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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1766505> since 2021-01-12T21:40:32Z

Published version:

DOI:10.1002/ijc.32324

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This is the peer reviewed version of the following article:

Assi N, Rinaldi S, Viallon V, et al. Mediation analysis of the alcohol-postmenopausal breast cancer relationship by sex hormones in the EPIC cohort. *Int J Cancer* (2019)

which has been published in final form at: <https://doi.org/10.1002/ijc.32324>

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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.

Word count: 3,949 after revision.

Table count: 4 tables and 4 supplemental tables after revision.

Conflict of Interests Statement

The authors declare no potential conflicts of interest.

Sources of Support:

N. Assi was financially supported by a grant from the Fondation de France (FdF) supporting her postdoctoral research (grant number: 00069254). T. Key was funded through a grant from Cancer Research UK (grant number: CRUK C8221/A19170). The steroids measurements used in this study were (partly) funded by a grant from the German Research Foundation, Graduiertenkolleg 793: Epidemiology of communicable and chronic non-communicable diseases and their interrelationships and through financial support from the National Cancer Institute (USA) (grant no. 1U01CA98216-01). The coordination of EPIC is financially supported by the European Commission (DG-SANCO) and the International Agency for Research on Cancer. The national cohorts are supported by Danish Cancer Society (Denmark); Ligue Contre le Cancer, Institut Gustave Roussy, Mutuelle Générale de l'Éducation Nationale, Institut National de la Santé et de la Recherche Médicale (INSERM) (France); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum and Federal Ministry of Education and Research (Germany); the Hellenic Health Foundation (Greece); Associazione Italiana per la Ricerca sul Cancro-AIRC-Italy and National Research Council (Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS), Netherlands Cancer Registry (NKR), LK Research Funds, Dutch Prevention Funds, Dutch ZON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF), Statistics Netherlands (The Netherlands); Nordic Centre of Excellence programme on Food, Nutrition and Health. (Norway); Health Research

Fund (FIS), PI13/00061 to Granada), Regional Governments of Andalucía, Asturias, Basque Country, Murcia (no. 6236) and Navarra, ISCIII RETIC (RD06/0020) (Spain); Swedish Cancer Society, Swedish Scientific Council and County Councils of Skåne and Västerbotten (Sweden); Cancer Research UK (14136 to EPIC-Norfolk; C570/A16491 and C8221/A19170 to EPIC-Oxford), Medical Research Council (1000143 to EPIC-Norfolk and MR/M012190/1 to EPIC-Oxford) (United Kingdom).

This study was done independently and with no input from the funders. The funders were not involved in the design, implementation, analysis or interpretation of the data.

"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.

1 **Abstract** (250 words unstructured)

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

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

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

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

24

- 25 **Keywords:** sex steroids, alcohol, breast cancer, mediation analysis, hormonal signature,
- 26 EPIC.

27 **Introduction**

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

38 A positive dose-response association between alcohol intake and BC risk, consistently
39 across hormonal receptor status, was shown in a study based on 11,576 incident BC cases
40 within the European Prospective Investigation into Cancer and nutrition (EPIC) cohort (10).
41 Little is known on the mechanisms through which alcohol exerts its carcinogenic effect
42 during BC development, yet accumulating evidence suggests that the association between
43 alcohol intake and breast carcinogenesis may be partly mediated through endogenous sex
44 steroids (11–15). Estrogens and androgens are well-known activators of cellular proliferation,
45 and are associated with an increased BC risk (15). Findings from the EPIC study and the
46 Endogenous Hormones and Breast Cancer Collaborative Group supported the association
47 between elevated pre-diagnostic serum concentrations of oestrogens, androgens and low
48 serum levels of sex hormone binding globulin (SHBG) and higher postmenopausal BC risk
49 (16–18). It has been suggested that alcohol consumption increases the concentrations of sex
50 steroids in serum in both pre- and post-menopausal women (15). In EPIC, higher
51 concentrations of androgens including testosterone and free testosterone, and lower

52 concentrations of SHBG were observed in postmenopausal women who consumed more than
53 25 g/day of alcohol (i.e. 2 glasses) compared with women who were non consumers (19). A
54 review suggested that estrogens could mediate the relationship between alcohol and BC as
55 alcohol elevates concentrations of circulating oestrogens (15). In postmenopausal women,
56 nearly 100% of estrogens are synthesized from aromatization of androgens in peripheral
57 tissues, with SHBG regulating their circulating concentrations and that of their free fractions
58 (20).

