Urinary 6-Sulfatoxymelatonin Levels and Risk of Breast Cancer in Postmenopausal Women

Eva S. Schernhammer, Franco Berrino, Vittorio Krogh, Giorgio Secreto, Andrea Micheli, Elisabetta Venturelli, Sabina Sieri, Christopher T. Sempos, Adalberto Cavalleri, Holger J. Schünemann, Sabrina Strano, Paola Muti

Background Low urinary melatonin levels have been associated with an increased risk of breast cancer in premenopausal women. However, the association between melatonin levels and breast cancer risk in postmenopausal women remains unclear.

Methods We investigated the association between melatonin levels and breast cancer risk in postmenopausal women in a prospective case–control study nested in the Hormones and Diet in the Etiology of Breast Cancer Risk cohort, which included 3966 eligible postmenopausal women. The concentration of melatonin's major metabolite, 6-sulfatoxymelatonin, was measured in a baseline 12-hour overnight urine sample from 178 women who later developed incident breast cancer and from 710 matched control subjects. We used multivariable-adjusted conditional logistic regression models to investigate associations. Relative risks are reported as odds ratios (ORs). All statistical tests were two-sided.

Results Increased melatonin levels were associated with a statistically significantly lower risk of invasive breast cancer in postmenopausal women (for women in the highest quartile of total overnight 6-sulfatoxymelatonin output vs the lowest quartile, multivariable OR also adjusted for testosterone = 0.56, 95% confidence interval [CI] = 0.33 to 0.97; P_{trend} = .02). This association was strongest among never and past smokers (OR = 0.38, 95% CI = 0.20 to 0.74; P_{trend} = .001) and after excluding women who were diagnosed with invasive breast cancer within 4 years after urine collection (OR = 0.34, 95% CI = 0.15 to 0.75; P_{trend} = .002). We did not observe substantial variation in relative risks by hormone receptor status of breast tumors. Among the 3966 women in the cohort, 40 of the 992 women in the highest quartile of 6-sulfatoxymelatonin developed breast cancer during follow-up, compared with 56 of the 992 women in the lowest quartile of 6-sulfatoxymelatonin.

Conclusion Results from this prospective study provide evidence for a statistically significant inverse association between melatonin levels, as measured in overnight morning urine, and invasive breast cancer risk in postmenopausal women.

J Natl Cancer Inst 2008;100:898–905

Many biologic functions in humans follow distinct, approximately 24-hour patterns (1) that are driven by a circadian pacemaker that is located in the hypothalamus (2). Secretion of melatonin, an indoleamine hormone that is produced primarily by the pineal gland, also follows a circadian rhythm of approximately 24 hours; melatonin is secreted exclusively during the dark phase of a light–dark cycle (3). The urine concentration of the major metabolite of melatonin, 6-sulfatoxymelatonin, is strongly associated with melatonin levels in blood and saliva (4–10). Urinary 6-sulfatoxymelatonin levels, as measured in the first morning urine specimen, accurately reflect plasma melatonin levels measured during the previous night (5,11).

Results of previous studies [reviewed in (12)] indicate that night-shift work, a surrogate for exposure to light at night, is associated with an increased risk of breast cancer (13). From a previous suggestion by Cohen et al. (14) that melatonin appears to be involved in the induction of breast tumors and on the basis of various results from laboratory and animal experiments (15–17),

Affiliations of authors: Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA (ESS); Department of Epidemiology, Harvard School of Public Health, Boston, MA (ESS); LBI-ACR and ACR-ITR VIEnna/CEADDP, Vienna, Austria (ESS); Department of Preventive and Predictive Medicine (FB, GS, EV, AC), Nutritional Epidemiology Unit (VK, S. Sieri), and Descriptive Epidemiology and Health Planning Unit (AM), Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Istituto Nazionale Tumori, Milan, Italy; Department of Social and Preventive Medicine, SPHHP, University at Buffalo (CTS, HJS); Molecular Chemoprevention Unit, Scientific Director Department (S. Strano), Istituto Nazionale Tumori Regina Elena IRCCS (HJS, PM), Rome, Italy; Department of Epidemiology and Biostatistics, McMaster University, Hamilton, Ontario, Canada (HJS).

Correspondence to: Eva S. Schernhammer, MD, DrPH, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Ave, Boston, MA 02115 (e-mail: eva.schernhammer@channing.harvard.edu).

See "Funding" and "Notes" following "References."

DOI: 10.1093/jnci/djn171

light-induced suppression of melatonin secretion has been hypothesized to be a major cause of this association. However, only two prospective studies (18,19), both among primarily premenopausal women, have investigated the association between circulating melatonin and breast cancer risk. Results of these studies were inconsistent: one study (18) found no evidence that 24-hour urinary levels of melatonin are associated with the risk of breast cancer, whereas the other study (19) found that melatonin levels in the first morning urinary specimen were strongly and inversely associated with the risk of breast cancer. No previous study, to our knowledge, has evaluated this association in postmenopausal women.

We used a nested case–control design to conduct a prospective study of the association between melatonin levels in a 12-hour overnight urine specimen and breast cancer risk in a large cohort of postmenopausal women enrolled in the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) cohort. We evaluated the association between the total amount of 6-sulfatoxymelatonin produced between 7:00 PM and 7:00 AM as assessed in a urine sample obtained at baseline and breast cancer risk. We also evaluated the association between the creatinine-adjusted 6-sulfatoxymelatonin level as measured in an overnight urine specimen and breast cancer risk.

CONTEXT AND CAVEATS

Prior knowledge

Low levels of urinary melatonin have been associated with an increased risk of breast cancer in premenopausal women, but the risk in postmenopausal women remains unclear.

Study design

Prospective case–control study nested in the Hormones and Diet in the Etiology of Breast Cancer Risk cohort.

Contribution

Increased melatonin levels were associated with a statistically significantly lower risk of invasive breast cancer in postmenopausal women. Among the 992 women in the highest 6-sulfatoxymelatonin quartile, 40 developed breast cancer during follow-up, compared with 56 of the 992 women in the lowest 6-sulfatoxymelatonin quartile.

