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Mediation analysis of the alcohol-postmenopausal breast cancer relationship by sex hormones in the EPIC Cohort

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Running Title

Mediation by sex hormones of the alcohol and breast cancer association

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List of abbreviations used

1-SD: one standard deviation; 95%CI: 95% Confidence Intervals; AICR: American Institute of Cancer Research; BC: Breast Cancer; BMI: Body Mass Index; DHEAS: dehydroepiandrosterone sulfate; DKFZ: German Cancer Research Center; EPIC: European Prospective Investigation into Cancer and nutrition; ER(+ or -): Estrogen Receptor (positive or negative); IARC: International Agency for Research on Cancer; ICD-10: International Classification of Diseases 10th Revision; NDE: Natural Direct Effect; NIE: Natural Indirect

Effect; OR: Odds ratio; PLS: Partial Least Squares; RD: Risk Difference; SHBG: Sex Hormone Binding Globulin; TE: Total Effect; WCRF: World Cancer Research Fund.

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"For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php"

What's new? (74 words – limit: 75)

We examined whether sex hormones and SHBG, individually or through a composite hormonal signature, act as mediators on the pathway between alcohol intake and postmenopausal BC. While limited evidence suggested a mediated proportion of 19% of the total effect through free estradiol, mediation by individual sex-hormone levels suggested a borderline significant indirect effect through free estradiol accounting for 19% of the mediated proportion, However, the hormonal signature mediated about 24% of the alcohol-BC association, suggesting that any potential mechanism of sex-steroids in the alcohol and BC relationship is likely to involve interplay of hormones, beyond the action of single hormonal levels.

Abstract (250 words unstructured)

Alcohol consumption is associated with higher risk of breast cancer (BC); however, the biological mechanisms underlying this association are not fully elucidated, particularly the extent to which this relationship is mediated by sex hormone levels.

Circulating concentrations of estradiol, testosterone, their free fractions and sex-hormone binding globulin (SHBG), were examined in 430 incident BC cases and 645 matched controls among alcohol-consuming postmenopausal women nested within the European Prospective Investigation into Cancer and Nutrition. Mediation analysis was applied to assess whether individual hormone levels mediated the relationship between alcohol intake and BC risk. An alcohol-related hormonal signature, obtained by Partial Least Square (PLS) regression, was evaluated as a potential mediator. Total (TE), natural direct (NDE) and natural indirect effects (NIE) were estimated.

Alcohol intake was positively associated with overall BC risk and specifically with estrogen receptor positive tumours with respectively TE=1.17(95%CI: 1.01,1.35) and 1.36(1.08,1.70) for a 1-SD deviation increase of intake. There was no evidence of mediation by sex steroids or SHBG separately except for a weak indirect effect through free estradiol where NIE=1.03(1.00,1.06). However, an alcohol-related hormonal signature negatively associated with SHBG and positively with estradiol and testosterone, was associated with BC risk (OR=1.25 (1.07,1.47)) for a 1-SD higher PLS score, and had a statistically significant NIE accounting for a mediated proportion of 24%.

There was limited evidence of mediation of the alcohol-BC association by individual sex hormones. However, a hormonal signature, reflecting lower levels of SHBG and higher levels of sex steroids, mediated a substantial proportion of the association.

Keywords: sex steroids, alcohol, breast cancer, mediation analysis, hormonal signature, EPIC.

Introduction

Breast cancer (BC) is the most frequent type of cancer accounting for nearly a quarter of all cancers in women worldwide with about 2.08 million incident breast cancer cases diagnosed in 2018 (1). BC incidence is expected to continue rising with increases in obesity, reductions in fertility and aging of the population, in particular in developing countries (2). BC is a multifactorial disease and its aetiology includes dietary, lifestyle, hormonal, and reproductive risk factors (3). Among these, alcohol intake has been consistently associated with higher BC risk and has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen (Group 1) (4). The evidence is considered strong both in pre- and post-menopausal women (5–8), as confirmed by in a comprehensive analysis by the World Cancer Research Fund (WCRF) (9).

