

## ENDOGENOUS SEX HORMONES AND SUBSEQUENT BREAST CANCER IN PREMENOPAUSAL WOMEN

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**Because of large intra-individual variation in hormone levels, few studies have investigated the relation of serum sex hormones to breast cancer (BC) in premenopausal women. We prospectively studied this relation, adjusting for timing of blood sampling within menstrual cycle. Premenopausal women (5,963), recruited to the Hormones and Diet in the Etiology of Breast Tumors (ORDET) cohort study, provided a blood sample in the 20–24th day of their menstrual cycle. After 5.2 years of follow-up, 65 histologically confirmed BC cases were identified and matched individually to 4 randomly selected controls. Sera, stored at –80°C, were assayed blindly for dehydroepiandrosterone sulfate, total and free testosterone (FT), androstenedione, androstanediol-glucuronide, progesterone, 17-OH-progesterone, sex hormone-binding globulin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Fifty-five cases had information for multivariate analyses. Compared to controls, BC cases had shorter cycles and intervals between blood sampling and bleeding, and lower LH and FSH. FT was significantly associated with BC risk: relative risk (RR; adjusted for age, body mass index and ovarian cycle variables) of highest vs. lowest tertile was 2.85 [95% confidence interval (CI) = 1.11–7.33, *p* for trend = 0.030]. Progesterone was inversely associated with adjusted RR for highest vs. lowest tertile of 0.40 (95% CI = 0.15–1.08, *p* for trend = 0.077), significantly so in women with regular menses, where adjusted RR was 0.12 (95% CI = 0.03–0.52, *p* for trend = 0.005). These findings support the hypothesis that ovarian hyperandrogenism associated with luteal insufficiency increases the risk of BC in premenopausal women.**

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**Key words:** breast cancer; hormones; premenopausal women; cohort study

The hypothesis that breast cancer (BC) is related to ovarian function dates back over a century.<sup>1</sup> Epidemiological, *in vitro* and *in vivo* studies conducted in the second half of the last century made it clear that steroid sex hormones regulate cell proliferation and play a major role in promoting BC.<sup>2,3</sup> Several mechanistic hypotheses for the development of BC have been proposed,<sup>2,4</sup> but until recently, hormone measurements by epidemiological studies have failed to corroborate any of them. Over the last decade, however, several prospective cohort studies in postmenopausal women have shown that BC development is preceded by alterations in levels of circulating sex hormones.<sup>5</sup> High serum levels of free and total estradiol, total testosterone and other estrogens and androgens, as well as low serum levels of sex hormone-binding globulin (SHBG), have been found to be implicated in the risk of BC.<sup>5</sup> Our own study also indicated that high serum levels of free testosterone are associated with the risk of BC.<sup>6</sup> These prospective investigations were carried out with the help of thousands of healthy women who provided blood samples for storage and future nested-in-the-cohort case-control analyses. Compared to case control studies in clinical settings, the strengths of prospective studies are that control subjects belong to the same cohort that generates the incident disease cases and that blood is collected before the diagnosis of cancer thereby excluding abnormal values that may be due to overt illness.

Hormone measurements in premenopausal women are difficult to interpret because serum levels change with the menstrual cycle and because cycle length varies inter- and intra-individually. Only a few prospective investigations have addressed the role of sex hormone levels in BC before the menopause;<sup>7–10</sup> all considered small numbers of case women and did not produce clear results. The endocrine basis of BC in premenopause is therefore the subject of several disparate hypotheses. These include the hypothesis of Grattarola, advanced in the 1960s,<sup>11–12</sup> that hyperandrogenism with luteal inadequacy plays a role in the induction of BC, and of Henderson *et al.*<sup>13</sup> and Key *et al.*<sup>2</sup> 20 years later, that excess of estrogen plus progesterone can be responsible.

The present prospective study was designed to investigate whether luteal inadequacy and hyperandrogenism increase the risk of BC in premenopausal women. We collected blood samples from premenopausal women participating in the study on Hormones and Diet in the Etiology of Breast Tumors (ORDET).<sup>6,14</sup> Samples were taken between the 20th and 24th day of the cycle (theoretically during the mid luteal phase). The first day of menstrual bleeding subsequent to sampling was also recorded to provide an additional data point for correctly locating the sampling day within the cycle.

In these women, we analyzed the relationship between BC and serum levels of the androgens dehydroepiandrosterone sulfate (DHEAS), total testosterone, free testosterone, androstenedione and androstanediol-glucuronide (Adiol-G), and also progesterone, 17-OH-progesterone, SHBG, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Estradiol was not considered in the present analysis because of its extraordinary intra-individual variation in premenopausal women.<sup>15</sup>

**Abbreviations:** Adiol-G, androstanediol-glucuronide; BC, breast cancer; BMI, body mass index; CI, confidence interval; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; FT, free testosterone; ICD-9, International Classification of Diseases, 9th edition; LH, Luteinizing hormone; ORDET, Hormones and Diet in the Etiology of Breast Tumors cohort study; RIA, Radioimmunoassay; RR, Relative risk; SHBG, Sex hormone-binding globulin.