Implications

Further investigation into the primary mechanisms for the association between melatonin and breast cancer risk is warranted.

Limitations

Information on light exposure at night was lacking. The authors could not correct for errors in laboratory measurement of melatonin and within-person variability in melatonin measurements.

Study Subjects and Methods

Study Subjects

The ORDET cohort was established in northern Italy between June 1, 1987, and June 30, 1992, with 10 786 healthy women aged 35–69 years (20,21). All women were residents of the Varese province—an area covered by the Lombardy Cancer Registry (22)—who had heard about the study through the media, at public meetings, or at centers for the early detection of breast cancer and who volunteered to participate. All women gave their written informed consent. At enrollment, each participant responded to a questionnaire that collected information about a number of baseline characteristics, including demographics and dietary intake; several anthropometric variables, including height and weight, were measured directly; and blood and urine specimens were collected. Because the focus of the study was endogenous hormones and their relationship with breast cancer risk, stringent inclusion criteria were established and highly standardized conditions for collecting biological samples were applied. Women were excluded if they reported a bilateral ovariectomy, were currently breast-feeding or pregnant, had used oral contraceptives or hormone replacement therapy in the last 3 months, had a metabolic disease, or reported a history of cancer.

Cancer incidence information available from the local cancer registry (Varese Cancer Registry) was linked to the ORDET cohort database to identify case subjects with incident breast cancer through December 31, 2003. The Varese Cancer Registry is a high-quality registry, in which less than 2% of patients with breast cancer have been identified by the registry through death certificates alone and the histology and cytology of 96.3% of all breast tumors has been confirmed through pathology reports (20,23). The ORDET database was also linked to the Varese residents' database to check the vital status of participants.

After exclusion of women with a history of cancer (except for nonmelanoma skin cancer) and women who, immediately after baseline, were lost to follow-up (observed time = 0), 10 633 participants remained to form the ORDET base population. For this study, we further restricted the ORDET cohort to the 3966 participants who were postmenopausal at baseline. Women were considered postmenopausal if they reported not having had menses over the past 12 months. Participants were censored at the time of cancer diagnosis, death, or loss to follow-up, whichever came first. Median follow-up time was 13.5 years.

Selection of Case Subjects and Control Subjects

Case subjects and control subjects were selected from among all 3966 eligible postmenopausal women. Case subjects for this analysis were defined as women who developed breast cancer after their recruitment into the ORDET cohort but before the end of the study period (December 31, 2003). We identified a total of 184 case subjects with incident breast cancer. Of these, we eliminated three women because breast cancer was not their first cancer and another three women because no urine specimen had been collected. Among the remaining 178 case subjects, seven had in situ breast cancer.

For each case subject with breast cancer, four control subjects were randomly chosen from among appropriate risk sets defined as consisting of all cohort members who satisfied the matching criteria and were alive and free of cancer (except for nonmelanoma skin cancer) at the time of diagnosis of the index case subject. This incidence density sampling protocol for control subject selection allowed the control group to include women who later became a case subject (14 women became case subjects after their selection as a control subject) and each control subject to be sampled more than once (42 women were sampled more than once). A total of 710 control subjects were selected. Matching characteristics were

age at enrollment (±3 years), date of recruitment (±180 days), and laboratory batch.

Specimen Collection

Women were instructed to collect their urine over the previous night. They followed a collection protocol that called for them to discard the last void at 7:00 PM and to collect urine during the night up to 7:00 AM. The overnight urine specimen was kept at room temperature during collection. After delivery to the ORDET recruitment center on the day after overnight collection at between 7:30 AM and 9:00 AM, all urine specimens were processed immediately and stored at −80°C until biochemical determinations were done. To separate urine from mucosal residues and other secretion components that could interfere with bioassays, all samples were paper filtered and separated into 2-mL aliquots. No preservatives were added at collection or during storage. Blood samples were also collected after overnight fasting between 7:30 AM and 9:00 AM and stored at −80°C. This study was approved by the Ethical Review Board of the National Cancer Institute of Milan (Italy).

Laboratory Methods

Stability and reliability of the ORDET collection method for urinary 6-sulfatoxymelatonin have been demonstrated to be reasonable, although storage temperature has been shown to affect specimens such that urine in long-term storage at −30°C had consistently lower 6-sulfatoxymelatonin levels than urine in long-term storage at −80°C (24). Urine in our study was stored at −80°C, as specified by the study protocol.

Urine specimens from case subjects with breast cancer and matched control subjects were handled identically and assayed together on the same day and in the same run. All samples were taken out of the freezer simultaneously and sent to the laboratory in the same parcel on dry ice. All specimens had been stored at −80°C for an average of 17 years. Laboratory personnel were blinded to the case–control status of all specimens. Analytic error was controlled for by including two standard samples in each assay.

Urinary 6-sulfatoxymelatonin in each specimen was assayed by the Hormone Research Laboratory, Fondazione Istituti di Ricovero e Cura a Carattere Scientifico (IRCCS), Istituto Nazionale dei Tumori (Milan, Italy), with an enzyme-linked immunosorbent assay (product EK-M6S, Bühlmann Laboratories AG, Allschil, Switzerland); this assay has a lower detection limit for 6-sulfatoxymelatonin of 0.8 ng/mL.

Creatinine levels were also measured in each sample by the Medical Laboratory of the Department of Oncology, Fondazione IRCCS Istituto Nazionale Tumori (Milan, Italy) with a Hitachi Modular Automatic Analyzer and optimized reagents (F. Hoffmann-La Roche Ltd, Basel, Switzerland) (25). The average between-batch coefficients of variation were 5.3% and 10.3% for urinary 6-sulfatoxymelatonin (by use of high- and low-standard quality-control samples, respectively) and 2.7% and 2.1% for creatinine concentrations of 1.2 and 4.37 mg/dL, respectively. The within-batch coefficients of variation for 6-sulfatoxymelatonin, derived from the quality-control urine specimens included in the assays, were 1.8% and 9.8% (by use of high- and low-standard quality-control samples, respectively).

