# Sex Hormone Levels, Breast Cancer Risk, and Cancer Receptor Status in Postmenopausal Women: the ORDET Cohort

**Sabina Sieri,[1] Vittorio Krogh,[1] Gianfranco Bolelli,[7] Carlo Alberto Abagnato,[7] Sara Grioni,[1] Valeria Pala,[1] Alberto Evangelista,[1] Claudia Allemani,[2] Andrea Micheli,[3] Giovanna Tagliabue,[4] Holger J. Schunemann,[8] Sylvie Menard,[5] Franco Berrino,[6] and Paola Muti[8]**

[1]Nutritional Epidemiology Unit, [2]Analytical Epidemiology Unit, [3]Descriptive Epidemiology and Health Planning Unit, [4]Cancer Registry Division, [5]Laboratories and Department of Experimental Oncology, Molecular Biology Unit, and [6]Etiological and Preventive Epidemiology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; [7]Centro Medico Diagnostico Emilia, Bologna, Italy; and [8]Epidemiology and Prevention Unit, Istituto Regina Elena, Rome, Italy

## Abstract

Background: **Endogenous sex hormone levels have been associated with increased breast cancer risk in postmenopausal women in several prospective studies. However, it remains unclear to what extent serum hormone-breast cancer associations differ with receptor status.**

Methods: **Associations between serum sex hormone levels and breast cancer risk were assessed in a nested case-control study on postmenopausal women of the ORDET cohort. After a median follow-up of 13.5 years, 165 women developed breast cancer. Relative risks of developing breast cancer were estimated by conditional logistic regression.**

Results: **Total and free testosterone levels were directly associated with breast cancer risk [relative risk, 3.28 (95% confidence interval, 1.93-5.55) and 2.86 (95% confidence interval, 1.66-4.94), respectively, for highest versus lowest quartile]. When relations between hormone level and risk of breast cancer expressing various receptor combinations were assessed, high total testosterone was significantly associated with increased risk of estrogen receptor–positive cancers, irrespective of progesterone receptor status. High total testosterone was also associated with increased risk of both human epidermal growth factor receptor 2 (HER2)–negative ($HER2^-$) and $HER2^+$ cancers. High estradiol tended to be associated with increased risk of $HER2^-$ cancer and inversely associated with $HER2^+$ cancer, with significant ($P$ = 0.027) heterogeneity between $HER2^+$ and $HER2^-$ cancers. However, there were relatively few $HER2^+$ cases.**

Conclusions: **This study provides further evidence that high levels of circulating testosterone increase the risk of developing breast cancer in postmenopausal women. The cancers that develop are mainly estrogen receptor positive. Although $HER2^+$ and $HER2^-$ breast cancers were both associated with high total testosterone, they showed opposing associations with estrogen.** (Cancer Epidemiol Biomarkers Prev 2009;18(1):169–76)

## Introduction

Hormone-related factors such as early menarche, late menopause, and use of postmenopausal hormone therapy have been known for some time to be associated with increased risk of breast cancer (1). The extensive review of Bernstein and Ross (2) found substantial evidence that endogenous sex hormones play a role in the development of breast cancer. Subsequently, high serum levels of estrogens and androgens in postmenopausal women were related to increased breast cancer risk in several prospective studies (3-6), and a pooled analysis of nine prospective studies found a strong association of breast cancer risk with serum concentrations of endogenous sex hormones in postmenopausal women (7). The Nurses Health Study (8) and the large European Prospective Study into Cancer and Nutrition (9) reached similar conclusions.

However, it remains unclear to what extent serum hormone-breast cancer associations differ with breast cancer subtype, such as those defined by estrogen receptor (ER) or progesterone receptor (PR) status, in part because few studies have investigated this area (8, 10, 11). It is known that tumors overexpressing human epidermal growth factor receptor 2 (HER2) constitute a distinct subtype (comprising 20-25%) of breast cancers (12, 13); however, we are aware of only one study that addressed the role of hormonal risk factors in $HER2^+$ cancers (14).

To further investigate the influence of sex hormone levels in promoting specific breast cancer subtypes, we expanded our previous nested case-control study on the ORDET cohort (6), extending follow-up to the end of 2003, by which time 165 postmenopausal breast cases had been diagnosed. We assessed associations between endogenous hormone levels and breast cancer risk and also investigated whether associations varied with the hormone receptor and HER2 status of the cancers diagnosed.

Received 8/29/08; revised 10/15/08; accepted 10/22/08.

**Grant support:** Department of Defense grant W81 XWH 04 1 0195 and National Cancer Institute grant CA98344.

**Requests for reprints:** Vittorio Krogh, Nutritional Epidemiology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, I-20133 Milan, Italy. Phone: 39-02-23903508; Fax: 39-02-23903510. E-mail: vittorio.krogh@istitutotumori.mi.it



doi:10.1158/1055-9965.EPI-08-0808

## Materials and Methods

Between June 1987 and June 1992, 10,786 healthy women, aged 35 to 69 y, residents of Varese province, Northern Italy, participated in the prospective ORDET study. All members of the cohort were volunteers recruited from the general population through public meetings; radio, television, and newspaper advertising; and from among those attending early diagnosis units for breast cancer. Women were excluded if they had undergone hormone treatment in the 3 mo before admission, had chronic or acute liver disease, or had received bilateral ovariectomy.

These women completed dietary and lifestyle questionnaires, had their anthropometric measurements recorded by trained nurses, and gave blood and urine samples. This study was approved by the Ethical Review Board of the National Cancer Institute of Milan.

Cancer incidence information, available from the local cancer registry (Varese Cancer Registry), was linked to the ORDET database to identify breast cancer cases incident up to end of December 2003. Less than 0.3% of breast cancer cases are known to Varese Cancer Registry by death certificate only, and 87.3% of all but female skin cancer cases are confirmed microscopically through pathology reports (95.0% for breast cancer cases; ref. 15). The completeness of reporting to the cancer registry is 98.7% for all but skin cancer sites and 99.2% for breast cancer (16).

