

Research Article

Postmenopausal Serum Sex Steroids and Risk of Hormone Receptor-Positive and -Negative Breast Cancer: a Nested Case-Control Study

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Abstract

Prediagnostic endogenous sex steroid hormone levels have well established associations with overall risk of breast cancer. While evidence toward the existence of distinct subtypes of breast cancer accumulates, few studies have investigated the associations of sex steroid hormone levels with risk of hormone receptor [estrogen receptor (ER) and/or progesterone receptor (PR)] defined breast cancer. In a case-control study nested within the EPIC cohort (European Prospective Investigation into Cancer and Nutrition), estradiol, testosterone, and sex hormone-binding globulin levels were measured in prediagnostic serum samples from postmenopausal women not using hormone replacement therapy at blood donation. A total of 554 women who developed invasive breast cancer with information on receptor status were matched with 821 control subjects. Conditional logistic regression models estimated breast cancer risk with hormone concentrations according to hormone receptor status of the tumor. Sex steroid hormones were associated with risks of not only ER+PR+ breast cancer [estradiol OR for highest vs. lowest tertile = 2.91 (95% CI: 1.62–5.23), $P_{\text{trend}} = 0.002$; testosterone OR = 2.27 (95% CI: 1.35–3.81), $P_{\text{trend}} = 0.002$] but also of ER-PR- breast cancer [estradiol OR = 2.11 (95% CI: 1.00–4.46), $P_{\text{trend}} = 0.05$; testosterone OR = 2.06 (95% CI: 0.95–4.46), $P_{\text{trend}} = 0.03$], with associations appearing somewhat stronger in the receptor-positive disease. Serum androgens and estrogens are associated with risks of both hormone receptor-negative as well as receptor-positive breast tumors. Further research is needed to establish through which molecular pathways, and during which evolutionary stages of development, androgens and estrogens can promote the occurrence of both receptor-positive and -negative clinical breast tumors. *Cancer Prev Res*; 4(10); 1626–35. ©2011 AACR.

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Introduction

Breast cancer is a complex and heterogeneous disease, with a variety of histopathologic and molecular subforms that have diverse clinical outcomes and risk factors (1, 2). One important classification of clinical breast tumors into subtypes is based on the presence or absence of estrogen receptor (ER) and progesterone receptor (PR), as well as HER2 receptors, and the routine identification of these receptors currently guides targeted therapies and provides important prognostic information (2, 3). The presence or absence of hormone receptors also broadly corresponds to more detailed molecular subclassification of breast tumors, as determined by microarray-based gene expression profiling coupled to hierarchical clustering analyses (4–6). ER-positive disease accounts for about 80% and PR positive for about 65% of breast cancer cases (7, 8).

Epidemiologic studies have shown that risk factors associated with increased lifetime exposures to estrogen such as early age at menarche, late age at menopause, postmenopausal hormone replacement therapy (HRT), and postmenopausal adiposity levels are associated with hormone receptor–positive breast cancer particularly, more than with risk of ER-negative tumors (2). Although the associations of endogenous sex hormone levels with the risk of breast cancer overall, all subtypes combined, are well established (9, 10), only few prospective studies have thus far investigated this association by hormone receptor status (2, 11–15). In the latter studies, estradiol and testosterone levels have shown direct relationships with ER-positive, PR-positive, and joint ER+PR+ tumors (14, 15). Meanwhile, prospective studies investigating associations with hormone receptor–negative or HER2-positive breast cancer have been limited by relatively small numbers of ER-negative tumors (14, 15).

Previously, the relationship between postmenopausal sex steroid hormone and sex hormone binding–globulin (SHBG) levels with breast cancer risk overall was investigated in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (10). The current analysis expands on this nested case–control study with additional breast cancer cases, with a particular focus on receptor status and with oversampling of ER-negative cases. To our knowledge, this is the largest prospective study to date to investigate the association of estradiol, testosterone, their non-SHBG bound fractions, and SHBG with the risk of breast cancer by hormone receptor status, with a total of 172 ER-negative and 382 ER-positive cases.

Materials and Methods

EPIC is a multicenter prospective cohort study designed to investigate the relationships between diet, nutrition and metabolic factors, and cancer, consisting of 366,521 women and 153,457 men aged mostly between 25 to 70 years (16, 17). All participants were enrolled between 1992 and 2000 from 23 centers in 10 European countries: Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, The Netherlands, and the United Kingdom.

Baseline anthropometric measurements and questionnaire data on habitual diet, reproductive and menstrual history, exogenous hormone use [oral contraceptive (OC) and HRT use], medical history, lifetime smoking and alcohol consumption history, occupation, level of education, and physical activity were collected. Blood samples were also collected for most participants. Details about the standardized procedures for recruitment, measuring baseline anthropometry (height, weight, waist, and hip circumferences), questionnaires and biological sample collection at study centers are given elsewhere (16, 17). Sweden and Norway were not included in the current analysis because independent studies were being completed on breast cancer risk, or because a blood serum sample was not available. All subjects gave written informed consent to use their questionnaire data and future analyses of their blood samples. The Internal Review Boards of International Agency for Research on Cancer (IARC) and all EPIC recruitment centers approved the analyses of serum samples.