A positive dose-response association between alcohol intake and BC risk, consistently across hormonal receptor status, was shown in a study based on 11,576 incident BC cases within the European Prospective Investigation into Cancer and nutrition (EPIC) cohort (10). Little is known on the mechanisms through which alcohol exerts its carcinogenic effect during BC development, yet accumulating evidence suggests that the association between alcohol intake and breast carcinogenesis may be partly mediated through endogenous sex steroids (11–15). Estrogens and androgens are well-known activators of cellular proliferation, and are associated with an increased BC risk (15). Findings from the EPIC study and the Endogenous Hormones and Breast Cancer Collaborative Group supported the association between elevated pre-diagnostic serum concentrations of oestrogens, androgens and low serum levels of sex hormone binding globulin (SHBG) and higher postmenopausal BC risk (16–18). It has been suggested that alcohol consumption increases the concentrations of sex steroids in serum in both pre- and post-menopausal women (15). In EPIC, higher concentrations of androgens including testosterone and free testosterone, and lower

concentrations of SHBG were observed in postmenopausal women who consumed more than 25 g/day of alcohol (i.e. 2 glasses) compared with women who were non consumers (19). A review suggested that estrogens could mediate the relationship between alcohol and BC as alcohol elevates concentrations of circulating oestrogens (15). In postmenopausal women, nearly 100% of estrogens are synthetized from aromatization of androgens in peripheral tissues, with SHBG regulating their circulating concentrations and that of their free fractions (20).

To our knowledge, only one study conducted within the Women's Health Initiative has explored a causal pathway from alcohol to postmenopausal BC operating through serum estrogen, but no significant evidence was found(21).

Here, we examine whether estradiol, testosterone, their free fractions, and SHBG, as well as a composite hormonal signature, mediated the relationship between alcohol intake and postmenopausal BC in a nested-case control study within EPIC, among alcohol drinkers.

Material and Methods

The EPIC study. EPIC is a multicentre prospective cohort designed to investigate the associations of diet, lifestyle, environmental and metabolic factors with cancer and other disease outcomes. Over 360,000 women and 150,000 men aged 20-85 years were recruited between 1992 and 2000 from 23 centres spanning 10 European countries: Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, The Netherlands and the United Kingdom (22). The rationale, study design and methods of the EPIC study have been extensively described (22–24). Biological samples were collected at recruitment prior to disease onset in approximately 80% of the cohort and were stored at IARC (Lyon, France) in −196ºC liquid nitrogen for all countries, except from Denmark (nitrogen vapour, −150ºC) and Sweden

(freezers, −80ºC) where samples were stored locally. All participants gave their written informed consent to use their questionnaire data and biological samples for future analyses.

Exposure assessment. During the enrolment period, information on socio-demographic characteristics including education, occupational and recreational levels of physical activity, tobacco smoking, medical and reproductive history, exogenous hormone use, anthropometric measures as well as alcohol consumption habits were gathered using lifestyle questionnaires. Dietary intake over the 12 months was assessed at baseline using validated country-specific dietary questionnaires (self-administered, food frequency questionnaires, semi-quantitative or interviewer-performed) designed to specifically capture local habits with high compliance as detailed elsewhere (22–24). Baseline alcohol intake was calculated from the number of glasses of beer and/or wine, cider, sweet liquors, fortified wines, distilled spirits consumed per day or week in the year preceding recruitment. The individual average daily alcohol intake, expressed in grams per day (g/d), was computed based on the standard glass volume and ethanol content as the sum of the ethanol content of all alcoholic beverages consumed obtained through country-specific food composition tables per alcoholic beverage type. This calculation was done based on data collected through 24-hr dietary recalls from a subgroup of the cohort containing detailed information on alcohol intake distribution during the day in relation to main meals (25,26).

Ascertainment of cancer outcome. Incident BC cases were identified through record linkage with regional cancer and pathology registries with the exception of Naples (Italy), Germany, Greece and France where a combination of methods was employed including: cancer and pathology registries, health insurance records, active follow-up through direct contact with study subjects or next of kin, and collection of clinical records. Vital status was ascertained from municipal, regional or national-level mortality registries. For this study, the closure date was the last date of complete follow-up, both for cancer incidence and vital status, ranging

from 2003 to 2006, depending on each EPIC study centre (16,22,27–29). All the self-reported BC cases were systematically verified from clinical and pathologic records. Cancer incidence data were classified according to the International Classification of Diseases for Oncology (ICD-O), as first primary invasive BC, ICD-O codes C50. Information on hormone receptor status (estrogen and progesterone) as well as the laboratory methods and quantification descriptions used to determine the receptor status, were collected by the EPIC centres and criteria were retained to harmonize positive receptor identification across centres (28).

