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Research Article

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Urinary Melatonin in Relation to Postmenopausal Breast Cancer Risk According to Melatonin 1 Receptor Status

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Abstract

Background: Urinary melatonin levels have been associated with a reduced risk of breast cancer in postmenopausal women, but this association might vary according to tumor melatonin 1 receptor (MT1R) expression.

Methods: We conducted a nested case–control study among 1,354 postmenopausal women in the Nurses' Health Study, who were cancer free when they provided first-morning spot urine samples in 2000 to 2002; urine samples were assayed for 6-sulfatoxymelatonin (aMT6s, a major metabolite of melatonin). Five-hundred fifty-five of these women developed breast cancer before May 31, 2012, and were matched to 799 control subjects. In a subset of cases, immunohistochemistry was used to determine MT1R status of tumor tissue. We used multivariable-adjusted conditional logistic regression to estimate the relative risk (RR) of breast cancer [with 95% confidence intervals (CI)] across quartiles of creatinine-standardized urinary aMT6s level, including by MT1R subtype.

Results: Higher urinary melatonin levels were suggestively associated with a lower overall risk of breast cancer (multivariable-adjusted RR = 0.78; 95% CI = 0.61–0.99, comparing quartile 4 vs. quartile 1; $P_{trend} = 0.08$); this association was similar for invasive vs. *in situ* tumors ($P_{heterogeneity} = 0.12$). There was no evidence that associations differed according to MT1R status of the tumor (e.g., $P_{heterogeneity\ for\ overall\ breast\ cancer} = 0.88$).

Conclusions: Higher urinary melatonin levels were associated with reduced breast cancer risk in this cohort of postmenopausal women, and the association was not modified by MT1R subtype.

Impact: Urinary melatonin levels appear to predict the risk of breast cancer in postmenopausal women. However, future research should evaluate these associations with longer-term follow-up and among premenopausal women. *Cancer Epidemiol Biomarkers Prev; 26(3); 413–9. ©2016 AACR.*

Introduction

Light exposure during the biologic night has been hypothesized to increase risk of breast cancer (1). Melatonin, an indolamine hormone, is a molecular marker of the circadian system; it is entrained to the 24-hour environmental light-dark cycle, released by the pineal gland, and suppressed by light (2). In addition, melatonin has been shown to mediate numerous cell-signaling pathways involved in breast cancer, including estrogen-dependent pathways (3, 4). Some epidemiologic studies (5–7), although not all (8–11), have reported that lower levels of urinary melatonin are associated with greater risk of breast cancer—in line with the notion that light exposure at night, which suppresses melatonin secretion, promotes carcinogenesis.

Moreover, two melatonin receptors have been identified, melatonin 1 receptor (MT1R) and melatonin 2 receptor (MT2R), and MT1R has been found on the surface of breast tumors in cell culture (12–14). In rodents, MT1R overexpression has been linked to reduced breast tumor incidence (15), whereas age-related decline in MT1R expression has been shown to reduce the sensitivity of melatonin for these receptors, leading to enhanced tumor growth (16, 17). Indeed, experimental work indicates that melatonin's anticarcinogenic effects may be largely mediated by MT1R (18), but this potential mechanism has been understudied in human populations. In the Nurses' Health Study (NHS), previous research found that higher urinary melatonin levels were strongly associated with lower risk of breast cancer based on 357 cases that developed over six years of follow up (7); however, the combined role of endogenous melatonin levels and MT1R in breast cancer has not been explored.

To extend our previous study, we evaluated the association of urinary melatonin and breast cancer risk in the Nurses' Health Study, utilizing ~200 additional breast cancer cases with twice the length of follow-up. Furthermore, we examined whether this association differed according to MT1R status of the breast tumor.