Plasma sex steroid measurements (testosterone, free testosterone, sex hormone–binding globulin, and estradiol) were conducted by Centro Medico Diagnostico Emilia (Bolgona, Italy). For testosterone and free testosterone, we used the "coat-a-count" procedure, which is a solid-phase radioimmunoassay (Diagnostic Product Corporation, Los Angeles, CA). For sex hormone–binding globulin, we used a solid-phase chemiluminescent immunometric assay (Diagnostic Product Corporation) with an IMMUNOLITE 1000 analyzer. For estradiol, we used a SPECTRIA Estradiol Sensitive coated-tube radioimmunoassay (Orion Diagnostica Oy, Espoo, Finland). The between-batch coefficients of variation, derived from quality controls that were included in the analytic assays, ranged from 5.8% to 18.3% for all four analytes (high- and low-standard quality-control samples).

Statistical Analyses

In total, 178 case subjects with invasive or in situ breast cancer and 710 matched control subjects were included in our analyses. Nine women had 6-sulfatoxymelatonin levels that were below the limit of detection for the assay (ie, <0.8 ng/mL), and their values were determined by extrapolation from the standard curve. We multiplied 6-sulfatoxymelatonin concentration (expressed as nanogram per milliliter) by 12-hour urine volume (expressed as milliliter) to obtain the total amount of 6-sulfatoxymelatonin produced between 7:00 PM and 7:00 AM (expressed as microgram per 12 hours). In secondary analyses, 6-sulfatoxymelatonin levels were normalized to the creatinine level of the sample to account for any differences arising from variations in urine concentrations (expressed as nanogram of 6-sulfatoxymelatonin per milligram of creatinine).

To test for differences in hormone levels between case subjects and control subjects, we used mixed-effects regression models for clustered data to adjust for possible confounding due to the matching factors and for any residual correlation between case subjects and control subjects within the matched set (26). We used conditional regression models to estimate the relative risks of breast cancer (reported as odds ratios [ORs] with 95% confidence intervals [CIs]) by quartile of urinary 6-sulfatoxymelatonin concentrations as derived from urinary 6-sulfatoxymelatonin values for all control subjects. Multivariable models were adjusted for known risk factors for breast cancer and circulating levels of testosterone, because circulating testosterone was the hormonal risk factor that was most strongly associated with breast cancer risk in postmenopausal women in our dataset (data not shown). Odds ratios were adjusted for the following breast cancer risk factors: body mass index in six categories (≤21, 21.1–23, 23.1–25, 25.1–27, 27.1–30, or >30 kg/m^2), history of benign breast disease (yes or no), first-degree family history (ie, mother or sister) of breast cancer (yes or no), smoking history (never, past, or current), age at menarche in three categories (≤13, 14, or ≥15 years), age at menopause in four categories (≤45, 46–49, 50–52, or ≥53 years), alcohol consumption (none or ≤12 or >12 g/day), duration of oral contraceptive use (never or ≤1 or >1 year), duration of hormone replacement therapy use (never or ≤1 or >1 year), parity (nulliparous or 1–2 or ≥3 children), age at birth of first child (<25, 25–27, or ≥28 years), and educational level (≤5, >5 to 8, or >8 years).

We tested for trends by modeling natural 6-sulfatoxymelatonin concentrations continuously and calculating the Wald statistic.

To evaluate the presence of an interaction between smoking (binary; current vs past or never smokers) and 6-sulfatoxymelatonin level (continuous), we added an interaction term to our logistic regression model and used the likelihood ratio test for interaction to determine statistical significance.

Because the menopausal status of women with a simple hysterectomy may have been misclassified in secondary analyses, we excluded seven case subjects and 20 control subjects who had hormone levels indicative of premenopausal women (follicle-stimulating hormone levels of less than 5 μg/mL or luteinizing hormone levels of less than 3 μg/L). We used SAS version 9.1.3 (Cary, NC) for all analyses. All *P* values were two-sided.

Results

Baseline Characteristics

Baseline characteristics of the 178 case subjects and 710 control subjects are shown in Table 1. The mean time between urine collection and diagnosis was 80 months (±50 months, SD), with a range of 1–182 months. Study participants were all postmenopausal women aged 41–70 years at urine collection. Most baseline characteristics did not differ by case–control status (Table 1). However, the age-adjusted mean 12-hour overnight urinary 6-sulfatoxymelatonin level was slightly lower for the case subjects than for the control subjects (11.1 μg of 6-sulfatoxymelatonin vs 12.1 μg of 6-sulfatoxymelatonin; 21.0 ng of 6-sulfatoxymelatonin per mg of creatinine vs 23.5 ng of 6-sulfatoxymelatonin per mg of creatinine). Age and age-adjusted baseline characteristics were also calculated by quartile of urinary overnight 6-sulfatoxymelatonin among the 710 control subjects (Table 2). Several of the baseline characteristics of control subjects, including age, family history of breast cancer, history of benign breast disease, smoking, and body mass index, differed modestly by 6-sulfatoxymelatonin quartile. None of the sex steroids tested, including circulating plasma testosterone, free testosterone, sex hormone–binding globulin, and estradiol, appeared to vary substantially by 6-sulfatoxymelatonin quartile.

Overnight Urinary 6-Sulfatoxymelatonin Output and Breast Cancer Risk

Overall, we observed an inverse association between 12-hour urinary overnight 6-sulfatoxymelatonin output and breast cancer risk (for highest vs lowest quartile of urinary 6-sulfatoxymelatonin output, OR = 0.68, 95% CI = 0.42 to 1.11; P_{trend} = .09; Table 3), with little change in these estimates after additional adjustment for breast cancer risk factors, including current smoking status. Night work and melatonin have been more strongly related with risk of invasive than in situ breast cancer (19,27–29), and so we excluded seven case subjects who were diagnosed with in situ breast cancer and their matched control subjects. The inverse association with invasive breast cancer was slightly stronger than that with breast cancer overall (for highest vs lowest quartile of urinary 6-sulfatoxymelatonin output, multivariable OR = 0.59, 95% CI = 0.35 to 1.00; P_{trend} = .04).