The nested case-control study. The current study is based upon data available from two nested case-control studies within EPIC on postmenopausal BC risk and endogenous hormone levels ("study phase 1" (27) and "study phase 2" (28)). Norway and Sweden were not included in these analyses either because a blood serum sample was not available or because independent studies were being completed on BC risk. In both study phases, postmenopausal women were included. Postmenopausal women were defined as women who had no menstruations in the 12 months preceding study enrolment, or were older than 55 years of age if the menstrual cycle information was not available, or who had undergone a bilateral oophorectomy. Only women with available blood samples who were not using any menopausal hormone therapy at the time of blood collection (as the use of exogenous hormones influences the endogenous concentrations and some may be the same as endogenous), and who did not have any prevalent cancer at baseline (with the exception of non-melanoma skin cancer) were included into the study. For each case, up to two controls with a blood sample available were chosen at random among appropriate risk sets consisting of all postmenopausal cohort members alive and free of cancer at the time of diagnosis of the case. This was done using an incidence density sampling protocol allowing the inclusion of subjects who became a case later in time, while each control could be sampled more than once (28). Controls were matched to the cases on study recruitment centre, age at blood donation (± 6 months), follow-up time since blood

donation (± 3 months), time of the day of blood collection (± 1 hour), and fasting status (<3 hours, 3–6 hours, >6 hours). Analyses were also conducted stratifying on estrogen receptor (ER) status. Over the two study phases, there were 798 BC and 1294 matched controls with 387 cases being oestrogen receptor positive (ER+) tumours, 153 oestrogen negative tumours (ER-) and 258 with unknown hormonal receptor status (estrogen and/or progesterone). After excluding case-sets in which the case or her control(s) were non-drinkers (daily intake <0.1g/day), the final study sample included 430 cases and 645 matched controls with 218 ER+ (62% from "study phase 1" and 38% from "study phase 2"), 105 ER- (27% from "study phase 1" and 73% from "study phase 2") and 107 with unknown hormonal receptor status (estrogen and/or progesterone). The ethical review boards of the participating institutions/countries/study centres and the International Agency for Research on Cancer (IARC) approved each of the two phases of the study.

Hormone concentrations. For all women in "study phase 1", hormone measurements of estradiol (pmol/L), testosterone (nmol/L) and SHBG (nmol/L) were performed at IARC, while for "study phase 2" they were performed at the German Cancer Research Center (DKFZ). The same assay methods were used whenever possible in the two phases of the study, as detailed elsewhere (16,28). Cases and their matched controls were analysed within the same analytical batch and laboratory technicians were blinded to the case–control status of the study participants. Serum concentrations of free estradiol (pmol/L) and free testosterone (nmol/L) were computed from mass action law equations using absolute concentrations of each sex steroid and SHBG assuming a constant concentration of 43g/L for albumin(28,30,31).

Statistical analyses. In all analyses, baseline alcohol intake (g/day), sex hormones and SHBG levels were log-transformed to normalize their distributions (ln(alcohol+1)). In addition, for the sex hormones and SHBG, residuals on centre were computed to account for variability

that lie in phase of study, distribution across batches of each sex steroid, and the differences of study protocols for sample collection and preparation including treatment and sample handling e.g. thaw-freeze cycles. The residuals were calculated for each biomarker in univariate linear regression models. Geometric means for sex steroids and SHBG in alcohol consumers as well as 2.5^{th} and 97.5^{th} quantiles were computed for cases and controls by study phase. The difference in hormone and SHBG levels between cases and controls was assessed through two-sample t-tests computed on the log-transformed concentrations.

Partial Least Square (PLS) analysis. With the aim of deriving a hormonal signature associated with alcohol intake, we applied PLS analysis, a multivariate dimension-reduction method that generalises features of principal component analysis with those of multiple linear regression (32). The mathematical and computational details of the PLS method have been thoroughly described in our previous studies within EPIC (33,34). In brief, one PLS factor was retained after performing PLS analysis only amongst controls in the subset of alcohol consumers and a linear combination of the response variables (i.e. estradiol, testosterone and SHBG), was extracted that had maximum covariance with the predictor variable (alcohol intake). Using the loadings derived from the analysis, i.e. the coefficients quantifying the contribution of each hormone to the PLS factor, the PLS score was computed and subsequently extrapolated to the cases. The score was tested as a composite mediator in the alcohol-BC association using mediation analysis as described below. Similarly, a PLS sensitivity analysis was performed including free fractions of estradiol and testosterone from the response set as both these free fractions were computed from SHBG and estradiol or testosterone. The PLS score was calculated as formerly detailed and successively used in a mediation analysis. As the results were virtually unchanged, we did not report the results for this sensitivity analysis.