Blood samples were collected according to standardized protocols. From each subject, 30 mL of blood was drawn and after centrifugation, blood fractions (serum, plasma, buffy coat, and red blood cells) were aliquoted in 28 plastic straws of 0.5 mL each (12 plasma, 8 serum, 4 erythrocytes, and 4 buffy coat for DNA), which were heat sealed and stored under liquid nitrogen (-196°C). Half of the 28 aliquots were stored locally and the other half centrally at the IARC, with the exception of Denmark blood samples which were stored locally in 1 mL tubes at -150°C .

In all countries (except for France, Greece, and Germany) incident breast cancer cases were identified using a combination of methods employing record linkage with cancer and pathology registries. Vital status was collected from regional or national mortality registries. In Greece, Germany, and France, active follow up of cancer was through health insurance records and direct contact of participants and their next of kin was used. Self-reported breast cancer cases were all systematically verified from clinical and pathologic records. The closure date for this follow-up period was the date of last complete follow-up for both cancer incidence and vital status, which ranged between 2003 and 2006, depending on the center. Cancer incidence data were classified according to International Classification of Diseases 10th Revision (ICD-10).

Information on receptor status of participants, as well as the available laboratory methods and quantification descriptions used to determine receptor status was collected by 20 centers using the same techniques to collect incident breast cancer cases. At the time of this study, insufficient information on HER2 status ($n = 37$) had been collected to be included in the current analysis. To standardize the quantification of receptor status received from the EPIC centers, the following criteria for a positive receptor status were used; $\geq 10\%$ cells stained, any "plus-system" description, ≥ 20 fmol/mg, an Allred score of ≥ 3 , an IRS ≥ 2 , or an H-score ≥ 10 (18–22).

The present analysis was based upon postmenopausal female participants with a blood sample, after a priori

excluding women with prevalent cancer at any organ site (except nonmelanoma skin cancer) at baseline examination. Women were considered postmenopausal at the time of blood collection if they had had no menstrual cycles in the last 12 months, were older than 55 years (if the menstrual cycle history was missing), or had reported a bilateral oophorectomy. All women included in this analysis were not using HRT at the time of blood donation.

This study expands on a previous case-control study nested within EPIC on postmenopausal breast cancer risk and endogenous hormone concentrations (10). Additional cases were women who subsequently developed breast cancer after blood donation and before the end of the study period. For each case subject, up to 2 control subjects with a blood sample were chosen at random among appropriate risk sets consisting of all cohort members alive and free of cancer at the time of diagnosis of the index case. An incidence density sampling protocol was used, such that controls could include subjects who became a case later in time, while each control could be sampled more than once. The matching criteria were the study recruitment center, age at blood donation (± 6 months), time of the day of blood collection (± 1 hour), and fasting status (<3 hours, 3–6 hours, >6 hours).

Among the cases included in the study conducted in 2004 (10), only 49% had information on hormone receptors and were included in the present analysis (329 cases plus 596 matched controls; ref. 10); this part of our previous study is now referred to as "study phase 1." After completing a new round of follow-up in EPIC, all newly identified ER-negative cases, plus an equal number of ER-positive cases along with their matched controls were included (225 cases and 225 controls; this is now referred to as "study phase 2"). Overall, a total of 554 (382 ER-positive and 172 ER-negative) cases and 821 matched controls were thus included in this analysis.

Hormone assays in study phase 1 were carried out at IARC, whereas in study phase 2 hormone assays were done at the German Cancer Research Center (DKFZ), using the same assays whenever possible.

In study phase 1, estradiol concentrations were determined using a radioimmunoassay with a double-antibody system for the separation of free and bound antigen [Diagnostic Systems Laboratories Inc. (DSL)]. Because this assay was no longer produced by the company when study phase 2 started, estradiol was assayed using a similar double-antibody radioimmunoassay (DiaSorin). In both study phases, testosterone concentrations were measured with the same radioimmunoassay (Immunotech). A solid phase "sandwich" immunoradiometric assay (CIS Bio International) was used for the analysis of SHBG levels.

Serum samples were thawed once and all hormone measurements done on one day to avoid additional freeze-thaw cycles. Laboratory technicians were blinded to the case-control status of the study subjects. Cases and their individually matched controls were always analyzed within the same analytical batch.

Mean intrabatch coefficients of variation were 5.8% and 11.4% for estradiol, 10.8% and 8.2% for testosterone, and 8.0% and 3.1% for SHBG for study phases 1 and 2, respectively. Interbatch coefficients of variation were 13.1% and 13.4% for estradiol, 15.3% and 14.0% for testosterone, and 16.5% and 7.1% for SHBG for study phases 1 and 2, respectively. Serum concentrations of free testosterone and free estradiol (unbound to SHBG or albumin) were calculated from mass action equations using absolute concentrations of each steroid and SHBG, and assuming a constant serum albumin concentration of 43 g/L (23, 24).