After excluding women diagnosed with cancer before enrollment (except nonmelanoma skin cancer) and women who emigrated or were lost to follow-up immediately after recruitment, 10,633 participants were followed, 3,966 of whom were recruited after menopause (defined as absence of menstruation over the previous 12 mo). Cases and controls were obtained from these postmenopausal women. Women were censored at date of cancer diagnosis, death ($n$ = 474), loss to follow-up ($n$ = 329), or end of follow-up, whichever came first.

**Case Selection.** Cases were women diagnosed with breast cancer after recruitment to ORDET but before December 31, 2003 (a median follow-up of 13.5 y). One hundred eighty-four breast cancer cases were identified. Of these, three were eliminated because breast cancer was not their first cancer (they were censored at the date of first cancer diagnosis) and another five were eliminated because serum was not available. A further seven cases were eliminated because levels of follicle-stimulating hormone (<5 μg/mL) and luteinizing hormone (<3 μg/L) were in the premenopausal range. Thus, 169 cases were analyzed, including 7 diagnosed with *in situ* breast cancer. All cases were confirmed by pathology reports.

**Control Selection.** Up to four controls, matched to cases by age (±3 y) and length of storage of serum samples (±180 d), were chosen at random from all cohort members alive and free of breast cancer at the time of diagnosis of the matched case. An incidence density sampling protocol for control selection was used, such that controls could include women who later became cases (14 women), and could also serve as controls more than once (42 women). A total of 703 controls were selected initially by the sampling protocol. However, 20 were eliminated because follicle-stimulating hormone, luteinizing hormone, and progesterone levels were in the premenopausal range, so 683 women served as controls.

**Serum Analysis.** Serum samples were stored at −80°C for a mean of 17 y. There were no thawing accidents. Case and control samples were removed from the freezer together (within a few minutes) and sent to laboratory together in a single container packed with dry ice. Case samples were assayed together with their matched control samples (i.e., in the same batch) and all laboratory personnel were blind to case versus control status. The samples were analyzed by the Centro Medico Diagnostico Emilia (Bologna, Italy).

Total estradiol was determined using the Orion Diagnostica Spectra Estradiol Sensitive RIA kit (Orion Diagnostica Oy). Total and free testosterone were analyzed by solid-phase RIA (Coat-A-Count procedure from Siemens Medical Solutions Diagnostics Ltd.). Sex hormone–binding globulin (SHBG) was analyzed by automated solid-phase chemiluminescent immunoassay (Immulite 1000 Analyzer from Siemens Medical Solutions Diagnostics).

Quality control was done at three concentrations for SHBG and total and free testosterone and four concentrations for total estradiol. In each batch, quality control samples were evaluated in quadruplicate. Within-batch quality control coefficients of variation were 1.7% (high concentration) and 21.3% (low concentration) for estradiol; 6.2% and 11.2%, respectively, for total testosterone; 6.9% and 12.8%, respectively, for free testosterone; and 3.8% and 4.3%, respectively, for SHBG. Average between-batch coefficients of variation were 6.6% (high) and 21.4% for estradiol (low), 7.7% and 15.0% for total testosterone, 15.7% and 22.4% for free testosterone, and 4.6% and 8.3% for SHBG.

**ER, PR, and HER2 Status.** Information on ER, PR, and HER2 status was collected from pathology records. When the information was not available (ER in 66 cases, PR in 67, and HER2 in 69), immunohistochemical determinations were carried out on paraffin-embedded blocks of tumor tissue archived in pathology laboratories in the Province of Varese. The determinations were done at the Molecular Biology Unit Laboratories of the National Cancer Institute, Milan. The following antibodies were used: anti-HER2 CB11 (Ylem), anti-ER (diluted 1:200, clone 1D5; DBA), and anti-PR MAb1A6 (diluted 1:100; DBA). An automated immunostainer (Dako TechMate 1000) and peroxidase-streptavidin revelation were used. Negative controls were incubated with nonimmune serum from species in which the primary antibody was produced. Sections of tumor with known reactivity to each antibody were used as positive controls. Sections were considered $ER^+$ or $PR^+$ when >10% of cancer cells were labeled in the nucleus. Sections were considered $HER2^+$ when intense membrane immunostaining was present in over 10% of cancer cells. In Italy, the HER2 assay and result interpretation are standardized (17).

**Statistical Analysis.** Descriptive statistics of breast cancer risk factors and mean serum levels of hormones and SHBG were used to compare cases and controls. The significance of case-control differences in mean hormone exposure levels was evaluated by paired comparisons ($t$ tests) of case values with the average of the four matched controls in each case-control set. Relative risks (RR) for developing breast cancer were calculated by

**Table 1. Baseline characteristics of postmenopausal case and control subjects**

| | Controls ($n$ = 672) | Cases ($n$ = 165) |
|---|---|---|
| | $n$ (%) | $n$ (%) |
| Education (y) | | |
| ≤5 | 445 (66.12) | 102 (61.45) |
| 6-8 | 127 (19.02) | 45 (27.11) |
| >8 | 100 (14.86) | 19 (11.45) |
| Hormone therapy use* | | |
| Yes | 135 (20.30) | 40 (24.54) |
| No | 530 (79.70) | 123 (75.46) |
| Oral contraceptive use* | | |
| Yes | 84 (12.50) | 13 (7.88) |
| No | 588 (87.50) | 152 (92.12) |
| Breast-feeding | | |
| Yes | 511 (75.93) | 120 (72.29) |
| No | 162 (24.07) | 46 (27.71) |
| Age at first birth | | |
| Nulliparous | 65 (10.42) | 15 (9.04) |
| Age <25 | 274 (43.91) | 68 (40.96) |
| Age >25 | 285 (45.67) | 83 (50.00) |
| Family breast cancer | | |
| Yes | 50 (7.43) | 22 (13.25) |
| No | 622 (92.57) | 144 (86.75) |

| | $n$ | Mean ± SD | $n$ | Mean ± SD |
|---|---|---|---|---|
| Age at baseline | 671 | 58.10 ± 5.33 | 165 | 58.02 ± 5.31 |
| Smoking (pack-years) | 670 | 2.91 ± 7.38 | 163 | 3.42 ± 8.61 |
| Age at menarche | 672 | 13.29 ± 1.56 | 165 | 13.24 ± 1.55 |
| Age at menopause | 672 | 48.86 ± 4.76 | 165 | 49.21 ± 4.49 |
| Full-term pregnancy | 672 | 2.09 ± 1.25 | 165 | 2.05 ± 1.23 |
| BMI* at baseline | 670 | 26.31 ± 4.14 | 165 | 26.46 ± 4.36 |