Alcohol and BC association. The association between alcohol intake and BC risk was first evaluated in multivariable conditional logistic regression models within the total nested case-control study. Since no statistically significant association was found for a 1-SD higher log-transformed alcohol intake and risk of overall BC, ER+ and ER- tumours (**Table 2**), the investigation was restricted to the case-sets of alcohol consumers.

Mediation analysis. The mediating role of each sex steroids and SHBG (mediator) in the association between alcohol consumption (exposure) and BC (outcome) was examined and mediating effects were assessed separately for each of the considered mediators, and for their composite signature, among alcohol consumers. Estimates of the natural direct effect (NDE), the natural indirect effect (NIE), the total effect (TE) as well as the effect of the mediator adjusted for alcohol, i.e. the exposure, and for confounding variables were computed using a counterfactual approach adapted to dichotomous outcomes (35). Formulae from VanderWeele and Vansteelandt(36) were adapted to accommodate continuous exposures and use of conditional logistic regression models for our matched study design. In brief, two models were specified to obtain NDE and NIE and the odds ratio for the mediator effect adjusted for the exposure. In the outcome model, the exposure and the mediator were related to the BC indicator in a conditional logistic regression. In models for each mediator of interest, the mediator was linearly regressed on the exposure. This was done only on the subset of controls to account for the nested case-control design (37). The total effect was obtained from a conditional logistic regression relating alcohol intake to BC risk. The formulae detailing how to obtain estimates and their associated 95%CI and p-value as well as the notations used have been extensively detailed in our previous work (33). Assuming the outcome was rare, we computed the proportion mediated which is measure defined on the risk difference scale and captures the importance of the mediating pathway (37). Based on the estimated odds ratios, this quantity was calculated using the following formula: $\frac{NDE*(NIE-1)}{(NDE*NIE-1)}$ (36). Since mediation

analysis was applied to the nested case-control study restricted to alcohol consumers, the interpretation of the causal effects is for an increase of one standard deviation in the exposure (log transformed alcohol intake) among alcohol consumers. All models were adjusted for a list of potential confounders including body mass index (BMI, continuous), age at menopause (continuous), and the following categorical variables: smoking status (never, former, current, unknown), education level (none, primary school, technical/professional school, secondary school, longer education including university degree, unknown/unspecified), physical activity index (inactive, moderately inactive, moderately active, active, unknown), use of menopausal hormone therapy (ever vs. never), use of contraceptive pill (ever vs. never), age at first full-term pregnancy (nulliparous, <23 years, 24-25 years, 26-28 years, >29 years), number of full term pregnancies (nulliparous, 1 full-term birth, 2 full-term births, 3 full-term births, 4 or more full-term births), and age at menarche (<12 years, 12 years, 13 years, 14 years, >14 years). The mediator model was additionally adjusted for study phase (phase 1 vs. phase 2). Interactions between the exposure and each of the confounders were tested both in the outcome model and in the mediator model among controls; with an additional test for exposure-mediator interaction in the former and a term testing interaction between exposure and age at blood collection in the latter. None of the interactions were statistically significant and therefore were not included the final mediation analyses.

All statistical tests were two-sided, p-values below 0.05 were considered statistically significant. All analyses were performed using the R statistical software, with the package 'plsgenomics' used for PLS analysis and mediation computed with an in-house macro.

Results

The study population characteristics by case-control status are presented in **Table 1** for the case-sets of alcohol consumers that were examined in this study. Overall, cases had a higher average alcohol intake compared with controls (11.3 vs. 9.5g/day) and a higher total energy

intake (1970 vs. 1919 kcal/day). **Supplemental Table 1** shows the characteristics of the whole population of the nested case-control study. Hormone concentration levels for the study population at baseline restricted to drinker case-sets are shown in **Supplemental Table 2** by study phase. Concentrations of testosterone and free testosterone were significantly higher in cases than in controls in "study phase 2", whereas concentrations of estradiol and its free fraction were significantly higher in cases than in controls in "study phase 1". Additionally, the concentration values for estradiol were on average higher in "study phase 1" than in "study phase 2" (respectively 99.1 and 89.2 for cases and controls vs. 45.0 and 41.7) likely due to differences in assays between phases.

