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Original Contribution

Urinary Melatonin Concentration and the Risk of Breast Cancer in Nurses' Health Study II

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Experimental and epidemiologic data support a protective role for melatonin in breast cancer etiology, yet studies in premenopausal women are scarce. In a case-control study nested within the Nurses' Health Study II cohort, we measured the concentration of melatonin's major urinary metabolite, 6-sulfatoxymelatonin (aMT6s), in urine samples collected between 1996 and 1999 among 600 breast cancer cases and 786 matched controls. Cases were predominantly premenopausal women who were diagnosed with incident breast cancer after urine collection and before June 1, 2007. Using multivariable conditional logistic regression, we computed odds ratios and 95% confidence intervals. Melatonin levels were not significantly associated with total breast cancer risk (for the fourth (top) quartile (Q4) of aMT6s vs. the first (bottom) quartile (Q1), odds ratio (OR) = 0.91, 95% confidence interval (CI): 0.64, 1.28; $P_{trend} = 0.38$) or risk of invasive or in situ breast cancer. Findings did not vary by body mass index, smoking status, menopausal status, or time between urine collection and diagnosis (all $P_{interaction}$ values ≥ 0.12). For example, the odds ratio for total breast cancer among women with ≤5 years between urine collection and diagnosis was 0.74 (Q4 vs. Q1; 95% CI: 0.45, 1.20; $P_{trend} = 0.09$), and it was 1.20 (Q4 vs. Q1; 95% CI: 0.72, 1.98; $P_{trend} = 0.70$) for women with >5 years. Our data do not support an overall association between urinary melatonin levels and breast cancer risk.

breast cancer; melatonin; 6-sulfatoxymelatonin

Abbreviations: aMT6s, 6-sulfatoxymelatonin; BMI, body mass index; CI, confidence interval; ER, estrogen receptor; NHS II, Nurses' Health Study II; OR, odds ratio; ORDET, Hormones and Diet in the Etiology of Breast Cancer Risk; PR, progesterone receptor; Q, quartile.

Recent meta-analyses of epidemiologic studies suggest that women who work the night shift have a 19%–51% increased risk of breast cancer (1–3). Decreased melatonin production due to greater light exposure at night is a potential biological mechanism underlying this relationship. Melatonin (*N*-acetyl-5-methoxytryptamine) is a naturally occurring hormone produced primarily by the pineal gland (4). The synthesis and release of melatonin is stimulated by darkness and suppressed by light, with low circulating levels observed during the day and the highest levels being found at night between 2 AM and 4 AM (4). Melatonin is metabolized through the liver and excreted in the urine, and 6-sulfatoxymelatonin (aMT6s) is the main metabolite of melatonin measured in urine for estimation of circulating melatonin levels (4).

Multiple lines of evidence support potential antiestrogenic, antioxidant, and antiproliferative properties of melatonin (4–6). Melatonin may influence estrogen signaling directly at the tissue level through interaction with estrogen receptor or indirectly via down-regulation of the hypothalamic-pituitary-gonadal axis, resulting in reduced levels of circulating estrogens (7, 8). Further, melatonin has been shown to down-regulate aromatase expression, thereby reducing local estrogen production and suppressing tumor growth (8).

In addition to the potential estrogen-mediated pathways, the activation of melatonin receptors, which bind melatonin,

has been shown to inhibit breast tumor initiation, growth, and cell proliferation (9). Further, in an in vitro study, melatonin receptor 1 was associated with suppressed breast tumor growth (10). Finally, the antioxidant properties of melatonin may combat oxidative stress by suppressing tumor initiation and promoting apoptosis (11).

Eight prospective epidemiologic studies (12–19) have examined this relationship, with conflicting results, due in part to the relatively small sample sizes and short follow-up periods in prior studies, as well as the variation in methods of assessing aMT6s levels. We conducted a prospective nested case-control study of urinary aMT6s levels and breast cancer risk among predominantly premenopausal women in the Nurses' Health Study II (NHS II) cohort. The current analysis was an extension of our previous report (13), with 6 additional years of follow-up and triple the sample size.

