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Urinary Melatonin Levels and Risk of Postmenopausal Breast Cancer in the Women's Health Initiative Observational Study

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URINARY MELATONIN LEVELS AND RISK OF POSTMENOPAUSAL BREAST CANCER IN THE WOMEN'S HEALTH INITIATIVE OBSERVATIONAL STUDY

A Thesis Presented

by

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ABSTRACT

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MAY 2012

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Prior studies have observed a link between night shift work and increased risk of breast cancer. Melatonin, a hormone related to circadian rhythm, has been proposed to lower breast cancer risk by inhibiting cell proliferation. The disruption of peak melatonin that occurs during night shift work could explain the increase in risk observed. Several studies have assessed whether higher melatonin levels are associated with decreased breast cancer risk, but results have been conflicting. We examined the relationship between urinary melatonin levels and breast cancer risk in a nested case-control study conducted within the Women's Health Initiative Observational Study. First morning urine samples collected at baseline were assayed for melatonin levels in 258 women diagnosed with invasive breast cancer and 515 matched controls from three enrollment sites. Using conditional logistic regression to adjust for matching factors and established

risk factors, results indicate no association between urinary melatonin levels and breast cancer risk. The mean creatinine adjusted melatonin levels for cases and controls were 16.30 ng/mg and 16.05 ng/mg, respectively. Compared to the lowest quartile of creatinine adjusted melatonin, the odds of breast cancer did not vary by quartile of creatinine adjusted melatonin, adjusted for known breast cancer risk factors: second quartile 0.84 (95% CI 0.52-1.38), third quartile 1.05 (95% CI 0.65-1.72) and fourth quartile 1.09 (95% CI 0.66-1.81). This study does not suggest that melatonin is protective against breast cancer and suggests that reasons other than melatonin suppression may explain the increased risk of breast cancer seen in night shift workers.

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CHAPTER I

INTRODUCTION

The American Cancer Society has estimated that 226,870 women will be diagnosed with breast cancer and 39,510 women will die of breast cancer in 2012 in the United States.[1] On January 1, 2008, 2,632,005 U.S women had a history of breast cancer. From 2003 to 2007, the annual age-adjusted death rate in the United States for breast cancer was 24 per 100,000 women per year.[2] The median age of death for breast cancer was 68 years old.[2] A vast majority of breast cancers occur in postmenopausal women, with an estimated 78.2% of breast cancer diagnoses in women over the age of 55 from 2003-2007.[2]

Previous literature has found a link between night shift work and breast cancer.[4, 5] Interruption of melatonin production caused by night shift work may explain the increase in breast cancer risk.[5,6] Melatonin is a hormone found in the pineal gland that circulates throughout the body and influences circadian rhythm. Melatonin may affect breast cancer risk by decreasing cell proliferation and limiting tumor growth. Current epidemiological literature points towards a relationship between melatonin levels and breast cancer risk. A majority of prospective studies have found an inverse association between urinary melatonin levels and breast cancer risk[7-11], though not all results have been statistically significant.[7-9,12] Prospective studies focusing on postmenopausal women using the more reliable first morning urine samples will aid this growing field of research. Therefore, we conducted a nested case-control study within the Women's Health Initiative Observational Study to assess urinary melatonin, as measured through first morning urine, and subsequent postmenopausal breast cancer risk.

CHAPTER II

REVIEW OF THE LITERATURE

Shift work and Breast Cancer Risk

In 2010, the International Agency for Research on Cancer (IARC) published a report that concluded shift work that disrupts circadian rhythm is probably carcinogenic to humans.[5] The IARC assesses a variety of data sources, including studies conducted in human and animals. There was limited evidence in humans for this relationship and sufficient evidence from experiments conducted with animals. The variety of definitions of shift work is one difficulty in assessing the relationship between cancer and shift work. In general, shift work is defined as working irregular hours, typically outside the standard daylight hours of 8 am to 6 pm. In the United States, shift workers make up approximately 15% of the fulltime work force work.[5]

A total of eight studies have looked specifically at shift work and breast cancer risk. A meta-analysis analyzing six of these eight studies was conducted by Megdal et al. (2005).[13] The authors found a 51% increased risk of breast cancer in these studies among night shift workers excluding airline cabin crew, including both pre- and postmenopausal breast cancer (RR 1.51, 95% CI 1.36-1.68). A majority of women included in these studies were postmenopausal. Airline cabin crew were excluded from this analysis, but when included the results were similar (RR=1.48, 95% CI 1.36-1.61). A limitation of this meta-analysis is that each study used varying definitions of shift work, which limits the comparability of the studies.[5] Due to the fact that shift work disrupts circadian rhythm and peak melatonin production, the hormone melatonin is often cited as a potential biological mechanism for the association between shift work and breast cancer.

Biological Mechanisms of Melatonin and Breast Cancer Risk

Melatonin is a hormone that influences circadian rhythms in bacteria, plants and a variety of vertebrates, including humans.[5] As light enter the eyes, the information is processed by the hypothalamus sending a signal to the pineal gland, which secretes melatonin. The peak melatonin secretion occurs during the night, between 2-3 am, but can be suppressed by light.[5,6] In experimental studies, it has been found that animals that are either exposed to light during typical dark periods or who have received a pinealectomy, the removal of the pineal gland, can experience disruption in the timing and the duration of melatonin secretion.[5] A growing body of literature suggests that night shift work is related to breast cancer risk through nighttime light exposure, and perhaps lower melatonin levels. There has been some conflicting evidence in this regard, as a recent study conducted within Canadian nurses by Grundy et al. (2011) has found no association between night shift light exposure and melatonin levels.[14] No significant decrease in melatonin levels was found between female nurses working the night shift and those working the day shift on a rapidly rotating shift schedule. The authors did address the fact that light exposure experienced by the nurses within the study, 37.2 lux, was well below the level found to decrease melatonin levels in experimental studies, 200 lux. The effects of melatonin on breast cancer are only recently beginning to be understood, but it is hypothesized that melatonin is able to effect breast cancer risk through suppression of cell growth and proliferation.[5,6]

Studies have shown that melatonin is able to suppress the proliferation of both estrogen receptor alpha-positive and estrogen receptor negative human breast cancer cells

in vitro.[6] A linear dose response relationship has been observed between melatonin and suppression of these cells, indicating that higher levels of melatonin is associated with increased cancer cell suppression.[6] Melatonin is able to block estrogen receptors, thus decreasing the likelihood that the breast cancer will be able to form.