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Materials and Methods

Study population

We conducted a nested case–control study within the NHS cohort. The NHS cohort began in 1976, when 121,701 female nurses, who were 30 to 55 years old and living in the United States, returned an initial mailed questionnaire with information on breast cancer risk factors and major medical diagnoses. This information has been updated using similar mailed questionnaires every 2 years, and participation has exceeded 90% at every questionnaire cycle. Between 2000 and 2002, 18,643 participants provided spot urine samples and completed a short supplemental questionnaire, including information on date and time of urine collection, confirmation of first-morning urine, current weight, and recent postmenopausal hormone (PMH) use; 93% of urine samples were first-morning samples. These samples were returned on ice, by overnight mail, after which they were aliquoted and stored in nitrogen freezers (at −130°C) until they were assayed for melatonin.

Women were eligible for the present analysis if they provided a urine sample and had no history of cancer (except nonmelanoma skin cancer) prior to urine collection; cases were women who developed breast cancer after urine collection and before May 31, 2012. These cases were matched either 1:1 (for postmenopausal cases with recent PMH use) or 1:2 (all other cases) to controls who did not develop breast cancer during the follow-up period. Matching factors included: birth year, menopausal status, recent PMH use (defined as use $\leq$3 months from the time of urine collection), timing of urine collection (month and time of day), and fasting status at urine collection. Women were considered postmenopausal if they reported natural menopause or bilateral oophorectomy, or if they reported hysterectomy without bilateral oophorectomy and were ages $\geq$56 years and a nonsmoker, or ages $\geq$54 years and a current smoker (90% of women in the cohort had reached natural menopause by these ages). We focused on postmenopausal women in this study. The Institutional Review Board of Brigham and Women's Hospital approved this study, and informed consent was implied by voluntary return of mailed questionnaires and biological specimens.

Breast cancer case ascertainment

Participants self-reported a breast cancer diagnosis on one of the biennial cohort questionnaires. All self-reported cases were asked for permission to review their medical records, which provided confirmation of their diagnosis. When medical records were unavailable, breast cancer cases were defined as probable and included in the analysis if corroborated by phone interview or written confirmation from the participant. In some cases, breast cancer was reported during death follow-up, when family members or the postal service reported a participant's death to study personnel. The National Death Index was reviewed after each questionnaire cycle to determine the status of women who were unresponsive to cohort questionnaires. In this cohort, self-reported breast cancer was >98% accurate compared to pathology reports (19).

Urinary melatonin assessment

Urine samples for breast cancer cases and controls were removed from the freezer simultaneously, handled identically, and each batch was shipped in one parcel. Three batches were sent for laboratory analysis based on the biennial questionnaire cycle in which women reported incident breast cancer. The first batch (i.e., incident cases reported on the 2002 and 2004 biennial questionnaires and their matched controls) and second batch (i.e., incident cases reported on the 2006 biennial questionnaire and their matched controls) were sent to the Endocrine Core Laboratory of M. Wilson (Yerkes National Primate Center at Emory University, Atlanta, GA). The third batch (i.e., incident cases reported on the 2008 and 2010 biennial questionnaires and their matched controls) were sent to the Specialty Assay Research Core Laboratory of R. Carroll (Brigham and Women's Hospital, Boston, MA). 6-Sulfatoxymelatonin (aMT6s) was assayed using the Buhlmann ELISA, with a lower limit of detection of 0.8 ng/mL; creatinine levels were also assayed, which enabled creatinine standardization to account for differences in urine concentration. Laboratory personnel were blinded to case–control status, and case–control samples were assayed together on the same day and in the same run; quality control samples were included in each batch. In this analyses, within-batch coefficients of variation were: 9.5%, 10.3%, and 6.2% for aMT6s and 8.5%, 3.1%, and 1.1% for creatinine across the three batches, respectively.

The reproducibility of creatinine-adjusted aMT6s levels over 3 years has been previously established, using the collection and assessment method described for this cohort (intraclass correlation = 0.72; ref. 20).

MT1 receptor assessment

A detailed description of the breast tumor tissue block collection, microarrays (TMAs) construction, and immunohistochemical analyses performed can be found elsewhere (21–23). Briefly, we collected archived formalin-fixed, paraffin-embedded breast cancer blocks from incident breast cancer cases through 2006. Hematoxylin and eosin sections from those cases were reviewed to confirm the diagnosis, classify the cancer according to histological type and grade, and identify the area from which the cores for the TMAs would be taken. TMAs were constructed in the Dana-Farber Harvard Cancer Center Tissue Microarray Core Facility (Boston, Massachusetts). Three cores 0.6 mm in diameter were obtained from each breast cancer sample and inserted into the recipient TMA blocks.

