Markers of circadian disruption and reproductive risk factors for breast cancer

by

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Abstract

Background: Circadian disruption is hypothesized to impact reproductive signaling, which in turn has long been linked to breast cancer. The melatonin hypothesis holds that increased exposure to artificial light at night and resulting inhibition of nocturnal melatonin may account for recent increased breast cancer incidence. Coregulation of melatonin, circulating reproductive hormones and precursor gonadotropins has been well documented in animal models, particularly those of seasonal breeders, yet elucidation of these complex hormonal relationships in humans has proven challenging. This work investigates the link between circadian disruption and female gonadal activity and reproductive-related outcomes in the context of breast cancer risk.

Methods: We examined the association between rotating shift work and age at menopause among the Nurses' Health Study II cohort, estimating cause-specific hazards using proportional hazards models with time-dependent covariates. The cross-sectional associations between nocturnal melatonin production, menstrual cycle length and circulating sex steroid hormones and prolactin were examined in two additional populations using ordinary least squares regression and generalized estimating equations. Effect modification of nocturnal melatonin and prolactin by age and diurnal preference was additionally investigated.

Results: The hazard of natural menopause was observed to be lower in women who had worked >10 years of rotating shifts since baseline (HR: 0.76; 95% CI: 0.69-0.85), in the direction of our hypothesis. The strength of this effect was not maintained upon inclusion of prior exposure. Nocturnal melatonin levels were not found to be associated with menstrual cycle length, nor with daytime circulating levels of estradiol, progesterone or prolactin. However, we observed effect modification of nocturnal melatonin and circulating daytime prolactin by age.

Conclusion: Our findings do not conclusively support or refute an association between circadian disruption and reproductive risk factors for breast cancer. Future work will benefit from more precise measurement and observation of the interplay between endocrine markers of circadian activity and reproductive function over time.

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Chapter 1 Introduction and Objectives

1.1. Thesis Overview

Humans rely on internal clocks to regulate the timing of various physiological processes. The central circadian clock or "pacemaker", residing in the suprachiasmatic nucleus (SCN) of the hypothalamus, governs the periodicity of a multitude of downstream homeostatic functions, either directly or in concert with circadian clocks in peripheral tissues[1]. Through regulation of timing of gene expression involved in endocrine signaling and other fundamental biological functions, these clocks enable physiological anticipation of activity and rest at opposing times of the day, dynamically effecting such behavioral attributes as alertness or propensity for sleep[2]. Examples of processes under circadian regulation include those related to cardiovascular function (e.g., blood pressure and heart rate)[3], core temperature[4], activity of renal[5] and multiple organs involved in digestion[6], immune function[7] and reproduction[8,9].

Circadian periodicity of biological rhythms is modified to varying degrees by multiple exogenous and endogenous factors, which have been termed "chronodisruptors"[10]. Empirical evidence, together with the observation that the SCN receives direct innervation from non-visual receptors in the retina[11], suggests that central pacemaker is chiefly entrained by external photic cues. Over the course of evolution, these cues were predominated by exposure to sunlight during the day and the lack thereof at night. Exposure to such stimuli, on the whole, has recently become more and more erratic due to the demands of modern society. Wake and sleep schedules stray increasingly from the light and dark cycles set by earth's 24 hour periodic rotation, enabled by the ubiquity of electric light. Light exposure during naturally dark periods, commonly termed "light at night" in the literature, may be typically attributable to the desire to maximize leisure and family time in juxtaposition with regimented work (or school) schedules. Such schedules not only predispose toward extended light at night exposure, but may additionally result in attenuation of photic cues during the daytime due to reduced exposure to intense natural light (ranging between 10,000 to more than 100,000 lux) and extended exposure to indoor artificial light that is typically orders of magnitude dimmer (400 lux or less)[12]. The question of how harmful this trend is, and the degree to which altered exposure to natural circadian cues translates into disease burden, remains the subject of intense scrutiny and debate.

Evidence from animal models suggests that desynchronization from such cues can drastically alter biological function, inducing or exacerbating disease processes. Yet the observation that humans self-select toward circadian misalignment while other animals do not[13] alone raises the question of how well these models extend to humans. Perhaps the most compelling support is found from indications of moderately increased risks of chronic disease among highest exposure groups such as night shift workers[14]. Regardless, the recognition that the disruption of these oscillatory rhythms inherent in many biological processes may precipitate broad-spectrum homeostatic dysfunction suggests its role in the etiology of a wide range of chronic conditions. Circadian disruption may contribute to illness related to energy metabolism [15] and cardiovascular disease[16], among others. Even if circadian disruption is insufficient to induce clinically relevant disease single-handedly in most cases (i.e., attributable risk is small), the potentially high prevalence of this exposure, together with its implication in multiple disease processes, supports its candidacy as a factor responsible for a significant proportion of morbidity and mortality in modern society.

Observational epidemiologic evidence and that from various animal models have additionally supported a link between circadian disruption and cancer, particularly those related to digestion (i.e., colorectal cancer) and reproduction, the most widely studied being female breast cancer[17]. Circadian regulation of reproduction has long been recognized and described in animal models. The idea that sufficient disruption of these processes may lead to increased cancer risk for sites whose neoplastic initiation and progression have been observed to respond to reproductive signaling (e.g., estrogen receptor positive tumours) underpins the biological model for this research.

This dissertation investigates the link between circadian disruption and female gonadal activity and reproductive-related hormonal signaling in the context of breast cancer risk. To accomplish this, we examined the association between rotating shift work exposure and age at menopause among the Nurses' Health Study II cohort. The cross-sectional association between nocturnal melatonin production, menstrual cycle length and circulating sex steroid hormones were examined in a second sample of young, healthy cycling women who were not taking oral contraceptives. The cross-sectional association between nocturnal melatonin and circulating prolactin levels was assessed among a third study sample of women, with a focus on examining effect modification by age and diurnal preference, that is, whether one feels more alert toward

the beginning or end of the day.

1.1 Thesis Objectives

This dissertation is divided into three main objectives, each investigated among separate female populations:

1. **Objective 1 assessed the association between rotating shift work and age at menopause.** Rationale: The underlying biological model maintains that circadian disruption may convey increased breast cancer risk through dysregulation of reproductive hormone signaling. Alteration of gonadal activity through suppression and/or phase-shifting of nocturnal melatonin secretion resulting from night work may induce lifelong exposure to a hormonal environment which augments breast tissue proliferation, increasing cancer risk.