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## MATERIAL AND METHODS

*Cohort recruitment*

Between June 1987 and June 1992, 10,786 women aged 35–69, resident in Varese province, northern Italy, the area covered by the Lombardy Cancer Registry,<sup>16</sup> were recruited to the ORDET study.<sup>6,14</sup> The women volunteered their participation having heard about the study through radio, television and newspaper advertising, at public meetings and at centers for BC early diagnosis. Women who were pregnant or breastfeeding in the 6 months prior to presentation, those on hormone treatment over the preceding 3 months and those with chronic or acute liver disease, a history of cancer or bilateral ovariectomy were not recruited. Recruited women signed a consent form after being informed about the aims and requirements of the study, which had been approved by the Ethical Review Board of the National Cancer Institute of Milan.

Recruited women were classified as menopausal, perimenopausal or premenopausal according to ORDET rules;<sup>14</sup> menopausal women were defined as those who had not menstruated for at least 12 months preceding recruitment, perimenopausal as those who had not menstruated for the previous 6 to 11 months and premenopausal as those who had at least 1 menstruation in the 6 months prior to recruitment. Of 10,786 participants, 138 women did not furnish sufficient information to permit classification and were excluded from the present study, 37 were later excluded after linkage with Lombardy Cancer Registry files showed they had a cancer (any type) at recruitment and an additional 13 were excluded because they could not be traced during follow-up. According to the above definitions, 5,963 women in the cohort were premenopausal and were included in our study.

Premenopausal women were asked to provide a blood sample between the 20th and 24th day after menstrual bleeding. When recruitment was too late in the cycle to obtain blood between the 20th and 24th day, the women were called back the next month and the date of last menses was updated. Only 1.5% of women had a sample taken before the 20th day and 1.3% gave a sample after the 24th day. All women were given a postcard to note the date of next bleeding: 5,593 sent the completed card back to the ORDET center. Antecubital venous blood (40 ml) was drawn between 8 am and 9.30 am after overnight fasting. Women were recruited at 2 centers in Varese Province, 1 of which processed all the blood samples. Sera were placed in  $-80^{\circ}\text{C}$  freezers for long-term storage. No thawing accidents occurred. The effects of cryoconservation on hormone concentrations in serum were studied on ORDET samples. Pearson's  $r$  coefficients between values at sampling and those determined after 3 years of storage were 0.80 for total testosterone, 0.89 for free testosterone (based on 16 women) and 0.98 for progesterone, (based on 8 premenopausal women).<sup>17</sup>

The ORDET procedures for obtaining information on the enrolled women have been extensively described elsewhere.<sup>6,14,18</sup> Trained nurses acquired information on menstrual and reproductive history and other potential risk factors for BC, filling in the ORDET questionnaire according to standardized procedures, adherence to which was checked periodically. The nurses also encouraged participants to remember menstruation dates over the 6 months preceding blood donation. Anthropometric measurements were taken with the women in light clothes and shoeless. Body mass index (BMI) was calculated as weight in kg divided by height in meters squared. The repeatability of data was checked on 223 cohort members a year after enrolment. The correlation coefficients (Pearson's  $r$ ) were 0.90 for age at menarche, 0.98 for number of births, 0.99 for age at first birth, 0.95 for weight and 0.98 for height.<sup>6</sup> The repeatability of some hormone measurements was checked in 60 ORDET premenopausal participants who donated blood a year later on the same day of their cycle and same time of day. Intraclass correlation coefficients were 0.85 for DHEAS, 0.66 for free testosterone, 0.60 for total testosterone but only 0.06 for estradiol, which was not therefore considered in our study.<sup>15</sup>

*Identification of breast cancer cases and selection of control women*

The ORDET file was linked with Lombardy Cancer Registry files to identify BC case women diagnosed after blood sampling up to June 30, 1995 and to the regional file of Varese Province residents to determine vital status. The Lombardy Cancer Registry data are of high quality and completeness: only 1.3% of BC cases are known by death certificate only, and 96% of BC cases are histologically confirmed.<sup>16</sup>

In 1993 after a mean 3.5 years of follow-up, there was sufficient power to perform an ORDET case-control nested-in-the-cohort-analysis of post-menopausal women.<sup>6</sup> The analysis of premenopausal cases was performed later when the number cases we expected indicated sufficient study power. For an assumed relative risk (RR) of 2.5 or above<sup>14</sup> for BC in the highest compared to the lowest tertile of exposed subjects in terms of endogenous hormone levels, 38 cases were required assuming power level of  $(1 - \beta) = 0.80$  and  $\alpha$  error of 5% (1-sided test).<sup>19</sup>

In 1995, among the 5,963 premenopausal women followed for 31,240 woman-years (average 5.2 years; range 3–8 years), 65 invasive BC cases (ICD-9 code 174; mean age at diagnosis 46.9 years, range 37–58 years) were identified through the Registry, while 52.0 invasive BC cases were expected based on Registry incidence data. Two other women, initially classified as invasive BC cases, were excluded from the analysis because the histological diagnosis was *in situ* carcinoma.