Immunohistochemical staining for the MT1 receptor was performed on 5-μm paraffin sections cut from TMA blocks, using the following antibody: melatonin receptor (MT2/MTNR1B) from LS Bio (Catalog No.: LS-A930; Lot. No.: 7195/7196AP3-1; 1:1,000 dilution). A maximum of three cores were reviewed for each tumor, and degree of MT1 receptor staining in the epithelium was assessed using image analysis software (Definiens). Mean staining across available cores was used to define the outcome; epithelial staining was scored as positive if the mean percentage of stained cells was $\geq$50%; it was otherwise considered negative. This cut point was determined by maximizing the accuracy of image analysis software results compared to those of an expert pathologist, who manually reviewed one of the TMAs (n = 126 participants, with concordance of MT1R positive and negative ratings = 89% and 79%, respectively). Because there is no established cut point for MT1R positivity, we also defined alternative cut points based on the absolute distribution of mean epithelial staining ($\leq$1/3, >1/3 to $\leq$2/3, and >2/3 of cells positive) and relative distribution of mean epithelial staining (in tertiles).

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Statistical analysis

Of the 18,643 cohort participants with urine samples, 606 women without a prior history of cancer developed incident breast cancer during the follow-up period; they were matched to 1,085 cancer-free controls. We excluded 279 women whose urine sample was not assayed for aMT6s, nine women whose aMT6s values were statistical outliers (according to the generalized extreme studentized deviate many-outlier detection approach; ref. 24), and 23 women who were not postmenopausal at the time of urine collection. In addition, we excluded 26 women whose matched pair was removed based on these exclusions. The remaining 1,354 women (555 breast cancer cases and 799 matched controls, with 158 having MT1R subtype information) comprised our analytic sample.

For these analyses, aMT6s levels below the limit of detection ($n = 29$) were conservatively set to 0.8 ng/mL (i.e., the lower limit of detection), and aMT6s levels were standardized to creatinine. Because absolute aMT6s levels assayed by the Wilson laboratory were consistently lower compared to the Carroll laboratory, we used a previously described method to recalibrate these measurements by accounting for batch-to-batch variability that was not explained by age and body-mass index (BMI; i.e., the strongest predictors of aMT6s levels; refs. 25, 26).

Using logistic regression models conditioned on matching factors, we estimated simple and multivariable-adjusted odds ratios to approximate relative risks (RR) of breast cancer (with corresponding 95% confidence intervals [CI]) across quartiles of creatinine-standardized urinary aMT6s levels (based on recalibrated values). Our analyses extend a previous study of urinary melatonin and incident breast cancer in this cohort, which included follow-up time up until May 31, 2006 (7); thus, to facilitate a comparison with previous findings, we also calculated effect estimates based on the second half of follow up (i.e., cases occurring between June 1, 2006 and May 31, 2012, and their matched controls). In the subset of case–control pairs for which MT1R subtyping was available in the cases, we obtained separate estimates for MT1R- positive vs. negative tumors (defined as a tumor with $\geq$50% vs. <50% of cells with MT1R staining) using similar models. We used batch-corrected quartiles based on the creatinine-standardized aMT6s distribution in the controls; the lowest quartile served as the reference category. Tests of linear trend were calculated across quartiles using the Wald statistic, and tests of heterogeneity were calculated across breast cancer subtypes (invasive vs. *in situ* and MT1R-positive vs. MT1R-negative) using polytomous conditional logistic regression.