METHODS

Study population

NHS II is an ongoing prospective cohort study of 116,430 US female registered nurses aged 25–42 years at baseline in 1989. Self-administered questionnaires are completed biennially to update information on lifestyle factors, health behaviors, medical history, and incident disease. Between 1996 and 1999, a total of 29,611 women aged 32–54 years provided blood and urine samples and completed a short questionnaire to record the date and time of urine collection, the number of night shifts worked in the past 2 weeks, and the participant's current weight, smoking status, and other lifestyle variables. Among these women, 18,521 premenopausal women who had not used oral contraceptives, been pregnant, or breastfed within the past 6 months and had no personal history of cancer provided a single urine sample and 2 blood samples timed with their menstrual cycle. The remaining 11,090 women (e.g., postmenopausal, using hormonal contraception, or not able to provide timed samples) contributed an untimed sample. Urine samples were collected without preservatives and were shipped overnight on ice to our laboratory. Ninety-three percent of samples were received within 26 hours of collection, and we have previously demonstrated that levels of urinary aMT6s remain stable when processing is delayed for 24–48 hours (20). Samples have been stored in the vapor phase of liquid nitrogen freezers (≤−130°C) since collection.

Follow-up of the blood and urine substudy cohort was close to 95%. The institutional review boards of Brigham and Women's Hospital (Boston, Massachusetts) and the University of Massachusetts, Amherst (Amherst, Massachusetts) approved this analysis.

Assessment of breast cancer cases

We identified incident invasive and in situ breast cancer cases by self-report on biennial questionnaires. Deaths were reported by family members, reported by the US Postal Service, or ascertained through the National Death Index. A study physician performed medical record review to confirm breast cancer cases and to abstract information on invasiveness and hormone receptor status. If medical record confirmation was not possible, the nurse participant confirmed her diagnosis, and these cases ($n = 19$) were included in this analysis given that 99% of self-reported breast cancer cases in this cohort are confirmed upon medical record review. A total of 600 breast cancer cases were diagnosed after urine collection and before June 1, 2007. As previously reported (13), participants diagnosed with breast cancer by June 2001 ($n = 192$) were matched with 2 controls, and the present study additionally included case women diagnosed after June 2001 ($n = 408$) who were matched with 1 control. All cases were matched with controls by year of birth (±2 years), menopausal status at urine collection (premenopausal vs. not), month/year (±2 months) and time (±2 hours) of urine sample collection, luteal day of the menstrual cycle at urine collection if the sample was timed (±1 day), fasting status at urine collection (yes, no), and ethnicity (African-American, Asian, Caucasian, Hispanic, or other).

Assessment of melatonin secretion

Nocturnal melatonin secretion was estimated by measuring the concentration of the major urinary metabolite of melatonin, aMT6s, in urine samples (80% first morning void; 20% randomly timed spot urine sample). In 2001, urinary aMT6s was measured at the Endocrine Core Laboratory of Dr. M. Wilson (Yerkes National Primate Research Center, Emory University, Atlanta, Georgia) using a competitive enzyme-linked immunosorbent assay (ALPCO Diagnostics, Windham, New Hampshire) with a lower detection limit of 0.8 ng/mL. Urinary creatinine concentration was measured in the same laboratory using a modified Jaffe method. From 2003 through 2007, urinary melatonin was measured at the Ricchuiti Laboratory (now the Carroll Laboratory, Boston, Massachusetts) using commercially available enzyme-linked immunosorbent assay kits with a lower detection limit of 0.8 ng/mL (IBL International GmbH, Hamburg, Germany), and urinary creatinine levels were measured using the COBAS Integra 400 assay (Roche Diagnostics, Indianapolis, Indiana). For each participant, urinary aMT6s was divided by the urinary creatinine level to account for differences in urine concentration, resulting in normalized urinary aMT6s values expressed as ng/mg creatinine.