For each case woman, 4 matched control women were chosen randomly from cohort members who had not developed BC at the time of diagnosis of the case. The control women were matched by age ( $\pm 5$  years), menopausal status at recruitment, recruitment date ( $\pm 89$  days), daylight saving period at recruitment and recruitment center. Serum samples of case women and their matched controls were extracted from the freezers; each case sample was placed with its control samples in another freezer; thereafter all case and control samples were processed identically. Of the 325 premenopausal women (65 case women and 260 control women), 16 women, all controls, were excluded immediately as their serum FSH levels were  $\geq 30$  mIU/ml, indicating they were not premenopausal. One control woman was reclassified as perimenopausal and was therefore also excluded. Thus, 308 premenopausal women (65 case women and 243 matched control women) were considered eligible. Their characteristics are shown in Table I.

The questionnaires of the women were checked for recent menstrual irregularities suggesting perimenopausal status. The checkers were blind to case vs. control status. The following subsets of women were defined: group a, case and control women who reported menstrual cycles at fairly regular intervals in the 6 months prior to blood donation (48 cases and 152 matched controls); and group b, case and control women without marked menstrual irregularities in the 6 months prior to blood donation, specifically, women of group a plus those with 1 missing period but at least 5 periods in the 6 months prior to blood donation (59 cases and 197 matched controls). In order to reduce the effect of the intra-individual variation in hormone levels, some analyses were restricted to either group a or b. Further restriction was necessary because 6 cases and 15 controls lacked sufficient serum for all the analyses, the date of menses prior to blood sample was missing in 1 control, the postcard stating date of menses after blood sampling was missing in 4 cases and 14 controls and information on BMI was missing in 3 controls and on FSH in 3 controls. Furthermore, the 29 control women matched to the 10 case women excluded for insufficient serum or missing data were also excluded from the matched analyses. Thus, the conditional logistic analyses with all variables were performed on 55 cases and 175 matched controls (40 cases and 108 matched controls for group a; 50 cases and 142 matched controls for group b).

**TABLE I**—CHARACTERISTICS OF 65 BREAST CANCER CASE WOMEN AND 243 MATCHED CONTROL WOMEN RECRUITED IN PREMENOPAUSE FROM THE ORDET COHORT

Characteristic	Cases Mean $\pm$ SD <sup>1</sup> (n)	Controls Mean $\pm$ SD <sup>1</sup> (n)	<i>p</i> value <sup>2</sup>
Age (years)	44.3 $\pm$ 4.9 (65)	43.4 $\pm$ 4.4 (243)	0.006
Age at menarche (years) <sup>3</sup>	12.7 $\pm$ 1.6 (64)	12.6 $\pm$ 1.4 (235)	0.807
Age at first childbirth (years) <sup>4</sup>	25.1 $\pm$ 4.0 (57)	25.1 $\pm$ 3.8 (185)	0.893
Number of births <sup>5</sup>	1.8 $\pm$ 1.1 (65)	1.9 $\pm$ 1.1 (240)	0.623
Weight (kg) <sup>6</sup>	60.4 $\pm$ 9.5 (65)	60.2 $\pm$ 9.4 (239)	0.712
Height (m) <sup>7</sup>	1.58 $\pm$ 0.07 (65)	1.58 $\pm$ 0.06 (238)	0.550
BMI (kg/m <sup>2</sup> )	24.2 $\pm$ 3.6 (65)	24.2 $\pm$ 3.7 (238)	0.902

<sup>1</sup>Standard deviation.—<sup>2</sup>Student's *t*-test for paired data was used for normally distributed variables (height and BMI); the Wilcoxon test for paired data was used for the other variables with nonnormal distribution.—<sup>3</sup>Information from 4 control and 1 case are missing; the 4 control women for the missing case were excluded.—<sup>4</sup>Based on parous women.—<sup>5</sup>Information for 3 control women missing.—<sup>6</sup>Information for 4 control women missing.—<sup>7</sup>Information for 5 control women missing.

### Analysis of serum samples

In the spring of 2000, case and matched control serum samples were analyzed together in batches by technicians blind to disease status. Each batch contained samples from 7 case women, their matched control women and lyophilized serum samples of known low, medium and high concentrations for each hormone (Lypochek, Bio-Rad, Milan, Italy) placed at the beginning, middle and end of each batch, respectively. The lyophilized sera were not assayed for SHBG, Adiol-G or 17-OH-progesterone; however serum samples from the laboratory with known low and high levels of each of these substances were used for quality control. These samples were placed at the beginning and middle of each batch.