In all analyses, levels of estradiol (pmol/L), free estradiol (pmol/L), testosterone (nmol/L), free testosterone (pmol/L), and SHBG (nmol/L) were \log_2 -transformed to normalize their variable distributions. Statistical significance of baseline case-control differences was evaluated using conditional logistic regression. Correlations between hormones and anthropometric indices, adjusting for age at blood donation and laboratory batch were calculated using Pearson's correlation coefficient. Statistical significance of case-control differences in geometric mean hormone levels were evaluated using paired *t* tests of case values versus the average of the two matched controls.

Conditional logistic regression models were used to estimate ORs and 95% CIs for breast cancer subclassified by single hormone receptor status (ER-positive/negative or PR-positive/negative) or joint hormone receptor status (ER+PR+, ER+PR-, and ER-PR-) at different serum hormone concentrations. There were too few breast cancer cases with the joint ER-PR+ receptor pattern ($n = 11$) to be considered as a separate outcome category. The risk associated with serum levels of the sex steroid hormones was examined both on the \log_2 continuous scale and in tertiles.

To statistically account for the differences observed between study phase 1 and study phase 2, tertile cut-points were based on the study phase-specific hormone distributions in control subjects.

Likelihood ratio tests were used to assess linear trends in ORs with increasing exposure level with assigned quantitative scores 1, 2, and 3 for the tertile categories. Heterogeneity between breast cancer subtypes was assessed using a log likelihood ratio test to assess conditional logistic regression models with and without interaction terms for breast cancer subtype outcome (ER-positive ER-negative, PR-positive, PR-negative or ER+PR+, ER+PR-, ER-PR-). Interaction terms were created by multiplying breast cancer subtype with the linear trend over the tertile score of hormone levels.

The effects of potential confounders (other than those accounted for by the matching criteria) were examined by adding regression terms into the logistic regression models. The categorical variables included age at menarche (<12 years, 12 years, 13 years, 14 years, and >14 years), age at first childbirth (nulliparous, <23 years, 24–25 years, 26–28 years, >29 years), number of full-term births (nulliparous, 1 full-term birth, 2 full-term births, 3 full-term births, and 4 or more full-term births), history of breastfeeding (ever

vs. never), OC (ever vs. never), hormone therapy use (ever vs. never), age at menopause, smoking status (current, former never), alcohol consumption (<1.5 g/d, 1.5–10 g/d, 10–20 g/d, and >20 g/d), physical activity (active, moderately active, moderately inactive, and inactive; ref. 25), education level (none, primary school, technical/professional school, secondary school, longer education including university degree, or not specified) and body mass index (BMI; continuous). Missing values (generally <2%) were accounted for by creating an extra category in each covariate.

To assess differences in risk estimates by subgroups, analyses based on the continuous log₂ scale were stratified by study phase, time between blood donation, and diagnosis less than and greater than 2 years, age at diagnosis (greater than/less than 64 years), and BMI above and below the median. Analyses by subgroups of BMI were done using unconditional logistic regression, adjusting for matching factors. Sensitivity analyses excluding past users of OC or HRT were also done. Heterogeneity between the subgroups was assessed using Cochran's Q-statistic (26).

Results

The mean age at blood donation was 59.8 and 60.1 years, for cases and controls, respectively (Table 1). At the

time of blood donation, the mean time since menopause was 10.7 years for cases and 11.1 years for age-matched controls. The mean years since menopause to breast cancer diagnosis was 14.4 years in study phase 1 and 15.9 in study phase 2. There was a mean time lag of 4.3 years after blood donation until breast cancer diagnosis. Compared with control subjects, cases had a higher BMI (26.4 vs. 26.0, $P = 0.02$). Due to the study design, 112 (50%) of the 225 breast cancer cases from study phase 2 were ER-negative.

Generally, in both study phases, geometric mean levels of all sex steroids were significantly higher among cases compared with controls (Table 2). Differences in absolute estrogen levels were observed between the two study phases, which may be explained by the different radioimmunoassays used to measure estradiol, as indicated also by measurements on quality control samples. SHBG mean levels were only lower in cases than in controls in study phase 2. Likewise, differences in absolute SHBG levels observed between the study phases 1 and 2 may be explained by changes over time in antibodies, standard curves, and standards of the assay kit used for this study (CIS Bio International).

Correlations between all sex steroids and SHBG levels adjusted for batch and age at blood donation were similar in magnitude in both study phases, with the exception of weaker correlations observed in study phase 2 for testosterone with estradiol and of free testosterone with free estradiol (Supplementary Table S1).