*Past use.

conditional logistic regression (18). Serum levels of the various hormones and SHBG were examined both as continuous variables and by quartiles based on the frequency distribution in controls. The potential confounders age at menarche, age at first birth (nulliparous, <25, >25 y old), age at menopause, and history of breast cancer in first-degree relatives (yes/no) were included in the fully adjusted model. We excluded a further 4 cases and 11 controls from the fully adjusted model because values of confounder variables were missing (165 cases and 672 controls analyzed). Tests of linear trend were done using the median of each quartile.

Separate analyses were done to examine the association between levels of sex hormones/SHBG and breast cancer risk by receptor status. To do this, we categorized cases by receptor status ($ER^+$, $ER^-$, $PR^+$, and $PR^-$) and also identified three groups based on joint ER/PR status: $ER^+/PR^+$, $ER^+/PR^-$, and $ER^-/PR^-$. There were too few $ER^-/PR^+$ cases ($n$ = 5), so this category was eliminated. For these analyses, hormones/SHBG were categorized into tertiles; age, age at menarche, age at first birth, breast-feeding (yes/no), age at menopause, and history of breast cancer in first-degree relatives (yes/no) were included as confounders.

To test for differences in hormone/SHBG levels by receptor status, we used polychotomous logistic regression (19) with three end points for ER status ($ER^+$, $ER^-$, and no breast cancer), PR status ($PR^+$, $PR^-$, and no breast cancer), and HER2 status ($HER2^+$, $HER2^-$, and no breast cancer) and four end points for combined receptor status ($ER^+/PR^+$, $ER^+/PR^-$, $ER^-/PR^-$, and no breast cancer). Heterogeneity was investigated by the Wald test on tertile trends. The analyses were done with Stata version 7.

## Results

Table 1 shows the characteristics of the 165 cases and 672 controls. Compared with controls, cases had a higher frequency of family history of breast cancer, lower frequency of breast-feeding, and lower past use of oral contraceptives. Cases were also slightly older at menopause,

**Table 2. Geometric means (95% CI) of serum sex hormone concentrations in case and control subjects**

| | Controls | Cases | $P$ for difference |
|---|---|---|---|
| | Mean | Mean | |
| Total testosterone (nmol/L) | 0.86 (0.83-0.90) | 0.97 (0.90-1.05) | 0.006 |
| Free testosterone (pmol/L) | 1.86 (1.74-2.00) | 1.93 (1.72-2.17) | 0.629 |
| Estradiol (pmol/L) | 22.1 (20.4-24.0) | 19.2 (17.0-21.8) | 0.031 |
| SHBG (nmol/L) | 94.1 (88.9-99.6) | 82.2 (75.7-89.3) | 0.002 |

younger at menarche, and smoked more (median pack-years).

Levels of sex hormones and SHBG varied markedly between cases and controls, being higher in cases for total testosterone, free testosterone, and estradiol and lower for SHBG (Table 2). Table 3 shows adjusted RRs of developing breast cancer by quartiles of hormone/SHBG levels. Women in the highest quartile of total testosterone had a significantly greater risk [RR, 3.13; 95% confidence interval (95% CI), 1.86-5.28] of developing breast cancer than those in the lowest quartile (reference). After adjustments for age at menarche, age at first birth, age at menopause, and family history of breast cancer, the association between total testosterone and breast cancer risk was slightly stronger (RR, 3.28; 95% CI, 1.93-5.55 in the highest quartile). The test for trend was significant ($P < 0.001$).

Women in the highest quartile of free testosterone had a significantly greater risk (RR, 2.74; 95% CI, 1.60-4.69) of developing breast cancer than those in the lowest quartile (reference). After adjustments for the same confounders used for total testosterone, the RR increased to 2.86 (95% CI, 1.66-4.94) in the highest quartile. The trend was again significant ($P < 0.001$).

No association of estradiol levels with breast cancer risk was found, although RR estimates were in the same direction as those for total and free testosterone. Furthermore, RRs of breast cancer did not vary significantly with SHBG. RR estimates remained unchanged after adjustment for body mass index (BMI; data not shown).

We examined whether the increased breast cancer risks related to high levels of total and free testosterone were independent of estradiol levels. We found that estradiol-adjusted RRs of breast cancer were slightly greater than non–estradiol-adjusted RRs (data not shown). When the analyses were restricted to cases diagnosed with breast cancer 6 months or more after blood sampling, RRs were unchanged compared with those given by the main analysis (data not shown).

Table 4 shows the results of the analysis of the risk of developing breast cancer expressing specified receptors, in relation to hormone/SHBG levels. The strongest associations were with total testosterone in $ER^+$ cases (RR, 2.65; 95% CI, 1.62-4.32, highest versus lowest tertile), $PR^+$ cases (RR, 2.32; 95% CI, 1.33-4.06, highest versus lowest tertile), and $PR^-$ cases (RR, 2.47; 95% CI, 1.29-4.75, highest versus lowest tertile). Tests for heterogeneity between $ER^+$ and $ER^-$, and between $PR^+$ and $PR^-$, were not significant.

When relations between hormone/SHBG level and risk of breast cancer expressing various combinations of receptor were assessed, risk of $ER^+/PR^+$ and $ER^+/PR^-$ cancers were significantly associated with high total testosterone (Table 5). The RR for $ER^+/PR^+$ cancer was 2.33 (95% CI, 1.31-4.15) in the highest total testosterone tertile versus the lowest and the RR for $ER^+/PR^-$ cancer was 3.06 (95% CI, 1.31-7.16). Tests for heterogeneity to assess differences between $ER^-/PR^-$ and $ER^+/PR^+$ and $ER^+/PR^-$ subgroups, respectively, were not statistically significant.