The anti-proliferative effect of melatonin is believed to occur through at least four pathways.[5] First, the prevention of the uptake of linoleic acid has been shown to have an inhibitory effect, preventing the growth of tumor cells.[5,15] Linoleic acid is thought to be an energy source for tumor growth, so inhibition of linoleic acid by melatonin would prevent excess cell growth.[15] Second, in experimental studies done in vitro, melatonin has been shown to inhibit the mitogenic action of hormones and growth factors, e.g. estradiol and prolactin. This again prevents the growth of cancerous cells. Third, melatonin increases the expression of the tumor suppressor gene TP53. Finally, melatonin down regulates aromatase expression. This down regulation diminishes the rate of tumor growth and proliferation.[5]

Overall, melatonin has been found to suppress cell proliferation. This inhibition of cell proliferation, through the blockage of estrogen and linoleic acid receptors, inhibition of growth factors and hormones, increase of TP53 and down regulation of aromatase, is the mechanism through which higher levels of melatonin might protect women against breast cancer.[5,6,15]

Epidemiology of Melatonin and Breast Cancer Risk

Relatively few studies have examined the relationship between urinary melatonin levels and breast cancer risk.[7-12] Five prior studies were nested case-control studies in which the cases and controls came from large prospective cohort studies [8-12] and one was

a retrospective case-control study.[7] The following are the three studies which included information on postmenopausal breast cancer.[9,10,12]

In the first prospective study to consider the association of urinary melatonin levels and breast cancer risk, Travis et al. conducted a matched nested case-control study of 127 cases and 353 controls participating in the Guernsey Cohort, a prospective cohort study of 5,093 women living on the island of Guernsey in the British Isles from 1977 to 1985.[12] This is the only study to use the 24-hour method of urine collection. Women answered a baseline questionnaire that included information on reproductive history, menopausal status, past use of oral contraceptives and other hormones and breast cancer screening history. After recruitment, women were asked to provide a 24-hour urine sample. Breast cancer cases were obtained through the Wessex Cancer Registry, death certificates, and pathology reports through October 31, 2001. Each case was matched to three controls on the basis of age, date of recruitment and menopausal status.

Urine samples were thawed and assayed by radioimmunoassay with ^{125}I-labeled tracer in 2002. Among postmenopausal women only, relative to the women in the first tertile, those in the second tertile of creatinine adjusted melatonin had 24% lower risk of breast cancer (OR = 0.76, 95% CI 0.31-1.84) and women in the third tertile of melatonin had 1.09 times the risk of breast cancer compared to those in the lowest tertile of creatinine adjusted melatonin (OR=1.09, 95% CI 0.46-2.6). When restricted to only women with invasive breast cancer, the results did not change significantly. Although there was a wide confidence interval, this study suggests there is no association between melatonin and breast cancer risk. This study, however, may be subject to nondifferential misclassification of the exposure, as the 24-hour sample method has not been found to be

reliable for measuring peak melatonin levels that occur during the night.[4,7] The study also had a small sample size, limiting its power to find a modest association between creatinine adjusted melatonin levels and breast cancer risk.

In the largest study to date, using first morning urine collection, Schernhammer et al. conducted a nested case-control study of 357 cases and 533 matched controls to evaluate the relationship between creatinine adjusted urinary melatonin and breast cancer risk in the Nurses' Health Study, a prospective cohort that began in 1976, following 121,700 registered nurses ages 30 to 55 in the United States.[10] At baseline, eligible nurses completed a questionnaire about their health status, medical history and known or suspected risk factors for cancer and heart disease. These questionnaires were administered every two years to identify new risk factors and diagnosis of disease. In December 2002, 18,706 women provided a blood sample as well as a first morning urine sample. Time of menstrual cycle was not recorded, as most of the cohort was postmenopausal at the time of collection. Cases were defined as women who were not diagnosed with any cancer, except for non-melanoma skin cancer, prior to urine collection and were diagnosed with breast cancer during follow-up through May 31, 2006. Two controls were matched for each case on year of birth, menopausal status, recent postmenopausal hormone use, month and time of day of urine collection and fasting status at urine draw. The exceptions were postmenopausal breast cancer cases with hormone use who were matched with only 1 control.

Relative to women in the lowest quartile of creatinine adjusted melatonin, women in the second quartile had 1.17 times the risk of invasive breast cancer (OR 1.17, 95% CI 0.75-1.82), women in the third quartile had a 30% lower risk of invasive breast cancer

(OR=0.70, 95% CI 0.43-1.12) and women in the fourth quartile of melatonin had a 26% lower risk of invasive breast cancer (OR=0.74, 95% CI 0.46-1.21), (p_{trend} = 0.03). The associations seen between in situ breast cancer and creatinine adjusted melatonin, though based on small numbers, were stronger than those seen for invasive breast cancer, with women in the highest quartile at 77% lower risk of in-situ breast cancer compared to those in the lowest quartile (OR=0.23, 95% CI 0.08-0.69). There were no significant differences in the results when restricted to women with ER+ receptor status. One limitation of the study is the relatively short follow-up time. Urine collection occurred in 2002 and all cases were diagnosed by 2006, with an average follow-up time of 2.5 years for the cases. Restricting analysis to those women who were diagnosed after 2 years did not significantly alter the results. The short follow-up time could mean that melatonin levels were already being affected by undiagnosed breast cancer.