In the conditional logistic regression analyses, levels of total estradiol showed direct associations with risks of both ER-positive breast cancer [OR for upper vs. lower tertiles: 2.58 (95% CI: 1.69–3.93), $P_{\text{trend}} < 0.0001$] and ER-negative breast cancer subtypes [OR = 1.65 (95% CI: 0.91–2.98), $P_{\text{trend}} = 0.09$], with associations showing no significant heterogeneity ($P_{\text{het}} = 0.88$; Fig. 1). Likewise, total testosterone also showed direct associations with risks of both ER-positive [OR = 1.68 (95% CI: 1.16–2.44), $P_{\text{trend}} = 0.006$] and ER-negative breast cancer subtypes [OR = 1.75 (95% CI: 0.94–3.35), $P_{\text{trend}} = 0.04$], which also did not show significant heterogeneity ($P_{\text{het}} = 0.99$). There was a significant negative association of SHBG with the risk of ER-positive breast cancer [OR = 0.71 (95% CI: 0.51–1.00), $P_{\text{trend}} = 0.04$] but not with ER-negative cancer (OR = 0.73 [95% CI: 0.43–1.25] $P_{\text{trend}} = 0.25$), however this was not heterogeneous ($P_{\text{het}} = 0.98$). A similar degree of heterogeneity between breast cancer subtypes was seen when defined by PR status (Supplementary Fig. S1).

Further differentiation of the ER-positive tumors by including PR receptor information showed the strongest direct risk associations among all subtypes with ER+PR+ cancer [estradiol OR = 2.91 (95% CI: 1.62–5.23), $P_{\text{trend}} = 0.0002$; testosterone OR = 2.27 (95% CI: 1.35–3.81), $P_{\text{trend}} = 0.002$; Fig. 2). Interestingly, however, stronger observations were also seen in the ER-PR- subtypes [estradiol OR = 2.11 (95% CI: 1.00–4.46), $P_{\text{trend}} = 0.05$; testosterone OR = 2.06 (95% CI: 0.95–4.46) $P_{\text{trend}} = 0.03$] in comparison

Table 1. Baseline characteristics of case and control subjects, all centers combined, the EPIC cohort, 2007

Covariate	Cases	Controls	P_{diff}^a
Numbers of subjects	554	821	
ER-positive	382	602	
ER-negative	172	219	
PR-positive	211	345	
PR-negative	197	285	
ER+PR+	200	328	
ER+PR-	80	133	
ER-PR-	117	152	
Age at blood donation	59.8	60.1	
Years since menopause (blood donation)	10.7	11.1	
Years since menopause (diagnosis)–study phase 1	14.4	–	
Years since menopause (diagnosis)–study phase 2	15.9	–	
Years between blood donation and diagnosis	4.3	–	
Age at menarche	13.3	13.3	0.86
Age at menopause	49.3	49.0	0.50
Body-mass index (kg/m ²)	26.4	26	0.02
Waist circumference (cm)	83.1	82.5	0.11

^a P for difference from paired t test.

Table 2. Geometric mean and 5th and 95th percentile ranges of case and control subjects, the EPIC cohort, 2007

Hormones	Cases	Controls	P_{diff}^a
Study phase 1	$n = 329$	$n = 596$	
Estradiol (pmol/L)	97.26 (50.39–209.74)	88.63 (43.78–189.41)	0.001
Free estradiol (pmol/L)	2.53 (1.26–6.13)	2.27 (1.01–5.01)	0.001
Testosterone (nmol/L)	1.25 (0.52–2.85)	1.08 (0.38–2.64)	0.0002
Free testosterone (pmol/L)	20.31 (7.92–57.26)	17.12 (4.60–49.02)	0.0002
SHBG (nmol/L)	34.26 (15.41–77.23)	35.15 (12.56–86.91)	0.46
Study phase 2	$n = 225$	$n = 225$	
Estradiol (pmol/L)	46.02 (26.31–140.27)	40.66 (25.91–79.27)	0.003
Free estradiol (pmol/L)	1.08 (0.56–3.62)	0.93 (0.52–1.92)	0.001
Testosterone (nmol/L)	1.39 (0.56–3.16)	1.18 (0.52–2.60)	0.0001
Free testosterone (pmol/L)	18.89 (7.11–51.23)	14.80 (5.90–37.64)	<0.0001
SHBG (nmol/L)	45.67 (18.68–105.0)	51.69 (22.99–112.8)	0.01

^a P for difference from paired t test.

with the single receptor ER-negative defined breast cancer ORs. Relative risk estimates for hormone levels with the discordant ER+/PR- receptor breast cancer subtype showed no distinct associations. Similar risk estimates of SHBG were observed with joint ER+PR+ breast cancer [OR = 0.74 (95% CI: 0.46–1.21), $P_{trend} = 0.22$] and ER-PR- tumors [OR = 0.89 (95% CI: 0.48–1.68), $P_{trend} = 0.73$, $P_{het} = 0.34$].

With the exception of SHBG, relative risk estimates did not change after adjustment for BMI (data not shown). After adjusting for BMI, the significant negative risk association of SHBG with ER-positive disease was no longer significant [OR = 0.75 (95% CI: 0.52–1.08), $P_{trend} = 0.10$]. Risk estimates for estradiol and testosterone were stronger in ER-positive tumors after adjusting for age at menopause and age at first childbirth [estradiol OR = 2.69 (95% CI: 1.73–4.20), $P_{trend} < 0.0001$ and testosterone OR = 1.78 (95% CI: 1.22–2.62), $P_{trend} = 0.003$]. Risk associations of all hormones with ER-negative breast tumors were slightly attenuated after adjusting for age at menopause and age at first childbirth [estradiol OR = 1.26 (95% CI: 0.65–2.44), $P_{trend} = 0.44$ and testosterone OR = 1.65 (95% CI: 0.85–3.21), $P_{trend} = 0.10$].