In our final study population, alcohol intake was statistically significantly associated with higher BC risk with TE OR=1.17(1.01,1.35) for a 1-SD higher log-transformed alcohol intake (**Table 2**). The association was stronger in ER+ tumours with TE OR=1.36(1.08,1.70, n cases=218). There was no association found for ER- BC (TE OR=1.29(0.87, 1.91, n cases=105)). A positive association with BC risk overall and for ER+ BC was observed for 1-SD increase in log-transformed hormones with OR=1.38(1.12,1.70) and 1.83(1.28,2.63) for estradiol, 1.32(1.08,1.61) and 1.51(1.10,2.08) for free estradiol and 1.21(1.03,1.41) and 1.45(1.13,1.87) for testosterone, respectively. There was an inverse association between SHBG and ER- BC risk (**Supplemental Table 3**).

Results from individual mediation analyses are presented in **Table 3** with estimates for the direct and indirect effects. In the ER+ subset, the NDE estimates for the direct association of alcohol with BC risk considering estradiol, free estradiol, testosterone, free testosterone and SHBG as the mediator, were statistically significant with NDE=1.35(1.07,1.70) 1.34(1.07,1.69), 1.30(1.02,1.64), 1.33(1.05,1.68) and 1.35(1.08,1.70), respectively (**Table 3**). None of the NIE estimates were statistically significant suggesting that the four sex steroids and SHBG did not mediate the alcohol and BC association individually (**Table 3**). However,

the NIE was borderline significant for free estradiol (1.03 (1.00,1.06)) suggesting a weak mediation by free estradiol corresponding to a mediated proportion of 19%.

PLS analysis provided a composite signature of estradiol, testosterone and SHBG, as the first PLS factor, with positive loadings for estradiol (0.007) and testosterone (0.070) and a high negative loading for SHBG (-0.141) (**Supplemental Table 4**). A 1-SD increase in the PLS score was associated with a higher BC risk with OR=1.23 (1.05,1.43) and statistically significantly mediated the association between alcohol intake and overall BC risk with NIE=1.04(1.01,1.07), accounting for a mediated proportion of 24% of the total effect (**Table 4**). For ER+ and ER- BC subtypes, the hormonal signature did not mediate the alcohol-BC association as the NIE, corresponding to a proportion mediated of 12 and 36%, were not statistically significant. The identified signature was however associated with high ER- risk with OR=1.69(1.03,2.69) (**Table 4**).

Discussion

In this study restricted to alcohol consumers, a candidate mechanism of the association between alcohol intake and postmenopausal BC development was investigated with mediation analysis. Overall, there was limited evidence that this association was mediated by individual sex-hormone levels with a weak mediation by free estradiol, however, a composite score summarizing information from the individual hormones and SHBG showed that 24% of the relationship between alcohol and BC risk is mediated by a hormonal signature negatively associated with SHBG and positively related to sex steroids.

Alcohol is an established risk factor for BC (4,9), both in pre- and postmenopausal women (6,38). Evidence from a reanalysis of 53 epidemiological studies suggested that the relative risk of BC increased linearly by 7% for each additional 10 g/day intake of alcohol (unit of alcohol as defined by WHO)(39). A dose response association was observed in EPIC,

irrespective of beverage type, with a higher risk attaining 25% (17-35%) for the highest intakes compared to moderate alcohol use (from 0.1 to 5 g/day) (10).

Despite this, the biological pathways that link alcohol with BC development are not well delineated. Hormones and SHBG are involved in complex biological pathways that regulate a host of metabolic functions (20,40). It had previously been suggested that sex-hormones could be involved in the underlying mechanism of the alcohol and BC association (41–43). Several controlled feeding studies (44,45) and observational studies (14,17,19,46) reported associations between alcohol intake and increased sex-hormone blood concentrations in both pre- and postmenopausal women. Compared with non-drinkers, concentrations of estrone, estradiol, and dehydroepiandrosterone sulfate (DHEAS) were higher in women consuming more than 25 g/day of alcohol in a cross-sectional study in EPIC(14). Findings of similar magnitude were reported in a study of 1,291 postmenopausal controls from a nested case-control study in EPIC (19) with 10-20% larger levels of testosterone and free testosterone and 15% lower SHBG concentrations in alcohol consumers compared with non-drinkers. Similar associations were reported in a recent cross-sectional analysis examining BC risk factors including alcohol and circulating sex hormones measured in over 6,000 postmenopausal controls from 13 prospective studies (17).