Multivariable-adjusted models were controlled for possible breast cancer risk factors: age at menarche (<12, 12, 13, $\geq$14 years), age at menopause ($\leq$45, 46–50, 51–53, $\geq$54 years), parity (nulliparous, 1–2, 3–4, or $\geq$5 children), family history of breast cancer (mother or sister: yes, no), BMI (continuous in kg/m^2), alcohol intake (0, 1–14.9, $\geq$15 g/day), smoking status (current: yes, no), history of benign breast disease (yes, no), PMH use (current: yes, no), and type and duration of PMH use (estrogen-only: 0, <5, $\geq$5 years; estrogen + progesterone: 0, <5, $\geq$5 years). Antidepressant use (current: yes, no) and first morning urine (yes, no) variables were included in our models to reduce variation in urinary melatonin levels.

We conducted several secondary analyses. First, we repeated our overall analysis of urinary melatonin and incident breast cancer while restricting to women with first-morning urine samples. Second, we repeated our analysis restricting our sample to estrogen- and progesterone-positive cases (i.e., the majority of breast cancer cases in our sample) because melatonin might influence breast cancer development via hormone-dependent pathways (3, 4). Third, we re-evaluated the association between urinary melatonin and incident breast cancer while implementing a 2-year lag between time of urine collection and breast cancer diagnosis to minimize the possibility of subclinical breast tumors affecting urinary melatonin levels. Finally, in analyses incorporating the MT1R status of breast tumors, we explored alternative categories for defining MT1R-positivity (low, medium, and high) based on the absolute and relative distributions of mean epithelial staining, as described above.

Statistical tests were two sided and were considered statistically significant at $P < 0.05$. All analyses were conducted using SAS version 9.3 (SAS Institute).

Results

When we examined the distribution of breast cancer risk factors, we observed several expected patterns in our sample: breast cancer cases were more likely to have a family history of breast cancer, and a history of benign breast disease compared to controls (Table 1). In addition, cases had a higher mean intake of alcohol relative to controls. Among 158 cases with MT1R subtyping, women with MT1R-positive tumors were less likely to be current smokers, recent PMH users, and estrogen-only PMH users for $\geq$5 years compared to women with MT1R-negative tumors; MT1R-positive cases also had higher alcohol consumption compared to MT1R-negative cases.

There were few meaningful differences in participant characteristics at the time of urine collection according to quartiles of urinary melatonin level; however, as expected, mean age and BMI decreased across increasing melatonin quartiles (Table 2).

In our main analyses, we observed a suggestive trend of decreasing risk of overall breast cancer (including invasive and in situ cases) across increasing melatonin quartiles during a mean of 4.0 years of follow up in simple and multivariable-adjusted models ($P_{trend} = 0.08$ for both models; Table 3). Point estimates were similar in simple versus multivariable-adjusted models, with significantly reduced risk comparing quartile 4 versus quartile 1 in the fully adjusted model (i.e., the 95% CI for this comparison did not include the null value of 1.00; RR = 0.78; 95% CI, 0.61–0.99). When we examined invasive and *in situ* cases separately, there were similar trends with decreasing risk of breast cancer observed over increasing quartiles of melatonin ($P_{heterogeneity} = 0.12$), although neither of these individual trends reached statistical significance (P_{trend} for final models = 0.19 for invasive cases and 0.09 for *in situ* cases). When we evaluated these associations based on cases occurring during the second half of follow up (and their matched controls), we found that urinary melatonin levels were not related to risk of breast cancer during that period (multivariable-adjusted RR = 1.05; 95% CI, 0.66–1.67 comparing quartile 4 vs. quartile 1 of urinary melatonin, $P_{trend} = 0.75$; Supplementary Table).

In the subset of 158 case–control pairs with MT1R subtyping in the cases, we found a similar pattern of overall association between urinary melatonin and breast cancer (Table 4). In multivariable-adjusted models, associations were also similar when we separately examined MT1R-negative cases (RR = 0.60; 95% CI, 0.35–1.06 comparing tertile 3 versus tertile 1, $P_{trend} = 0.07$) and

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Table 1. Baseline characteristics (at urine collection in 2000) of breast cancer cases ($n = 555$), including by MT1R subtype ($n = 158$), and matched controls ($n = 799$)