**Table 3. Crude and adjusted RRs of developing breast cancer with 95% CI for quartiles of total testosterone, free testosterone, estradiol, and SHBG in serum of postmenopausal women of the ORDET cohort**

| | Cases/controls | Crude RR (95% CI) | Adjusted RR* (95% CI) |
|---|---|---|---|
| Total testosterone (median nmol/L) | | | |
| 1 (0.48) | 25/181 | 1.00 | 1.00 |
| 2 (0.73) | 31/153 | 1.50 (0.85-2.66) | 1.48 (0.83-2.64) |
| 3 (0.97) | 45/148 | 2.36 (1.37-4.06) | 2.39 (1.38-4.13) |
| 4 (1.34) | 64/160 | 3.13 (1.86-5.28) | 3.28 (1.93-5.55) |
| *P* for trend † | | <0.001 | <0.001 |
| Continuous ‡ | | 1.54 (1.27-1.88) | 1.58 (1.29-1.93) |
| Free testosterone (median pmol/L) | | | |
| 1 (0.66) | 26/162 | 1.00 | 1.00 |
| 2 (1.32) | 40/163 | 1.55 (0.90-2.69) | 1.60 (0.92-2.78) |
| 3 (2.15) | 38/162 | 1.57 (0.89-2.77) | 1.56 (0.88-2.78) |
| 4 (3.68) | 61/155 | 2.74 (1.60-4.69) | 2.86 (1.66-4.94) |
| *P* for trend † | | <0.001 | <0.001 |
| Continuous ‡ | | 1.34 (1.11-1.62) | 1.36 (1.13-1.65) |
| Estradiol (median pmol/L) | | | |
| 1 (8.1) | 32/161 | 1.00 | 1.00 |
| 2 (16.5) | 47/160 | 1.50 (0.90-2.49) | 1.47 (0.88-2.44) |
| 3 (23.5) | 40/161 | 1.32 (0.77-2.24) | 1.33 (0.78-2.28) |
| 4 (36.5) | 46/160 | 1.46 (0.87-2.46) | 1.50 (0.88-2.54) |
| *P* for trend † | | 0.267 | 0.216 |
| Continuous ‡ | | 1.09 (0.90-1.33) | 1.09 (0.90-1.33) |
| SHBG (median nmol/L) | | | |
| 1 (48.6) | 47/161 | 1.00 | 1.00 |
| 2 (77.4) | 35/160 | 0.71 (0.42-1.19) | 0.69 (0.41-1.17) |
| 3 (102.0) | 50/161 | 1.00 (0.61-1.63) | 0.98 (0.59-1.61) |
| 4 (149.0) | 33/160 | 0.66 (0.39-1.12) | 0.66 (0.39-1.13) |
| *P* for trend † | | 0.253 | 0.267 |
| Continuous ‡ | | 0.86 (0.70-1.05) | 0.86 (0.71-1.05) |

*Adjusted for age at first birth, age at menarche, age at menopause, and family history of breast cancer.
†*P* value for trend from model with the median of quartiles entered as continuous variables.
‡Log transformed.

**Table 4. Adjusted RRs of developing ER$^+$, ER$^-$, PR$^+$, and PR$^-$ breast cancer in relation to hormone levels in postmenopausal women of the ORDET cohort**

| Total testosterone (median nmol/L) | ER$^+$ | | ER$^-$ | | PR$^+$ | | PR$^-$ | |
|---|---|---|---|---|---|---|---|---|
| | Cases/ controls | RR* (95% CI) | Cases/ controls | RR* (95% CI) | Cases/ controls | RR* (95% CI) | Cases/ controls | RR* (95% CI) |
| 1 (0.56) | 28/231 | 1.00 | 10/231 | 1.00 | 22/231 | 1.00 | 15/231 | 1.00 |
| 2 (0.87) | 38/200 | 1.58 (0.93-2.67) | 7/200 | 0.83 (0.31-2.22) | 32/200 | 1.72 (0.97-3.08) | 14/200 | 1.06 (0.50-2.25) |
| 3 (1.25) | 61/193 | 2.65 (1.62-4.32) | 13/193 | 1.62 (0.69-3.80) | 42/193 | 2.32 (1.33-4.06) | 30/193 | 2.47 (1.29-4.75) |
| *P* for trend † | | <0.001 | | 0.237 | | 0.003 | | 0.004 |
| Continuous ‡ | | 1.61 (1.30-2.00) | | 1.18 (0.80-1.74) | | 1.52 (1.20-1.92) | | 1.50 (1.13-2.00) |
| *P* heterogeneity§ | | | | 0.39 | | | | 0.64 |
| Free testosterone (median pmol/L) | | | | | | | | |
| 1 (0.80) | 34/208 | 1.00 | 7/208 | 1.00 | 25/208 | 1.00 | 16/208 | 1.00 |
| 2 (1.67) | 36/208 | 1.05 (0.63-1.75) | 10/208 | 1.45 (0.54-3.89) | 28/208 | 1.14 (0.64-2.03) | 18/208 | 1.10 (0.55-2.23) |
| 3 (3.28) | 57/208 | 1.64 (1.03-2.62) | 13/208 | 1.89 (0.74-4.83) | 43/208 | 1.71 (1.00-2.91) | 25/208 | 1.56 (0.81-3.01) |
| *P* for trend † | | 0.023 | | 0.192 | | 0.036 | | 0.156 |
| Continuous ‡ | | 1.30 (1.06-1.59) | | 1.24 (0.85-1.82) | | 1.35 (1.08-1.70) | | 1.20 (0.91-1.59) |
| *P* heterogeneity§ | | | | 0.92 | | | | 0.82 |
| Estradiol (median pmol/L) | | | | | | | | |
| 1 (10.1) | 38/208 | 1.00 | 11/208 | 1.00 | 25/208 | 1.00 | 24/208 | 1.00 |
| 2 (19.3) | 41/208 | 1.09 (0.67-1.77) | 9/208 | 0.83 (0.33-2.04) | 35/208 | 1.41 (0.81-2.46) | 13/208 | 0.56 (0.28-1.13) |
| 3 (32.5) | 48/208 | 1.26 (0.79-2.02) | 10/208 | 0.92 (0.38-2.23) | 36/208 | 1.46 (0.84-2.54) | 22/208 | 0.92 (0.50-1.69) |
| *P* for trend † | | 0.321 | | 0.887 | | 0.214 | | 0.893 |
| Continuous ‡ | | 1.12 (0.91-1.38) | | 0.92 (0.66-1.28) | | 1.24 (0.96-1.60) | | 0.91 (0.73-1.15) |
| *P* heterogeneity§ | | | | 0.54 | | | | 0.35 |
| SHBG (median nmol/L) | | | | | | | | |
| 1 (54.9) | 41/208 | 1.00 | 11/208 | 1.00 | 29/208 | 1.00 | 22/208 | 1.00 |
| 2 (91.6) | 47/209 | 1.10 (0.69-1.76) | 10/209 | 0.86 (0.36-2.09) | 39/209 | 1.28 (0.75-2.16) | 19/209 | 0.84 (0.44-1.60) |
| 3 (141.0) | 39/207 | 0.97 (0.60-1.57) | 9/207 | 0.81 (0.33-2.00) | 28/207 | 0.98 (0.56-1.72) | 18/207 | 0.83 (0.43-1.60) |
| *P* for trend † | | 0.871 | | 0.655 | | 0.881 | | 0.595 |
| Continuous ‡ | | 0.89 (0.74-1.07) | | 0.91 (0.63-1.30) | | 0.86 (0.69-1.06) | | 0.91 (0.70-1.19) |
| *P* heterogeneity§ | | | | 0.74 | | | | 0.74 |