Assays were conducted in a total of 3 batches: 1 at Emory University (2001) and 2 at Carroll Laboratory (2003/2005 and 2007 samples). Because the original data showed considerable differences in absolute levels of aMT6s across batches, we completed a drift recalibration project. A total of 45 urine samples (15 control participants from each cycle: 2001, 2003/2005, and 2007) that represented low ($n = 5$), medium ($n = 5$), and high ($n = 5$) tertiles of melatonin values per cycle were sent to the Carroll Laboratory in 2013 and assayed as described above. The correlation between the original assay results and the reanalyzed results (samples analyzed in 2013) was greater than 0.90 for all follow-up cycles, indicating that the different assays were measuring the same analyte, though with differing absolute levels. We used samples from the original batches and the reanalyzed set of 45 samples to statistically account for laboratory drift over time. As described elsewhere (21), we performed linear regression within each

batch to regress the rerun values on the original laboratory values, and the resulting intercept and slope were used to predict recalibrated values for participants in that batch. Using the recalibrated data, we created quartiles of creatinine-adjusted melatonin levels based on the distribution in controls for all analyses.

Masked replicate quality control samples (10% of samples) were included in each batch to assess the coefficient of variation. Within-batch coefficients of variation in 3 total batches ranged from 2.4% to 13.9% for melatonin and from 1.2% to 9.2% for creatinine. Samples for cases and controls were treated identically, case-control sets were assayed together, and laboratory personnel were masked to the case/control status of all specimens.

Statistical analysis

For this study, we selected NHS II participants who were cancer-free at the time of urine collection (1996–1999) and diagnosed with breast cancer between urine collection and June 2007, as well as their matched controls. A total of 1,386 participants were eligible for this analysis after exclusion of 6 observations that were either missing melatonin or creatinine values or were identified as statistical outliers on the log scale using the extreme Studentized deviate many-outlier procedure (22). Urinary aMT6s measurements below the lower detection limit of the assay ($n = 10$) were set equal to the detection limit to produce conservative estimates.

We used conditional logistic regression to estimate odds ratios and 95% confidence intervals in our primary analyses. For subanalyses, we used conditional logistic regression if the stratification variable was a case characteristic (i.e., invasive breast cancer vs. in situ breast cancer) and sufficient numbers were available (i.e., estrogen receptor (ER)-positive/progesterone receptor (PR)-positive (ER+/PR+) status, time between urine collection and diagnosis). For other subanalyses with limited numbers (i.e., ER+/PR− status, ER−/PR− status, and stratification by smoking, body mass index (BMI; weight (kg)/height $(m)^2$), and menopausal status), we used unconditional logistic regression with adjustment for matching factors to maximize statistical power. Tests for trend were performed using melatonin as a continuous variable, and P values were calculated using the Wald statistic.

Data on lifestyle factors and other characteristics were taken from the biennial questionnaire completed closest to the time of urine collection, as well as the questionnaire completed at the time of blood and urine collection. In addition to the matching factors (i.e., simple models), multivariable models adjusted for age at menarche (≤11, 12, 13, or ≥14 years); parity and age at first birth combined (nulliparous, 1–2 children and <25 years at first birth, 1–2 children and 25–29 years at first birth, 1–2 children and ≥30 years at first birth, ≥3 children and <25 years at first birth, or ≥3 children and ≥25 years at first birth); age at menopause (premenopausal, ≤45 years, or >45 years); alcohol intake (none, <5 g/day, 5–9 g/day, or ≥10 g/day); family history of breast cancer (yes, no); history of benign breast disease (yes, no); and BMI (continuous). To explore whether there were differing influences of BMI on premenopausal women and postmenopausal women, we included an interaction term for BMI (<25, ≥25) and menopausal status (premenopausal, postmenopausal) in our model; however, this did not significantly affect our estimates, and therefore the term was not retained in our final models. Additional adjustment for oral contraceptive use (never, past, or current), hormone replacement therapy (never, past, or current), physical activity (metabolic equivalents/week), chronotype (morning type, evening type, or neither), smoking status (never, past, or current), breastfeeding (ever or never), and current use of antidepressant medication (yes, no) did not alter our estimates; thus, these variables were not retained in our final models.

To evaluate whether the association between melatonin levels and breast cancer risk varied across strata of smoking status at urine collection (never smoker or past/current smoker), BMI at urine collection (<25, ≥25), menopausal status at diagnosis (premenopausal or postmenopausal), and time between urine collection and diagnosis (dichotomized at the median as ≤5 years or >5 years), we added an interaction term for each potential effect modifier (multiplying the dichotomous effect modifier by the midpoint of each quartile of melatonin) to our model and used the likelihood ratio test for interaction to determine statistical significance.