Characteristic	Controls ($n = 799$)	All breast cancer cases ($n = 555$)	MT1R negative cases ($n = 91$)	MT1R positive cases ($n = 67$)
Mean melatonin level, ng/mg creatinine (SD)	29.8 (30.7)	26.6 (25.9)	29.1 (28.7)	24.2 (34.0)
Mean age, years (SD)[a]	66.9 (6.8)	66.6 (6.8)	67.4 (7.1)	66.9 (6.5)
Mean age at menarche, years (SD)	12.6 (1.4)	12.5 (1.4)	12.6 (1.3)	12.4 (1.4)
Mean age at menopause, years (SD)	49.6 (4.7)	50.3 (5.0)	50.4 (4.3)	50.5 (4.7)
Mean number of births (SD)	3.2 (1.6)	3.1 (1.4)	3.2 (1.5)	3.1 (1.3)
Family history of breast cancer, %	16	23	23	21
Mean BMI, kg/m^2 (SD)	26.4 (5.2)	26.8 (5.0)	26.8 (4.7)	27.3 (4.8)
Mean alcohol intake, g/day (SD)	5.3 (8.5)	6.3 (9.8)	5.8 (9.1)	6.8 (11.8)
Current smoker, %	4	6	9	3
History of benign breast disease, %	59	65	63	60
Recent PMH use, %[a]	56	66	73	66
Duration of PMH use (estrogen only), %				
None	58	63	54	72
<5 years	13	10	11	15
≥5 years	29	27	35	13
Duration of PMH use (estrogen + progesterone), %				
None	62	54	56	52
<5 years	15	14	6	15
≥5 years	23	32	38	33
First-morning urine, %	95	93	97	97
ER+/PR+ tumor, %	n/a	74	78	75

[a]These are matching factors.

MT1R-positive cases (RR = 0.76; 95% CI, 0.39–1.51 comparing tertile 3 vs. tertile 1, $P_{trend} = 0.39$; $P_{heterogeneity} = 0.88$). When we restricted to invasive cases only (there were not enough *in situ* cases to examine them separately), these results were largely unchanged, and there were no differences of association found comparing MT1R-positive vs. MT1R-negative cases ($P_{heterogeneity} = 0.57$).

In secondary analyses, our results were very similar when we restricted our analysis of urinary melatonin and incident breast cancer to women with first-morning urine samples (multivariable-adjusted estimate comparing quartile 4 vs. quartile 1 of urinary melatonin: RR = 0.79; 95% CI, 0.61–1.03; $P_{trend} = 0.09$). Results were also similar when we examined estrogen- and progesterone-positive cases only (multivariable-adjusted estimate comparing quartile 4 vs. quartile 1 of urinary melatonin: RR = 0.82; 95% CI, 0.60–1.11; $P_{trend} = 0.19$), and after excluding cases diagnosed within 2 years of urine collection (multivariable-adjusted estimate comparing quartile 4 vs. quartile 1 of urinary melatonin: RR = 0.79; 95% CI, 0.58–1.06; $P_{trend} = 0.22$; data not shown in tables). For analyses based on MT1R status of tumors, results were similar when we utilized alternative cut points for the definition of MT1R positivity. For example, use of cut points based on absolute values of the distribution yielded the following results: multivariable-adjusted estimates comparing tertile 3 vs. tertile 1 of urinary melatonin: RR = 0.59; 95% CI, 0.21–1.65; $P_{trend} = 0.41$, for low MT1R positivity; RR = 0.67; 95% CI, 0.33–1.33; $P_{trend} = 0.14$, for medium MT1R positivity; RR = 1.00; 95% CI, 0.44–2.29; $P_{trend} = 0.90$, for high MT1R positivity; $P_{heterogeneity} = 0.80$; data not shown in tables.