When we evaluated the influence of sex steroid hormones on these associations, no hormone associated with the risk of breast cancer risk in postmenopausal women was strongly correlated with urinary 6-sulfatoxymelatonin level (all Spearman rank correlations ≤ 0.15: r = 0.05 and P = .20 for testosterone; r = −0.01 and P = .87 for free testosterone; r = 0.02 and P = .62 for estradiol; and r = 0.15 and

Table 1. Baseline characteristics of 178 postmenopausal women with invasive (n = 171) or in situ (n = 7) breast cancer and 710 matched control subjects*

Characteristics	Case subjects (n = 178)	Control subjects (n = 710)
Age, y	57.7 (5.9)	57.8 (5.6)
Urinary aMT6s, ng/mL of creatinine	21.0 (1.10)	23.5 (0.55)
Urinary aMT6s per 12 h, μg	11.1 (0.55)	12.1 (0.27)
Age at menarche, y	13.2 (1.6)	13.3 (1.6)
Age at menopause, y	48.8 (4.9)	48.6 (5.0)
Parity (among parous women only), No. of children	2.2 (1.1)	2.3 (1.1)
Age at first child's birth (among parous women only)	26.9 (4.2)	26.6 (4.4)
Positive family history of breast cancer, %	12.9	7.2
HRT use, %	19.1	16.8
Duration of HRT use (among HRT users only), y	2.5 (3.1)	1.6 (2.2)
OC use, %	10.7	13.2
BMI, kg/m^2	26.2 (4.4)	26.1 (4.1)
Alcohol consumption, g/day	11.3 (14.6)	10.4 (13.6)
Positive history of benign breast disease, %	9.6	8.2
Education beyond 8 y, %	12.9	14.9
Smoking history		
Current smoker, %	16.3	14.9
Past smoker, %	11.2	11.5
Never smoker, %	72.5	73.6
Pack-years among ever smokers	12.9 (12.3)	11.3 (11.0)
Sex hormone levels		
Sex hormone–binding globulin, nmol/L	94.2 (3.86)	100.5 (1.94)
Testosterone, ng/mL	0.32 (0.02)	0.26 (0.01)
Free testosterone, pg/mL	0.75 (0.06)	0.61 (0.03)
Estradiol, pg/mL	9.66 (1.73)	10.7 (0.91)

* Data are the mean (SD). aMT6s = 6-sulfatoxymelatonin; HRT = hormone replacement therapy; OC = oral contraceptive; BMI = body mass index.

Table 2. Age and age-adjusted baseline characteristics of 710 control subjects by quartile of 12-hour overnight urinary 6-sulfatoxymelatonin level*

Characteristic	Q1 (n = 177)	Q2 (n = 178)	Q3 (n = 178)	Q4 (n = 177)
Urinary aMT6s output per 12 h, μg	<6.5	6.5–10.8	10.8–16.5	≥16.5
Age, y	59.2	57.3	58.1	56.0
Age at menarche, y	13.4	13.2	13.3	13.3
Age at menopause, y	48.4	49.0	48.8	48.4
Parity (among parous women only), No. of children	2.5	2.4	2.1	2.3
Age at first child's birth (among parous women only), y	26.0	26.5	27.3	26.6
Positive family history of breast cancer, %	5.1	6.2	7.3	10.1
HRT past use, %	21.5	17.0	14.6	14.0
OC use, %	12.1	15.8	16.4	9.7
BMI, kg/m^2	26.4	26.2	26.2	25.8
Alcohol consumption, g/day	9.1	10.1	12.8	9.7
Positive history of benign breast disease, %	6.8	6.8	6.7	12.4
Education beyond 8 y, %	9.0	16.4	15.2	19.1
Smoking history				
Current smoker, %	16.9	16.8	12.0	12.5
Past smoker, %	8.1	12.9	10.7	10.0
Never smoker, %	75.0	70.3	77.3	77.5
Pack-years among ever smokers	13.6	10.2	10.4	9.3
Sex hormone levels				
Sex hormone–binding globulin, nmol/L	97.4	99.8	95.8	109.2
Testosterone, ng/mL	0.24	0.26	0.27	0.27
Free testosterone, pg/mL	0.53	0.62	0.62	0.65
Estradiol, pg/mL	10.3	11.1	9.6	11.7

* Data are the mean. Q = quartile; aMT6s = 6-sulfatoxymelatonin; HRT = hormone replacement therapy; OC = oral contraceptive; BMI = body mass index.

$P < .001$ for sex hormone–binding globulin). Further adjustment for testosterone, free testosterone, estradiol, or sex hormone–binding globulin did not alter the estimates substantially, although the association between 6-sulfatoxymelatonin level and risk of breast cancer increased in statistical significance after adjustment for circulating testosterone (OR = 0.56, 95% CI = 0.33 to 0.97; $P_{trend} = .02$) (Table 3). In secondary analyses, we excluded the seven case subjects and 20 control subjects with a hysterectomy whose hormonal status indicated that they were still premenopausal. However, the exclusion of these women left our results virtually unchanged (data not shown).