Associations for estradiol and testosterone with the risk of ER-positive breast cancer did not remarkably change after excluding women who were diagnosed within 2 years of blood donation (Supplementary Fig. S1). Restricting analyses to women diagnosed within 2 years of blood donation, significant risk associations were no longer observed for total estradiol and testosterone for the ER-positive breast cancer. ER-negative breast cancer risk estimates in total estradiol and testosterone did not differ between women diagnosed before and after 2 years of blood donation. For women diagnosed within 2 years of blood donation, SHBG concentrations on a continuous \log_2 scale showed an unexpected risk association with ER-positive breast cancer [OR = 1.75 (95% CI: 1.13–2.742)], statisti-

cally heterogeneous from the association seen for breast cancer diagnosed after 2 years ($P_{het} = 0.0004$). For study phase 1, a maximum follow-up time of 13.2 years (median 8.3 years) was observed, thus extending well beyond the 2-year cut-point. A similar heterogeneity of circulating sex hormones by study phase and duration of follow-up greater or less than 2 years was also seen for the 2 study phases combined.

Relative risk estimates were of similar magnitude when breast cancer cases were stratified into those diagnosed before age 64 (the median age at diagnosis) and after 64 years (data not shown). Furthermore, there was no clear heterogeneity between relative risk estimates in the subgroups of women with a BMI below and above the median (25.5 kg/m²). Excluding women who had reported previous use of HRT or OC did not affect the associations in each subtype.

Discussion

Prospective cohort studies, including our earlier study in the EPIC cohort, have shown an increased risk of breast cancer risk overall with increases in postmenopausal blood concentrations of estrogens and androgens (10). Our present, extended analysis within the EPIC cohort shows that serum levels of total and bioavailable testosterone and estradiol are associated with risks of ER-positive, PR-positive, and joint ER+PR+, as well as of ER-negative, PR-negative, and joint ER-PR- breast tumors.

To our knowledge, 6 previous prospective studies have so far reported the risk association of sex steroid hormone levels with breast cancer risk by receptor status, and they are summarized in Table 3. As can be seen from this table, these studies have generally shown direct risk associations with estrogen and/or testosterone with ER-positive, PR-positive, and joint ER+PR+ breast cancer subtypes (27). However,