Table 2. Baseline characteristics (at urine collection in 2000) of matched controls across quartiles of creatinine-standardized urinary melatonin level ($n = 799$)

	Quartiles of urinary melatonin levels (in ng/mg creatinine)			
Characteristic	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Mean melatonin level, ng/mg creatinine (SD)	6.9 (3.0)	17.6 (3.4)	30.3 (4.3)	64.8 (44.2)
Mean age, years (SD)	67.9 (6.6)	67.5 (6.6)	66.1 (6.9)	66.2 (6.9)
Mean age at menarche, in years (SD)	12.6 (1.4)	12.6 (1.3)	12.6 (1.3)	12.6 (1.4)
Mean age at menopause, years (SD)	49.4 (4.4)	49.4 (5.3)	49.7 (4.2)	49.8 (4.9)
Mean number of births (SD)	3.3 (1.6)	3.2 (1.8)	3.2 (1.5)	3.3 (1.5)
Family history of breast cancer, %	14	17	13	19
Mean BMI, in kg/m^2 (SD)	27.6 (5.8)	26.9 (5.4)	25.9 (4.9)	25.3 (4.1)
Mean alcohol intake, g/day (SD)	5.5 (9.0)	4.9 (8.8)	5.9 (8.7)	4.9 (7.3)
Current smoker, %	7	3	3	4
History of benign breast disease, %	61	61	50	61
Recent PMH use, %	54	58	56	58
Duration of PMH use (estrogen only), %				
None	58	57	57	59
<5 years	13	15	12	12
≥5 years	29	28	31	29
Duration of PMH use (estrogen + progesterone), %				
None	62	61	62	63
<5 years	19	13	17	12
≥5 years	19	26	21	25
First-morning urine, %	94	97	96	95

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Table 3. RR and 95% CIs of breast cancer across quartiles of creatinine-standardized urinary melatonin level ($n = 1{,}354$)

	Quartiles of urinary melatonin levels (in ng/mg creatinine)				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	**P_{trend}**
Invasive + *in situ* cases combined					
No. of cases/no. of controls	162/200	148/199	136/201	109/199	
Simple RR[a]	1.00 (ref)	0.95 (0.76–1.19)	0.90 (0.72–1.13)	0.79 (0.62–1.01)	0.08
Multivariable RR[b]	1.00 (ref)	0.96 (0.77–1.20)	0.92 (0.73–1.16)	0.78 (0.61–0.99)[c]	0.08
Invasive cases only					
No. of cases/no. of controls	121/156	114/154	111/150	87/161	
Simple RR[a]	1.00 (ref)	0.97 (0.75–1.26)	0.97 (0.75–1.26)	0.80 (0.61–1.06)	0.15
Multivariable RR[b]	1.00 (ref)	0.99 (0.76–1.28)	1.01 (0.78–1.31)	0.80 (0.61–1.07)	0.19
In situ cases only					
No. of cases/no. of controls	41/44	34/45	25/51	22/38	
Simple RR[a]	1.00 (ref)	0.89 (0.57–1.41)	0.68 (0.42–1.12)	0.76 (0.45–1.28)	0.29
Multivariable RR[b]	1.00 (ref)	0.84 (0.52–1.35)	0.63 (0.38–1.06)	0.62 (0.36–1.07)	0.09

[a]Simple conditional logistic regression model.

[b]Multivariable conditional logistic regression model adjusted for the following: age at menarche (<12, 12, 13, ≥14 years), age at menopause (≤45, 46–50, 51–53, ≥54 years), parity (nulliparous, 1–2, 3–4, or ≥5 children), family history of breast cancer (mother or sister: yes, no), BMI (continuous in kg/m^2), alcohol intake (0, 1–14.9, ≥15 g/day), smoking status (current: yes, no), history of benign breast disease (yes, no), antidepressant use (current: yes, no), PMH (current: yes, no), type and duration of PMH (estrogen only: 0, <5, ≥5 years; estrogen + progesterone: 0, <5, ≥5 years), and first-morning urine (yes, no).

[c]This estimate is statistically significant because the 95% CI does not include the null value of 1.00.

Discussion

Overall, we found a suggestion that higher levels of urinary melatonin were related to lower risk of breast cancer risk over twelve years of follow up, which is consistent with previous findings with shorter follow up in this cohort. However, this association appeared to be driven by results from the first half of follow up, as there was no association between melatonin levels and breast cancer risk during the second half of follow up. In a subset of women with MT1R subtyping, we found no evidence that the association between urinary melatonin and incident breast cancer differed comparing MT1R-positive versus MT1R-negative tumors. To our knowledge, this is the first epidemiologic study to evaluate this association according to MT1R subtype of the breast tumor.