2. **Objective 2 assessed the cross-sectional associations between nocturnal urinary melatonin metabolite (aMT6s) concentrations, menstrual cycle duration and circulating reproductive steroid hormone levels.**

    2.1. **Association between urinary aMT6s concentrations and menstrual cycle duration.** Rationale: An association between menstrual cycle length and nocturnal melatonin production may be suggestive of a regulating effect of melatonin on gonadal function.

    2.2. **Association between urinary aMT6s and steroid reproductive hormone levels.** Rationale**:** Only one or two studies have assessed these associations in larger samples using frequentist analyses and rigorous adjustment for potential confounders. Though they have failed to report significant correlations between overnight aMT6s concentrations and serum estrogens, the replication of these associations will help confirm these previous findings.

3. **Objective 3 assessed the cross-sectional association between nocturnal urinary aMT6s concentrations and circulating prolactin levels.** Rationale: There has only been one other study that has assessed this association in larger samples, adjusting for potential confounders. Replication of these associations will help confirm these findings.

3.1. **Exploratory assessment of effect modification by diurnal preference and age.** Rationale: There is some evidence that people who feel more alert later in the day may more easily adapt to circadian disruptive environments. Changes in reproductive function and endocrine metabolism have additionally been observed with age. Variation in the degree to which circulating hormone levels are affected by nocturnal melatonin may identify those at greatest risk of adverse reproductive-related outcomes attributable to circadian disruptive stimuli.

1.2 Roles and Responsibilities

Analyses addressing each thesis objective were performed on previously collected data. Objective 1 was conducted using self-reported questionnaire data from the Nurses' Health Study (NHS) II cohort. Objectives 2 and 3 were conducted using data previously collected by the thesis supervisor on two separate samples of women for the investigation of the effects of light, physical activity and other environmental exposures on hormonal factors related to cancer risk.

In preparation for work pertaining to objective 1, I, the doctoral candidate, developed a proposal, reviewed by the thesis supervisor and NHS investigator Dr. Eva Schernhammer, which was submitted and approved at a NHS research meeting. Subsequently, I received onsite training and experience related to the manipulation and analysis of NHS data. I identified the research question for each objective, reviewed the literature as summarized in Chapter 2, identified and further developed definitions for all main exposures, outcomes and covariates investigated in this research. I selected all analytic strategies and conducted all analyses employed in this research, under advisement from the thesis committee. I completed original drafts of all manuscripts and thesis chapters. Subsequent revisions were based on iterative feedback from the thesis committee.

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Chapter 2 Literature Review

This chapter reviews the current literature on the link between circadian disruption and female reproduction in the context of breast cancer etiology. It begins by defining circadian disruption and introduces the light at night hypothesis and the integral role of melatonin and its most common surrogate measure in observational studies, night shift work. An overview of potential mechanisms by which circadian disruption may impact gonadal activity, steroid hormone and prolactin production and ovulation via the hypothalamic-pituitary-gonadal axis (HPGA), with emphasis on melatonin-dependent mechanisms, is provided. The second half of the chapter summarizes the literature linking circadian disruption, gonadal activity and reproductive signaling to breast cancer risk, with a focus on the observational evidence. The chapter concludes with a rationale for this research: the investigation of the effect of circadian disruption on potential reproductive precursors of breast cancer.

2.1 Defining Circadian Disruption

The term circadian disruption is becoming increasingly common in scientific literature, yet its definition is not always consistent or clear[1]. For example, it can be used interchangeably to describe the alteration of function of biological systems under circadian governance[2], or the desynchronization of biological clocks from an underlying 24-hour periodicity that causes this alteration of function[3]. Most frequently it is used as an umbrella term for both concepts[4,5]. Here, circadian disruption is defined as the former, more specifically, the alteration of function of biological systems due to interruption of governing regulatory processes exhibiting 24-hour periodicity (circadian rhythm) generally induced by changes in the timing and intensity of exposure to entraining stimuli (i.e., light). For clarification, it should be noted that processes exhibiting circadian rhythms are not dependent on these entraining stimuli to function as they have been observed to be conserved in their absence[6,7]. The timing and function of circadian-regulated processes experience greatest disruption when governing cues are inconsistent. For example, it is not exposure to light at night alone, but exposure to light at night in conjunction with light during the day, potentially together with other conflicting circadian cues, that makes it

a powerful chronodisruptor. Fluctuating schedules that result in inconsistent exposure patterns to these cues are thought to exacerbate the disruptive effect as this undermines the ability to adapt to consistent circadian stimuli and why, for example, night workers who work days in combination with nights, rather than nights alone, are considered to be at greater risk to the deleterious effects of circadian disruption[8].

2.2 An Overview of Mammalian Circadian Regulation

The following description of how exposure to light at night impacts reproductive function begins with an overview of the mammalian circadian system. Numerous tissues exhibit approximate 24-hour periodicity. These rhythms originate from local biological clocks established by auto-feedback mechanisms at the level of gene expression. The master clock, or central pacemaker, comprised of neurons within the suprachiasmatic nucleus (SCN) of the hypothalamus, exhibits the same autoregulatory periodicity, driven by similar molecular mechanisms. From the murine model, timing of neuronal activity within the SCN can be attributed to clock gene expression, regulated by negative and positive feedback loops coupled by the transcription factor heterodimer CLOCK-BMAL1 (reviewed in Reppert and Weaver[9]). Briefly, in the negative feedback loop, CLOCK-BMAL1 heterodimers induce transcription of multiple *Period* (*Per*) and *Cryptochrome* (*Cry)* genes. Their protein products, PERs and CRYs, translocate back into the nucleus where CRY proteins interact with CLOCK and/or BMAL1 to downregulate transcription of *Per* and *Cry* in a cycle that takes approximately 24 hours to complete. In the positive feedback loop, CLOCK and BMAL1 heterodimers, in addition to driving *Per* and *Cry* transcription, activate transcription of a third gene, the protein product of which suppresses *Bmal1*. Finally, when CRY enters the nucleus to suppress *Per* and Cry transcription in the negative loop, activation of this third protein is also suppressed, resulting in the reactivation of *Bmal1* transcription. The circadian rhythmicity of clock gene expression (e.g., *Per1*) within the mammalian SCN has been confirmed *in vivo* in transgenic mouse models using recombinant fluorescent protein techniques[10].