All samples were assayed in duplicate using commercially available kits, following the manufacturers' instructions. Testosterone, DHEAS and progesterone were measured by radioimmunoassay (RIA) and SHBG by immunoradiometric assay using kits from Orion Diagnostica (Espoo, Finland). Androstenedione, 17-OH-progesterone and Adiol-G were assayed by RIA kits from ICN Biomedical (Opera, Italy). Free testosterone was assayed by a coated-tube RIA kit from Diagnostic Products Corporation (Medical Systems, Genoa, Italy). LH and FSH were assayed using a microparticle enzyme immunoassay kit (IMX System; Abbott, Rome, Italy). Detection limits were 0.03 ng/ml for testosterone, 11 ng/ml for DHEAS, 0.094 ng/ml for progesterone, 0.5 nmol/l for SHBG, 0.02 ng/ml for androstenedione, 0.1 ng/ml for 17-OH-progesterone, 0.4 ng/ml for Adiol-G, 0.5 mIU/ml for LH and 0.2 mIU/ml for FSH. Intra- and inter-assay coefficients of variation were, respectively, 4.2–4.6% and 8.0–9.1% for total testosterone; 4.3–8.6% and 6.0–15.2% for free testosterone; 2.2–7.0% and 6.8–13.8% for DHEAS; 6.3–8.7% and 5.3–10.6% for progesterone; 2.7–3.5% and 4.4–9.6% for SHBG; 1.5–2.7% and 2.4–4.8% for androstenedione; 6.8–9.1% and 7.4–9.2% for 17-OH-progesterone; 6.8–9.1% and 6.7–11.6% for Adiol-G; 2.6–3.5% and 2.8–8.1% for LH and 1.7–4.0% and 3.7–7.0% for FSH.

### Statistical analysis

We applied logarithmic transformations to the data for Adiol-G, LH, FSH and SHBG; and square root transformations to total testosterone, free testosterone and DHEAS, as the raw data distributions of control women were not normal. Differences between case and control means were tested using either the *t*-test for paired data or the Wilcoxon signed-rank test, as appropriate. To test differences between mean hormone levels in groups of control women by length of cycle, we used the Fisher test for independent samples. However we used the nonparametric Kruskal-Wallis test to assess differences in LH and FSH in groups by length of cycle because variances for these 2 hormones varied significantly with group (criterion of homoscedasticity not satisfied).

### Controlling for hormone variation due to the ovarian cycle

We divided the hormone and SHBG distributions of control women into tertiles (independently for each subset) and calcu-

lated odds ratio estimate of RRs of BC, using the lowest tertile as reference. Matching variables and potential confounding factors were controlled for by conditional logistic regression analysis. As found in previous studies,<sup>20–21</sup> case women and control women had different cycle lengths (Table II). Because hormone concentrations vary over the cycle, sampling on the same day of the cycle is likely to introduce a systematic bias into differences between cases and controls. We therefore included the length of the cycle in which blood was sampled (length-of-cycle variable) and the time between the day on which blood was sampled and the subsequent menses (days-from-sampling-to-next-menses variable) in the multivariate models as adjusting variables. Moreover, variations in cycle length are due mainly to variations in the length of the follicular phase, while the luteal phase is more stable.<sup>22</sup> Therefore, samples taken between the 20th and 24th day of the cycle will be more distant from the gonadotropin surge in women with short cycles (cases) than those with long cycles (controls). So in order to better *synchronize* the day of sampling of case women with that of control women, LH and FSH were inserted into the final models. LH, FSH and age were treated as continuous variables in all models. The length-of-cycle and days-from-sampling-to-next-menses variables were also continuous; however in models incorporating progesterone and 17-OH-progesterone, these time variables were categorized (dummy variables) because of the nonmonotonic trends of these hormones in mid luteal phase. BMI was always categorical (dummy variable).

Ninety-five percent confidence intervals (CI) of RRs and *p* values for linearity trends were calculated. For the linearity trend analyses, hormone and SHBG levels were treated as categorical variables of 3 levels. All *p* values refer to 2-sided statistical tests; *p*  $\leq$  0.05 was considered statistically significant. The analyses were performed with the Stata statistical software package, release 7.0 (2001) (Stata Corporation, College Station, TX).

## RESULTS

Table I shows, for case and control women, several known BC risk factors that may confound any association of BC with sex hormone levels. Although case women and their controls were age matched ( $\pm 5$  years), case women were still older than control women in the whole group (mean 44.3 years vs. 43.4 years; *p* = 0.006, Table I), in subgroup without marked irregularities (group b: mean 43.8 years vs. 42.5 years; *p* = 0.014, data not in Table I) and in the subgroup with regular menses (group a: mean 43.1 years vs. 41.8 years; *p* = 0.079, data not in Table I), so age was always retained in the regression models. Mean BMI was very similar in case and control women (Table I), but women in the 2 upper tertiles of BMI had a slightly lower cancer risk than women in the lowest tertile [age-adjusted RRs in group a were 0.78 (95% CI = 0.36–1.70) for the middle tertile and 0.75 for the highest tertile (95% CI = 0.34–1.64), data not in Table I]. Since BMI is asso-

**TABLE II**—MEAN VALUE AND STANDARD DEVIATION (SD) OF VARIABLES USED TO SYNCHRONIZE THE DAY OF BLOOD COLLECTION WITHIN THE CYCLE IN WOMEN WITH REGULAR MENSES (GROUP A),<sup>1</sup> WOMEN WITHOUT MARKED MENSTRUAL IRREGULARITIES (GROUP B)<sup>2</sup> AND ALL STUDY WOMEN