All statistical tests were 2-sided; $P < 0.05$ was used to define statistical significance. Analyses were conducted in SAS, version 9.2 (SAS Institute, Inc., Cary, North Carolina).

Table 1. Baseline Characteristics of 600 Cases and 786 Matched Controls in Nurses' Health Study II, 1996–2007

Characteristic[a]	Cases ($n = 600$)		Controls ($n = 786$)	
	Mean (SD)	%	Mean (SD)	%
Urinary aMT6s concentration, ng/mg creatinine	48.9 (31.8)		47.9 (29.6)	
Age, years[b]	43.9 (4.2)		44.0 (4.1)	
Age at menarche, years	12.4 (1.3)		12.4 (1.4)	
Body mass index[c]	25.0 (5.0)		25.7 (5.9)	
Alcohol consumption, g/day	4.2 (7.2)		3.6 (6.3)	
Age at first birth, years[d]	26.7 (4.7)		26.3 (4.6)	
Parity[d]	2.2 (0.9)		2.3 (0.9)	
Nulliparous		21.4		19.1
Caucasian ethnicity[b]		96.5		97.5
History of benign breast disease		27.4		19.2
Family history of breast cancer		16.5		10.1
Premenopausal at urine collection[b]		78.8		79.5

Abbreviations: aMT6s, 6-sulfatoxymelatonin; SD, standard deviation.

[a] Values are standardized to the age distribution of the study population. All characteristics except age were adjusted for age.

[b] Matching variables included age, ethnicity, and menopausal status at the time of urine collection.

[c] Weight (kg)/height $(m)^2$.

[d] Among parous women only.

Table 2. Baseline Characteristics of 786 Control Participants by Quartile of Urinary 6-Sulfatoxymelatonin Concentration in Nurses' Health Study II, 1996–2007

Characteristic[a]	Quartile of Urinary aMT6s Concentration[b]							
	Q1 ($n = 197$)		Q2 ($n = 196$)		Q3 ($n = 196$)		Q4 ($n = 197$)	
	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%
Age, years[c]	44.8 (3.8)		44.1 (4.1)		43.2 (4.3)		43.8 (4.2)	
Age at menarche, years	12.3 (1.5)		12.5 (1.4)		12.5 (1.3)		12.4 (1.4)	
Body mass index[d]	26.9 (6.3)		25.9 (6.5)		25.7 (5.1)		24.0 (4.9)	
Alcohol consumption, g/day	3.5 (5.6)		3.9 (6.4)		2.8 (5.5)		4.3 (7.0)	
Age at first birth, years[e]	26.6 (4.5)		26.3 (4.8)		26.3 (4.7)		26.0 (4.3)	
Parity[e]	2.3 (0.9)		2.3 (1.0)		2.3 (0.9)		2.4 (0.9)	
Nulliparous		18.7		20.6		17.8		16.5
Caucasian ethnicity[c]		94.9		98.9		97.6		98.9
History of benign breast disease		17.6		22.1		18.3		19.3
Family history of breast cancer		8.5		10.6		11.3		10.4
Premenopausal at urine collection[c]		77.2		74.9		84.7		80.0

Abbreviations: aMT6s, 6-sulfatoxymelatonin; Q, quartile; SD, standard deviation.

[a] Values are standardized to the age distribution of the study population. All characteristics except age were adjusted for age.

[b] Quartile ranges were as follows: Q1, ≤26.6 ng/mg creatinine; Q2, 26.7–42.5 ng/mg creatinine; Q3, 42.6–61.8 ng/mg creatinine; Q4, ≥61.9 ng/mg creatinine.

[c] Matching variables included age, ethnicity, and menopausal status at the time of urine collection.

[d] Weight (kg)/height $(m)^2$.

[e] Among parous women only.