*Adjusted for age, age at first birth, and family history of breast cancer.
†*P* value for trend from model with the median of tertiles entered as continuous variables.
‡Log transformed.
§Wald test for heterogeneity.

Finally, the association of hormone/SHBG level with the risk of developing breast cancers expressing/not expressing the HER2 receptor was evaluated (Table 6). The increased risk of breast cancer associated with high total testosterone was independent of the HER2 status of the cancer (RR, 3.49; 95% CI, 1.09-11.2 for HER2$^+$ cancer; RR, 2.24; 95% CI, 1.39-3.60 for HER2$^-$; in both cases for highest total testosterone tertile versus lowest). High free testosterone was significantly associated with increased risk of HER2$^-$ cancer (RR, 1.70; 95% CI, 1.06-2.73).

High estradiol levels tended to be associated with increased risk of HER2$^-$ cancer and inversely associated with HER2$^+$ cancer. The test for heterogeneity between HER2$^+$ and HER2$^-$ cancers was statistically significant ($P = 0.027$). When SHBG was considered as a continuous variable, a decreased risk of HER2$^-$ breast cancer was observed. We consider this an artifact due to the not normal distribution because there is no trend and no theoretical justification.

We further investigated the association of sex hormone/SHBG with the risk of so-called triple-negative cancers (ER$^-$/PR$^-$/HER2$^-$, only 14 cases) but found no significant association between levels of hormones/SHBG and the risk of this poor-prognosis breast cancer subtype.

## Discussion

In 1996, we published a case-control study using the first 24 postmenopausal breast cancer cases diagnosed in the ORDET cohort; we found that high levels of testosterone and of estradiol in serum were associated with increased breast cancer risk (6). Ten years on, with 165 cases diagnosed, the results of this present study support the original finding that high prediagnostic serum testosterone is significantly associated with increased risk of breast cancer. The strongest effect was that of high total testosterone on the risk of developing ER$^+$/PR$^+$ and ER$^+$/PR$^-$ cancers. Other studies have implicated testosterone in breast cancer risk, including a pooled analysis of nine prospective cohort studies (7).

The present analysis failed to confirm the significant association of high serum estradiol with increased breast cancer risk, found in our previous study. However, confidence intervals were wide and compatible with those of previous cohort studies, which reported RRs of ~2 for prediagnostic estrogen levels in the upper quartile or quintile (7, 9). The lack of significant association in the present study might also be explained by the fact that estradiol was measured using a different analytic technique to that in our previous study (20). The new technique has a higher coefficient of variation, but it is more practicable for analyses of large numbers of samples and might have limited our ability to detect an association. We also found that high estradiol was associated (non significantly) with increased risk of HER2$^-$, but not HER2$^+$ cancers, with significant heterogeneity between these two cancer types; however, we only had 23 HER2$^+$ cancers.

We found no association of BMI with breast cancer risk or with estradiol, total testosterone, or free testosterone levels. These findings are in direct contrast to those of a collaborative analysis of eight prospective studies (21), which indicated increased breast cancer risk with increasing BMI in postmenopausal women, which was largely explained by an increase in estrogens with BMI.

Whereas the mechanism by which estrogens promote breast cancer seems fairly well established (22), the mechanism of action of androgens is more obscure and it is unclear whether they affect breast cancer by directly stimulating the proliferation of breast cells or whether they serve simply as estrogen precursors. Another possible link between high androgens and breast cancer could be via metabolic syndrome, a condition characterized by high insulin levels that stimulate ovarian synthesis of androgen (23). In women, metabolic syndrome is associated with increased testosterone levels (24, 25). Other characteristics of metabolic syndrome may independently increase breast cancer risk (26).

We tried to address the issue of whether testosterone exerts its effect via circulating estrogens by doing a multivariate analysis in which serum testosterone levels were adjusted for estradiol levels. We found that the association of testosterone with breast cancer not only persisted but also became slightly stronger after adjusting for estradiol level, clearly suggesting that the androgen acts by mechanisms other than simply increasing estrogens. It is important to note, however, that in previous studies, adjustment for estrogen levels resulted in attenuation of the RR associated with serum testosterone (3, 7, 9).