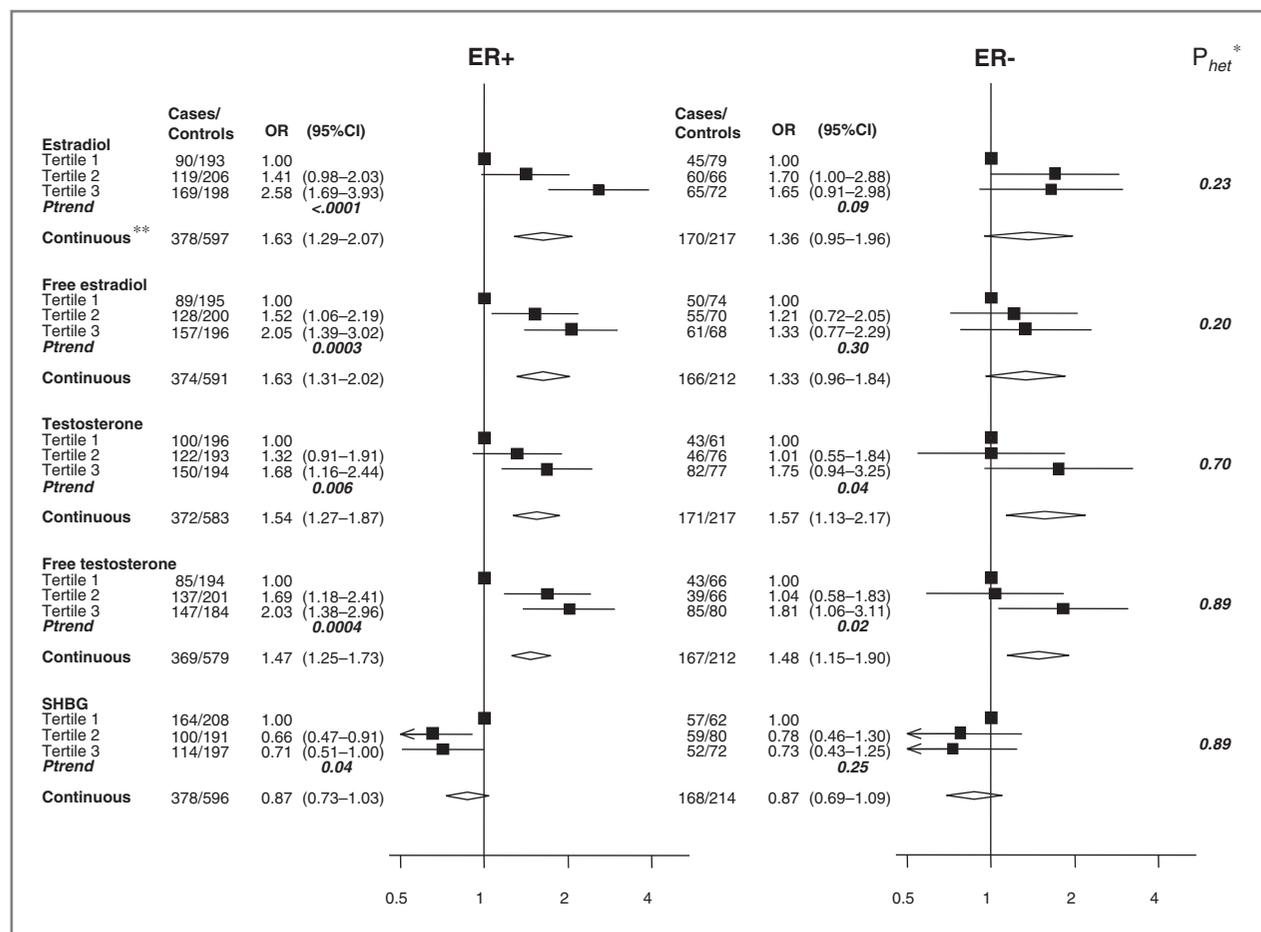


Figure 1. ORs estimates for ER breast cancer subtypes in postmenopausal women according to tertiles of sex steroid hormone and SHBG levels. Cases and controls matched for study center, batch, age at blood donation, time of blood collection, and fasting status. *, test for heterogeneity between ER-positive and ER-negative breast cancer was assessed using a log likelihood ratio test with and without an interaction term for breast cancer subtype outcome. **, Log₂ continuous increase in circulating hormone level.

due to the small numbers of hormone receptor–negative cases, each of these previous studies had little power to find significant associations within the ER-negative, PR-negative, or joint ER-PR- breast cancer subtypes. Our present study, although itself of limited size, includes substantially more ER-negative cases of breast cancer than most previous prospective studies and shows significant direct associations of both serum estradiol and testosterone with ER-negative, PR-negative, and joint ER-PR-negative breast cancer.

Although receptor status determination of breast tumors has become a standard part of breast cancer diagnosis, there is variation between laboratory methods and quantification systems for determining receptor status, and this was reflected also across the EPIC centers. Nevertheless, a number of studies have shown that the classification of receptors is relatively robust and have only moderate dependence on the assay used, especially for medium and high-expressing tumors (28). With respect to our hormone measurements, we observed some important differences in the absolute scales of measurement between

phases 1 and 2 of our study, most likely due to changes in the (commercial) assays used. However, cases and controls were matched by laboratory batch, and further standardization across the 2 phases was achieved by using phase-specific quartile cut-points for the estimation of ORs of breast cancer.

Whether estrogens promote the early development of breast tumors or whether increased circulating estrogens mostly promote late-stage growth of an established tumor, or both, is still unclear (11, 29). Observations from studies relating breast cancer risk and population incidence rates with the use of postmenopausal hormone (estrogen alone, or estrogen plus progestin) therapy suggest that effects of estrogens on breast tumor development could be essentially late stage (29, 30). In our study, interestingly, serum estradiol concentrations were associated more strongly with risk of breast cancer diagnosed at least 2 years after blood donation than for breast cancer diagnosed within a less than a 2-year interval. These latter observations would thus seem to argue against the concept that estrogens play a tumor-promoting role, especially in the very last stages.