The circadian signal originating in the SCN orchestrates the timing of local clocks, in the central nervous system and periphery, through neuronal transduction and endocrine signaling and indirectly through behavioral modification. An example of the latter is the promotion of alertness

at specific times of the day, which, through entrainment of meal times, regulates timing of local clocks within the digestive system[11]. Exogenous and endogenous signals entrain the timing of these clock gene cycles in the SCN, evolutionarily, to align timing of multiple biological processes to anticipate recurring diurnal patterns of activity and rest. The most influential factor is light, transduced via direct innervation from non-visual receptors in the retina and perhaps indirectly via serotonergic fibres originating from the raphe nuclei[12]. The translation of light stimuli into chemical messengers governing the activity of SCN neurons has not been fully deciphered[13] and further description of these molecular mechanisms is beyond the scope of this chapter. The SCN outputs the circadian signal via innervation to the surrounding hypothalamus. From there, the message is relayed by endocrine and autonomic neural projections. The first and most prominent pathway relaying a neuroendocrine circadian signal to be elucidated was the sympathetic link between the hypothalamus and pineal gland, producing the hormone melatonin[14]. The effects of a distorted circadian signal mediated by melatonin on gonadal activity (and resulting circulating steroid hormone output) and prolactin production, and the potential impact of this modified reproductive signaling on the initiation and promotion of breast cancer, form the underlying biological model of this dissertation.

2.3 Melatonin: A Circadian Endocrine Signal

The production and secretion of melatonin has a distinct circadian rhythm, reaching its peak in the middle of the night and dropping to nadir throughout the day. Unlike other circadian endocrine messengers (e.g., corticosterone), melatonin release has been observed to be predominantly dependent on light stimuli. Distributed via the circulatory system, circulating melatonin levels are thought to provide a reliable measure of the phase of the SCN central clock. The hormone relays the circadian message to targets through binding of melatonin-1 (MT1) and/or melatonin-2 (MT2) receptors. The degree to which melatonin itself performs entrainment of local clocks is not straightforward, the elucidation of which is hampered due to redundancies of pathways conveying the SCN signal and tissue-specific variation in the generation and regulation of circadian rhythmicity[14]. Yet the fact that melatonin seems to directly induce rhythmic activity in some central tissues, rather than simply entraining a self-sufficient local clock, cements its role as an important circadian messenger. In the pars tuberalis of the pituitary,

melatonin directly promotes the expression of certain genes, including some clock genes: pinealectomy results in loss of such rhythmic expression in these tissues in animal models[15,16].

2.4 Circadian Disruption and Nocturnal Melatonin Suppression

2.4.1 The Light at Night hypothesis of breast cancer

The "light at night" (LAN) or melatonin-breast cancer hypothesis, holds that women who work night shifts are potentially at greater risk primarily due to exposure to artificial electric light during normally dark periods[17]. The hypothesis, as first championed by Stevens, held that reduced nocturnal melatonin might explain a portion of the rising breast cancer risk in western countries through modulation of ovarian estrogens and pituitary prolactin production[18]. More recently, melatonin suppression has been implicated in breast cancer risk through additional direct local mechanisms after observed native anti-oncogenic actions of the pineal hormone in animal models[19,20]. The ability of LAN to both suppress and phase-shift nocturnal melatonin secretion has been demonstrated in humans, the degree to which is dependent on timing, duration, intensity and wavelength of light stimuli (reviewed in Brainard *et al*[21]). The suppressive effect of LAN on melatonin was subsequently reported from a sample of 16 healthy, cycling young women[22].

2.4.2 Circadian disruption and night work

Reduced circulating melatonin in night working women, relative to exclusive day workers, has been reported from observational studies[8,23-26]. A recent cross-sectional study among 345 nurses and midwives on rotating shifts did not observe lower melatonin compared to 370 day workers, though they noted significantly lower melatonin in rotating shift workers working eight or more nights per month[27]. An explanation for the null findings may lie in how melatonin was measured. The authors used the melatonin urinary metabolite, 6-sulfatoxymelatonin (aMT6s), as a marker for circulating melatonin, measured in a morning urine spot check. While they are correct that urinary aMT6s has been validated as an indicator of pineal melatonin secretion (reviewed in Mirick and Davis[4]), it has been done so in studies comparing nocturnal plasma melatonin to urinary melatonin or aMT6s collected over the entire night[28,29], or in first morning voids representative of entire nightly urine volume[30]. It is not clear whether participants were permitted

to void prior to morning collection, potentially jeopardizing the ability to capture melatonin metabolized throughout the entire night and particularly that produced during peak pineal secretory output. Interestingly, two studies that have tracked melatonin production following light exposure among rotating shift workers failed to note compelling evidence of an inverse association between light exposure during the night shift and morning[31] or overnight[32] urinary aMT6s. However, in the latter, 24-hour urinary melatonin metabolite concentrations were inversely associated with light intensity during the night[32]. It is possible that light intensity was insufficient to cause immediate melatonin suppression in a large proportion of these samples. For example, in the latter study, light intensity ranges during the night shift were reported be 29-223 lux[32]. From experimental findings, only 17% of a small sample of men and women exhibited melatonin suppression at 200 lux[33], with a threshold of 350 lux reported elsewhere[34]. Yet the absence of immediate melatonin suppression does not preclude more subtle phase-shifting of the nocturnal melatonin signal[21], with potentially disruptive effects on homeostatic function and reproductive signaling.

2.5 Melatonin and Gonadal Activity

2.5.1 Evidence from seasonal breeders

The majority of mechanistic evidence for melatonin as a central modulator of gonadal function is found from research on seasonally-breeding (i.e., able to reproduce only for a limited interval annually) animals spanning the last five decades. There have been numerous reviews of this literature. Most recently, Scherbath and Steinlechner provide a historical overview[35] with more detailed summaries of novel discoveries in this field given by Revel *et al*[36] and Dardente[37], the latter focusing on ovine models. While the mechanisms have yet to be fully elucidated, it is undisputed that melatonin is responsible for conveying photoperiod (i.e., day light length) information to central reproductive targets. Through what is becoming to be recognized as a complex integration of hypothalamic targets, the melatonin signal serves to either suppress or stimulate reproductive function via the HPGA, depending on the mammalian model in question and photoperiod. The duration of the nocturnal melatonin signal, proportional to photoperiod length, relays information on time of year, affecting gonadotropin releasing hormone (GnRH) and gonadotropin secretion from the hypothalamus and pituitary, respectively. In turn, gonadal

activity is either triggered or suppressed, often with accompanying morphological changes, leading to alterations in steroid hormone levels and control of reproductive endpoints such as ovulation.