The first nested case-control study to look at only postmenopausal breast cancer was conducted by Schernhammer at al. among 178 cases and 710 controls within the ORDET cohort, an Italian study that recruited women from 1987 through 1992.[9] At baseline, women completed a questionnaire of health characteristics, as well as provided a blood and urine sample. Each case was matched with 4 controls that were free of cancer at the time of the diagnosis for the case, matching on age at enrollment, date of recruitment and laboratory batch. Urine collection was completed through a twelve-hour overnight collection. Relative to women in the first quartile of melatonin levels, women in the second quartile of melatonin levels had a 30% lower risk of invasive breast cancer (OR=0.70, 85% CI 0.43-1.14), women in the third quartile of melatonin levels had 18% lower risk of invasive breast cancer (OR=0.82, 95% CI 0.50-1.34) and women in the

fourth quartile of melatonin levels had a 41% lower risk for invasive breast cancer (OR=0.59. 95% CI 0.35-1.00), (p_{trend} = 0.04). This study did not include any information on sleep disturbance or night shift work, which could have been an important factor to include in the analysis. A subanalysis was conducted using creatinine adjusted melatonin levels, which did not significantly change the results. Inclusion of the 7 cases of in situ breast cancer also did not change the results. Removal of current smokers strengthened the results, while restriction to current smokers caused an increase in risk when comparing the highest tertile of melatonin to the lowest (OR=3.55, 95% CI 0.61-20.8). The results were limited due to small number of current smokers. Exclusion of those diagnosed within 2 years and 4 years of urine collection strengthened the results (highest versus lowest quartile of melatonin OR=0.35, 95% CI 0.17-0.71, OR=0.34, 95% CI 0.15-0.75, respectively).

Overall, the literature is conflicting on the association between urinary melatonin levels and breast cancer risk. Conflicting results may be due to poor assessment of urinary melatonin, such as through 24-hour samples as opposed to the more reliable first morning sample. Nested case-control studies that used the overnight and first morning urinary analysis[9-11] found a non-significant inverse relationship between urinary melatonin and breast cancer risk, while those studies using 24-hour samples found no association between creatinine adjusted melatonin and breast cancer risk.[8] Additionally, retrospective case-control studies are unable to assess urinary melatonin until after the diagnosis of breast cancer, which could have altered melatonin level and may explain the lack of significant results in this study design.[7] One study has limited follow-up and

could potentially be finding the effect of undiagnosed breast cancer altering melatonin levels.[10]

Summary

The Surveillance Epidemiology and End Results (SEER) program of the National Cancer Institute estimates that 1 out of 8 woman born today will be diagnosed with breast cancer in their lifetime.[2] With increased amount of light exposure and sleep disruption, through night shift work or otherwise, there is a possibility that melatonin levels could be suppressed and potentially lead to an increased risk of breast cancer via less inhibition of breast cancer cells and their receptors.[6] Current studies lack lengthy follow-up periods and adequate collection of urine samples from women prior to their diagnosis of breast cancer. More prospective research needs to be conducted in order to assess the relationship between melatonin and breast cancer.

CHAPTER III

METHODS

Study Aims and Hypothesis

Specific Aim: We evaluated the association between urinary levels of melatonin and risk of postmenopausal invasive breast cancer through a nested case-control study among participants from the Women's Health Initiative Observational Study.

Hypothesis: There is an inverse association between urinary melatonin levels and risk of postmenopausal invasive breast cancer.

Study Design and Population

To examine the association between urinary melatonin levels and risk of breast cancer, we conducted a nested case-control study within the Observational Study (OS) arm of the Women's Health Initiative (WHI) among total of 258 cases of invasive postmenopausal breast cancer and 515 matched control subjects.

The OS recruited women from September 1993 to December 1998, enrolling a total of 93,676 women who were followed for eight to twelve years. Eligible women were between the ages of 50-79 years old, postmenopausal at enrollment (no menstrual cycles for at least twelve months prior to enrollment if 54 years old or younger and six months if 55 years old or older) with the intention to reside in the area for at least 3 years after enrollment. Exclusion criteria included any medical condition that had a predicted survival rate of less than 3 years, as well as any conditions that may have limited the ability to comply or stay within the study, such as alcohol or drug dependency, mental illness, dementia or active participation in another randomized control trial.[3] Both cases and controls also had the following exclusion criteria: self-reported history of any cancer

prior to WHI enrollment (other than non-melanoma skin cancer), self-report and/or adjudicated cancer during WHI follow-up (other than non-melanoma skin cancer or breast cancer for cases) and inadequate urine from screening visit 1.

Exposure Assessment

The exposure of interest is first morning urinary melatonin. Women who enrolled in the OS provided a first morning urine sample at one of three bone density sites (Birmingham, Pittsburgh and Tucson) at screening visit 1. When the liver metabolizes melatonin, it becomes the metabolite 6-sulfatoxymelatonin (aMT6s) found in the urine. Urinary melatonin level, aMT6s, was assessed through competitive enzyme-linked immunosorbent immunoassay (ELISA) and adjusted for creatinine levels to control for urine volume. The aMT6s levels were assessed as both a continuous and ordinal variable, divided into quartiles based upon levels within the controls.

Validity of Exposure Assessment

ELISA has been shown to be reliable for measuring aMT6s levels, with intra-assay and interassay correlation coefficients of 7.1 and 11.9% respectively, meaning that there is little variation between and within samples, thus small chances for laboratory errors.[16] Baskett et al. found that urinary melatonin levels were correlated with plasma melatonin levels in elderly populations ($R^2 = 0.797$).[17] Urinary melatonin can be used to measure peak melatonin levels, as opposed to having to use a more invasive nighttime blood draw.[17] Additionally, this metabolite has been found to be highly correlated with peak overnight melatonin levels when measured as a first morning urine void.[18,19] Data from the Nurses' Health Study also found an intra-class correlation coefficient of 0.72 for

first morning urine void over a three-year period, indicating that melatonin levels are relatively stable over time.[4]

Outcome Assessment

Cases and controls were individually matched on enrollment date, age at enrollment and randomization clinic, with a ratio of 1:2 cases to controls. Cases are women who had adjudicated diagnosis of invasive breast cancer at one of three enrollment sites during their WHI follow-up (Birmingham, Pittsburgh and Tucson). Cases reported their breast cancer diagnosis through the annual questionnaire. Those who reported a breast cancer diagnosis had their medical records reviewed to adjudicate the diagnosis.[20] Controls were women from these enrollment sites without a breast cancer diagnosis (invasive or non-invasive) during their WHI follow-up.