Two recent meta-analyses reported that higher levels of urinary melatonin were associated with a reduced risk of breast cancer in women based on published studies (27, 28). The summary relative risk of one meta-analysis indicated an approximately 18% decreased risk comparing quantile 4 versus quartile 1 of urinary melatonin, with borderline significance [RR = 0.82; 95% CI, 0.68–0.99 (27)]; this is very similar to the 22% risk reduction that we identified comparing quartile 4 versus quartile 1 in our present study (OR = 0.78; 95% CI, 0.61–0.99). Results of the second meta-analysis are also similar to our results, with an inverse association observed between urinary melatonin and breast cancer incidence, which appeared to be confined to postmenopausal women (RR = 0.81; 95% CI, 0.70–0.92 for each 15 ng/mg creatinine increase in aMT6s; ref. 28); however, the effect estimate comparing quantile 4 versus quartile 1 was not given, making it more difficult to compare these results to those of this study.

Indeed, one of the studies included in these meta-analyses is our previous analysis of the association between urinary melatonin levels and breast cancer risk in the NHS cohort (7). With shorter follow up, our prior study identified a 38% lower risk of breast cancer among women in the quartile 4 versus quartile 1 of urinary melatonin (RR = 0.62; 95% CI, 0.41–0.95); the P_{trend} was

Table 4. Multivariable-adjusted RR and 95% CIs of breast cancer, according to MT1R subtype, across tertiles of creatinine-standardized urinary melatonin level [$n =$ 403 (245 controls, 91 MT1R-negative cases, 67 MT1R positive cases)]

	Tertiles of urinary melatonin levels (in ng/mg creatinine)				
	Tertile 1	**Tertile 2**	**Tertile 3**	**P_{trend}**	**$P_{heterogeneity}$**
Invasive + *in situ* cases combined					
No. of cases/no. of controls	61/77	59/74	38/94		
MT1R negative + positive[a]	1.00 (ref)	1.01 (0.70–1.47)	0.67(0.44–1.02)	0.05	
No. of cases/no. of controls	32/42	35/34	24/61		
MT1R negative[b]	1.00 (ref)	1.08 (0.64–1.81)	0.60 (0.35–1.06)	0.07	0.88
No. of cases/no. of controls	29/35	24/40	14/33		
MT1R positive[b]	1.00 (ref)	0.95 (0.53–1.70)	0.76 (0.39–1.51)	0.39	
Invasive cases only					
No. of cases/no. of controls	49/64	44/57	32/73		
MT1R negative + positive[a]	1.00 (ref)	0.98 (0.64–1.50)	0.72 (0.45–1.14)	0.14	
No. of cases/no. of controls	29/38	29/32	23/54		
MT1R negative[b]	1.00 (ref)	1.00 (0.57–1.75)	0.65 (0.37–1.17)	0.14	0.57
No. of cases/no. of controls	20/26	15/25	9/19		
MT1R positive[b]	1.00 (ref)	0.88 (0.40–1.95)	0.97 (0.39–2.42)	0.79	

[a]Multivariable conditional logistic regression model adjusted for the following: age at menarche (<12, 12, 13, ≥14 years), age at menopause (≤45, 46–50, 51–53, ≥54 years), parity (nulliparous, 1–2, 3–4, or ≥5 children), family history of breast cancer (mother or sister: yes, no), BMI (continuous in kg/m^2), alcohol intake (0, 1–14.9, ≥15 g/day), smoking status (current: yes, no), history of benign breast disease (yes, no), antidepressant use (current: yes, no), PMH use (current: yes, no), type and duration of PMH use (estrogen only: 0, <5, ≥5 years; estrogen + progesterone: 0, <5, ≥5 years), and first-morning urine (yes, no).

[b]Multivariable conditional polytomous logistic regression model with same covariates as listed above for conditional logistic regression.