Empirically, photoperiod renders seasonal breeders fertile during the time of year corresponding to the best chance of survival due to a variety of exogenous and endogenous pressures[38] that will not be discussed further here. Almost 50 years ago it was observed that the pineal gland, its major endocrine product being melatonin, was crucial for maintaining functional gonadal morphology in seasonally-breeding hamsters[39]. Initially, findings from long-day breeders, mammals who are fertile during seasons with longer photoperiods (i.e., spring), suggested that melatonin had an inhibitory effect on gonadal activity[40]. Subsequent research in these models[41,42], and in the short-day breeding (fertile when photoperiod is short, i.e., fall) sheep[43], indicated that melatonin could also be gonadotropic. It soon became evident that regulatory control of the HPGA by melatonin was not straightforward: duration of the nocturnal melatonin signal[44], which is longer during short photoperiods, and change in duration[45], is crucial for reproductive potentiation. In the short-day breeding sheep it has been demonstrated that the photoperiodic impact on gonadal activity occurs through regulation of GnRH pulse and luteinizing hormone (LH) released from the pars tuberalis of the anterior pituitary[46] and similar regulation of gonadotropins in response to artificial photoperiod or direct melatonin administration has been observed in other animals[47,48]. There is evidence that photoperiodic history has bearing on annual timing of gonadal activity: ewes exposed to the same photoperiod could either be rendered reproductively active or inactive via selective stimulation of LH secretion dependent on their photoperiodic trajectory (i.e., going from long to short days versus short to long) and similar findings have been observed in other animals[49]. These lines of inquiry in sheep have indicated that waxing and waning photoperiods at key times of the year entrains an endogenous circannual reproductive rhythm[50] and differences in the timing of reproductive activity across individuals or species is due to heterogeneity in both exogenous and endogenous factors such as social cues and genetic variability, respectively[51]. Despite the sizable body of research in this area, the pathways by which the melatonin signal exerts these reproductive pressures remain poorly understood. Still, the role of melatonin as a regulator of gonadal activity in mammals cannot be denied.

2.5.2 Is melatonin anti-gonadotropic in non-seasonal breeders?

In non-seasonally breeding mammals, such as humans, direct melatonin stimulation seems to exert an overall inhibitory effect on reproduction, as demonstrated in the rat[52]. Melatonin has been shown to inhibit pituitary secretion of gonadotropins, though this effect appears to last only a short time after birth due to reduction in melatonin receptor density in this region[53]. In neonatal gonadotrophs of rats, melatonin receptors on gonadotropin releasing neurons are involved in the regulation of GnRH gene expression[54], though again, most of these receptors are not conserved in maturity[55]. Neurons within the SCN and the pituitary have been the only central G-protein coupled MT1 and MT2 receptor sites to have been identified consistently across species, confirmed in humans (and rhesus monkeys) with *in vitro* audioradiography techniques[56]. More recently, MT1 localization has been discovered in multiple regions of the human hypothalamus, in addition to the pituitary, using immunocytochemical techniques[57]. Yet this expression has been observed to vary substantially across mammals,[58,59] to be highly plastic throughout the day[60], and, in the pars tuberalis, to be negatively regulated by melatonin itself[61,62], all of which pose challenges to discovering the central sites and mechanisms by which melatonin exerts pressure on gonadal activity. Biological models have been proposed linking the receptor-mediated nocturnal melatonin signal in the pars tuberalis to gonadotropic control via the GnRH pulse from the median eminence[59,63]. Though none have been robustly demonstrated, melatonin has been observed to suppress GnRH gene expression *in vitro* through MT1-recpetor mediated signal transduction[54]. Recent discovery of genes in the mammalian hypothalamus, the expression of which appear to be regulated by the melatonin signal, is suggestive of yet undiscovered pathways by which the hormone may mediate central gonadotropic control via the GnRH pulse. Novel candidates include kisspeptin, RFamide-related peptide gene (*Rfrp*) products and type 2 and 3 deiodinases, each implicated in the central control of gonadal activity[36,37]. Despite this, the regulation of the GnRH pulse signal is multifactorial, receiving input from afferent neurons, steroid hormone feedback, and a growing number of identified neuromodulators[64,65]. As such, it remains unclear how much of an influence melatonin may have on gonadal activity in humans in the presence of other exogenous and endogenous influences.

2.5.3 Evidence in humans

The complexity of the central regulation of gonadal function, together with the multi-potent activity of melatonin, has thwarted the elucidation of the degree to which melatonin signaling governs these processes. Despite this, observations of seasonal variation in fertility in humans have implied that its role in human reproduction is more than vestigial. Higher conception rates have been observed during the summer in more seasonally photoperiod-diverse environments[66] or by latitude[67] corresponding to seasonal variation in circulating melatonin[68]. More than one study has reported enhanced HPGA activity during longer photoperiods, suggesting an overall inhibitory effect of melatonin on fertility, and in particular, ovulation[69,70]. Further evidence implicating melatonin in regulation of reproduction in humans is found from observations of women with menstrual disorders.

2.5.3.1 Menstrual disorders

Amenorrhea, anovulation and polycystic ovarian syndrome are coincident with abnormally high levels of melatonin (reviewed in Barron[71]). Amenorrheic women exhibit reduced GnRH neuron activity in combination with higher peak levels and longer duration of nightly melatonin[72-74]. In one study, researchers noted an inverse relationship between nocturnal serum melatonin and estradiol concentrations[73]. However, it is uncertain whether higher levels of melatonin cause GnRH deficiency and/or lower circulating estrogen levels rather than the other way around through estrogenic hypothalamic feedback. While exogenous estrogen supplementation has been reported to be correlated with reduced nocturnal melatonin in amenorrheic women, this did not occur in normally cycling participants[73]. In addition, natural changes in circulating estradiol throughout the menstrual cycle[75,76] or increases during the onset of pregnancy[77] have not been associated with corresponding changes in nocturnal melatonin secretion. On the other hand, melatonin supplementation has been observed to affect gonadotropin and reproductive steroid hormone levels.

