizability of findings. Even so, at the DISC06 follow-up visit only 16 (7.6%) had elevated serum LDL-C according to current guidelines[32] and one was taking cholesterol lowering medication. Usual alcohol intake was self-reported, which could have led to misclassification of exposure, since people tend to under-report intake,[56] but if misclassification occurred it would likely have been non-differential. Metabolomics assays were performed using a single blood sample from each participant. Even though we adjusted for menstrual cycle phase as well as time of day of blood collection in addition to multiple potential confounders in analysis, other sources of variation could have inflated variances and diminished power. The Metabolon platform does not include estrogens, steroid hormones that are associated with breast cancer risk[28, 47] and previously have been associated with alcohol consumption in both pre- and postmenopausal women.[22, 39, 48, 63, 64] Finally, the cross-sectional study design limits our ability to infer causality.

In summary, the current analysis identified several novel metabolites associated with alcohol consumption. Most participate in pathways hypothesized to mediate the alcohol-breast cancer association including hormonal, one-carbon metabolism and oxidative stress pathways, but they could also affect risk via alternative pathways. Independent replication of alcohol-metabolite associations and prospective evaluation of confirmed associations with breast cancer risk are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Manhattan plot associations of alcohol with metabolites by superclass

Table 1.

Participant Characteristics (N=211)

Continuous Variables	Mean	SD
Age, y	27.22	1.06
BMI (kg/m ²)	25.53	5.46
Alcohol (drinks/wk)	4.93	6.33
Moderate-intense physical activity (MET·hr·wk ⁻¹)	24.61	21.68
Diet		
Energy (kcal/day)	1735	487
Protein (%kcal)	16.90	4.02
Carbohydrate (%kcal)	50.23	9.00
Fat (%kcal)	31.46	7.47
Categorical Variables	N	%
Race		
White	190	90.05
Other	21	9.95
Education		
High school	23	10.90
Some college	47	22.27
Bachelor's degree	106	50.24
Graduate school	35	16.59
Current Smoker		
No	156	73.93
Yes, <5 cigarettes/day	23	10.90
Yes, ≥5 cigarettes/day	32	15.17
Full-Term Pregnancies		
0	156	73.93
1	32	15.17
2+	23	10.90
Current Hormonal Contraceptive Use		
None	100	47.39
Combined oral contraceptive pill	88	41.71
Other	23	10.90
Menstrual Cycle Phase at Blood Collection		
Luteal	172	81.52
Follicular	27	12.80
Unknown	12	5.69
Time of Day at Blood Collection		
7-8am	37	17.54
9am	108	51.18
10am	47	22.27

Continuous Variables	Mean	SD
11am-2pm	19	9.00
DISC Treatment Group		
Intervention	107	50.71
Usual care	104	49.29

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Table 2.

Percent Change in Serum Metabolite Concentrations Associated with an Increase of One Alcoholic Beverage per Week for Metabolites Associated with Alcohol in a Multivariable Model at FDR $p < 0.10$

Metabolite	Unadjusted ^a			Multivariable Adjusted Excluding Diet ^b			Multivariable Adjusted Including Diet ^c		
	% Change/Drink/Wk	Pvalue	FDR Pvalue	% Change/Drink/Wk	Pvalue	FDR Pvalue	% Change/Drink/Wk	Pvalue	FDR Pvalue
Amino Acid									
<i>Glycine, Serine and Threonine Metabolism</i>									
sarcosine	3.86	4.05E-05	0.005	4.52	8.40E-06	0.001	3.89	3.98E-04	0.045
<i>Lysine Metabolism</i>									
3-methylglutaryl carnitine	-2.25	1.73E-02	0.152	-2.58	3.34E-03	0.094	-2.04	3.30E-02	0.463
Carbohydrate									
<i>Pentose Metabolism</i>									
arabitol/xylitol	0.88	2.05E-04	0.013	0.85	8.46E-04	0.032	0.82	2.67E-03	0.133
Energy									
<i>TCA Cycle</i>									
2-methylcitrate/homocitrate	1.04	5.66E-04	0.020	1.22	2.31E-04	0.013	1.16	8.93E-04	0.067
Lipid									
<i>Medium Chain Fatty Acids</i>									
10-undecenoate (11:1n1)	1.84	5.14E-05	0.005	1.65	4.91E-04	0.022	1.47	3.73E-03	0.159
<i>Polyunsaturated Fatty Acid (n3 and n6)</i>									
eicosapentaenoate (EPA; 20:5n3)	2.60	9.98E-06	0.001	2.76	2.27E-05	0.003	2.58	2.76E-04	0.045
<i>Inositol Metabolism</i>									
myo-inositol	0.79	2.92E-04	0.013	0.77	9.84E-04	0.034	0.50	4.63E-02	0.520
<i>Phospholipid Metabolism</i>									
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	0.99	9.39E-05	0.007	0.78	5.28E-04	0.022	0.52	2.72E-02	0.459
Steroid									
4-androsten-3beta,17beta-diol monosulfate (2)	5.34	1.21E-08	<0.001	4.37	2.47E-06	0.001	3.78	1.64E-04	0.045
4-androsten-3beta,17beta-diol disulfate	4.46	4.15E-07	<0.001	4.18	4.89E-06	0.001	3.36	5.97E-04	0.054
4-androsten-3beta,17beta-diol monosulfate (1)	1.97	1.35E-02	0.140	2.50	1.07E-03	0.034	2.01	1.51E-02	0.340
etiocholanolone glucuronide	2.86	1.60E-03	0.036	3.48	2.20E-04	0.013	3.23	1.71E-03	0.110
Nucleotide									
3-ureidopropionate	1.04	2.41E-03	0.049	1.05	3.19E-03	0.094	0.96	1.41E-02	0.333
Cofactors and Vitamins									
<i>Tocopherol Metabolism</i>									
gamma-CEHC	-2.33	2.36E-04	0.013	-2.54	4.24E-05	0.004	-2.40	3.94E-04	0.045