	Group a <sup>1</sup> Mean ± SD (n) <sup>3</sup>	Group b <sup>2</sup> Mean ± SD (n) <sup>3</sup>	All study women Mean ± SD (n) <sup>3</sup>
Time between previous menses and blood sampling (days)			
Cases	20.7 ± 1.4 (48)	20.7 ± 1.4 (59)	20.6 ± 1.4 (65)
Controls	20.4 ± 1.4 (151)	20.4 ± 1.4 (196)	20.3 ± 1.4 (241)
<i>p</i> <sup>4</sup>	0.272	0.357	0.340
Time from sampling to next menses (days) <sup>5</sup>			
Cases	6.2 ± 3.0 (45)	6.7 ± 3.6 (56)	7.1 ± 4.2 (61)
Controls	7.7 ± 5.2 (141)	8.9 ± 18.7 (183)	9.1 ± 17.4 (228)
<i>p</i> <sup>4</sup>	0.080	0.190	0.213
Length of the cycle in which blood was sampled (days) <sup>6</sup>			
Cases	26.8 ± 2.8 (45)	27.3 ± 3.3 (56)	27.7 ± 3.9 (61)
Controls	28.1 ± 5.2 (140)	29.3 ± 18.9 (182)	29.4 ± 17.6 (226)
<i>p</i> <sup>4</sup>	0.214	0.361	0.349
LH (mIU/ml)			
Cases	4.95 ± 2.81 (44)	5.12 ± 2.90 (53)	5.94 ± 5.16 (59)
Controls	7.47 ± 9.96 (144)	7.24 ± 9.06 (187)	7.46 ± 9.12 (228)
<i>p</i> <sup>7</sup>	0.037	0.034	0.187
FSH (mIU/ml)			
Cases	4.76 ± 1.93 (44)	5.41 ± 3.51 (53)	6.23 ± 4.37 (59)
Controls	5.76 ± 4.04 (141)	6.09 ± 4.96 (182)	6.43 ± 5.02 (222)
<i>p</i> <sup>7</sup>	0.086	0.374	0.520

<sup>1</sup>Women with regular menses, *i.e.*, those with menstrual cycles at fairly regular intervals in the 6 months prior to blood collection.—<sup>2</sup>Women without marked menstrual irregularities, *i.e.*, those with menstrual cycles at fairly regular intervals in the 6 months prior to blood collection or women with one missing period but at least 5 periods. Note: group b includes women of group a.—<sup>3</sup>Owing to missing data or control women excluded because the matched case was missed, the numbers differed through the different variables.—<sup>4</sup>Wilcoxon-test for paired data.—<sup>5</sup>Days-from-sampling-to-next-menses variable.—<sup>6</sup>Length-of-cycle variable.—<sup>7</sup>*t*-test for paired data.

**TABLE III**—MEAN SERUM LEVELS AND STANDARD DEVIATION (SD) OF HORMONES AND SHBG ON 20–24TH DAY OF CYCLE IN CONTROL WOMEN WITH REGULAR MENSES (GROUP A),<sup>1</sup> BY TERTILES OF LENGTH OF CYCLE IN WHICH BLOOD WAS SAMPLED

	Length of cycle (mean ± SD)			<i>p</i> value, Fisher's test
	≤26 days (n = 72)	27–28 days (n = 49)	≥29 days (n = 54)	
Total testosterone (ng/ml) <sup>2</sup>	0.41 ± 0.14	0.43 ± 0.16	0.47 ± 0.19	0.181
Free testosterone (pg/ml) <sup>2</sup>	0.91 ± 0.44	1.10 ± 0.55	1.17 ± 0.62	0.028
Progesterone (ng/ml)	10.37 ± 5.65	13.57 ± 5.22	10.10 ± 6.30	0.003
17-OH-progesterone (ng/ml)	1.62 ± 0.77	2.13 ± 0.64	2.01 ± 0.79	0.001
Adiol-G (ng/ml) <sup>3</sup>	2.43 ± 1.44	2.55 ± 1.21	2.99 ± 2.24	0.251 <sup>5</sup>
Androstenedione (ng/ml)	1.61 ± 0.70	1.80 ± 0.77	2.03 ± 0.84	0.013
LH (mIU/ml) <sup>3</sup>	4.59 ± 2.27	5.08 ± 2.89	12.65 ± 15.58	<0.001 <sup>5</sup>
FSH (mIU/ml) <sup>3,4</sup>	5.07 ± 1.86	4.32 ± 2.25 <sup>6</sup>	8.27 ± 6.70 <sup>7</sup>	<0.001 <sup>5</sup>
DHEAS (ng/ml) <sup>2</sup>	1420 ± 707	1627 ± 682	1558 ± 736	0.180
SHBG (nmol/l) <sup>3</sup>	58.3 ± 31.3	45.0 ± 19.2	49.9 ± 19.4	0.011

<sup>1</sup>Control women (both those matched and not matched) with regular menses, *i.e.*, those with menstrual cycles at fairly regular intervals in the 6 months prior to blood collection.—<sup>2</sup>Square root transformation of not normally distributed data.—<sup>3</sup>Logarithmic transformation of not normally distributed data.—<sup>4</sup>Data missing for 3 control women.—<sup>5</sup>Because of high variance in highest tertile of length of cycle compared with other tertiles (criterion of homoscedasticity not met), the nonparametric Kruskal-Wallis test was used.—<sup>6</sup>Datum for 1 control woman missing.—<sup>7</sup>Data for 3 control women missing.

ciated with both ovarian function and BC risk,<sup>23</sup> it was also retained in all regression models.