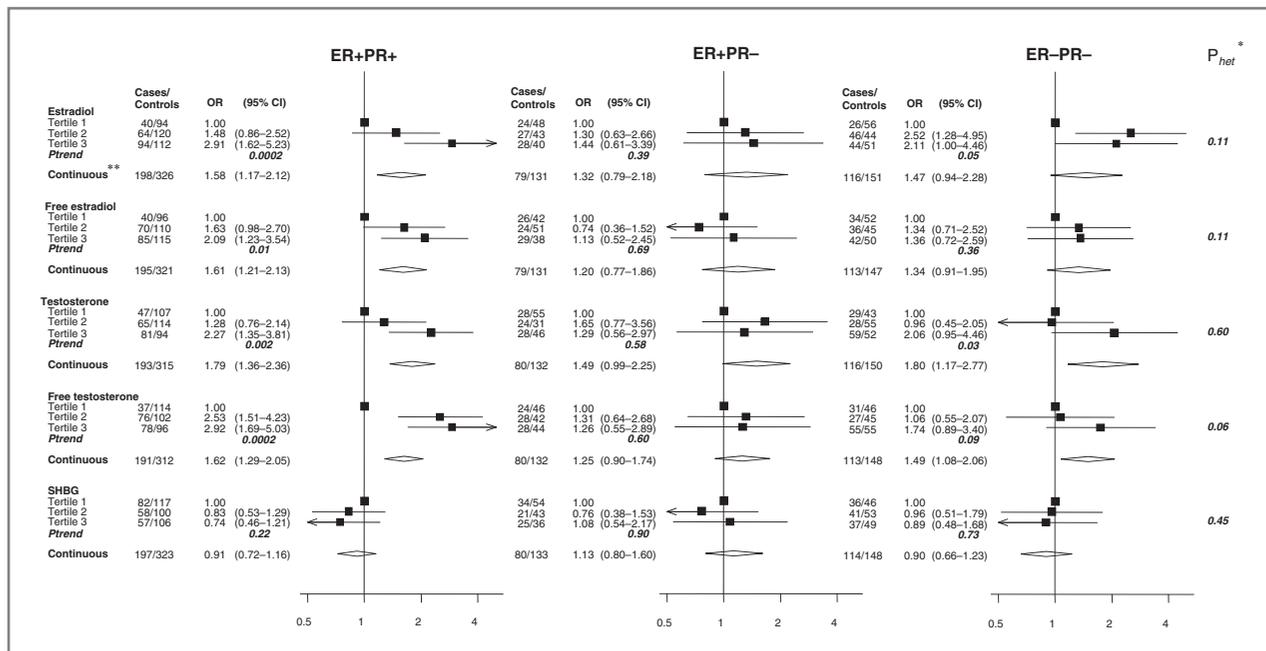


Figure 2. ORs estimates for joint ERPR breast cancer subtypes in postmenopausal women according to tertiles of sex steroid hormone and SHBG levels. Cases and controls matched for study center, batch, age at blood donation, time of blood collection, and fasting status. *, test for heterogeneity between ER+PR+, ER+PR-, and ER-PR- breast cancer was assessed using a log likelihood ratio test with and without an interaction term for breast cancer subtype outcome. **, Log₂ continuous increase in circulating hormone level.

Other studies, however, did not report the same heterogeneity of association of serum endogenous estrogens with breast cancer risk by lag-time between blood donation and breast tumor diagnosis (1, 31). Furthermore, the negative risk association with increasing SHBG, and its inverse relationship with bioavailable estradiol and testosterone could be an artifact due to inverse causation. For example, weight loss relatively shortly before breast cancer diagnosis could have led to an increase in SHBG levels and to decreases in bioavailable (non-SHBG bound) estradiol and testosterone.

Estrogens are believed to play key roles in the development of normal breast tissue as well as in breast cancer progression (32, 33). Estrogen binding to ER- α results in the stimulation of cell proliferation and inhibition of apoptosis, thereby stimulating tumor growth (34, 35), and clinical studies have shown that, indeed, only patients with ER-positive tumors respond favorably to antiestrogenic adjuvant therapy (3, 36). It thus seems paradoxical that, in our analysis, estrogens do also show a direct association with ER-negative, PR-negative, and joint ER-PR- breast cancer, with an effect size that was only moderately smaller than that seen for hormone receptor-positive disease. A direct, but statistically non-significant, association for estrogens with hormone receptor-negative breast cancer was also observed in 2 previous prospective cohort studies (11, 13) and, taken together, these various observations could be interpreted as to suggest that estrogens act through molecular pathways that do not directly involve the ER- α receptor.

However, breast cancer development is a slow process that evolves through several evolutionary stages. Estrogens may not only have late-stage effects on growth promotion of tumors (especially of ER-positive type) but probably also play an important role in earlier evolutionary stages of development, and indeed, available evidence suggests that most ER-negative ductal carcinomas *in situ* or invasive breast cancers arise from ER-positive precursors or cells that stop expressing the receptor (37).

The specific role that androgens may have in breast cancer etiology has long been debated (33, 38). One concept is that androgens (androstenedione and testosterone), convert into estrogens (estrone and estradiol, respectively), thus stimulating the growth and division of breast cells (33). Estradiol concentrations are higher in tumors than in nonmalignant tissue, suggesting increased aromatase activity in mammary cancers and (39, 40), possibly, further increasing breast cancer risk through local estrogen synthesis. This local conversion of androgens into estrogens within breast tissue is likely to be a relevant physiologic mechanism especially with respect to the development of ER-responsive tumors which, as discussed above, may also include some fraction of tumors that do not express ER- α any longer at the time of their clinical manifestation. Almost all ER-positive tumors are also androgen receptor (AR) positive (40), however, and the same is true for a considerable proportion of ER-negative breast carcinomas (41). Furthermore, the ER-negative/PR-negative/AR-positive breast tumor

Table 3. Characteristic of nested matched case control studies on postmenopausal hormone concentrations and breast cancer risk by receptor status