Metabolite	Unadjusted ^a			Multivariable Adjusted Excluding Diet ^b			Multivariable Adjusted Including Diet ^c		
	% Change/ Drink/W k	Pvalue	FDR Pvalue	% Change/ Drink/W k	Pvalue	FDR Pvalue	% Change/ Drink/W k	Pvalue	FDR Pvalue
Vitamin A Metabolism									
retinol (Vitamin A)	1.13	8.76E-04	0.026	1.09	2.76E-04	0.014	0.84	9.16E-03	0.280
Xenobiotic									
Food Component/Plant									
piperine	3.73	3.90E-03	0.063	4.99	1.25E-04	0.009	4.04	3.88E-03	0.159

^aResults from simple linear regression of individual metabolite concentrations on alcohol intake (drinks/wk).

^bResults from linear mixed effects regression of individual metabolite concentrations on alcohol intake (drinks/wk) adjusted for clinic as a random effect and BMI (kg/m²;continuous), race (white/non-white), college graduate (yes/no), current smoker (no/yes <5 cigarettes per day/yes >5 cigarettes per day), current hormonal contraceptive use (none/combined oral contraceptive pill/other), parous (yes/no), physical activity (MET·hr·wk⁻¹;continuous), menstrual cycle phase (luteal/follicular/missing), time of blood collection (7-8am/9am/10am/11am-2pm), and DISC treatment group (intervention/usual care) as fixed effects.

^cResults from linear mixed effects regression of individual metabolite concentrations on alcohol intake (drinks/wk) adjusted as for b above plus energy (kcal/day; continuous), fat (%kcal/day; continuous) and carbohydrate (%kcal/day; continuous) as fixed effects.

Table 3.

Multivariable Diet Adjusted Percent Change in Metabolite Concentrations for Categories of Alcohol Consumption^a

	Drinks per Week				
	0	>0 and <1	1 and <5	5 and <10	10 and 40
N	17	41	81	46	26
Sarcosine					
% change	-5.44	ref	-1.90	21.49	138.02
95% CI ^b	-39.36, 47.43		-27.23, 32.24	-14.97, 73.57	55.52, 264.29
Pvalue	0.815		0.905	0.312	<0.001
Eicosapentaenoate					
% change	-1.56	ref	10.46	23.52	59.87
95% CI	-26.80, 32.39		-9.50, 34.80	-2.64, 56.71	20.36, 112.36
Pvalue	0.921		0.355	0.101	0.002
4-Androsten-3beta,17beta-diol monosulfate					
% change	-8.43	ref	6.01	50.68	97.88
95% CI	-38.58, 36.53		-18.96, 38.66	9.32, 107.67	34.96, 190.15
Pvalue	0.682		0.687	0.019	0.001
4-Androsten-3beta,17beta-diol disulfate					
% change	10.58	ref	3.13	53.52	123.64
95% CI	-26.31, 65.92		-21.50, 35.48	10.82, 112.68	51.60, 229.93
Pvalue	0.646		0.834	0.015	<0.001
Gamma-CEHC					
% change	-13.26	ref	-27.84	-33.63	-41.50
95% CI	-34.91, 14.89		-40.75, -13.19	-47.44, -17.04	-55.72, -23.68
Pvalue	0.355		0.002	0.001	<0.001
2-Methylcitrate					
% change	-4.85	ref	-7.58	-3.25	19.51
95% CI	-17.84, 9.56		-16.03, 1.90	-13.96, 8.41	4.09, 37.14
Pvalue	0.522		0.133	0.595	0.017

^aResults from linear mixed effects regression of individual metabolite concentrations on alcohol intake modeled as a factor with 5 levels and adjusted as described in Table 2 footnote c.

^bCI=confidence interval

Table 4.

Mediation of Alcohol - Metabolite Associations by Dietary Fat and Carbohydrate Intakes

Metabolite	Dietary Fat				Dietary Carbohydrate			
	Proportion Mediated		ACME ^a	Sensitivity	Proportion Mediated		ACME ^a	Sensitivity
	Proportion	95% CI ^b	p-value	ρ^c	Proportion	95% CI ^b	p-value	ρ^c
Lipids								
myo-inositol	0.29	0.03 - 0.72	0.03	-0.2	0.33	0.06 - 0.79	0.02	-0.2
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	0.33	0.11 - 0.88	0.002	-0.2	0.29	0.02 - 0.87	0.04	-0.2
Cofactors and Vitamins								
retinol	0.15	-0.08 - 0.50	0.19	-0.1	0.20	-0.04 - 0.61	0.09	-0.1

^aACME = average causal mediation effects^bCI = confidence interval^c ρ at ACME=0