RESULTS

Our study comprised 1,386 participants, including 600 cases and 786 matched controls. Cases and controls were similar with regard to most breast cancer risk factors, including age at menarche, parity, age at first birth, and BMI (Table 1). However, cases were more likely than controls to have a history of benign breast disease and a family history of breast cancer. Participants were predominantly premenopausal at diagnosis (79%), and the urine sample provided by the majority of women was a first morning sample (80%).

Among the 786 controls, the distributions of most baseline characteristics, including age at menarche, age at first birth, and parity, were similar across quartiles of creatinine-adjusted aMT6s (Table 2). Controls in the highest quartile of urinary aMT6s had a lower BMI than controls in the lowest quartile (24 vs. 27). In addition, compared with those in the lowest quartile of urinary aMT6s, controls in the highest quartile were less likely to be past or current smokers (29.5% vs. 42.5%).

Urinary aMT6s was not associated with the risk of breast cancer overall (Table 3). Compared with women in the bottom quartile of urinary aMT6s concentrations, the multivariable odds ratio for women in the top quartile was 0.91 (95% confidence interval (CI): 0.64, 1.28; $P_{trend} = 0.38$). No significant associations were observed when we examined invasive and in situ tumors separately. For invasive tumors, the odds ratio comparing the top quartile of urinary aMT6s levels with the bottom quartile was 0.94 (95% CI: 0.62, 1.43; $P_{trend} = 0.52$), and for in situ tumors, the comparable odds ratio was 0.96 (95% CI: 0.48, 1.89; $P_{trend} = 0.67$). In secondary analyses, we restricted the data to women providing first morning urine samples and excluded current night-shift workers, since night-shift work may alter first morning urinary aMT6s levels; however, our results were unchanged (data not shown).

In analyses stratified by tumor hormone receptor status, we observed no association between urinary melatonin level and ER+/PR+ tumors ($n = 286$ cases; for quartile 4 (Q4) vs. quartile 1 (Q1), odds ratio (OR) = 0.94, 95% CI: 0.56, 1.58; $P_{trend} = 0.59$). Further, no significant association or trend emerged between urinary melatonin level and ER+/PR− breast cancer risk ($n = 45$ cases; for Q4 vs. Q1, OR = 1.07, 95% CI: 0.42, 2.72; $P_{trend} = 0.78$) or ER−/PR− breast cancer risk ($n = 78$ cases; for Q4 vs. Q1, OR = 0.96, 95% CI: 0.47, 1.97; $P_{trend} = 0.96$).

Next, we evaluated the association between urinary aMT6s and breast cancer risk by duration of follow-up and other factors (Table 4). A nonsignificant 26% reduced risk of breast cancer was observed among women diagnosed ≤5 years after urine collection (for Q4 vs. Q1, OR = 0.74, 95% CI: 0.45, 1.20; $P_{trend} = 0.09$), whereas a nonsignificant increase in risk was observed in women with >5 years between urine collection and diagnosis (for Q4 vs. Q1, OR = 1.20, 95% CI: 0.72, 1.98; $P_{trend} = 0.70$) ($P_{interaction} = 0.12$). The suggestion of an inverse trend emerged among postmenopausal women (for Q4 vs. Q1, OR = 0.71, 95% CI: 0.38, 1.34; $P_{trend} = 0.08$), although the interaction by menopausal status at diagnosis was not significant ($P_{interaction} = 0.64$). Finally, no significant variation in the association was observed across strata of BMI ($P_{interaction} = 0.33$) or smoking status ($P_{interaction} = 0.27$).

Table 3. Odds Ratios for Breast Cancer by Cancer Type and Quartile of Urinary 6-Sulfatoxymelatonin Concentration in Nurses' Health Study II, 1996–2007