Effect Modification

On the basis of a previous study (30) that indicated that an increased level of nocturnal plasma melatonin was inversely associated with tumor estrogen receptor concentration, we conducted analyses stratified on estrogen receptor status. Hormone receptor status, including HER2 status, was available for more than 93% of all case subjects with breast cancer; 139 (78.5%) of the 177 tumors for which estrogen receptor status was available were estrogen receptor positive (only 38 women had estrogen receptor–negative breast tumors), and 135 (81.3%) of the 166 women for whom HER2 status was available were HER2 negative (only 31 women had HER2-positive breast tumors). The inverse association between 6-sulfatoxymelatonin and breast cancer risk remained by and large unchanged when we restricted the analysis to women with estrogen receptor–positive breast tumors (for highest vs lowest quartile of urinary 6-sulfatoxymelatonin, multivariable OR = 0.59, 95% CI = 0.30 to 1.13) and was virtually the same when we restricted the analysis to women with HER2-negative tumors (for highest vs lowest quartile of urinary 6-sulfatoxymelatonin, multivariable OR = 0.60, 95% CI = 0.32 to 1.13). Although there were only six case subjects in the highest quartile of 6-sulfatoxymelatonin, the risk of estrogen receptor–negative breast cancer appeared lowest among women in the highest quartile of 6-sulfatoxymelatonin concentration (data not shown).

We found no effect modification by age (stratified along the median, <59 vs ≥59 years old) or body mass index (stratified along the median, 25.6 kg/m^2). Because a previous study (31) indicated that cigarette smoking affects melatonin production in premenopausal women, we further stratified by smoking status. Among never and past smokers, we observed a statistically significant inverse association between urinary melatonin level and breast cancer risk after adjustment for testosterone (highest vs lowest quartile of urinary 6-sulfatoxymelatonin output, OR = 0.38, 95% CI = 0.20 to 0.74; $P_{trend} = .001$) (Table 3). By contrast, we did not observe an inverse association among women who reported cigarette smoking at the time of urine collection (highest vs lowest tertile of urinary 6-sulfatoxymelatonin output, age-adjusted OR = 3.55, 95% CI = 0.61 to 20.8; $P_{trend} = 0.07$; χ^2 from log likelihood ratio test for interaction between smoking and 6-sulfatoxymelatonin = 9.22, P [1 *df*] = .002). The power of these analyses was limited, however, with only 28 case subjects with breast cancer among current smokers.

Lag-Time Analyses

To exclude the possibility of preclinical tumors influencing 6-sulfatoxymelatonin levels, we carried out subset analyses in which we excluded case subjects who were diagnosed shortly after urine collection. In these subset analyses, the strength of the association between urinary 6-sulfatoxymelatonin level and breast cancer risk was stronger after excluding case subjects who were diagnosed with invasive breast cancer within 2 years (for highest vs lowest quartile

Table 3. Association between risk of breast cancer by quartile of total 12-hour overnight 6-sulfatoxymelatonin output*

Group and parameter	Q1	Q2	Q3	Q4	P_{trend}†
Urinary aMT6s output per 12 h, μg	<6.5	6.5–10.8	10.8–16.5	≥16.5	
Case subjects with invasive and in situ breast cancer					
No. of case subjects/No. of control subjects	56/177	37/177	45/178	40/178	
Simple OR (95% CI)‡	1.00 (ref.)	0.65 (0.41 to 1.04)	0.79 (0.51 to 1.23)	0.68 (0.42 to 1.11)	.09
Multivariable OR (95% CI)§	1.00 (ref.)	0.68 (0.42 to 1.11)	0.84 (0.53 to 1.35)	0.65 (0.39 to 1.09)	.08
Case subjects with invasive breast cancer					
No. of case subjects/No. of control subjects	55/171	37/170	42/169	37/173	
Simple OR (95% CI)‡	1.00 (ref.)	0.67 (0.42 to 1.07)	0.76 (0.48 to 1.20)	0.63 (0.39 to 1.04)	.05
Multivariable OR (95% CI)§	1.00 (ref.)	0.70 (0.43 to 1.14)	0.82 (0.50 to 1.34)	0.59 (0.35 to 1.00)	.04
Multivariable OR (95% CI)§, adjusted for testosterone	1.00 (ref.)	0.69 (0.41 to 1.14)	0.84 (0.51 to 1.38)	0.56 (0.33 to 0.97)	.02
Excluding current smokers					
No. of case subjects/No. of control subjects	51/158	31/133	36/138	25/148	
Simple OR (95% CI)‡	1.00 (ref.)	0.69 (0.41 to 1.15)	0.72 (0.44 to 1.20)	0.44 (0.25 to 0.80)	.004
Multivariable OR (95% CI)§	1.00 (ref.)	0.72 (0.41 to 1.26)	0.74 (0.42 to 1.29)	0.40 (0.21 to 0.76)	.002
Multivariable OR (95% CI)§, adjusted for testosterone	1.00 (ref.)	0.69 (0.39 to 1.22)	0.71 (0.40 to 1.26)	0.38 (0.20 to 0.74)	.001

* Total 12-hour overnight 6-sulfatoxymelatonin output was the concentration of 6-sulfatoxymelatonin (ng/mL) multiplied by the 12-hour volume (mL). Quartiles are based on the distribution in control subjects. Q = quartile; aMT6s = 6-sulfatoxymelatonin; OR = odds ratio; CI = confidence interval; ref. = referent.

† We tested for trends by modeling 6-sulfatoxymelatonin output continuously and calculating the Wald statistic. All statistical tests were two-sided.

‡ Simple conditional logistic regression model adjusting for the matching variables (year of birth, month and year of urine collection, and laboratory batch) was used.

§ Multivariable conditional logistic regression models were used. Relative risks (expressed as odds ratios) were adjusted for the following breast cancer risk factors: body mass index in six categories (≤21, 21.1–23, 23.1–25, 25.1–27, 27.1–30, or >30 kg/m^2), history of benign breast disease (yes or no), family history (mother or sister) of breast cancer (yes or no), smoking history (never, past, or current), age at menarche in three categories (≤13, 14, or ≥15 years), age at menopause in four categories (≤45, 46–49, 50–52, or ≥53 years), alcohol consumption (none or ≤12 or >12 g/day), duration of oral contraceptive use (never or ≤1 or >1 year), duration of hormone replacement therapy use (never or ≤1 or >1 year), parity (nulliparous or 1–2 or ≥3 children), age at first child's birth (<25, 25–27, or ≥28 years), and participant's educational level (≤5, >5 to 8, or >8 years).

of urinary 6-sulfatoxymelatonin output, OR = 0.35, 95% CI = 0.17 to 0.71; P_{trend} < .001) or 4 years (for highest vs lowest quartile of urinary 6-sulfatoxymelatonin output, OR = 0.34, 95% CI = 0.15 to 0.75; P_{trend} = .002) after urine collection.