We found that high levels of testosterone were primarily associated with increased risk of $ER^+/PR^+$ and $ER^+/PR^-$ cancers, as also found by the Nurses Health Study (8) and a cohort study that examined only $ER^+$ tumors (10). This association of testosterone with $ER^+$ cancers is biologically plausible because testosterone maybe converted by aromatase within tumor cells into estrogens to directly stimulate tumor cell proliferation (27). Much less is known about the influence of testosterone on PR; however, PRs are induced by ER, and the presence of PR is a marker of a functional ER (28). Although few studies have assessed the receptor expression of subsequent tumors in relation to hormone levels (8, 10, 11, 29), more have investigated tumor receptor status in relation to hormone-related factors such as nulliparity, early menarche, and delayed childbearing. From these studies too, it seems that the risk is confined to receptor-positive tumors ($ER^+PR^+$) with no appreciable elevation in risk of receptor-negative cancers ($ER^-PR^-$; refs. 30-33). These factors have been proposed to confer risk by increasing systemic exposure to cycling reproductive hormones (30, 34).

To our knowledge, this is the first prospective study to investigate whether sex hormone/SHBG levels are associated with the risk of $HER2^+$ or $HER2^-$ breast

**Table 5. Adjusted RRs of developing $ER^+PR^+$, $ER^+PR^-$, and $ER^-PR^-$ breast cancers in relation to hormone levels in postmenopausal women of the ORDET cohort**

| Total testosterone (median nmol/L) | $ER^+PR^+$ | | $ER^+PR^-$ | | $ER^-PR^-$ | |
|---|---|---|---|---|---|---|
| | Cases/controls | RR* (95% CI) | Cases/controls | RR* (95% CI) | Cases/controls | RR* (95% CI) |
| 1 (0.56) | 20/231 | 1.00 | 8/231 | 1.00 | 7/231 | 1.00 |
| 2 (0.87) | 30/200 | 1.76 (0.97-3.21) | 8/200 | 1.11 (0.41-3.04) | 6/200 | 1.01 (0.33-3.08) |
| 3 (1.25) | 39/193 | 2.33 (1.31-4.15) | 20/193 | 3.06 (1.31-7.16) | 10/193 | 1.80 (0.67-4.84) |
| *P* for trend † | | 0.005 | | 0.005 | | 0.227 |
| Continuous ‡ | | 1.53 (1.20-1.95) | | 1.76 (1.23-2.50) | | 1.16 (0.75-1.80) |
| *P* heterogeneity§ | | 0.77 | | 0.35 | | — |
| Free testosterone (median pmol/L) | | | | | | |
| 1 (0.80) | 22/208 | 1.00 | 12/208 | 1.00 | 4/208 | 1.00 |
| 2 (1.67) | 27/208 | 1.24 (0.68-2.26) | 9/208 | 0.71 (0.29-1.73) | 9/208 | 2.30 (0.70-7.61) |
| 3 (3.28) | 40/208 | 1.79 (1.02-3.14) | 15/208 | 1.20 (0.54-2.64) | 10/208 | 2.59 (0.80-8.43) |
| *P* for trend † | | 0.033 | | 0.495 | | 0.153 |
| Continuous ‡ | | 1.36 (1.08-1.73) | | 1.13 (0.80-1.59) | | 1.33 (0.86-2.05) |
| *P* heterogeneity§ | | 0.79 | | 0.49 | | — |
| Estradiol (median pmol/L) | | | | | | |
| 1 (10.1) | 22/208 | 1.00 | 16/208 | 1.00 | 8/208 | 1.00 |
| 2 (19.3) | 33/208 | 1.51 (0.85-2.69) | 7/208 | 0.45 (0.18-1.12) | 6/208 | 0.76 (0.26-2.24) |
| 3 (32.5) | 34/208 | 1.54 (0.87-2.74) | 13/208 | 0.82 (0.38-1.76) | 9/208 | 1.10 (0.41-2.91) |
| *P* for trend † | | 0.179 | | 0.690 | | 0.793 |
| Continuous ‡ | | 1.26 (0.97-1.64) | | 0.92 (0.69-1.23) | | 0.90 (0.63-1.28) |
| *P* heterogeneity§ | | 0.68 | | 0.64 | | — |
| SHBG (median nmol/L) | | | | | | |
| 1 (54.9) | 28/208 | 1.00 | 12/208 | 1.00 | 10/208 | 1.00 |
| 2 (91.6) | 34/209 | 1.17 (0.68-2.01) | 13/209 | 1.08 (0.48-2.43) | 6/209 | 0.56 (0.20-1.59) |
| 3 (141.0) | 27/207 | 0.98 (0.56-1.72) | 11/207 | 0.97 (0.41-2.25) | 7/207 | 0.67 (0.25-1.81) |
| *P* for trend † | | 0.892 | | 0.467 | | 0.451 |
| Continuous ‡ | | 0.80 (0.55-1.18) | | 1.00 (0.71-1.40) | | 0.80 (0.55-1.18) |
| *P* heterogeneity§ | | 0.53 | | 0.59 | | — |

*Adjusted for age, age at first birth, and family history of breast cancer.
†*P* value for trend from model with the median of tertiles entered as continuous variable.
‡Log transformed.
§Wald test for heterogeneity.

**Table 6. Adjusted RRs of HER2$^+$ and HER2$^-$ breast cancer in relation to hormone levels in postmenopausal women of the ORDET cohort**