Cancer Type and Quartile of Urinary aMT6s Level[a]	No. of Cases	No. of Controls	Multivariable OR[b]	95% CI
Total breast cancer[c,d]	600	786		
Q1	145	197	1.00	Referent
Q2	170	196	1.02	0.75, 1.40
Q3	125	196	0.79	0.56, 1.11
Q4	160	197	0.91	0.64, 1.28
P for trend			0.38	
Invasive breast cancer	422	551		
Q1	103	138	1.00	Referent
Q2	117	144	0.92	0.64, 1.33
Q3	92	140	0.82	0.55, 1.23
Q4	110	129	0.94	0.62, 1.43
P for trend			0.52	
In situ breast cancer	159	193		
Q1	36	48	1.00	Referent
Q2	51	38	1.90	0.93, 3.91
Q3	27	47	0.68	0.33, 1.42
Q4	45	60	0.96	0.48, 1.89
P for trend			0.67	
ER+/PR+ breast cancer	286	414		
Q1	73	105	1.00	Referent
Q2	80	98	0.98	0.62, 1.55
Q3	58	110	0.67	0.41, 1.10
Q4	75	101	0.94	0.56, 1.58
P for trend			0.59	

Abbreviations: aMT6s, 6-sulfatoxymelatonin; CI, confidence interval; ER+, estrogen receptor-positive; OR, odds ratio; PR+, progesterone receptor-positive; Q, quartile.

[a] Quartiles were based on the distribution in control subjects. Ranges were as follows: Q1, ≤26.6 ng/mg creatinine; Q2, 26.7–42.5 ng/mg creatinine; Q3, 42.6–61.8 ng/mg creatinine; Q4, ≥61.9 ng/mg creatinine.

[b] Multivariable conditional logistic regression models, in addition to matching variables, included adjustment for the following breast cancer risk factors: age at menarche (≤11, 12, 13, or ≥14 years); parity and age at first birth combined (nulliparous, 1–2 children and <25 years at first birth, 1–2 children and 25–29 years at first birth, 1–2 children and ≥30 years at first birth, ≥3 children and <25 years at first birth, or ≥3 children and ≥25 years at first birth); age at menopause (premenopausal, ≤45 years, or >45 years); alcohol intake (none, <5 g/day, 5–9 g/day, or ≥10 g/day); family history of breast cancer (yes, no); history of benign breast disease (yes, no); and body mass index (weight (kg)/height $(m)^2$; continuous).

[c] The "total breast cancer" analysis included 422 invasive cases and matched controls, 159 in situ cases and matched controls, and 19 self-reported cases and matched controls.

[d] Simple OR and 95% CI for total breast cancer by quartile: Q1, OR = 1.00 (referent); Q2, OR = 1.13 (95% CI: 0.84, 1.52); Q3, OR = 0.81 (95% CI: 0.58, 1.12); Q4, OR = 0.98 (95% CI: 0.71, 1.34).

DISCUSSION

In this prospective study, we did not observe a significant association between urinary melatonin levels and breast cancer risk overall. In our previous report, which included 147 invasive breast cancer cases and 291 matched controls from the current expanded data set, women with the highest levels of aMT6s had a 41% reduced risk of invasive breast cancer (for Q4 vs. Q1, OR = 0.59, 95% CI: 0.36, 0.97) (13). In this updated analysis with longer follow-up time and a greater sample size, adding cases that occurred farther from the time of urine collection, we observed an attenuation of our previously published results.

In total, 8 prospective studies of the melatonin–breast cancer relationship (12–19) have been conducted to date, including 2 in premenopausal women (13, 16), 3 in postmenopausal women (14, 15, 19), and 3 in pre- and postmenopausal women combined (12, 17, 18). A meta-analysis of 5 of the 8 previously published studies (18) found that women with the highest aMT6s levels had a significantly reduced risk of breast cancer overall (for Q4 vs. Q1, OR = 0.81, 95% CI: 0.66, 0.99), supporting a modest inverse association between urinary melatonin levels and breast cancer risk based on studies that used first morning or 12-hour urine collection methods (18). Further, a significant inverse association was observed in postmenopausal women (for Q4 vs. Q1, OR = 0.68, 95% CI: 0.49, 0.92), but no association was reported in premenopausal women (for Q4 vs. Q1, OR = 1.05, 95% CI: 0.71, 1.54) (18).