Table II shows the variables pertaining to the cycle in which blood was sampled for 3 sets of subjects. In group a, where intra-individual variation was low, cases and controls differed more clearly than in nongroup a women. In particular, compared to control women, group a case women had a shorter length of cycle (mean 26.8 days *vs.* 28.1 days, *p* = 0.214), less days from sampling to next menses (mean 6.2 days *vs.* 7.7 days, *p* = 0.080) and lower serum gonadotropins, significantly for LH (mean LH 4.95 mIU/ml *vs.* 7.47 mIU/ml, *p* = 0.037 and mean FSH 4.76 mIU/ml *vs.* 5.76 mIU/ml, *p* = 0.087).

Table III shows mean serum levels of hormones and SHBG (measured on 20–24th day of the cycle) in 3 subgroups of group a control women defined by different cycle lengths. Hormone mean levels differed in these subgroups, significantly, for free testosterone, progesterone, 17-OH-progesterone, androstenedione, LH, FSH and SHBG.

Table IV shows RRs for BC in relation to serum levels of hormones according to 2 models for women in group a: after adjusting for age and BMI (model 1), and after adjusting for age, BMI length-of-cycle variable, days-from-sampling-to-next-menses variable, LH and FSH (model 2). Free testosterone and progesterone showed to be related to BC risk, more evidently than

**TABLE IV**—RELATIVE RISKS (RR) OF BREAST CANCER IN GROUP A<sup>1</sup>, BASED ON 40 CASE WOMEN AND 108 MATCHED CONTROL WOMEN RECRUITED IN PRE-MENOPAUSE, BY SERUM SEX HORMONE AND SHBG TERTILES.<sup>2</sup>

	Serum concentration, tertiles			<i>p</i> for trend <sup>3</sup>
	Low	Middle	High	
Total testosterone, ng/ml	≤0.358	>0.358 to 0.510	>0.510	
RR (model 1)	1	0.95 (0.37–2.44)	1.14 (0.40–3.23)	0.823
RR (model 2)	1	1.06 (0.38–2.97)	2.16 (0.62–7.61)	0.281
Free testosterone, pg/ml	≤0.770	>0.770 to 1.201	>1.201	
RR (model 1)	1	1.60 (0.56–4.57)	2.39 (0.76–7.52)	0.135
RR (model 2)	1	1.86 (0.59–5.84)	3.07 (0.87–10.9)	0.082
Progesterone, ng/ml	≤9.01	>9.01 to 13.54	>13.54	
RR (model 1)	1	0.90 (0.38–2.13)	0.32 (0.10–1.06)	0.086
RR (model 2)	1	0.40 (0.13–1.20)	0.12 (0.03–0.52)	0.005
17-OH-Progesterone, ng/ml	≤1.602	>1.602 to 2.125	>2.125	
RR (model 1)	1	0.85 (0.35–2.06)	0.69 (0.23–2.13)	0.519
RR (model 2)	1	0.79 (0.28–2.22)	0.41 (0.11–1.56)	0.208
Androstenedione, ng/ml	≤1.357	>1.357 to 2.120	>2.120	
RR (model 1)	1	0.76 (0.33–1.77)	0.72 (0.26–1.99)	0.479
RR (model 2)	1	0.73 (0.30–1.80)	0.94 (0.30–2.97)	0.773
DHEAS, ng/ml	≤1219.8	>1219.8 to 1817.9	>1817.9	
RR (model 1)	1	0.87 (0.33–2.30)	1.17 (0.43–3.18)	0.780
RR (model 2)	1	0.93 (0.34–2.55)	1.18 (0.42–3.29)	0.764
Adiol-G, ng/ml	≤1.768	>1.768 to 2.966	>2.966	
RR (model 1)	1	1.23 (0.43–3.51)	1.89 (0.71–5.04)	0.199
RR (model 2)	1	1.50 (0.49–4.53)	1.61 (0.56–4.63)	0.384
SHBG, nmol/l	≤40.93	>40.93 to 58.28	>58.28	
RR (model 1)	1	0.88 (0.37–2.09)	0.41 (0.14–1.19)	0.122
RR (model 2)	1	0.97 (0.40–2.36)	0.48 (0.14–1.59)	0.288

<sup>1</sup>Women with regular menses.—<sup>2</sup>RR model 1, adjusted by age and BMI; RR model 2, adjusted by age, BMI, time from sampling to next menses (days-from-sampling-to-next-menses variable), length of the cycle in which blood was sampled (length-of-cycle variable), LH, and FSH.—<sup>3</sup>Linear trends in RRs by 3 level categorical variable.