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

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

65

66 **Material and Methods**

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

76 (freezers, -80°C) where samples were stored locally. All participants gave their written
77 informed consent to use their questionnaire data and biological samples for future analyses.

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

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

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

108 *The nested case-control study.* The current study is based upon data available from two nested
109 case-control studies within EPIC on postmenopausal BC risk and endogenous hormone levels
110 (“study phase 1” (27) and “study phase 2” (28)). Norway and Sweden were not included in
111 these analyses either because a blood serum sample was not available or because independent
112 studies were being completed on BC risk. In both study phases, postmenopausal women were
113 included. Postmenopausal women were defined as women who had no menstruations in the
114 12 months preceding study enrolment, or were older than 55 years of age if the menstrual
115 cycle information was not available, or who had undergone a bilateral oophorectomy. Only
116 women with available blood samples who were not using any menopausal hormone therapy at
117 the time of blood collection (as the use of exogenous hormones influences the endogenous
118 concentrations and some may be the same as endogenous), and who did not have any
119 prevalent cancer at baseline (with the exception of non-melanoma skin cancer) were included
120 into the study. For each case, up to two controls with a blood sample available were chosen at
121 random among appropriate risk sets consisting of all postmenopausal cohort members alive
122 and free of cancer at the time of diagnosis of the case. This was done using an incidence
123 density sampling protocol allowing the inclusion of subjects who became a case later in time,
124 while each control could be sampled more than once (28). Controls were matched to the cases
125 on study recruitment centre, age at blood donation (\pm 6 months), follow-up time since blood

126 donation (\pm 3 months), time of the day of blood collection (\pm 1 hour), and fasting status ($<$ 3
127 hours, 3–6 hours, $>$ 6 hours). Analyses were also conducted stratifying on estrogen receptor
128 (ER) status. Over the two study phases, there were 798 BC and 1294 matched controls with
129 387 cases being oestrogen receptor positive (ER+) tumours, 153 oestrogen negative tumours
130 (ER-) and 258 with unknown hormonal receptor status (estrogen and/or progesterone). After
131 excluding case-sets in which the case or her control(s) were non-drinkers (daily intake
132 $<$ 0.1g/day), the final study sample included 430 cases and 645 matched controls with 218
133 ER+ (62% from “study phase 1” and 38% from “study phase 2”), 105 ER- (27% from “study
134 phase 1” and 73% from “study phase 2”) and 107 with unknown hormonal receptor status
135 (estrogen and/or progesterone). The ethical review boards of the participating
136 institutions/countries/study centres and the International Agency for Research on Cancer
137 (IARC) approved each of the two phases of the study.

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

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

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

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

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

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

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

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

221 **Results**

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

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

234 In our final study population, alcohol intake was statistically significantly associated
235 with higher BC risk with TE OR=1.17(1.01,1.35) for a 1-SD higher log-transformed alcohol
236 intake (**Table 2**). The association was stronger in ER+ tumours with TE OR=1.36(1.08,1.70,
237 n cases=218). There was no association found for ER- BC (TE OR=1.29(0.87, 1.91, n
238 cases=105)). A positive association with BC risk overall and for ER+ BC was observed for 1-
239 SD increase in log-transformed hormones with OR=1.38(1.12,1.70) and 1.83(1.28,2.63) for
240 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
241 1.45(1.13,1.87) for testosterone, respectively. There was an inverse association between
242 SHBG and ER- BC risk (**Supplemental Table 3**).

243 Results from individual mediation analyses are presented in **Table 3** with estimates for
244 the direct and indirect effects. In the ER+ subset, the NDE estimates for the direct association
245 of alcohol with BC risk considering estradiol, free estradiol, testosterone, free testosterone
246 and SHBG as the mediator, were statistically significant with NDE=1.35(1.07,1.70)
247 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**).
248 None of the NIE estimates were statistically significant suggesting that the four sex steroids
249 and SHBG did not mediate the alcohol and BC association individually (**Table 3**). However,

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

252 PLS analysis provided a composite signature of estradiol, testosterone and SHBG, as
253 the first PLS factor, with positive loadings for estradiol (0.007) and testosterone (0.070) and a
254 high negative loading for SHBG (-0.141) (**Supplemental Table 4**). A 1-SD increase in the
255 PLS score was associated with a higher BC risk with OR=1.23 (1.05,1.43) and statistically
256 significantly mediated the association between alcohol intake and overall BC risk with
257 NIE=1.04(1.01,1.07), accounting for a mediated proportion of 24% of the total effect (**Table**
258 **4**). For ER+ and ER- BC subtypes, the hormonal signature did not mediate the alcohol-BC
259 association as the NIE, corresponding to a proportion mediated of 12 and 36%, were not
260 statistically significant. The identified signature was however associated with high ER- risk
261 with OR=1.69(1.03,2.69) (**Table 4**).