Creatinine-Adjusted Urinary 6-Sulfatoxymelatonin Concentration and Breast Cancer Risk

In secondary analyses, we also evaluated associations between the creatinine-adjusted 6-sulfatoxymelatonin level and breast cancer risk. Creatinine-adjusted and total 6-sulfatoxymelatonin were highly correlated (Spearman r = 0.93 and P < .001), and both measures also correlated well with crude 6-sulfatoxymelatonin concentration (r = 0.83 and P < .001). In multivariable analyses, we observed an inverse association between creatinine-adjusted urinary 6-sulfatoxymelatonin and risk of invasive breast cancer (for highest vs lowest quartile of total urinary 6-sulfatoxymelatonin, OR = 0.63, 95% CI = 0.37 to 1.07; P_{trend} = .02), a risk that was also markedly stronger among never and past smokers (highest vs lowest quartile of total 12-hour urinary 6-sulfatoxymelatonin, OR = 0.49, 95% CI = 0.26 to 0.91; P_{trend} = .003).

Urinary creatinine concentration is influenced by a number of factors, including sex, ethnicity, age, and body mass index (32). Although our study population included exclusively white women, differences in age and body mass index could have biased our creatinine-adjusted 6-sulfatoxymelatonin measure. We found no correlation between the creatinine-adjusted 6-sulfatoxymelatonin level and creatinine level (Spearman r = −0.02 and P = .70), however, indicating that no major bias appears to have been introduced by adjusting for creatinine.

Discussion

We found a statistically significant inverse association between overnight urinary 6-sulfatoxymelatonin level and breast cancer risk in postmenopausal women, a finding that was even stronger after current smokers were excluded. To our knowledge, our study is the first to report these associations. Among the 992 women in the highest 6-sulfatoxymelatonin quartile, 40 developed breast cancer during follow-up (average follow-up = 13.5 years), compared with 56 of the 992 women in the lowest 6-sulfatoxymelatonin quartile.

Few previous studies have evaluated the association between circulating melatonin levels and breast cancer risk, and most are limited by the fact that melatonin levels were measured after the subjects were diagnosed with breast cancer (6,11,30,33–41). The only two prospective studies have been conducted among primarily premenopausal women, and each used a different measure of melatonin. The first study (18) found no association between the circulating level of 6-sulfatoxymelatonin in 24-hour urine specimens and breast cancer risk. The other study (19) found a strong inverse association between levels of 6-sulfatoxymelatonin in first morning urine specimens and breast cancer risk in premenopausal women. It has been argued subsequently (42) that pooled urine samples collected over 24 hours cannot detect differences between subjects in the nocturnal duration or the peak of melatonin secretion, and such differences may be important for assessing an association between melatonin levels and breast cancer risk.

The best urine sample (eg, 24-hour urine, 12-hour overnight urine, or first morning voids) to reflect circulating levels of a hormone with substantial variation in secretion throughout the day

remains subject of research. Spot (ie, only one timed sample) urine samples are most practical, especially in large epidemiological studies. Depending on the amount of fluid intake, the dilution of spot urine samples (and, thus, the concentration of the substrate measured in urine) varies throughout the day. A common approach to correct for various dilutions among spot urine samples is to adjust for urinary creatinine (32). However, urinary creatinine concentration is influenced by many factors, including sex, ethnicity, and age (32). Thus, the independence of the effects of creatinine adjustment must be evaluated carefully in each individual study. In addition, as with the 24-hour urine specimen, a single untimed spot urine specimen is likely to be unable to accurately capture the concentration of a hormone whose secretion rate varies throughout the day. These limitations could be overcome by using timed 12-hour urine specimens, but few studies have examined the differences between various sampling methods. Results from studies of cortisol appear to indicate that measurements from overnight urine specimens, although comparable to those from 24-hour urine specimens, require a larger sample size to detect differences (43,44). Given the somewhat opposite physiological 24-hour secretion rhythm of cortisol and melatonin (ie, peak production in the morning and a nadir at night for cortisol; peak production at night and nadir during the day for melatonin), use of an overnight urine sample for assessing melatonin could increase the likelihood of finding a true association between melatonin levels and breast cancer risk, when compared with use of a 24-hour urine specimen, although additional studies are needed to determine the optimum approach.

We were able to consider most important breast cancer risk factors in our analyses. Excluding case subjects who were diagnosed within the first 2 or 4 years after urine collection did not alter our findings; in fact, the risk reduction associated with higher 6-sulfatoxymelatonin levels appeared slightly stronger in these analyses, indicating that 6-sulfatoxymelatonin levels are not simply a marker for tumor growth.

The interaction between smoking and 6-sulfatoxymelatonin levels in our data was novel and highlights additional challenges posed by using a marker that is measured in urine; that is, it is highly dependent on the metabolic rate of melatonin. Cytochrome P450 1A2 (CYP1A2) is the primary enzyme involved in the metabolization of melatonin to urinary 6-sulfatoxymelatonin (45), and smoking has been shown to induce CYP1A2 activity (45,46). Higher CYP1A2 activity, however, appears to be associated with breast cancer risk, particularly in postmenopausal women (47). The interaction between smoking and 6-sulfatoxymelatonin levels in our cohort could be indicative of the role of CYP1A2 activity in these associations and may ultimately support the hypothesis that smoking renders urinary 6-sulfatoxymelatonin levels a less useful marker for breast cancer risk prediction, particularly in postmenopausal women. Total nocturnal plasma melatonin output has been reported (6) to correlate with the level of 6-hydroxymelatonin sulfate in the first morning void urine specimen in 29 women aged 40–70 years; however, whether smoking may have differentially affected these findings was not reported. In a comparable dataset (19), no interaction between smoking and 6-sulfatoxymelatonin levels was observed (E. S. Schernhammer, MD, DrPH, S. E. Hankinson, ScD, unpublished data, 2008); however, 6-sulfatoxymelatonin was measured in first morning urine specimens, compared with the overnight urine specimen that we used. Clearly, more studies will be required to elucidate the effect of smoking on circulating levels of melatonin and its metabolites.