2.5.3.2 Melatonin supplementation in healthy cycling women

Morning melatonin supplementation has been associated with increased LH secretion in the follicular phase[78], though there was no evidence of elevated steroid ovarian hormones. While this appears to be at odds with the hypothesis that melatonin has an overall inhibitory influence on gonadal activity, it could be that melatonin supplementation during the day is itself disruptive of the endogenous circadian melatonin signal, perhaps having an opposite effect on gonadotropins. That is, the regulation of these hormones by melatonin may depend on time of day. Support for this is found in the investigation of melatonin supplementation on phase advances and delays of natural nocturnal melatonin secretion[79]. Nocturnal secretion was advanced most profoundly by afternoon melatonin supplementation and appeared to be delayed by exogenous melatonin taken in the morning. Diurnal variation in melatonin receptor density at central sites, as observed in rodent models[60], could explain opposing effects of spikes in circulating melatonin at opposite times of the day. Furthermore, long term daily high-dose melatonin supplementation, perhaps sufficient to override the natural endogenous circadian production rather than merely phase shifting it, has been reported to reduce circulating LH and estrogen levels in cycling women as is characteristic of inhibition of the HPGA axis[80]. Other intervention studies have observed associations between long-term supplementation and circulating reproductive hormones, but these have been limited largely to postmenopausal women[81-83].

2.5.3.3 Endogenous melatonin and circulating ovarian hormones

Earlier studies investigating the relationship between endogenous melatonin production and gonadotropin and/or sex steroid levels in healthy cycling women have been mostly quasi-experimental designs with small sample sizes. Of the larger of these, morning serum melatonin concentrations were inversely correlated with both FSH and estradiol in both the follicular and luteal phases of menstrual cycles of 20 healthy women aged 25 to 30 years[29]. The inverse correlation was somewhat stronger in the luteal phase due both to substantially increased estradiol levels as expected, but also due to significantly lower levels of melatonin.

The few larger observational studies assessing relationship between endogenous melatonin production and circulating reproductive hormones have been mostly null. Schernhammer *et al* found statistically significant correlations between urinary melatonin and both progesterone and bioavailable estradiol in a sample of 80 premenopausal women from the Nurses' Health Study (NHS) II cohort, though after adjustment for age and BMI in multivariable models, the associations were no longer statistically significant[8]. In a larger follow-up study first-morning urinary aMT6s was not associated with any of the reproductive hormones assessed, including estradiol and progesterone sampled from both the luteal and follicular phases, across aMT6s quartiles[84]. Finally, Langley *et al* did not find statistically significant associations between first morning aMT6s and plasma estradiol nor progesterone in multivariable regression models from a sample of 82 premenopausal shift-working nurses[85]. A significant inverse crude relationship between aMT6s and estradiol in a sub-analyses restricted to winter participants was reported but was not sustained in the multivariable model.

2.5.3.4 Melatonin and the menstrual cycle

Most studies have reported melatonin to vary negligibly over the menstrual cycle in healthy women [75,76,86-89], though conflicting findings exist[29,90-92]. Heterogeneity across studies stemming from small sample sizes, variation in melatonin measurement methods, and potential differences in participant characteristics, such as oral contraceptive use or nocturnal light exposure, pose challenges to drawing firm conclusions. Findings that melatonin is stable over the menstrual cycle upholds the idea that regulation of gonadal activity due to changes in nocturnal melatonin levels requires long-term entrainment. In support of this, two studies have reported inverse correlations between nocturnal melatonin and LH[93] and estradiol[68] during the ovulatory[68,93] and luteal phase[68], corresponding to seasonal fluctuations in melatonin levels. This may explain the lack of corresponding changes in circulating reproductive hormones in response to acute melatonin suppression due to magnetic field[94] or light at night[22] exposure or poor correlations between endogenous melatonin and circulating ovarian hormones[84,85] discussed above. Direct evidence for a delayed effect of melatonin on gonadal activity comes from experimental designs reporting shortened menstrual cycle lengths in women exposed to light at night for multiple consecutive days[95-97]. Shorter menstrual cycles have also been observed among shift workers[98-100].

2.6 Prolactin Regulation by Melatonin

2.6.1 Evidence from animal models

Though originally named for its ability to stimulate milk production in animals, prolactin has been reported to have more than 300 biological functions in vertebrates[101]. Produced by multiple tissues, including epithelial cells of lactating mammary glands, circulating prolactin is chiefly secreted by lactotrophs in the pars distalis of the anterior pituitary[102]. In mammals, pituitary prolactin production is chiefly under negative dopaminergic regulation from the hypothalamus, which inhibits the high basal secretory tone of lactotrophs, though other recognized modulators of prolactin output include serotonin, gamma-aminobutyric acid, estrogens, opioids and substance P[103]. The effect of melatonin on dopaminergic neurons in non-seasonally breeding rodent models, and consequentially on pituitary prolactin secretion, has been controversial (discussed in Chu *et al*[104]). In vitro, melatonin has been reported as an inhibitor of dopaminergic neuron activity (reviewed in Zisaspel[105]). Superficially, this might indicate that the decrease in nocturnal melatonin hypothesized to be associated with circadian disruption might lead to lowered pituitary prolactin, thereby potentially reducing prolactin-mediated breast cancer risk.

More recent evidence from rats in vivo, however, has been suggestive of an opposite effect. Administration of endogenous melatonin has been shown to increase activity in dopaminergic neurons regulating pituitary prolactin[104]. Acute exogenous melatonin was associated with suppression of circulating prolactin levels. Chronic melatonin supplementation, while having no bearing on the normal diurnal pattern of dopaminergic neuron activity, did attenuate the daily surge in circulating prolactin, perhaps indicative of more subtle long-term entrainment of dopaminergic tone by melatonin, or additional dopaminergic-independent mechanisms. Lower circulating prolactin and reduced prolactin gene expression following melatonin supplementation has been reported elsewhere[106].

Support of a direct pathway through which melatonin may impact prolactin secretion has been demonstrated in the seasonally-breeding sheep model. A series of in vivo experiments in hypothalamo-pituitary disconnected rams (i.e., innervations between the hypothalamus and pituitary surgically severed)[107,108] revealed that diurnal variation in prolactin secretion could be conserved in animals with implant-administered exogenous melatonin. A possible explanation of this dopaminergic-independent mechanism involves a yet to be identified “tuberlin” intermediary

relaying melatonin signaling from receptors in the pars tuberalis to prolactin secretion in the pars distalis of the anterior pituitary. It has been speculated that the generation of this tuberlin may be a consequence of the melatonin signal acting on local clock genes, or alternatively, that these clock genes modify the effect of melatonin in this tuberlin pathway[109]. A follow-up experiment in hypothalamo-pituitary disconnected rams confirmed that long-term photoperiodic regulation of prolactin, corresponding to higher levels during long day seasons when the nocturnal melatonin signal is weakest, is mediated by this direct pathway[110]. Chronic circadian disruption, induced by long photoperiods through exposure to light at night, may similarly lead to elevated levels of prolactin. However, the existence of these pathways in non-seasonally breeding humans is uncertain.