Validity of Outcome Assessment

The baseline questionnaire for enrollment into the WHI has very high reliability, with a reliability coefficient of 0.89 for reporting of a history of breast cancer.[13] Additionally, self-report of breast cancer has been found to be a reliable measure of breast cancer rates in observational studies.[22-24]

Covariate Assessment

Data on demographics, physical characteristics, behavioral characteristics, medication use and additional variables believed to be associated with breast cancer were collected through standardized questionnaires administered at baseline and updated over the course of followup.[21] The variables of interest for this study include age, ethnicity, education level, height, weight, body mass index, hormone use, history of breast biopsy and benign breast disease, age at menarche, age at menopause, type of menopause

(natural, hysterectomy, bilateral oophorectomy), age at first child's birth, number of pregnancies, breast feeding, marital status, prior needle aspiration of a breast lump, oral contraceptive use, current medication, first degree relative with breast cancer, energy expenditure from physical activity (MET hr/wk), sleep quality, alcohol intake and smoking status.

Statistical Analysis

Upon inspection of the data, it was found that one case had missing melatonin levels and one control had a melatonin level that was an obvious outlier (1971.28 ng/mg). These participants were removed from analysis, along with their matched pairs. This left 773 observations for analysis, 258 cases and 515 matched controls. We used t-tests and chi square tests as appropriate to test if there was a difference in the covariates of interest in cases and controls. A t-test was also conducted to see if there was a difference between mean melatonin levels in cases and controls (Table 1). Those categorical variables with small cell counts were collapsed. The distribution of continuous melatonin levels, adjusted for creatinine, was assessed for normality using the Shapiro-Wilk test. Due to the non-normal distribution of melatonin, Kruskal-Wallis tests were used to test whether the median creatinine adjusted melatonin levels in controls differed by covariates (Table 2).

As this was a matched study design, we used conditional logistic regression to determine if higher levels of creatinine adjusted urinary melatonin is associated with lower odds of invasive breast cancer. We divided creatinine adjusted melatonin levels into quartiles based on control levels, using the lowest level of melatonin as the referent group.

To address potential confounding, we included covariates identified in previous studies as known risk factors for breast cancer established within WHI[25], as well as covariates included in other studies assessing the relationship between melatonin and breast cancer risk. Due to small numbers, ethnicities collapsed into white, black or other race. Highest education levels were collapsed into high school or less, college, post graduate or missing. Marital status was collapsed into never married, divorced/separated, widowed, married or marriage like relationship, or missing. Age at menarche was collapsed into 11 or less, 12-13 years, 14-15, 16 or older or missing. Hours of sleep was collapsed into 5 or less hours, 6 hours, 7 hours, 8 hours, 9 or more hours, or missing. Estrogen and progesterone duration was collapsed into none, less than 5 years, 5 to less than 10 years, 10+ years or missing. Body mass index was collapsed into less than or equal to 24.9, 25-29.9, 30-34.9, or 35+. Quality of sleep was collapsed into restless, average quality, or restful. Hysterectomy, needle aspiration, bilateral oophorectomy were included as ever/never. Oral contraceptive use and female relative with breast cancer were included as yes/ no. Smoking status was included as never, past or current smoker. Number of live births was included as never pregnant, never had term pregnancy, 1, 2-4, or 5+ pregnancies. Age at first birth was included as never pregnant, less than 20 years, 20-29 years, 30+ years or missing. Alcohol intake, total energy expenditure and age of menopause were continuous variables. Age and region were controlled for by their use as matching factors.

Regression analyses began with the fitting of single predictor conditional regression models for each variable with the outcome of invasive breast cancer (Table 1). These results were used to assess the predictive significance of WHI breast cancer risk

factors and established risk factors used in other studies. An initial multiple predictor conditional logistic regression model was fit controlling for matching factors (enrollment date, age at enrollment and enrollment site) with the following predictors: ethnicity, age of menopause, needle aspiration of the breast, energy expenditure from recreational activities, duration of estrogen and progesterone use, time since quitting hormone replacement therapy, body mass index and highest education level. Adjusted odds ratios were calculated using the lowest creatinine adjusted melatonin quartile as the referent group (Table 3). Predictors in this initial multiple predictor conditional logistic regression model was retained for inclusion in subsequent models if its associated likelihood ratio test was statistically significant at $p < 0.10$. Another multiple predictor conditional logistic regression model was fit using creatinine adjusted melatonin as a continuous variable.

We conducted sensitivity analysis by removing current smokers from our analysis as a previous study had found an effect by smoking status.[9] We also estimated a model in which we restricted our analysis to only those with ER+ receptor status to see if there was any difference in this population as previous studies had looked at this population (Table 4). [9,10]

To assess the potential for a disease effect of undiagnosed breast cancer, another model was fit restricting the population into those diagnosed before and after 4 years from urine collection. Then, we divided the population into those diagnosed from 4-7 years and those diagnosed after 7 years (Table 5).

Finally, while previous studies have not found a significant relationship between sleep duration and melatonin levels[10], we constructed a model with sleep duration and

breast cancer risk, to see if there was any association (Table 6). All statistical analysis was conducted using Stata V. 12.1.