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also highly significant ($P = 0.004$), although the risk appeared to be equally lower in the top two quartiles. In addition, exclusion of breast cancer cases diagnosed within 1 to 2 years of urine collection somewhat attenuated the effect estimate and it became nonsignificant (RR = 0.72; 95% CI, 0.45–1.17, with a 1-year lag; and RR = 0.76; 95% CI, 0.44–1.31, with a 2-year lag). In this study, we observed that this association was attenuated further and became null during the period 6 to 12 years after urine collection. Taken together, these results suggest that endogenous melatonin levels could influence breast cancer incidence in the shorter but not longer term.

With regard to the melatonin receptor, to date, few studies have assessed MT1R status of breast tumors in women. One study with MT1R information identified an association between MT1R positivity and survival among women with triple-negative breast cancer (29). These findings suggest that greater numbers of MT1R may be related to better outcomes in breast cancer, but more specific interpretation is difficult due to the lack of urinary melatonin assessment and restriction to triple-negative breast tumors in this population. More directly related to our analyses, a second study examined the association between melatonin receptor genes and breast cancer incidence in Chinese women, and results suggested that genotype might influence risk of breast cancer (30). Nonetheless, differences in genetic variation across different ethnic populations could limit the generalizability of these findings, and no additional studies have been undertaken in other ethnic populations. Although this study does not suggest differences in the association between endogenous melatonin levels and breast cancer risk according to MT1R type, experimental evidence demonstrating an age-related decrease in sensitivity of these receptors for melatonin might explain this null finding (16, 17). Clearly, additional studies are needed to replicate our findings in postmenopausal women, as well as explore these associations in premenopausal women.

Our study is unique because it incorporates information on both urinary melatonin levels and MT1R subtype of breast tumors in women. An important limitation, however, is the relatively small number of women in our cohort (29%) with both of these measurements, which limits our power to detect differences in the association between urinary melatonin and breast cancer by MT1R subtype. Moreover, because of this small number of cases, we were unable to examine these associations for *in situ* cases separately. In addition, there are limitations with our measurements of urinary melatonin levels and MT1R subtyping. Because urine was collected at only one point in time, it is possible that melatonin levels at that time may not reflect melatonin levels over time; however, previous research has established that urinary melatonin levels are highly correlated over three years in this cohort (intraclass correlation = 0.72), which lends more credibility to the notion that one-time melatonin assessment might contain relevant information about longer-term melatonin levels (20). For MT1R subtyping, there is little established information on the optimal cut point for characterizing positivity of this tumor marker. Thus, in our study, we utilized several different approaches to this definition, including ones based on a comparison of pathologist vs. machine read values, as well as absolute and relative values of our distribution. Results were similar comparing these various approaches, and therefore this limitation is less likely to have affected our interpretation of results. Finally, we assessed MT1R expression of breast tumors because this receptor is thought to largely mediate melatonin's anticancer effects; however, it is possible that expression of MT2R and key downstream signaling proteins play an important role in mediating this association, and we have not assessed these tumor factors in our cohort (18).

In summary, higher melatonin levels were associated with reduced breast cancer risk in postmenopausal women—consistent with our previous finding based on shorter follow up in this cohort. Moreover, there was no difference in the association comparing MT1R-positive versus MT1R-negative breast tumors. Future research should evaluate these associations with longer-term follow up, a larger number of cases with MT1R subtyping, and among premenopausal women.

Disclosure of Potential Conflicts of Interest

A.H. Beck is employed as a president at PathAI, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: E.E. Devore, E.T. Warner, S.E. Hankinson, E.S. Schernhammer

Development of methodology: E.E. Devore, E.T. Warner, E.S. Schernhammer

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Heather Eliassen, A.H. Beck, S.E. Hankinson, E.S. Schernhammer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.E. Devore, E.T. Warner, A. Heather Eliassen, S.B. Brown, S.E. Hankinson, E.S. Schernhammer

Writing, review, and/or revision of the manuscript: E.E. Devore, E.T. Warner, A. Heather Eliassen, S.B. Brown, A.H. Beck, S.E. Hankinson, E.S. Schernhammer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Heather Eliassen

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