| | HER2$^+$ | | HER2$^-$ | |
|---|---|---|---|---|
| | Cases/controls | RR* (95% CI) | Cases/controls | RR* (95% CI) |
| Total testosterone (median nmol/L) | | | | |
| 1 (0.56) | 4/231 | 1.00 | 32/231 | 1.00 |
| 2 (0.87) | 7/200 | 2.02 (0.58-7.04) | 36/200 | 1.32 (0.79-2.21) |
| 3 (1.25) | 12/193 | 3.49 (1.09-11.2) | 58/193 | 2.24 (1.39-3.60) |
| *P* for trend † | | 0.030 | | 0.001 |
| Continuous ‡ | | 1.93 (1.26-2.95) | | 1.45 (1.18-1.79) |
| *P* heterogeneity§ | | | | 0.53 |
| Free testosterone (median pmol/L) | | | | |
| 1 (0.80) | 7/208 | 1.00 | 33/208 | 1.00 |
| 2 (1.67) | 7/208 | 0.98 (0.34-2.85) | 37/208 | 1.13 (0.68-1.88) |
| 3 (3.28) | 9/208 | 1.14 (0.40-3.20) | 56/208 | 1.70 (1.06-2.73) |
| *P* for trend † | | 0.785 | | 0.018 |
| Continuous ‡ | | 1.35 (0.87-2.12) | | 1.27 (1.04-1.55) |
| *P* heterogeneity§ | | | | 0.46 |
| Estradiol (median pmol/L) | | | | |
| 1 (10.1) | 12/208 | 1.00 | 34/208 | 1.00 |
| 2 (19.3) | 5/208 | 0.43 (0.15-1.26) | 41/208 | 1.21 (0.74-1.99) |
| 3 (32.5) | 6/208 | 0.43 (0.15-1.24) | 51/208 | 1.54 (0.95-2.48) |
| *P* for trend † | | 0.095 | | 0.087 |
| Continuous ‡ | | 0.86 (0.61-1.21) | | 1.15 (0.93-1.43) |
| *P* heterogeneity§ | | | | 0.027 |
| SHBG (median nmol/L) | | | | |
| 1 (54.9) | 7/208 | 1.00 | 42/208 | 1.00 |
| 2 (91.6) | 8/209 | 0.95 (0.32-2.77) | 48/209 | 1.11 (0.70-1.76) |
| 3 (141.0) | 8/207 | 1.18 (0.42-3.32) | 36/207 | 0.86 (0.53-1.40) |
| *P* for trend † | | 0.736 | | 0.514 |
| Continuous ‡ | | 1.30 (0.82-2.07) | | 0.82 (0.69-0.99) |
| *P* heterogeneity§ | | | | 0.52 |

*Adjusted for age, age at first birth, and family history of breast cancer.
†*P* value for trend from model with the median of tertiles entered as continuous variable.
‡Log transformed.
§Wald test for heterogeneity.

cancers in postmenopausal women. We found that high total testosterone was associated with increased risk of cancers both expressing and not expressing this epidermal growth factor receptor, whereas high free testosterone was only associated with increased risk of HER2$^-$ disease. The increased risk agrees with the finding of the only previous case-control study that investigated sex hormone levels and the HER2 status of the cancers (29).

We found that high estradiol levels tended to be associated with increased risk of HER2$^-$ cancer and inversely associated with the risk of HER2$^+$ cancer. It is interesting that in our study, a positive association between estradiol levels and breast cancer risk in the whole cohort was not observed but that HER2 receptor status conferred significant heterogeneity on the relation between breast cancer risk and estradiol levels. The absence of an association between estradiol levels and risk of HER2$^+$ cancer is consistent with the fact that HER2$^+$ cancers typically do not express ER or PR and do not respond to tamoxifen (35, 36). Evidence is growing that factors influencing hormonal status (e.g., parity, age at menarche, age at menopause, and pregnancy), and also the presence of BRCA1 or BRCA2 gene mutations, only influence the risk of developing HER2$^-$ breast cancers (14, 37).

The fact that testosterone was associated with both HER2$^+$ and HER2$^-$ cancers again suggests that testosterone acts to increase cancer risk by mechanisms other than via its conversion to estrogen. HER2 overexpression occurs in 15% to 30% of invasive breast cancers and is associated with worsened prognosis (38). HER2$^+$ cancers are usually also high grade, contain high numbers of proliferating cells (KI67$^+$), show chromosomal aneuploidy, and, as noted previously, do not express ER or PR (39).

When we investigated whether sex hormone/SHBG levels were associated with changed risk of developing so-called triple-negative cancers (ER$^-$, PR$^-$, and HER2$^-$), which are known to have a poor prognosis (40), we did not find significant associations, but the number of cases was very small.

The strengths of this study are its prospective design, the fact that reproductive history and other participant data were obtained before diagnosis (reducing problems of recall bias), and the fact that blood was also sampled before diagnosis. The main source of hormone receptor data was medical records, but when this information was missing, immunohistochemical determinations were done on archived tumor specimens. Although hormone receptor status was determined in most cases by immunohistologic methods, different laboratories may have used different cutoffs separating receptor-positive from receptor-negative tumors, which may have blurred differences between ER$^+$ and ER$^-$ tumors.

To conclude, this study provides further evidence that high levels of circulating testosterone increase the risk of developing breast cancer in postmenopausal women. The cancers that develop are mainly ER$^+$ (ER$^+$PR$^+$ or

$ER^+PR^-$); the risk of developing $ER^-PR^-$ cancers might not be increased. Although $HER2^+$ and $HER2^-$ breast cancers were both associated with high total testosterone, they showed opposing associations with serum estrogen. This is not surprising because breast cancer is not a single disease but a group of diseases distinguished by different behavioral and molecular characteristics whose pathogeneses are also likely to differ. Further studies, however, are required to elucidate how high levels of circulating sex hormones can promote particular subtypes of breast cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the 10,786 ORDET participants, Christofer Sempos for his invaluable contribution in the design of the study, Don Ward for help with the English language, D. Del Sette for technical support during cohort follow-up, and Franco Vecchi and Franca Franceschetti for conducting the assays.