**TABLE V**—RELATIVE RISKS (RR) OF BREAST CANCER IN WOMEN RECRUITED IN PRE-MENOPAUSE BY SERUM SEX HORMONE TERTILES ACCORDING TO MODEL 2.<sup>1</sup>

	Serum concentration, tertiles			<i>p</i> for trend <sup>3</sup>
	Low	Middle	High	
Free testosterone, pg/ml				
All study women	1	1.76 (0.75–4.09)	2.85 (1.11–7.33)	0.030
Group b <sup>2</sup>	1	1.69 (0.64–4.48)	3.53 (1.19–10.51)	0.023
Progesterone, ng/ml				
All study women	1	0.76 (0.34–1.69)	0.40 (0.15–1.08)	0.077
Group b <sup>2</sup>	1	0.81 (0.35–1.90)	0.40 (0.14–1.15)	0.101

<sup>1</sup>RR from all study women, based on 55 case women and 175 matched control women. RR from group b, based on 50 case women and 142 matched control women. Model 2: adjusted by age, BMI, days-from-sampling-to-next-menses and length-of-cycle variables, LH and FSH.—<sup>2</sup>Women without marked menstrual irregularities.—<sup>3</sup>Linear trends in RRs by 3 level categorical variable.

other hormones. In model 1, high free testosterone was associated, but not significantly, with increased BC risk [RR for the highest tertile 2.39 (95% CI = 0.76–7.52, *p* for trend 0.135)] and high progesterone was associated, but not significantly, with reduced risk of BC [RR for the highest tertile was 0.32 (95% CI = 0.10–1.06, *p* for trend was 0.086)]. In model 2, after synchronizing by day of sampling, the association between BC risk and free testosterone strengthened but did not become significant [RRs for the highest tertile was 3.07 (95% CI = 0.87–10.9, *p* for trend 0.082)], while the inverse association between BC risk and high progesterone became highly significant [RR for highest tertile was 0.12 (95% CI = 0.03–0.52, *p* for trend 0.005)]. When model 2 was changed to remove the adjustment for BMI, the association of BC risk with free testosterone was weakened, but the association with progesterone persisted and remained highly significant (data not shown). When the model 2 analysis was restricted to the 28 case women diagnosed before the age of 50 years and their 86 matched control women (data not shown in Table IV), the positive association with free testosterone [RR for upper tertile was 1.38 (95% CI = 0.31–6.11), *p* for trend 0.608] decreased, but the negative association with progesterone persisted and remained significant

[RR for upper tertile was 0.11 (95% CI = 0.02–0.64), *p* for trend 0.015].

Table V shows results obtained with model 2 in all study women and in women with menses without marked irregularities (group b). High free testosterone was significantly associated with increased BC risk in all study women and in group b, with upper tertile RRs of 2.85 (95% CI = 1.11–7.33, *p* for trend 0.030) and 3.53 (95% CI = 1.19–10.51, *p* for trend 0.023), respectively. Progesterone was inversely related to BC risk but not significantly; the association was weaker than in group a (Table IV). After adjusting for other potential confounders (parity, age at first childbirth and age at menarche), the RRs and the fit of the models did not change substantially (data not shown). None of the interactions between hormones and those confounder variables associated to the disease (*i.e.*, age and BMI) were significant (data not shown).

## DISCUSSION

The study was designed to provide information on the risk of BC in relation to serum levels of sex hormones that vary markedly over the menstrual cycle. In order to reduce this variation, blood

samples were collected within a narrow window (20th–24th day of the cycle) when most women were in mid luteal phase. Serum levels of androgens were higher in women with long cycles than in those with short cycles (Table III). This is probably because women with long cycles were those that gave blood nearest to their midcycle androgen surge,<sup>24</sup> since luteal phase length is relatively constant.<sup>22</sup> Similarly, progesterone levels (Table III) were highest in women with medium length cycles (27–28 days), likely reflecting the fact that progesterone peak occurs in mid luteal phase.<sup>25</sup> Mean gonadotropin levels were high only in women with a long cycle ( $\geq 29$  days), where the sample day is likely to be near the midcycle gonadotropin peak (Table III). In view of these findings, both ovarian cycle variables and gonadotropin levels were used in the multivariate models.

In 2 studies on the physiology of premenopausal ovarian hormones the data were *synchronized* by considering the day of the LH peak as day 0 of the cycle.<sup>22,26</sup> In our study, we applied adjustments for length-of-cycle, days-from-sampling-to-next-menses and gonadotropin variables, in order to achieve a similar *synchronizing* effect. Each *synchronizing* variable tended to improve the strength of the association between BC risk with both progesterone and free testosterone. Some misclassification, however, is likely to persist and the true association may be stronger than we found. ORDET did not recruit women who had had bilateral ovariectomy. However 2 cases and 2 controls had received unilateral ovariectomy. When these were excluded from the analyses, the results were substantially unchanged. We have not considered other potential confounding factors, such as fat intake and alcoholic beverages that may be associated with both BC risk and hormonal levels. The association of these factors with the BC risk, however, is relatively small, with RR less than 1.5<sup>27,28</sup> and could not conceivably explain the stronger association observed with free testosterone and progesterone in our study.