262 **Discussion**

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

270 Alcohol is an established risk factor for BC (4,9), both in pre- and postmenopausal women
271 (6,38). Evidence from a reanalysis of 53 epidemiological studies suggested that the relative
272 risk of BC increased linearly by 7% for each additional 10 g/day intake of alcohol (unit of
273 alcohol as defined by WHO)(39). A dose response association was observed in EPIC,

274 irrespective of beverage type, with a higher risk attaining 25% (17-35%) for the highest
275 intakes compared to moderate alcohol use (from 0.1 to 5 g/day) (10).
276 Despite this, the biological pathways that link alcohol with BC development are not well
277 delineated. Hormones and SHBG are involved in complex biological pathways that regulate a
278 host of metabolic functions (20,40). It had previously been suggested that sex-hormones
279 could be involved in the underlying mechanism of the alcohol and BC association (41–43).
280 Several controlled feeding studies (44,45) and observational studies (14,17,19,46) reported
281 associations between alcohol intake and increased sex-hormone blood concentrations in both
282 pre- and postmenopausal women. Compared with non-drinkers, concentrations of estrone,
283 estradiol, and dehydroepiandrosterone sulfate (DHEAS) were higher in women consuming
284 more than 25 g/day of alcohol in a cross-sectional study in EPIC(14). Findings of similar
285 magnitude were reported in a study of 1,291 postmenopausal controls from a nested case-
286 control study in EPIC (19) with 10-20% larger levels of testosterone and free testosterone and
287 15% lower SHBG concentrations in alcohol consumers compared with non-drinkers. Similar
288 associations were reported in a recent cross-sectional analysis examining BC risk factors
289 including alcohol and circulating sex hormones measured in over 6,000 postmenopausal
290 controls from 13 prospective studies (17).

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

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

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

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

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

331 *Conclusion*

332 Our findings suggested that alcohol intake was associated with higher postmenopausal BC
333 risk in alcohol consumers, overall and for ER+ tumours, with limited evidence of mediation
334 by sex steroids, when examined individually. However, the hormonal signature mediated
335 about 24% of the alcohol-BC association, suggesting that any potential mechanism of sex-
336 steroids in the alcohol and BC relationship is likely to involve an interplay of hormones,
337 beyond the action of single hormonal levels. Future replication of these findings is needed,
338 possibly in populations with larger amounts of alcohol intake and larger sample size. Finally,
339 our results suggest that sex hormones play a minor role in mediating the alcohol-BC relation
340 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/m ²)	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)
	12 years	85 (18.9)
	13 years	91 (21.2)
	14 years	104 (24.2)
	>14 years	86 (20.0)
	Unknown	10 (2.3)
Age at first full term pregnancy		
	Nulliparous	69 (16.0)
	<23 years	118 (27.4)
	24-25 years	62 (14.4)
	26-28 years	96 (22.3)
	>29 years	85 (19.8)
Use of contraceptive pill**		
	Ever	175 (40.7)
	Never	248 (57.7)
	Unknown	7 (1.6)
Use of hormonal menopause therapy**		
	Ever	87 (20.2)
	Never	342 (79.6)
	Unknown	1 (0.2)
Physical activity levels		
	Active	94 (21.9)
	Moderately active	71 (16.5)
	Moderately inactive	162 (37.7)
	Inactive	102 (23.7)
	Unknown	1 (0.2)
Smoking Status		
	Never	241 (56.0)
	Former	116 (27.0)
	Current smoker	71 (16.5)
	Unknown	2 (0.5)
Fasting status at the time of blood collection		
	No (<3h)	240 (55.8)
	In between (3-6h)	86 (20.0)
	Yes (>6h)	92 (21.4)
	Unknown	12 (2.8)
Education level		
	None	4 (0.9)
	Primary school completed	158 (36.7)
	Secondary school	69 (16.0)
	Technical / Professional school	110 (25.6)
	Longer education (including University degree)	58 (13.5)
	Unknown	31 (7.2)

*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)*
<i>In total nested case-control study</i>		
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)
<i>In nested case-control study restricted to alcohol consumers case-sets (>0.1g/day)</i>		
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 (± 6 months), 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 (± 6 months), 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.