## References

1. Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. Lancet Oncol 2001;2:133–40.
2. Bernstein L, Ross RK. Endogenous hormones and breast cancer risk. Epidemiol Rev 1993;15:48–65.
3. Zeleniuch-Jacquotte A, Shore RE, Koenig KL, et al. Postmenopausal levels of oestrogen, androgen, and SHBG and breast cancer: long-term results of a prospective study. Br J Cancer 2004;90:153–9.
4. Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 1995;87:190–7.
5. Dorgan JF, Longcope C, Stephenson HE, Jr., et al. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. Cancer Epidemiol Biomarkers Prev 1996;5:533–9.
6. Berrino F, Muti P, Micheli A, et al. Serum sex hormone levels after menopause and subsequent breast cancer. J Natl Cancer Inst 1996;88:291–6.
7. Key T, Appleby P, Barnes I, Reeves G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606–16.
8. Missmer SA, Eliassen AH, Barbieri RL, Hankinson SE. Endogenous estrogen, androgen, and progesterone concentrations and breast cancer risk among postmenopausal women. J Natl Cancer Inst 2004;96:1856–65.
9. Kaaks R, Rinaldi S, Key TJ, et al. Postmenopausal serum androgens, oestrogens and breast cancer risk: the European Prospective Investigation into Cancer and Nutrition. Endocr Relat Cancer 2005;12:1071–82.
10. Cummings SR, Lee JS, Lui LY, et al. Sex hormones, risk factors, and risk of estrogen receptor-positive breast cancer in older women: a long-term prospective study. Cancer Epidemiol Biomarkers Prev 2005;14:1047–51.
11. Zeleniuch-Jacquotte A, Toniolo P, Levitz M, et al. Endogenous estrogens and risk of breast cancer by estrogen receptor status: a prospective study in postmenopausal women. Cancer Epidemiol Biomarkers Prev 1995;4:857–60.
12. Menard S, Balsari A, Casalini P, et al. HER-2-positive breast carcinomas as a particular subset with peculiar clinical behaviors. Clin Cancer Res 2002;8:520–5.
13. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.
14. Balsari A, Casalini P, Bufalino R, Berrino F, Menard S. Role of hormonal risk factors in HER2-positive breast carcinomas. Br J Cancer 2003;88:1032–4.
15. Curado MP, Edwards B, Shin HR, et al., editors. Cancer incidence in five continents. Vol. IX. IARC Scientific Publications No. 160. Lyon: IARC; 2007.
16. Contiero P, Tittarelli A, Maghini A, et al. Comparison with manual registration reveals satisfactory completeness and efficiency of a computerized cancer registration system. J Biomed Inform 2008;41:24–32.
17. Italian Network for Quality Assurance of Tumor Biomarkers (INQAT) Group and SIAPEC-IAP. Interobserver reproducibility of immunohistochemical HER-2/neu assessment in human breast cancer: an update from INQAT round III. Int J Biol Markers 2005;20:189–94.
18. Breslow NE, Day NE. Statistical methods in cancer research. Volume I—the analysis of case-control studies. France: IARC Scientific Publications; 1980. p. 5–338.
19. Marshall RJ, Chisholm EM. Hypothesis testing in the polychotomous logistic model with an application to detecting gastrointestinal cancer. Stat Med 1985;4:337–44.
20. Payne DW, Holtzclaw WD, Adashi EY. A convenient, unified scheme for the differential extraction of conjugated and unconjugated serum C19 steroids on Sep-Pak C18-cartridges. J Steroid Biochem 1989;33:289–95.
21. Key TJ, Appleby PN, Reeves GK, et al. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst 2003;95:1218–26.
22. Hankinson SE, Eliassen AH. Endogenous estrogen, testosterone and progesterone levels in relation to breast cancer risk. J Steroid Biochem Mol Biol 2007;106:24–30.
23. Vona-Davis L, Howard-McNatt M, Rose DP. Adiposity, type 2 diabetes and the metabolic syndrome in breast cancer. Obes Rev 2007;8:395–408.
24. Pasanisi P, Berrino F, De Petris M, et al. Metabolic syndrome as a prognostic factor for breast cancer recurrences. Int J Cancer 2006;119:236–8.
25. Weinberg ME, Manson JE, Buring JE, et al. Low sex hormone-binding globulin is associated with the metabolic syndrome in postmenopausal women. Metabolism 2006;55:1473–80.
26. Xue F, Michels KB. Diabetes, metabolic syndrome, and breast cancer: a review of the current evidence. Am J Clin Nutr 2007;86:s823–35.
27. Dickson RB, Stancel GM. Estrogen receptor-mediated processes in normal and cancer cells. J Natl Cancer Inst Monogr 2000;27:135–45.
28. Lapidus RG, Nass SJ, Davidson NE. The loss of estrogen and progesterone receptor gene expression in human breast cancer. J Mammary Gland Biol Neoplasia 1998;3:85–94.
29. Kahan Z, Gardi J, Nyari T, et al. Elevated levels of circulating insulin-like growth factor-I, IGF-binding globulin-3 and testosterone predict hormone-dependent breast cancer in postmenopausal women: a case-control study. Int J Oncol 2006;29:193–200.
30. Enger SM, Ross RK, Paganini-Hill A, Carpenter CL, Bernstein L. Body size, physical activity, and breast cancer hormone receptor status: results from two case-control studies. Cancer Epidemiol Biomarkers Prev 2000;9:681–7.
31. Huang WY, Newman B, Millikan RC, et al. Hormone-related factors and risk of breast cancer in relation to estrogen receptor and progesterone receptor status. Am J Epidemiol 2000;151:703–14.
32. Potter JD, Cerhan JR, Sellers TA, et al. Progesterone and estrogen receptors and mammary neoplasia in the Iowa Women's Health Study: how many kinds of breast cancer are there? Cancer Epidemiol Biomarkers Prev 1995;4:319–26.
33. Yoo KY, Tajima K, Miura S, et al. Breast cancer risk factors according to combined estrogen and progesterone receptor status: a case-control analysis. Am J Epidemiol 1997;146:307–14.
34. Apter D, Reinila M, Vihko R. Some endocrine characteristics of early menarche, a risk factor for breast cancer, are preserved into adulthood. Int J Cancer 1989;44:783–7.
35. Carlomagno C, Perrone F, Gallo C, et al. c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J Clin Oncol 1996;14:2702–8.
36. Pietras RJ, Arboleda J, Reese DM, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 1995;10:2435–46.
37. Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001;344:539–48.
38. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
39. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Oncologist 1998;3:237–52.
40. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869–74.

# Sex Hormone Levels, Breast Cancer Risk, and Cancer Receptor Status in Postmenopausal Women: the ORDET Cohort

Sabina Sieri, Vittorio Krogh, Gianfranco Bolelli, et al.

*Cancer Epidemiol Biomarkers Prev* 2009;18:169-176.