CHAPTER IV

RESULTS

Mean creatinine adjusted melatonin levels were 16.29 ng/mg (sd=11.9) for cases and 16.05 ng/mg (sd=12.9) for controls. Characteristics of cases and controls are shown in Table 1 along with crude odds ratios from single predictor conditional logistic regression. At baseline, cases and controls differed in a few characteristics.

Contrary to what is typically seen in breast cancer studies, women who had a later age at first birth were at a lower risk of breast cancer relative to women who had their birth at less than 20 years of age. The risk of breast cancer was 26% lower for women who gave birth at 20-29 years old (95% CI 0.47-1.18), 42% lower for women who gave birth at 30 or older (0.28-1.21) and there was no association for women who never gave birth (OR=0.99, 95% CI 0.56-1.75) relative to women who had their first birth at less than 20 years old. All other known breast cancer risk factors indicated the correct direction of risk for breast cancer. With each one-point increase in body mass index there was a 1.12 times increased risk of breast cancer (95% CI 0.96-1.30). For each one-year increase in age of menopause, there was 1.02 times increased risk of breast cancer (0.99-1.04). Relative to women who never used estrogen and progesterone, women who used for 5-10 years had 1.75 times the risk of breast cancer (95% CI 1.01-3.01) and women who used for 10 or more years had 1.05 times the risk of breast cancer (0.54-2.05). Women who received a needle aspiration of the breast had 1.30 times the risk of breast cancer relative to women who had never received a needle aspiration (95% CI 0.87-1.95). Women who had a bilateral oophorectomy or a hysterectomy had a 46% (95% CI -.34-0.85) and 33% (95% CI 0.50-0.94) reduction in breast cancer risk, respectively. Women

with a family history of breast cancer had 1.85 times the risk of breast cancer relative to women with no family history (95% CI 1.67-2.92). Women who were past or current smokers had 1.08 (95% CI 0.80-1.46) and 1.32 (95% CI 0.72-2.40) times the risk of breast cancer relative to women who never smoked. Quality of sleep and hours of sleep were not significantly associated with breast cancer risk. Compared to those who only completed high school, those who completed a graduate education had 2.2 times the risk of breast cancer (95% CI 1.47-3.28).

Median levels of creatinine adjusted melatonin by breast cancer risk factors are presented in Table 2. Creatinine adjusted melatonin levels decreased as body mass index increased. Those who completed higher levels of education had higher levels of creatinine adjusted melatonin. There was a general decrease in creatinine adjusted melatonin as age increased. Women who had given birth to more children had higher levels of creatinine adjusted melatonin, as compared to women who had not given birth. Higher levels of quality of sleep and duration of sleep both led to higher levels of creatinine adjusted melatonin, though levels were fairly similar for those who slept 7, 8 or 9 hours. Never smokers appear to have lower levels of creatinine adjusted melatonin compared to past and current smokers, who have similar levels.

The initial multiple predictor conditional logistic regression model of breast cancer and creatinine adjusted melatonin (coded as quartiles) included matching factors plus the following confounders: age of menopause, needle aspiration of the breast, duration of estrogen and progesterone therapy, years since quitting hormone replacement therapy, body mass index, ethnicity, energy expenditure from physical activity and highest education level. Each covariate was taken out of the model one at a time and a

likelihood ratio test was conducted to see if the covariate was statistically significant. Any covariate with a p-value less than 0.05 were included in the final model. Age of menopause, needle aspiration, duration of estrogen and progesterone therapy, years since quitting hormone replacement therapy, ethnicity and energy expenditure from physical activity had p-values greater than 0.05, and were dropped from the model, leaving highest education level and body mass index. The odds ratio and 95% CI for the model adjusted for body mass index and education level are presented in Table 3 as the multivariable model. In the fully adjusted model, relative to the lowest quartile of creatinine adjusted melatonin, the risk of breast cancer was 0.85 (95% CI 0.52-1.37), 1.05 (95% CI 0.65-1.72), and 1.09 (95% CI 0.66-1.81) for women in the second, third and fourth quartile, respectively. In the model adjusted for body mass index and education level, relative to the lowest quartile of creatinine adjusted melatonin, the risk of breast cancer was 0.98 (95% CI 0.63-1.54), 1.26 (95% CI 0.80-1.97), and 1.20 (95% CI 0.76-1.90) for women in the second, third and fourth quartile, respectively. When analysis was run with creatinine adjusted melatonin as a continuous variable the results did not change (OR=1.00, 95% CI 0.99-1.01). After adjusting the continuous creatinine adjusted melatonin for known breast cancer risk factors the results did not change (OR=1.01, 95% CI 0.99-1.02).

In ancillary analysis, we explored whether there was an association between sleep duration and breast cancer risk (Table 4). From the fully adjusted model, adjusting for age of menopause, needle aspiration, duration of estrogen and progesterone therapy, years since quitting hormone replacement therapy, ethnicity and energy expenditure from physical activity, we found that compared to women who slept 5 hours or less, the risk of breast cancer was 0.87 (95% CI 0.43-1.75), 1.05 (95 % CI 0.53-2.11), 0.92 (95% CI 0.43-

1.97) and 0.71 (95% CI 0.26-1.92) for those who slept 6 hours, 7 hours, 8 hours and 9+ hours, respectively. The results did not significantly change was adjusting for creatinine adjusted melatonin.

We assessed differences in results by tumor receptor status and smoking status. The odds ratios for the analysis restricted to those who were ER+ and stratified on smoking status are presented in Table 5. Among the women with ER+ receptor status (n=176), relative to the lowest quartile, the risk of breast cancer was 0.69 (95% CI 0.39-1.23), 0.72 (95% CI 0.40-1.29) and 0.83 (95% CI 0.46-1.51) for the women in the second, third and fourth quartile, respectively. There were too few women who had ER- receptor status to complete an analysis (n=36). The remaining women had borderline (n=1), unavailable (n=9) or unknown receptor status (n=17).