2.6.2 Evidence in humans

Contrary to the above line of evidence in rats, experimental findings suggest that the net regulatory effect of melatonin on prolactin may not be dependent on dopaminergic pathways and may be positive overall in women. A study conducted among six cycling women demonstrated that administration of the dopaminergic antagonist domperidone was sufficient to significantly increase circulating prolactin[111]. In a later study, however, early afternoon oral melatonin induced a significant increase in circulating prolactin within three hours which was not blocked by coincident infusion with the dopamine antagonist naxolone[112]. Additional observations of a stimulatory action of afternoon and evening exogenous melatonin[113], or its receptor agonist ramelteon[114], on prolactin production in cycling women supports a positive relationship between melatonin and prolactin. Furthermore, elevated melatonin has been observed consistently among cases of hyperprolactinemia[115,116]. Finally, it has been long noted that the diurnal early morning prolactin peak in women reflects the preceding nocturnal melatonin acrophase[117]. Taken together, there is strong indication that pituitary prolactin production in women is under positive receptor-mediated control by melatonin of either endogenous or exogenous source, agnostic of time of day.

It follows that exposure to light at night[118] or working night shifts[23] have been observed to coincide with subsequent lower levels of prolactin. However, sample sizes in both studies were small, consisting of 11 and 27 cycling women, respectively, and conducted by the same group in

a population of Japanese women. As such, it is uncertain if similar trends in prolactin due to circadian disruption would be observed among other ethnicities. Breast cancer incidence has been substantially higher historically in the west compared to Japan. If elevated prolactin is indeed an important component in the increased risk attributable to circadian disruption, the generalizability of these findings across genetic and cultural heterogeneity may be called into question. At least one other study has reported alteration of the diurnal prolactin rhythm among female shift workers, though whether the result was a net increase in endogenous circulating prolactin is unclear[119]. Despite evidence of a stimulatory effect of melatonin on prolactin from smaller quasi-experimental studies, larger observational investigations have not upheld these findings: Schernhammer *et al* noted no significant difference in circulating prolactin levels across women who had never worked rotating shifts and those that had for less than or at least 15 years[8]. The only observational study to investigate the relationship between nocturnal melatonin secretion following daytime circulating prolactin levels recently found no association[85]. While it appears that acute attenuation of the melatonin signal has an immediate negative impact on endogenous prolactin production, the effect of chronic circadian disruption on cumulative endogenous prolactin exposure, potentially of greater importance in breast cancer risk, remains uncertain.

2.7 Circadian disruption and Female Breast Cancer Risk

2.7.1 Circulating melatonin

Both lower circulating levels of melatonin and night shift or rotating shift exposure have been linked to increased breast cancer risk. The first studies in humans to assess the relationship between melatonin and breast cancer have been inconsistent and of smaller sample sizes, comparing plasma melatonin or its urinary metabolite, aMT6s, across cases and controls. While many reported lower melatonin levels in patients[120-123], some reported the opposite[124,125] and still others, no difference[126,127]. However, the ability to make inferences about melatonin and breast cancer risk from such studies is dubious given that melatonin levels varied by disease and treatment characteristics. They were observed to decrease with increasing tumour size[121,123], increase with chemotherapy[121,124] and were dependent on estrogen and progesterone receptor status[120] or whether the breast tumour was primary or metastatic[122].

From studies wherein melatonin specimens were collected prior to diagnosis, there was an overall inverse association between nocturnal melatonin production and breast cancer risk, supporting a role for impaired melatonin secretion in disease etiology, though the association has been more consistent among postmenopausal women. A recent meta-analysis of data from four prospective cohorts combining both pre- and postmenopausal cases reported a 34 percent statistically significant risk reduction for women among the highest exposure category[128]. The authors opted to omit smokers and women diagnosed within two years of melatonin sample collection from the premenopausal *Hormones and Diet in the Etiology of Breast Cancer Risk* (ORDET) cohort data, resulting in a change in direction of association (see Table 1). In addition to ORDET which used overnight urinary melatonin, other included cohorts in the above analysis were Guernsey III, NHS I and NHS II, comparing 24-hour and first morning urinary aMT6s concentrations, respectively. Individual results by cohort and menopausal status are summarized in Table 2.1. The null findings from the Guernsey data may be due to the use of 24-hour urinary aMT6s, potentially less reflective of the nocturnal melatonin signal than the melatonin measures used in the other cohorts.

Table 2.1. Prospective urinary melatonin metabolite levels and breast cancer risk

Cohort	Cases	Controls	Urinary aMT6	Urinary aMT6s Categories	Summary RR (95% CI)
Premenopausal Breast Cancer					
Guernsey III[129]	77	214	24-hour	Tertiles	0.99 (0.45-2.17)
ORDET[128]	180	683	Overnight	Quartiles	1.43 (0.83-2.45)
ORDET[128]	120	302	Overnight	Quartiles	0.68 (0.32-1.44)[†]
NHS II[130]	147	291	First-morning	Quartiles	0.70 (0.47-1.06) 0.59 (0.39-0.97)[‡]
Postmenopausal Breast Cancer					
Guernsey III[129]	50	139	24-hour	Tertiles	1.09 (0.46-2.60)
ORDET[131]	178	710	Overnight	Quartiles	0.56 (0.33-0.97)
NHS I[132]	357	533	First-morning	Quartiles	0.61 (0.41-0.95)

[†] Subanalysis with smokers and cases diagnosed within two years of urinary melatonin collection excluded.
[‡] In situ cases (and matched controls) omitted.

2.7.2 Night work

Recent interest in the relationship between night shift and breast cancer risk has been spurred by the declaration of shift work as a "probable carcinogen" by the International Agency for Research on Cancer (IARC) in 2007[133]. This statement was based on "limited evidence in humans for the carcinogenicity of shift-work that involves night work" and "sufficient evidence in experimental animals for the carcinogenicity of light during the daily dark period (biological night)". Mechanistic evidence from multiple rodent studies has specifically involved various models of circadian disruption on tumourigenesis. Exposure to constant light, light at night, simulated "jet lag" or timing of exposure to carcinogens out of circadian phase have demonstrated increased tumour development in these animals due to these exposures. Further support has come from models demonstrating increased oncogenic potential following induced reduced nocturnal melatonin or pinealectomy.