Study	Cohort	No. of cases/controls	ER+/ER-	PR+/PR-	ER+PR+/ER+PR-	Hormones	Scale	Risk estimate						
								ER-positive	ER-negative	PR-positive	PR-negative	ER+PR+	ER+PR-	ER-PR-
Zeleniuch-Jacquotte et al. (11)	New York University Women's Health Study	130/248	53/23	-	-	E2	Log ₆	1.9 (0.9-4.0)	2.0 (0.7-5.6)	-	-	-	-	-
								3.3 (0.6-20.3)	9.2 (0.4-200.6)	-	-	-	-	-
								1.0 (0.7-1.4)	0.9 (0.9-1.0)	-	-	-	-	-
Mismner et al. (14)	Nurses Health Study	322/643	-	-	153/34/39	E2	Quartiles ^a	-	-	-	-	3.3 (2.0-5.4) ^c	1.0 (0.4-2.6)	1.0 (0.4-2.4)
								-	-	-	-	2.0 (1.2-3.4) ^c	1.9 (0.7-5.0)	0.7 (0.3-1.6)
								-	-	-	-	0.5 (0.3-0.8) ^c	1.8 (0.6-5.0)	1.1 (0.5-2.8)
Cummings et al. (12)	Osteoporotic Fractures Cohort ^b	208/378	196/-	-	-	E2	Quintiles ^a	2.9 (1.6-5.1)	-	-	-	-	-	-
								5.1 (2.5-10.3)	-	-	-	-	-	-
								-	-	-	-	-	-	-
Kahan et al. (27)	Hungarian Mammography Screening Program	102/102	-	-	64/23/10	T	Quartiles ^b	-	-	-	-	5.8 (2.1-16.2) ^c	-	-
								-	-	-	-	-	-	-
								-	-	-	-	-	-	-
Sieri et al. (15)	The ORDET Cohort	165/672	127/30	96/59	89/36/23	E2	Quartiles ^a	1.3 (0.8-2.0)	0.9 (0.4-2.2)	1.5 (0.8-2.5)	0.9 (0.5-1.7)	1.5 (0.9-2.7)	0.8 (0.4-1.8)	1.1 (0.4-2.9)
								2.7 (1.6-4.3) ^c	1.6 (0.7-3.8)	2.3 (1.3-4.1) ^c	2.5 (1.3-4.8) ^c	2.3 (1.3-1.5) ^c	3.1 (1.3-7.2) ^c	1.8 (0.7-4.8)
								1.6 (1.0-2.6) ^c	1.9 (0.7-4.8)	1.7 (1.0-2.9) ^c	1.6 (0.8-3.0)	1.8 (1.0-3.1) ^c	1.2 (0.5-2.6)	2.6 (0.8-8.4)
								1.0 (0.6-1.6)	0.8 (0.3-2.0)	1.0 (0.6-1.7)	0.8 (0.4-1.6)	1.0 (0.6-1.7)	1.0 (0.4-2.3)	0.7 (0.3-1.8)
Baglietto et al. (13)	Melbourne Collaborative Cohort ^b	197/857	132/45	87/89	83/48/41	E2	Log ₂	1.5 (0.9-2.5)	1.7 (0.7-4.0)	1.5 (0.8-2.8)	1.5 (0.8-2.9)	1.7 (0.9-3.1)	1.2 (0.5-2.8)	2.0 (0.8-5.0)
								1.7 (1.1-2.6)	1.7 (0.9-3.3)	2.0 (1.2-3.3)	1.5 (0.9-2.4)	2.3 (1.4-3.8)	1.1 (0.6-2.1)	2.1 (1.1-4.2)
								1.1 (0.9-1.5)	1.0 (0.7-1.5)	1.1 (0.8-1.5)	1.1 (0.8-1.5)	1.1 (0.8-1.6)	1.2 (0.8-1.7)	1.1 (0.7-1.6)
								0.4 (0.3-0.6)	0.4 (0.2-0.8)	0.3 (0.2-0.6)	0.5 (0.3-0.8)	0.3 (0.2-0.5)	0.6 (0.4-1.1)	0.4 (0.2-0.7)

^aEstimates in categories are highest vs. lowest.^bCase cohort study design.^cP < 0.05 on continuous trend.

E2: Estradiol.

Free E2: Free estradiol.

Free T: Free testosterone.

subtype has been further indicated as being androgen dependent in its growth (42). These observations suggest that large fractions of both ER-positive and ER-negative tumors could also respond directly to the growth-promoting effects of testosterone, through pathways that may not necessarily depend on the estrogen alpha receptor. A further interesting observation, in this context, is that also among premenopausal women, breast cancer risk is directly related to serum testosterone concentrations, although among premenopausal women serum androgen levels are not a primary determinant of breast and tissue serum concentrations of estradiol (43, 44), and no associations could be established so far, between premenopausal serum estrogen concentrations and breast cancer risk.

In summary, although breast cancer is a heterogeneous disease, we observed that widely reported associations of estradiol and its androgenic precursors with overall postmenopausal breast cancer risk were not statistically heterogeneous between receptor-positive tumors and hormone receptor-negative breast cancer. Further research is needed to establish through which molecular pathways and during which evolutionary stages of development, androgens and estrogens can promote the occurrence of both receptor-positive and -negative clinical breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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