When current smokers were excluded, the risk of breast cancer relative to women in the lowest quartile of creatinine adjusted melatonin is 1.01 (95% CI 0.60-1.70), 1.19 (95% CI 0.70-2.02) and 1.41 (95% CI 0.81-2.44) for women in the second, third and fourth quartile respectively. There were too few current smokers to analyze the association of melatonin and breast cancer in this subgroup (20 cases and 31 controls).

In another ancillary analysis, we considered the subgroup of women diagnosed within 4 years of urine collection, women diagnosed between 4 to 7 years after urine collection and those diagnosed 7 or more years after urine collection. The results of these analyses are presented in Table 6. In every subgroup we found no association between creatinine adjusted melatonin and breast cancer risk. For those diagnosed within 4 years, relative to women in the first quartile of creatinine adjusted melatonin, the odds ratios are 0.86 (95% CI 0.32-2.30), 1.42 (95% CI 0.54-3.75) and 1.40 (95% CI 0.51-3.81) for

women in the second, third and fourth quartile respectively. For those diagnosed between 4-7 years after diagnosis, relative to women in the first quartile of creatinine adjusted melatonin, the odds ratios are 0.52 (95% CI 0.18-1.52), 1.79 (95% CI 0.67-4.76) and 1.26 (95% CI 0.49-3.12) for women in the second, third and fourth quartile respectively. For those women diagnosed 7 or more years are urine collection, relative to women in the first quartile of creatinine adjusted melatonin, the odds ratios are 0.85 (95% CI 0.85-1.94), 0.81 (95% CI 0.33-1.95) and 0.94 (95% CI 0.42-2.13) for women in the second, third and fourth quartile respectively.

CHAPTER V

DISCUSSION

Contrary to our hypothesis, this study did not find a statistically significant relationship between urinary melatonin and breast cancer risk. The aim of this study was to examine the relationship between creatinine adjusted urinary melatonin levels and invasive breast cancer risk in postmenopausal women. The Women's Health Initiative Observational Study presented an opportunity to use a large prospective cohort with a long follow-up and first morning urine sample. Several studies have looked at urinary melatonin and breast cancer risk, but results have been conflicting.[7-12]

The mean creatinine adjusted melatonin found in our study, 16.29 ng/mg for cases and 16.05 ng/mg for controls, was different than what has been seen in previous studies. The means found within our study were higher compared to Travis et al., who found means of 6.47 ng/mg for cases and 6.57 ng/mg for controls.[12] The tertile cutoff points were also lower than our quartile cutoff points. The study conducted within the Nurses Health II also found lower mean creatinine adjusted melatonin levels, 10.8 ng/mg for cases and 12.7 ng/mg for controls.[11] The quartile cutoff points used were similar, but slightly higher for this study. The final three prospective studies conducted to date had higher mean creatinine adjusted melatonin levels, 29.3 ng/mg for cases and 27.6 ng/mg for controls[8], 21.0 ng/mg for cases and 23.5 ng/mg for controls[9], 24.5 ng/ng for cases and 28.8 ng/mg for controls.[10] Two of these studies did not use creatinine adjusted melatonin to create the quartiles[8,9]. The one study that did use creatinine adjusted melatonin quartiles had higher quartile cutoff points than our study.[10]

Of the five prospective studies conducted to date, one examined premenopausal women[8], two examined both pre- and postmenopausal women[11,12] and two examined only postmenopausal women.[9,10] A nested case-control conducted within the Nurses Health Study II among mostly premenopausal women found an inverse association between melatonin and breast cancer risk, the risk for invasive breast cancer for the highest quartile of creatinine adjusted melatonin compared to the lowest was 0.59 (95% CI 0.34-1.00).[11] This association was slightly weakened when those women with in situ breast cancer were included (OR=0.68, 95% CI 0.44-1.06). In comparison, the study conducted in the ORDET cohort among premenopausal women found a risk of 1.43 (95% CI 0.83-2.45) for all breast cancers comparing the fourth quartile of melatonin to the first.[8] A statistically significant inverse association was found in only one subgroup analysis, that of women who were diagnosed 8 years after urine collection. In the study conducted within the Guernsey cohort, no association was found between urinary melatonin levels and breast cancer risk in either pre or postmenopausal women.[12] The authors used a 24-hour collection of urine, which would not accurately measure the peak melatonin production, which was stated as a possible reason for the lack of association.[9,10,12] We used a first morning sample and were able to capture the peak melatonin level, so this is not a limitation for our study. The study conducted within the Nurses Health Study found an inverse association between creatinine adjusted melatonin and breast cancer risk (OR=0.62, 95% CI 0.41-0.95 for the fourth quartile compared to the first).[10] This association was weakened when in situ cancers were removed (OR=0.74, 95% CI 0.46-1.21 for the fourth quartile compared to the first), as compared to the Nurses Health II, which found less of an association with in situ breast cancer among premenopausal

women.[8] The ORDET cohort investigators found an inverse relationship between melatonin and breast cancer risk (OR=0.65, 95% CI 0.39-1.09 for the fourth quartile relative to the first quartile).[9] The association increased when current smokers were excluded from the analysis (OR=0.38, 95% CI 0.20-0.74).

The cases in our study were all diagnosed with invasive breast cancer, so we were unable to assess variations in the association between in situ and invasive breast cancer as seen in previous studies.[10-12] When we excluded current smokers the risk of breast cancer appears to have increased, but this was non-significant. Previous studies that restricted to ER+ receptor status saw no change in risk.[9,10] We saw an inverse relationship between creatinine adjusted melatonin and breast cancer risk when we restricted to ER+ receptor status, but this was non-significant. Unlike the study that found a significant inverse association when analysis was restricted to women diagnosed 8 years after urine collection[8], we found no difference when we limited the analysis to those women who were diagnosed 7 years after urine collection.