The most direct evidence in humans was based on epidemiologic studies indicating increased cancer risk, predominantly breast, among night shift or rotating shift health care workers and airline cabin crews. In addition to the first from 2005, there have been at least five additional meta-analyses published on the association between night work and breast cancer since the IARC announcement, four of which were published in 2013. All six are summarized in Table 2.2. Individual studies comprising the first two meta-analyses were highly conserved, consisting of those published until 2006, inclusive, while those comprising the 2013 meta-analyses were more heterogeneous. Of the nine individual studies published from 2007 onward, only two are included in all four 2013 meta-analyses, though approximately half are conserved across the last three. There was observed statistically significant heterogeneity across a substantial proportion of contributing studies (see Table 2.2). In all meta-analyses, multivariable adjusted effects were pooled over crude effects when available, though the complement of included potential confounders across individual studies was variable. Classifications of night work exposure defining pooled effects were also heterogeneous across meta-analyses. For example, the lower pooled effect sizes, many not statistically significant, in the meta-analysis by Kamdar *et al* is largely due to the difference in shift work exposure measures contributing to the pooled estimates[134]. In the first two meta-analyses, estimates representing comparisons between highest and lowest exposure groups were pooled[135,136]. In contrast, Kamdar *et al* used ever versus never risk estimates[134]. In Kamdar *et al*, most additional reported pooled estimates comparing

cumulative exposure levels of less than eight or eight or more years of night work to never night workers were not suggestive of an association[134]. Ijaz *et al* reported a statistically significant 9% increased risk (RR 1.09, 95% CI: 1.02-1.20) per five years of cumulative exposure from case-control studies, yet no corroborating pooled effect from cohort designs. However, it should be noted that the meta-analyses of Kamdar *et al* and Ijaz *et al* may have failed to capture increased risk associated with longer cumulative exposures. For example, in the NHS I[137] and II[138] cohorts, together contributing a substantial proportion of the total population data on this topic, definitive, statistically significant associations were only observed for women working rotating shifts for 30 (RR: 1.34, 95% CI:1.04-1.78) or 20 (RR: 1.79, 95% CI: 1.06-3.01) years or more, respectively. The inherent imprecision in measuring shift work exposure encountered in many of the included studies impedes its utility as a marker of exposure to circadian disruption and has likely contributed to attenuation of observed effect sizes. Despite this, there is a clear consensus of increased breast cancer risk among female night workers. The fact that poor reproductive outcomes[139] and altered menstrual cycles[98 99,100] have been associated with night shift work is suggestive that perhaps some of this risk is mediated through disruption of reproductive signaling.

Table 2.2. Meta-analyses on the association between night shift work and breast cancer risk

Study	Included Studies	Analytical Methodology	Effects of Interest	Pooled Estimates (95% CI)
Megdal *et al*, 2005[135]	7 cohort studies of airline cabin crews; exposure collected retrospectively in 3/7 studies. 6 studies on night shift work; 2 prospective cohort, 3 nested case-control and 1 case-control	Random effects pooled estimates calculated	Risk estimates contributing to pooled RR for night shift work compared highest to lowest exposure groups	Night shift work and airline cabin crews combined estimate: 1.48† (1.36-1.61) RR for night shift workers alone: 1.51† (1.36-1.68) SIR for airline cabin crews alone: 1.44† (1.26-1.65)
Erren *et al*, 2008[136]	7 studies on night shift workers; 2 prospective cohort and 3 nested case-control and 2 case-control	Fixed effects and random effects calculated	Risk estimates contributing to pooled RR for night shift work compared highest to lowest exposure groups (not explicitly reported)	All studies: 1.4† (1.3-1.6); 1.5‡ (1.2-1.8) Cohort studies (n=2): 1.4† (1.1-1.8); 1.4‡ (1.2-1.8) European studies only (n=3): 1.6† (1.3-1.8); 1.6‡ (1.2-2.22) North American Studies (n=4): 1.3† (1.1-1.6); 1.4‡ (1.1-1.8)
Kamdar *et al*, 2013[134]	Main ever versus never analysis included 8 studies on night shift workers and airline cabin crews; 3 cohort, 3 nested case-control and 2 case-control < 8 years and ≥ 8 years of ever versus never exposure included 13 and 9 studies, respectively	Fixed effects pooled estimates calculated	Risk estimates contributing to pooled RR compared ever versus never exposure. Additional comparisons of < 8 years and ≥ 8 years of ever versus never exposure	All studies: 1.21† (1.00-1.47)τ Cohort studies (n=3): 1.14† (0.85-1.53) τ European studies (n=4): 1.17† (0.84-1.63)τ North American Studies (n=3): 1.41† (0.97-2.03)τ All studies, < 8 years versus never : 1.13† (0.97-1.32)τ All studies, ≥ 8 years versus never: 1.04† (0.92-1.18) τ

Study	Included Studies	Analytical Methodology	Effects of Interest	Pooled Estimates (95% CI)
Jia *et al*, 2013[140]	13 studies on night work; 8 case-control and 5 cohort	Random and fixed pooled estimates calculated	Fixed RR effects reported when no indication of significant heterogeneity ($p > 0.10$)	All studies: 1.20‡ (1.08-1.33)τ Cohort studies (n=5): 1.08‡ (0.97-1.21)τ Case-control studies (n=8): 1.20† (1.17-1.50) ≥ 15 years versus never (n=6): 1.15†(1.03-1.29) European studies (n=8): 1.35† (1.15-1.67) Nurses (n=4): 1.15‡ (1.05-1.25)τ
Ijaz *et al*, 2013[141]	16 studies on night work; 12 case-control and 4 cohort	Random pooled estimates calculated	RR effects per 5 years of exposure, irrespective of continuity or intensity	Overall (n=12): 1.05‡ (1.01-1.10)τ Cohort studies (n=4): 1.01‡ (0.97-1.05) Case-control studies (n=12): 1.09‡ (1.02-1.20) Per 300 nights of shifts (n=8): 1.04‡ (1.00-1.10)
Wang *et al*, 2013[142]	10 studies on night work; 4 case-control; 3 nested case-control; 3 case-control	Fixed and random pooled effects calculated	RR effects for ever versus never and cumulative exposures. Random effects model used if I^2 >50%	Ever versus never (n=10): 1.19‡ (1.05-1.35) Per 5 years (n=10): 1.03‡ (1.01-1.05)τ Per 500 nights of shifts (n=4): 1.13 (1.07-1.21)τ