Our study is limited by the absence of information on light exposure at night, including night work status; thus, we cannot adjust for this factor. However, because the risk of breast cancer among the older women in our study (who were probably retired at the time of urine collection) and among the younger women (ie, we found no effect modification by age) was similar, some of that concern was alleviated. In addition, one would expect that our inability to adjust for night work would have biased our results only toward the null. Another potential limitation of our study is that we did not have information on the vitamin D status of our study subjects; vitamin D status is another possible breast cancer risk factor (48,49). The relationship between melatonin levels and vitamin D is unclear; however, if a relationship exists, it could have influenced our results. For example, women with low morning levels of melatonin (if due to an altered sleep–wake cycle) may also have particularly low levels of vitamin D because of low sun exposure. Finally, we were unable to correct for laboratory measurement error and within-person variability in our dataset; however, because of the random nature of this error, we expect that this correction would have strengthened our observed risks.

In summary, our findings show that melatonin secretion, as assessed by 6-sulfatoxymelatonin levels in 12-hour overnight urine specimens, is associated with an increased risk of developing breast cancer in postmenopausal women. These findings also indicate that factors affecting the metabolism of melatonin must be carefully taken into account in analyses that use this marker. Studies to confirm our findings should also address how melatonin levels measured in 24-hour urine specimens differ from those measured in 12-hour overnight or first morning urine specimens and investigate the primary mechanisms for the association between melatonin and breast cancer risk.

References

1. Czeisler CA, Klerman EB. Circadian and sleep-dependent regulation of hormone release in humans. *Recent Prog Horm Res.* 1999;54:97–130.
2. Czeisler CA, Duffy JF, Shanahan TL, et al. Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science.* 1999;284(5423):2177–2181.
3. Arendt J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. *Rev Reprod.* 1998;3(1):13–22.
4. Arendt J, Bojkowski C, Franey C, Wright J, Marks V. Immunoassay of 6-hydroxymelatonin sulfate in human plasma and urine: abolition of the urinary 24-hour rhythm with atenolol. *J Clin Endocrinol Metab.* 1985;60(6):1166–1173.
5. Baskett JJ, Cockrem JF, Antunovich TA. Sulphatoxymelatonin excretion in older people: relationship to plasma melatonin and renal function. *J Pineal Res.* 1998;24(1):58–61.
6. Cook MR, Graham C, Kavet R, Stevens RG, Davis S, Kheifets L. Morning urinary assessment of nocturnal melatonin secretion in older women. *J Pineal Res.* 2000;28(1):41–47.
7. Lang U, Kornemark M, Aubert ML, Paunier L, Sizonenko PC. Radioimmunological determination of urinary melatonin in humans: correlation with plasma levels and typical 24-hour rhythmicity. *J Clin Endocrinol Metab.* 1981;53(3):645–650.
8. Leibenluft E, Feldman-Naim S, Turner EH, Schwartz PJ, Wehr TA. Salivary and plasma measures of dim light melatonin onset (DLMO) in