The current study is the second of its kind to explore statistical mediation by sex steroids of the alcohol-BC relation. Previously, the mediating role of estradiol was examined in a case-cohort study with 600 cases (of which 401 ER+ and 163 ER-) in the Women's Health Initiative, where no indirect effect was observed suggesting no evidence for alcohol effect through estradiol, although a significant association between alcohol and BC risk overall and in ER+ tumours in postmenopausal women was reported (21). Our study had similar findings in terms of weak evidence of mediation through estradiol, and a strong association of alcohol intake with ER+ tumours. However, it expanded on the latter study by

exploring the mediating role of free estradiol, testosterone, free testosterone and SHBG in addition to a hormonal signature.

Strengths of this analysis include the use of harmonized standardised dietary questionnaires which were used to estimate alcohol at baseline. Further, we developed an alcohol-driven hormonal signature that was associated with BC risk and was robust to exclusion of free fractions of estradiol and testosterone from the PLS analysis. Our analyses focused on alcohol consumers, as baseline alcohol non-drinkers may be more health-conscious, may be former heavy drinkers or participants with underlying disease, thus potentially introducing concerns related to reverse causation and, particularly, exposure misclassification. The BC nested case-control study was relatively large in sample size, as it combined two successive rounds of acquisitions of sex steroids in EPIC.

Nevertheless, our study had limitations, amongst which the generally low alcohol intake of EPIC women, ~8 g/day on average with 80% below 15 g/d (10), but also among the participants of the BC nested case-control study ~10 g/day, which may limit the generalisability of our findings to populations with different alcohol consumption patterns. Another aspect pertains to a key assumption in mediation analysis, which requires a temporal ordering between exposure, mediator and outcome. In our study alcohol was assessed at baseline at the same time of biological samples' collection, estimating participants' alcohol intakes over the 12 months preceding enrolment, and endogenous hormones were measured in a single blood sample from each woman reflecting a limited time-frame. Although alcohol intake measurements indicated relatively high validity (24), and androgens, estrogens and SHBG concentrations in postmenopausal women show good reproducibility over time (47–49), both exposure and the mediators examined in this study may be subject to measurement errors. Under non-differential measurement error with a normally distributed mediator, the bias of the NIE is towards the null and if direct and indirect effects are in the same direction,

the bias of the NDE is away from the null (50). This may have contributed to an underestimation of the indirect effects and an overestimation of the direct effects in our study, resulting in a lower mediated proportion and possibly partially explaining the lack of mediation observed for sex steroids when examined separately. Lastly, in this study different radioimmunoassays were used to measure estradiol between phase 1 and phase 2 (28). For this reason, estradiol concentrations displayed between-studies variations, which we have tried to account for by adjusting for phase of study in the exposure-mediator models.

Conclusion

Our findings suggested that alcohol intake was associated with higher postmenopausal BC risk in alcohol consumers, overall and for ER+ tumours, with limited evidence of mediation by sex steroids, when examined individually. However, the hormonal signature mediated about 24% of the alcohol-BC association, suggesting that any potential mechanism of sex-steroids in the alcohol and BC relationship is likely to involve an interplay of hormones, beyond the action of single hormonal levels. Future replication of these findings is needed, possibly in populations with larger amounts of alcohol intake and larger sample size. Finally, our results suggest that sex hormones play a minor role in mediating the alcohol-BC relation and other, possibly unrecognized, pathways are likely involved.

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Tables

Table 1: Baseline characteristics of the study population of the EPIC nested case–control study on postmenopausal BC – casesets where both cases and controls are alcohol consumers at baseline (>0.1g/day).