One possible limitation of our study is nondifferential misclassification of exposure. The first morning urine samples were assayed for melatonin levels using the ELISA method, a validated method.[6] If melatonin levels were incorrectly measured, this misclassification would attenuate the results seen in this study. This is unlikely to occur, as the ELISA assay is a validated assay. Additionally, the lab personnel were blinded to the case status of the urine samples. It is possible that the urine samples were incorrectly collected or stored. This would also attenuate the results seen. Urinary melatonin levels have been found to be stable over time, allowing for one sample to be a representative

sample over many years, so the sample collected at baseline should be an accurate representation of melatonin exposure over time.[8]

Within the context of a nested case-control study, selection bias will occur if participants differentially drop out of the study based upon exposure and disease status. If a differential loss to follow-up occurred this could cause either an increase or decrease of the association seen. This is unlikely in our study, as all the women were followed very closely for many years and any women who did not complete a questionnaire was cross-referenced against breast cancer registries and the National Death Index to see if they had died or gone on to develop a disease. Additionally, the completion rates of the annual questionnaires were between 93-96%, indicating that there was a high degree of follow-up.[20]

The Women's Health Initiative is a large prospective cohort, which allowed for the collection of many potential confounding factors. Using information gathered from the baseline questionnaire, as well as the subsequent questionnaires, we were able to evaluate and control for many potential confounding factors. We assessed known breast cancer risk factors as identified from other WHI studies, as well as other factors considered in independent studies assessing urinary melatonin and breast cancer risk. While we were able to address many known confounders, it is possible that we have missed other lesser-known factors.

The results of this study is plausibly generalizable to all women at risk for postmenopausal breast cancer, as there is no reason to believe that the biological mechanism through which melatonin affects breast cancer risk varies. It is possible that menopausal status could alter the relationship between melatonin and breast cancer, as

pre- and postmenopausal breast cancers have different physiological mechanisms. Thus, more research will have to be conducted to see if the relationship between melatonin levels and pre- and postmenopausal breast cancer risk differ.

In summary, our study found no association between creatinine adjusted melatonin levels and invasive postmenopausal breast cancer risk. Relatively few studies have assessed the relationship between melatonin and risk of postmenopausal breast cancer and have provided inconsistent results.[7-12] Using a sample from the large Women's Health Initiative Observational Study cohort allowed for a large number of women to be assessed for a long period of follow-up. Additionally, the urine sample was collected as a first morning urine sample, believed to be a more reliable estimate than the 24-hour sample method. Analysis accounting for lag time, smoking status and ER+ receptor status did not significantly alter our findings. Additional studies will be needed that allow for long periods of follow-up, a large study population and first morning urine samples to confirm our results.

APPENDICES

APPENDIX A

HUMAN SUBJECT PROTECTION

All subjects recruited to the Women's Health Initiative signed an informed consent agreement understanding that they would participate in a longitudinal cohort study. Consenters also understood that they could discontinue their participation at any time. To protect confidentiality, all identifying factors were removed from the data set prior to our receipt. In our dataset, all participants are only identified by a study ID.

There were no known risks to participation in this study. The only potential concern is privacy and confidentiality, but we analyzed de-identified data and it was stored on a secure file. There were no known benefits for the study participants, other than contributing pertinent scientific data for this and other studies.

APPENDIX B

PERMISSION TO ACCESS DATA

I received permission to use Women's Health Initiative Observational Study data to assess the relationship between melatonin levels and risk of postmenopausal breast cancer.

APPENDIX C

TABLES

Table 1. Baseline characteristics of postmenopausal breast cancer cases and matched controls (N=773)

Covariate	Cases (n=258)	Controls (n=515)	p-value[a]	OR	95% CI
Creatinine adjusted melatonin level mean (sd)	16.29 ng/mg (11.9)	16.05 ng/mg (12.9)	0.801		
Age stratum at enrollment n (%)					
50-54	32 (12.40%)	70 (13.59%)	0.805	1.0 (referent)	
55-59	48 (18.60%)	89 (17.28%)		2.0	0.59-6.74
60-69	119 (46.12%)	250 (48.54%)		1.84	0.39-8.78
70-79	59 (22.87%)	106 (20.58%)		4.4	0.68-28.23
Ethnicity n (%)					
White	211 (81.78%)	418 (81.17%)	0.874	1.0 (referent)	
Black	31 (12.02%)	60 (11.65%)		1.02	0.61-1.72
Other race	16 (6.2%)	37 (7.18%)		0.77	0.35-1.70
Highest education level n (%)					
High school or less	88 (34.11%)	220 (43.72%)	0.001	1.0 (referent)	
College	81 (31.40%)	182 (35.34%)		1.13	0.78-1.64
Post graduate	86 (33.33%)	108 (20.97%)		2.20	1.47-3.28
Number of live births n (%)					
Never pregnant	31 (12.02%)	54 (10.49%)	0.068	1.0 (referent)	
Never had term pregnancy	7 (2.71%)	3 (0.58%)		5.7	1.10-29.58
1	23 (8.91%)	56 (10.87%)		0.72	0.38-1.39
2-4	148 (57.36%)	321 (62.33%)		0.83	0.52-1.33
5+	47 (18.22%)	78 (15.15%)		1.14	0.65-2.01
BMI categories n (%)					
<24.9	90 (34.88%)	203 (39.42%)	0.455	1.12	0.96-1.30
25-29.9	85 (32.95%)	171 (33.20%)			
30-34.9	45 (17.44%)	81 (15.73%)			
35+	36 (13.95%)	56 (10.87%)			
Quality of sleep n (%)					
Restless	36 (13.95%)	65 (12.62%)	0.211	1.0 (referent)	
Average quality	121 (46.90%)	212 (41.17%)		1.04	0.65-1.68
Restful	100 (38.76%)	232 (45.05%)		0.78	0.49-1.25

Notes: a. P-value for t-tests or chi-squared tests, as appropriate
Abbreviations: OR- Odds Ratio, sd- Standard Deviation, se- standard error, BMI- Body Mass Index, HRT- Hormone replacement therapy