Among individual prospective studies carried out among postmenopausal women, a significantly reduced (by 38%–44%) risk of breast cancer was observed among women in the highest quartile of melatonin level versus the lowest quartile in 2 studies (14, 15), whereas 1 study found no association (19). However, in the 3 studies that included both premenopausal and postmenopausal women (127–251 cases), no association between aMT6s levels and breast cancer risk was observed (12, 17, 18). Various urine collection methods were utilized in these studies, including 24-hour urine (12), randomly timed spot urine (17), and first morning urine samples (18). Despite the moderate correlation of urinary aMT6s levels between these methods (e.g., for first morning urine and 24-hour urine, $r = 0.66$ (18)), methods such as 24-hour urine collection may reduce interindividual variability and fail to capture the nocturnal melatonin peak (23), resulting in potential nondifferential exposure misclassification and accounting, at least in part, for the null findings observed.

As described previously, a significant inverse association was observed among predominantly premenopausal participants in our initial report of this relationship in NHS II (13). In the only other study of premenopausal women, conducted among women in the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) cohort (180 cases), a positive association was observed between melatonin and invasive breast cancer overall (for Q4 vs. Q1, OR = 1.43, 95% CI: 0.83, 2.45) (16). However, this association was attenuated among current nonsmokers (OR = 1.00, 95% CI: 0.52, 1.94) (16), which suggests that current smoking may alter rates of metabolization of urinary melatonin. This is of interest, because cytochrome P450 1A2 is the primary enzyme in the

Table 4. Odds Ratios for Breast Cancer by Quartile of Urinary 6-Sulfatoxymelatonin Concentration and Potential Effect Modifiers in Nurses' Health Study II, 1996–2007

Potential Effect Modifier	No. of Cases	No. of Controls	Quartile of Urinary aMT6s Concentration[a]								*P* for Trend	*P* for Interaction
			Q1		Q2		Q3		Q4			
			OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI		
Menopausal status at diagnosis[b,c]	536	710										0.64
Premenopausal	355	502	1.00	Referent	1.26	0.84, 1.88	0.86	0.57, 1.30	1.07	0.71, 1.62	0.85	
Postmenopausal	181	208	1.00	Referent	1.03	0.56, 1.90	0.81	0.41, 1.58	0.71	0.38, 1.34	0.08	
Body mass index[d] at time of urine collection[c]	600	786										0.33
<25	359	444	1.00	Referent	1.01	0.65, 1.57	0.96	0.61, 1.49	1.02	0.67, 1.57	0.95	
≥25	241	342	1.00	Referent	1.25	0.79, 1.98	0.61	0.36, 1.01	1.09	0.65, 1.83	0.82	
Smoking status at time of urine collection[c]	600	786										0.27
Never smoker	388	537	1.00	Referent	1.02	0.69, 1.50	0.68	0.45, 1.02	1.09	0.73, 1.62	0.95	
Past/current smoker	212	249	1.00	Referent	1.21	0.72, 2.04	1.09	0.61, 1.93	0.91	0.53, 1.58	0.61	
Time between urine collection and diagnosis, years[e]	600	786										0.12
≤5	261	447	1.00	Referent	0.93	0.60, 1.45	0.71	0.43, 1.18	0.74	0.45, 1.20	0.09	
>5	339	339	1.00	Referent	1.15	0.72, 1.82	0.88	0.54, 1.44	1.20	0.72, 1.98	0.70	

Abbreviations: aMT6s, 6-sulfatoxymelatonin; CI, confidence interval; OR, odds ratio; Q, quartile.

[a] Quartiles were based on the distribution in control subjects. Ranges were as follows: Q1, ≤26.6 ng/mg creatinine; Q2, 26.7–42.5 ng/mg creatinine; Q3, 42.6–61.8 ng/mg creatinine; Q4, ≥61.9 ng/mg creatinine.

[b] Women with missing or dubious data on menopausal status ($n = 140$) were excluded.

[c] For menopausal status, body mass index, and smoking status, multivariable unconditional logistic regression was used with adjustment for matching variables and the following breast cancer risk factors: age at menarche (≤11, 12, 13, or ≥14 years); parity and age at first birth combined (nulliparous, 1–2 children and <25 years at first birth, 1–2 children and 25–29 years at first birth, 1–2 children and ≥30 years at first birth, ≥3 children and <25 years at first birth, or ≥3 children and ≥25 years at first birth); age at menopause (premenopausal, ≤45 years, or >45 years); alcohol intake (none, <5 g/day, 5–9 g/day, or ≥10 g/day); family history of breast cancer (yes, no); history of benign breast disease (yes, no); and body mass index (continuous).