Characteristics*	**Cases**	**Controls**
Number of subjects	430	645
Casesets with ER positive tumours	218	318
Casesets with ER negative tumours	105	126
Age at blood collection (y)	59.9 (50.5,71.3)	60.1 (50.9,71.4)
Height (cm)	162.0 (150.5,174.6)	161.4 (149.1,174.0)
Weight (kg)	69.0 (50.7,98.4)	66.6 (49.2,90.5)
BMI (kg/m2)	26.3 (19.7,37.1)	25.6 (19.3,35.5)
Total energy intake (kcal/day)	1970.4 (1117.6,3063.4)	1919.1 (1077.7,3095.9)
Alcohol intake at recruitment (g/day)	11.3 (0.20,44.9)	9.5 (0.20,42.5)
Age at menopause (y)	49.3 (38.0,57.0)	48.9 (36.2,56.9)
Years between blood donation and diagnosis (y)	3.7 (0.6,9.5)	-
Age at menarche		
<12 years	54 (12.6)	69 (10.7)
12 years	85 (18.9)	128 (19.8)
13 years	91 (21.2)	140 (21.7)
14 years	104 (24.2)	153 (23.7)
>14 years	86 (20.0)	145 (22.5)
Unknown	10 (2.3)	10 (1.6)
Age at first full term pregnancy		
Nulliparous	69 (16.0)	88 (13.6)
<23 years	118 (27.4)	198 (30.7)
24-25 years	62 (14.4)	115 (17.8)
26-28 years	96 (22.3)	141 (21.9)
>29 years	85 (19.8)	103 (16.0)
Use of contraceptive pill**		
Ever	175 (40.7)	269 (41.7)
Never	248 (57.7)	374 (58.0)
Unknown	7 (1.6)	2 (0.3)
Use of hormonal menopause therapy**		
Ever	87 (20.2)	142 (22.0)
Never	342 (79.6)	502 (77.8)
Unknown	1 (0.2)	1 (0.2)
Physical activity levels		
Active	94 (21.9)	134 (20.8)
Moderately active	71 (16.5)	123 (19.1)
Moderately inactive	162 (37.7)	221 (34.3)
Inactive	102 (23.7)	164 (25.4)
Unknown	1 (0.2)	3 (0.5)
Smoking Status		
Never	241 (56.0)	345 (53.5)
Former	116 (27.0)	175 (27.1)
Current smoker	71 (16.5)	121 (18.8)
Unknown	2 (0.5)	4 (0.6)
Fasting status at the time of blood collection		
No (<3h)	240 (55.8)	360 (55.8)
In between (3-6h)	86 (20.0)	116 (18.0)
Yes (>6h)	92 (21.4)	145 (22.5)
Unknown	12 (2.8)	24 (3.7)
Education level		
None	4 (0.9)	9 (1.4)
Primary school completed	158 (36.7)	238 (36.9)
Secondary school	69 (16.0)	107 (16.6)
Technical / Professional school	110 (25.6)	167 (25.9)
Longer education (including University degree)	58 (13.5)	72 (11.2)
Unknown	31 (7.2)	52 (8.1)

*Values are presented as means and 2.5th and 97.5th percentiles in parentheses for continuous variables and as frequencies and percentages in parentheses for categorical variables. ** Women included in the study were not using any form of exogenous hormones at recruitment.

Table 2: Odds ratio and 95% confidence intervals (95%CI) for the Total Effects (TE) of alcohol on postmenopausal BC for 1-SD increase in log-alcohol intake.

Tumour type	**N cases/ N controls**	**TE (95%CI)***
In total nested case-control study		
Overall BC	798/1294	1.03 (0.93,1.14)
ER positive	387/612	1.04 (0.89,1.21)
ER negative	153/193	1.07 (0.80,1.45)
In nested case-control study restricted to alcohol consumers case-sets (>0.1g/day)		
Overall BC	430/645	**1.17 (1.01,1.35)**
ER positive	218/318	**1.36 (1.08,1.70)**
ER negative	105/126	1.29 (0.87,1.91)

*****Statistically significant TE are displayed in bold font.

Table 3: Results from the mediation analyses in case-sets of alcohol consumers (>0.1g/day), with ORs[*] and their associated 95% CIs for the Natural Direct Effect (NDE) and the Natural Indirect Effect (NIE) using residuals based on Centre for the log-transformed hormone levels.