Table 2. Median creatinine adjusted melatonin levels by baseline characteristics in 515 controls

Covariates	Median Creatinine Adjusted Melatonin (ng/mg)	P-value[a]
Age stratum at enrollment		
50-54 (n=70)	11.00	0.03
55-59 (n=89)	17.60	
60-69 (n=250)	13.24	
70-79 (n=106)	10.79	
Ethnicity		
White (n=418)	14.42	0.05
Black (n=60)	9.48	
Other (n=37)	10.45	
Highest education level		
High school or less (n=220)	11.60	0.06
College (n=182)	14.52	
Post graduate (n=108)	14.81	
Missing (n=5)		
Number of live births		
Never pregnant (n=54)	13.24	0.38
Never had term pregnancy (n=3)	6.69	
1 (n=56)	10.75	
2-4 (n=321)	13.90	
5+ (n=78)	15.39	
Missing (n=3)		
BMI categories		
<24.9 (n=203)	14.57	0.0068
25-29.9 (n=171)	14.07	
30-34.9 (n=81)	12.09	
35+ (n=56)	9.70	
Missing (n=4)		
Quality of sleep		
Restless (n=65)	11.30	0.33
Average quality (n=212)	12.42	
Restful (n=232)	14.64	
Missing (n=6)		
Hours of sleep		
5 or less hours (n=36)	10.20	0.48
6 hours (n=134)	11.23	
7 hours (n=209)	14.40	
8 hours (n=104)	15.47	
9 or more hours (n=28)	14.92	
Missing (n=4)		

Notes: a. P-value from Kruskal-Wallis test

Abbreviations: OR- Odds Ratio, BMI- Body Mass Index, HRT-Hormone replacement therapy

Table 3. Odds ratios (OR) and 95% confidence intervals of breast cancer by quartile of creatinine adjusted melatonin

	Number of cases /controls	Unadjusted OR (95% CI)	Fully Adjusted[a] OR (95% CI)	Multivariable[b] OR (95% CI)
Quartile 1	58/127	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
Quartile 2	60/130	1.02 (0.66-1.56)	0.85 (0.52-1.37)	0.98 (0.63-1.54)
Quartile 3	60/130	1.26 (0.82-1.94)	1.05 (0.65-1.72)	1.26 (0.80-1.97)
Quartile 4	74/129	1.13 (0.73-1.75)	1.09 (0.66-1.81)	1.20 (0.76-1.90)

Notes: a. Adjusted for body mass index, education level, age of menopause, needle aspiration, energy expenditure from recreational activity, years since quitting hormone replacement therapy, duration of estrogen and progesterone use and ethnicity

b. Adjusted for body mass index and education level

Table 4. Odds ratios (OR) and 95% confidence intervals of breast cancer by sleep duration

	Number of cases/ controls	Unadjusted OR (95% CI)	Fully Adjusted OR[a] (95% CI)	Fully Adjusted and Creatinine Adjusted Melatonin Levels[b] OR (95% CI)
5 hours or less	19/36	1.00 (referent)	1.00 (referent)	1.00 (referent)
6 hours	68/134	0.94 (0.50-1.79)	0.87 (0.43-1.75)	0.87 (0.43-1.77)
7 hours	107/209	0.96 (0.51-1.80)	1.05 (0.53-2.11)	1.06 (0.53-2.15)
8 hours	49/104	0.88 (0.45-1.73)	0.92 (0.43-1.97)	0.89 (0.41-1.92)
9+ hours	14/28	0.92 (0.38-2.21)	0.71 (0.26-1.92)	0.84 (0.51-1.38)

Notes: a. Adjusted for body mass index, education level, age of menopause, needle aspiration, energy expenditure from recreational activity, years since quitting hormone replacement therapy, duration of estrogen and progesterone use and ethnicity

b. Adjusted for creatinine adjusted melatonin level, body mass index, education level, age of menopause, needle aspiration, energy expenditure from recreational activity, years since quitting hormone replacement therapy, duration of estrogen and progesterone use and ethnicity

Table 5. Odds ratios (OR) and 95% confidence interval of breast cancer by smoking status and ER+ receptor status

	Number of cases/controls	Removed current smokers[a]	Number of cases/controls	ER+[a]
Quartile 1	50/116	1.0 referent	52/96	1.0 referent
Quartile 2	57/124	1.01 (0.60-1.70)	42/98	0.69 (0.39-1.23)
Quartile 3	70/121	1.19 (0.70-2.02)	52/96	0.72 (0.40-1.29)
Quartile 4	60/116	1.41 (0.81-2.44)	49/99	0.83 (0.46-1.51)

Notes: a. Adjusted for body mass index, education level, age of menopause, needle aspiration, energy expenditure from recreational activity, years since quitting hormone replacement therapy, duration of estrogen and progesterone use and ethnicity

Table 6. Odds ratios (OR) and 95% confidence intervals of breast cancer and creatinine adjusted melatonin by year of diagnosis

	Number of cases/ controls	Less than 4 years[a]	Number of cases/ controls	4-7 years[a]	Number of cases/ controls	7 or more years[a]
Quartile 1	15/40	1.00 (Referent)	20/39	1.00 (Referent)	20/39	1.00 (Referent)
Quartile 2	17/40	0.86 (0.32-2.30)	17/40	0.52 (0.18-1.52)	20/40	0.85 (0.37-1.94)
Quartile 3	26/40	1.42 (0.54-3.75)	24/39	1.79 (0.67-4.76)	20/40	0.81 (0.33-1.95)
Quartile 4	22/40	1.40 (0.51-3.81)	18/40	1.26 (0.49-3.12)	20/40	0.94 (0.42-2.13)

Notes: a. Adjusted for body mass index, age of menopause, needle aspiration, energy expenditure from recreational activity, years since quitting hormone replacement therapy, duration of estrogen and progesterone use and ethnicity

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