patients with rapid cycling bipolar disorder. *Biol Psychiatry.* 1996;40(8): 731–735.
9. Nowak R, McMillen IC, Redman J, Short RV. The correlation between serum and salivary melatonin concentrations and urinary 6-hydroxymelatonin sulphate excretion rates: two non-invasive techniques for monitoring human circadian rhythmicity. *Clin Endocrinol.* 1987;27(4):445–452.
10. Wetterberg L. Melatonin in humans physiological and clinical studies. *J Neural Transm Suppl.* 1978;13:289–310.
11. Graham C, Cook MR, Kavet R, Sastre A, Smith DK. Prediction of nocturnal plasma melatonin from morning urinary measures. *J Pineal Res.* 1998;24(4):230–238.
12. Schernhammer ES, Hankinson SE. Light at night: a novel risk factor for cancer in shift workers? *Clin Occup Environ Med.* 2003;3(2):263–278.
13. Stevens RG, Blask DE, Brainard GC, et al. Meeting report: the role of environmental lighting and circadian disruption in cancer and other diseases. *Environ Health Perspect.* 2007;115(9):1357–1362.
14. Cohen M, Lippman M, Chabner B. Role of pineal gland in aetiology and treatment of breast cancer. *Lancet.* 1978;2(8094):814–816.
15. Brzezinski A. Melatonin in humans. *N Engl J Med.* 1997;336(3):186–195.
16. Vijayalaxmi Thomas CR Jr, Reiter RJ, Herman TS. Melatonin: from basic research to cancer treatment clinics. *J Clin Oncol.* 2002;20(10): 2575–2601.
17. Blask DE, Brainard GC, Dauchy RT, et al. Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. *Cancer Res.* 2005;65(23): 11174–11184.
18. Travis RC, Allen DS, Fentiman IS, Key TJ. Melatonin and breast cancer: a prospective study. *J Natl Cancer Inst.* 2004;96(6):475–482.
19. Schernhammer ES, Hankinson SE. Urinary melatonin levels and breast cancer risk. *J Natl Cancer Inst.* 2005;97(14):1084–1087.
20. Berrino F, Muti P, Micheli A, et al. Serum sex hormone levels after menopause and subsequent breast cancer. *J Natl Cancer Inst.* 1996;88(5): 291–296.
21. Berrino F, Pisani P, Muti P. Prospective study of hormones and diet in the aetiology of breast cancer. In: Riboli E, Saracci R, eds. *Diet, hormones, and cancer: methodological issues for prospective studies*. Lyon, France: IARC; 1988:34–38.
22. Parkin DM, Whelan S, Ferlay J. *Cancer incidence in five continents*. Vol. VII. IARC Scientific Publications No. 143. Lyon, France: IARC; 1997.
23. Waterhouse J, Muir C, Shanmugaratnam K. *IARC Scientific Publications.* Lyon, France: IARC; 1982.
24. Barba M, Cavalleri A, Schunemann HJ, et al. Reliability of urinary 6-sulfatoxymelatonin as a biomarker in breast cancer. *Int J Biol Markers.* 2006; 21(4):242–245.
25. Bartels H, Bohmer M, Heierli C. Serum creatinine determination without protein precipitation [in German]. *Clin Chim Acta.* 1972;37:193–197.
26. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: a generalized estimating equation approach. *Biometrics.* 1988;44(4):1049–1060.
27. Schernhammer ES, Laden F, Speizer FE, et al. Rotating night shifts and risk of breast cancer in women participating in the Nurses' Health Study. *J Natl Cancer Inst.* 2001;93(20):1563–1568.
28. Davis S, Mirick DK, Stevens RG. Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst.* 2001;93(20):1557–1562.
29. Hansen J. Increased breast cancer risk among women who work predominantly at night. *Epidemiology.* 2001;12(1):74–77.
30. Tamarkin L, Danforth D, Lichter A, et al. Decreased nocturnal plasma melatonin peak in patients with estrogen receptor positive breast cancer. *Science.* 1982;216(4549):1003–1005.
31. Schernhammer ES, Kroenke CH, Dowsett M, Folkerd E, Hankinson SE. Urinary 6-sulfatoxymelatonin levels and their correlations with lifestyle factors and steroid hormone levels. *J Pineal Res.* 2006;40(2):116–124.
32. Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL. Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements. *Environ Health Perspect.* 2005;113(2):192–200.
33. Bartsch C, Bartsch H, Bellmann O, Lippert TH. Depression of serum melatonin in patients with primary breast cancer is not due to an increased peripheral metabolism. *Cancer.* 1991;67(6):1681–1684.
34. Bartsch C, Bartsch H, Fuchs U, Lippert TH, Bellmann O, Gupta D. Stage-dependent depression of melatonin in patients with primary breast cancer. Correlation with prolactin, thyroid stimulating hormone, and steroid receptors. *Cancer.* 1989;64(2):426–433.
35. Bartsch C, Bartsch H, Jain AK, Laumas KR, Wetterberg L. Urinary melatonin levels in human breast cancer patients. *J Neural Transm.* 1981;52(4): 281–294.
36. Bartsch C, Bartsch H, Karenovics A, Franz H, Peiker G, Mecke D. Nocturnal urinary 6-sulphatoxymelatonin excretion is decreased in primary breast cancer patients compared to age-matched controls and shows negative correlation with tumor-size. *J Pineal Res.* 1997;23(2):53–58.
37. Danforth DNJ, Tamarkin L, Mulvihill JJ, Bagley CS, Lippman ME. Plasma melatonin and the hormone-dependency of human breast cancer. *J Clin Oncol.* 1985;3(7):941–948.
38. Falkson G, Falkson HC, Steyn ME, Rapoport BL, Meyer BJ. Plasma melatonin in patients with breast cancer. *Oncology.* 1990;47(5):401–405.
39. Lissoni P, Bastone A, Sala R, et al. The clinical significance of melatonin serum determination in oncological patients and its correlations with GH and PRL blood levels. *Eur J Cancer Clin Oncol.* 1987; 23(7):949–957.
40. Lissoni P, Crispino S, Barni S, et al. Pineal gland and tumor cell kinetics: serum levels of melatonin in relation to Ki-67 labeling rate in breast cancer. *Oncology.* 1990;47(3):275–277.
41. Skene DJ, Bojkowski CJ, Currie JE, Wright J, Boulter PS, Arendt J. 6-sulphatoxymelatonin production in breast cancer patients. *J Pineal Res.* 1990;8(3):269–276.
42. Hrushesky WJ, Blask DE. Re: melatonin and breast cancer: a prospective study. *J Natl Cancer Inst.* 2004;96(11):888–889.
43. White IR, Brunner EJ, Barron JL. A comparison of overnight and 24 hour collection to measure urinary catecholamines. *J Clin Epidemiol.* 1995;48(2): 263–267.
44. Janicki-Deverts D, Zilles K, Cohen S, Baum A. Can a 15-hour (overnight) urinary catecholamine measure substitute for a 24-hour measure? *J Applied Biobehav Res.* 2006;11(2):69–78.
45. Faber MS, Jetter A, Fuhr U. Assessment of CYP1A2 activity in clinical practice: why, how, and when? *Basic Clin Pharmacol Toxicol.* 2005;97(3): 125–134.
46. Hartter S, Nordmark A, Rose DM, Bertilsson L, Tybring G, Laine K. Effects of caffeine intake on the pharmacokinetics of melatonin, a probe drug for CYP1A2 activity. *Br J Clin Pharmacol.* 2003;56(6):679–682.
47. Hong CC, Tang BK, Rao V, et al. Cytochrome P450 1A2 (CYP1A2) activity, mammographic density, and oxidative stress: a cross-sectional study. *Breast Cancer Res.* 2004;6(4):R338–R351.
48. Welsh J. Vitamin D and breast cancer: insights from animal models. *Am J Clin Nutr.* 2004;80(6):1721S–1724S.
49. Zhang SM. Role of vitamins in the risk, prevention, and treatment of breast cancer. *Curr Opin Obstet Gynecol.* 2004;16(1):19–25.

Funding

Department of Defense (W81XWH04-1-0195).

Notes

We are indebted to the 10786 ORDET participants. We would also like to thank Dr P. Crosignani and the staff of the Lombardy Cancer Registry for technical assistance; C. Agnoli for statistical support; Dr D. Morelli for conducting creatinine assays; and Drs G. Bolelli and F. Franceschetti for conducting sex steroid assays. The authors had full responsibility for all these activities: design of the study, the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

Manuscript received February 4, 2008; revised April 2, 2008; accepted April 25, 2008.