Our findings are consistent with the hypothesis, formulated 40 years ago by Grattarola,<sup>11,12</sup> that ovarian hyperandrogenism associated with anovulation or luteal inadequacy increases the risk of BC. Even if testosterone is synthesized also in the adrenal and, to a lesser extent, in peripheral tissues, the suggested anatomical basis for the hypothesis is hyperplasia of ovarian interstitial stromal tissue (where androgens are synthesized). Stromal hyperplasia is often associated with infertility, ovarian cyst formation, chronic anovulation and luteal insufficiency, as indicated by low progesterone.<sup>29</sup> Ovarian stromal hyperplasia was first described in BC patients in the 1950s.<sup>30</sup> The Grattarola hypothesis was strengthened by the observation in the 1960s that many premenopausal women with BC had a hyperplastic endometrium in the second phase of the cycle (an index of chronic anovulation) and elevated testosterone excretion.<sup>11,12</sup> High testosterone levels were often normalized by ovariectomy, implicating the ovaries as the testosterone main source.<sup>31</sup> On the other hand, the absence in our study of an association between androgens with mainly adrenal source, such as DHEAS and androstenedione,<sup>32</sup> and BC risk provides further support for the hypothesis of ovarian abnormality.

The findings of the present study do not support the estrogens plus progesterone hypothesis,<sup>2,13</sup> according to which exposure to endogenous progesterone should increase and not decrease the risk of BC. This hypothesis was inspired by the finding that the mitotic activity of the epithelial cells of the breast increases during luteal phase<sup>33</sup> and it is supported by other indirect evidence. First, a long period of menstrual irregularities after menarche with frequent anovulatory cycles, and therefore low progesterone exposure, has been found associated with low risk of subsequent BC.<sup>34,21</sup> Second, women with short menstrual cycles seem to be at increased risk for BC;<sup>20,21</sup> since it is mainly the follicular phase that varies in length, short cycles are associated with increased cumulative exposure to luteal hormones.<sup>13,20</sup> Third, prior to menopause obese women have a slightly lower risk of BC;<sup>23</sup> because obesity is

associated with anovulation, obese women may have a lower cumulative exposure to progesterone. Fourth, hormone replacement therapy with progestogens increases BC risk more than estrogen replacement therapy.<sup>35–37</sup> Hormone replacement therapy usually employs synthetic progestins that have androgenic activity,<sup>38</sup> while natural progesterone does not seem to increase BC risk.<sup>39</sup> Moreover the hypothesis that progesterone increases the risk of BC has never been corroborated by hormone measurements.

Analysis of the data in group a revealed a slightly increased risk of BC with low BMI. When BMI was introduced into the regression models the association of high testosterone with BC risk strengthened. In fact BMI was directly associated with total testosterone ( $r = 0.17$ ,  $p = 0.047$ ) and free testosterone ( $r = 0.26$ ,  $p = 0.001$ ).

Repeatability studies have shown fairly high intraclass correlation coefficients for testosterone and progesterone measurements.<sup>15,40</sup> We did not measure estrogen in our study because of the high intra-individual variation in estradiol levels in repeated blood samples taken on same day of subsequent cycles.<sup>15</sup> Other prospective studies that measured estrogens have provided inconsistent results,<sup>7–10</sup> although the 2 most recent studies<sup>8,9</sup> suggested that estradiol levels are associated with BC risk.

Two population-based case control studies, recently published, investigated the role of sex hormones in premenopause:<sup>41,42</sup> one found a significant positive association of dehydroepiandrosterone and a negative association of luteal progesterone with BC risk in sera,<sup>41</sup> while the second one found a significant association of testosterone with BC risk in plasma.<sup>42</sup>

Only a few previous prospective cohort studies have reported an association of luteal phase progesterone levels with subsequent BC. Thomas *et al.*<sup>9</sup> reported that mean serum progesterone was 9% lower in case than control women in early luteal phase (based on 12 case women). Wysowski *et al.*<sup>10</sup> found mean serum progesterone was 29% lower in case women than in control women matched for time since last menstrual period (based on 17 case women). By contrast Helzlsouer *et al.*<sup>7</sup> reported higher concentrations of luteal phase progesterone in case than control women, but again few subjects were studied (9 case women). None of the differences were statistically significant.

In conclusion, our prospective case-control study has shown that free testosterone was significantly associated with BC risk in premenopausal women and that progesterone was significantly inversely associated with BC risk in women with regular menses. The negative association with progesterone remained significant when the analyses were restricted to women up to 50 years of age. The sample size of the present study is relatively small; however we are not aware of any other prospective study of comparable magnitude that has addressed the same hypothesis. Furthermore, to our knowledge no other study has confined the collection of blood to a few days within luteal phase and also used a regression model taking into consideration a multidimensional adjustment with temporal and biological variables able to *synchronize* the day of the cycle on which blood was sampled, although several studies recorded the day of the cycle on which blood was taken, and a few also recorded the date of the following menses.<sup>43</sup> We suggest that methods similar to ours might be appropriate for analyzing data from other cohorts, to thus provide a test of the findings of the present study.

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