Hormone	NDE (95%CI)	NIE (95%CI)	% mediated – RD scale[†]
Overall BC			
Estradiol	1.15 (1.00,1.33)	1.02 (0.99,1.04)	13
Free Estradiol	1.15 (0.99,1.33)	1.03 (1.00,1.06)	19
Testosterone	1.12 (0.96,1.30)	1.02 (1.00,1.04)	16
Free Testosterone	1.13 (0.98,1.31)	1.01 (0.99,1.03)	8
SHBG	**1.16 (1.00,1.34)**	1.02 (0.99,1.05)	13
ER positive			
Estradiol	**1.35 (1.07,1.70)**	1.06 (0.98,1.14)	19
Free Estradiol	**1.34 (1.07,1.69)**	1.06 (0.99,1.13)	19
Testosterone	**1.30 (1.02,1.64)**	1.00 (0.95,1.05)	0
Free Testosterone	**1.33 (1.05,1.68)**	1.00 (0.98,1.01)	0
SHBG	**1.35 (1.08,1.70)**	1.00 (0.96,1.05)	0
ER negative			
Estradiol	1.31 (0.88, 1.95)	1.01 (0.97,1.06)	4
Free Estradiol	1.31 (0.86,2.00)	1.03 (0.95,1.10)	11
Testosterone	1.25 (0.83,1.87)	1.01 (0.98,1.04)	5
Free Testosterone	1.18 (0.76,1.82)	1.04 (0.96,1.13)	21
SHBG	1.19 (0.77,1.84)	1.08 (0.97,1.22)	33

[*] In the mediation analysis, the exposure was the log-transformed alcohol at baseline, the mediator was in turn each one of the log-transformed hormones (residuals on Centre), and the outcome was postmenopausal BC (subtypes listed above).The outcome models were computed through conditional logistic regressions. The mediator models were linear and additionally adjusted for phase of study. All models were adjusted for BMI (continuous), age at menopause (cont.), smoking status (categorical), education level (cat.), physical activity index (cat.), use of exogenous hormones (ever vs. never), use of pill (ever vs. never), number of full term pregnancies (cat.), age at full term pregnancy (cat.) and age at menarche (cat.). Cases and controls were matched on study recruitment centre, age at blood collection (±6months), time of the day at blood collection (±1 hour), fasting status (<3h, 3-6 h, >6h) and study phase (1 or 2).
[†] NDE and NIE, their 95% CIs and proportion mediated on the risk difference (RD) scale are computed from formulae as detailed in Materials and Methods. ORs are expressed for an increase in one standard deviation of the residuals on Centre of the log-transformed hormone variable. The NDE and NIE are expressed for an increase in one standard deviation of the log-transformed alcohol intake.

Bold font indicating statistically significant findings.

Table 4: Results from the mediation analyses: OR for the association between the PLS factor and postmenopausal BC and NDE and NIE and their respective 95%CI.

	OR (95%CI)	**NDE (95%CI)**	**NIE (95%CI)**	**% mediated – RD scale†**
Overall BC	**1.23 (1.05,1.43)**	1.14 (0.98,1.32)	**1.04 (1.01,1.07)**	24
ER positive	1.20 (0.95,1.51)	**1.33 (1.06,1.67)**	1.04 (0.99,1.09)	12
ER negative	**1.67 (1.03,2.69)**	1.15 (0.75,1.75)	1.07 (0.97,1.19)	36

* In the mediation analysis, the exposure was the log-transformed alcohol at baseline, the mediator was the log-transformed (residuals on Centre), and the outcome was postmenopausal BC (subtypes listed above).The outcome models were computed through conditional logistic regressions. The mediator models were linear and additionally adjusted for phase of study. All models were adjusted for BMI (continuous), age at menopause (cont.), smoking status (categorical), education level (cat.), physical activity index (cat.), use of exogenous hormones (ever vs. never), use of pill (ever vs. never), number of full term pregnancies (cat.), age at full term pregnancy (cat.) and age at menarche (cat.). Cases and controls were matched on study recruitment centre, age at blood collection (±6months), time of the day at blood collection (±1 hour), fasting status (<3h, 3-6 h, >6h) and study phase (1 or 2).
† NDE and NIE, their 95% CIs and proportion mediated on the risk difference (RD) scale are computed from formulae, see Materials and Methods. ORs are expressed for an increase in one standard deviation of the PLS hormonal signature score. The NDE and NIE are expressed for an increase in one standard deviation of the log-transformed alcohol intake.

Bold font indicating statistically significant findings.