[d] Weight (kg)/height $(m)^2$.

[e] For time between urine collection and diagnosis, conditional logistic regression was used with adjustment for matching variables and the breast cancer risk factors listed above.

metabolism of melatonin to urinary aMT6s, and smoking has been shown to stimulate cytochrome P450 1A2 activity (24, 25). In the present study, we did not observe substantial variation in analyses stratified by smoking status; however, the rate of current smoking in the NHS II cohort (7%) was much lower than that in the ORDET cohort (24.5%), which limited our ability to separately explore associations in these subgroups.

Several studies attempted to explore whether preclinical disease may influence melatonin levels in early follow-up cycles or whether melatonin levels in the more distant past may be more biologically relevant. Overall, 5 prior prospective studies examined the impact of time between urine collection and diagnosis on the association between urinary aMT6s levels and breast cancer risk (14–16, 18, 19). Results were inconclusive, with some investigators suggesting stronger inverse associations when excluding the first several years of follow-up after urine collection (14, 16), some reporting stronger positive associations for breast cancer cases that occurred closer to the time of urine collection (19), and others suggesting no difference in results by time between urine collection and breast cancer diagnosis (15, 18). Each of these lagged analyses was limited by relatively modest numbers of cases; thus, chance may be the most likely explanation for the observed inconsistencies. In the present study, with limited statistical power, we observed a nonsignificant inverse relationship between melatonin and breast cancer risk in women with 5 or fewer years between urine collection and diagnosis (for Q4 vs. Q1, OR = 0.71, 95% CI: 0.43, 1.17) and a nonsignificant positive association after more than 5 years (for Q4 vs. Q1, OR = 1.20, 95% CI: 0.72, 2.02). Although these differences by follow-up time were not significant ($P_{\text{interaction}} = 0.12$), they may still serve as a potential explanation for the discrepant findings between our current updated analyses (overall null results) and our earlier findings (13), in which aMT6s was significantly inversely associated with breast cancer risk. Therefore, a detailed reevaluation (e.g., a pooled analysis including all prospective studies) with both longer follow-up and greater power would be useful.

In line with prior studies (13–16, 19), we found that the association between urinary aMT6s levels and breast cancer risk does not vary by tumor estrogen receptor expression. A

moderate-to-strong inverse association between BMI and urinary melatonin levels has been reported (12, 20, 26, 27), but this relationship has not been consistently observed (14–18). In the present study, we found no evidence that the melatonin–breast cancer risk relationship varied by BMI or menopausal status (or a combination of the two variables) at diagnosis. Given that few studies have explored the potential for effect modification by BMI and/or menopausal status, confirmation of these findings is warranted.

To our knowledge, this is the largest prospective study to date to have examined the relationship between urinary aMT6s levels and breast cancer risk. We were able to account for most known breast cancer risk factors in our analyses, including lifestyle and personal characteristics. First morning urine measurements of aMT6s normalized to creatinine have been shown to provide reliable estimates of overnight melatonin production (28), and a validated enzyme-linked immunosorbent assay was utilized in this study. Furthermore, we had excellent laboratory coefficients of variation, and we recalibrated levels to account for variability in aMT6s levels across laboratories. Medical records and pathology reports were used to confirm self-reported breast cancer diagnoses, and 99% of self-reported breast cancer cases in this cohort are confirmed upon medical record review. Our study was limited because aMT6s was measured in urine that was collected only once per participant. However, the intraclass correlation over 3 years among premenopausal women from the NHS II cohort was high (intraclass correlation coefficient = 0.72), which supports a single morning urinary aMT6s measurement as a reasonable marker for long-term melatonin levels (20).

In summary, we did not observe an association between urinary melatonin levels and breast cancer risk overall in this large nested case-control study. Melatonin may play a role in various phases of carcinogenesis, which may account for the conflicting results observed in prospective studies to date. A pooled analysis of existing data and additional large prospective studies with long follow-up and consistent methods of measuring aMT6s are needed to confirm these findings.

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