†Fixed-effects estimate
‡Random-effects estimate
τSignificant heterogeneity among contributing studies
Standard errors for weighting of estimates derived from reported confidence intervals or standardized incidence ratios

2.7.3 Circulating steroid hormones

The etiologies of female-specific cancers (ovarian, endometrial and breast) have been widely-publicized to be linked to reproductive-related endocrine signaling. For breast and endometrial cancer in particular, the importance of increased gonadal activity on risk is immediately apparent from incidence as a function of age. For both sites, incidence increases rapidly after age thirty until menopause and then more gradually thereafter[143]. Further evidence is gleaned from the considerable observational data linking reproductive factors to breast cancer risk (for more recent reviews see Schindler[144]; Okobia and Bunker[145]; Bernstein[146]). Included are earlier menarche and later menopause, emphasizing a positive relationship between longer exposure to a hormonal milieu associated with gonadal activity and disease risk. A predominant theory holds that long-term exposure to elevated circulating steroid sex hormones, primarily estrogen or estrogen plus progesterone, may explain a significant proportion of risk due to stimulatory effects of these hormones on proliferation[143]. Numerous lines of evidence have established estrogen as stimulator of breast epithelial tissue growth[147], implicating elevated levels of the hormones in carcinogenesis through more rapid accumulation of genetic replication errors[148]. More recently, it has been suggested that estrogen may additionally exert a genotoxic effect through buildup of reactive oxygen species resulting from local estrogen metabolism[149]. Experimental exposure to exogenous progestogens, both alone and in combination with estrogens, have been associated with increased atypical hyperplasia and carcinomas of the breast in primates[150]. Interestingly, estrogen-only supplementation did not exhibit as great a stimulatory effect as that with lone progestogens. While conclusive mechanistic evidence is lacking in humans, the role of progesterone in breast carcinogenesis is implied due to its involvement in the proliferation and differentiation of breast tissue[151].

Evidence of estrogens and progestogens as promoters of increased breast cancer risk is found in epidemiologic studies of women on regimens of exogenous preparations. While the association between oral contraceptive use and breast cancer risk has been controversial, a recent meta-analysis compiling prospective data from 13 studies totaling 11,722 cases and 859,894 participants reported a modest eight percent increased risk (RR: 1.08, 95% CI: 0.99-1.17)[152]. Further pooled analysis from five studies demonstrated a statistically significant does response effect with increasing cumulative exposure: a 14 percent increase in risk (95% CI: 5-23%) with every 10 years of additional use. There is some question as to whether oral contraceptives

meaningfully increase exposure to circulating estrogens and progestogens[153], which may partially explain the small effect sizes. While women on oral contraceptives may be exposed to higher circulating levels of these hormones during the follicular phase, due to their mechanisms of action of inhibiting gonadotropin release, the result is reduced estradiol and progesterone output from the ovaries during the rest of the menstrual cycle.

It follows that hormone replacement therapy in postmenopausal women has been more strongly linked to breast cancer. In the multi-center Women's Health Initiative trial enrolling over 16,000 postmenopausal women, treatment with estrogen plus progestin was stopped early due to a statistically significant 24 percent increased breast cancer incidence relative to placebo in the intent-to-treat analysis[154]. Increased risk of invasive breast cancer was elevated to 49 percent once non-adherers were omitted from the analysis. Women on estrogen only supplements did not exhibit increased breast cancer risk, supporting elevated progestogens as being potentially carcinogenic, at least in combination with sufficient estrogens.

Elevated endogenous circulating levels of estrogens have also been linked to breast cancer risk in both premenopausal and postmenopausal women, though this effect has been stronger and more consistent in the latter. A recent reanalysis of seven prospective studies estimated a 19% increased odds of premenopausal breast cancer (OR: 1.19; 95% CI: 1.06-1.35) with doubling of circulating estradiol concentration, however no effect was observed for progesterone[155]. A meta-analysis combining six prospective studies among postmenopausal women reported a 15 percent (95% CI: 6-24%) increase in circulating estradiol in 329 women who proceeded to develop breast cancer compared to 1,105 women who remained disease free[156]. A following meta-analysis reanalyzing nine prospective studies reported a two-fold increased postmenopausal risk across highest and lowest estradiol quintiles (RR: 2.00; 95% CI:1.47-2.71)[157]. A subsequent investigation among 322 postmenopausal breast cancer cases from the NHS yielded similar findings[158]. Women who were in the highest quartiles of circulating estradiol had a borderline statistically significant 40 percent increased risk of breast cancer (RR: 1.4 95% CI: 0.9-2.1) compared to those in the lowest quartile. When restricted to cases with estrogen/progesterone-receptor positive tumours the risk increase between the lowest to highest quartiles was more than three-fold (RR: 3.3, 95% CI: 2.0-5.4).

2.7.4 Menstrual cycle and age at menopause

As mentioned, reproductive endpoints related to gonadal activity have been recognized as prognosticators of breast cancer risk. Of those not directly related to pregnancy, such as parity and breastfeeding, the timing and extent to which are largely self-selected, age at menopause stands out as a candidate marker for postmenopausal breast cancer risk attributed to chronic circadian disruption mediated through central reproductive signaling. A recent meta-analysis combining 117 observational studies including 118,964 women with invasive breast cancer and 425,055 women in total, reported a pooled, statistically significant three percent (95% CI: 2.6-3.4%) increased risk with each year older at menopause[159]. The authors noted larger effect sizes from subgroup analyses in women with lobular versus ductal tumours and estrogen receptor positive disease. This suggests that longer exposure to circulating reproductive hormones predispose toward certain disease subtypes and/or that certain types of malignant neoplasms are more responsive to these hormones, with the important implication that the annual increase in risk observed with menopausal age is higher among certain women.

Menstrual cycle metrics, such as life-long number of cycles[160,161], have also been associated with breast cancer risk. In a study examining the association between lifelong number of menstrual cycles and breast cancer risk, women with more than 490 cycles had a 1.80-fold (95% CI: 1.09-2.96) increased risk compared to those that had 415 or less[161]. Over half of the women that had more than 490 cycles reported shorter cycle lengths (26 days or less), indicating a correlation between increased risk and menstrual cycle length. Positive associations between shorter menstrual cycles and breast cancer risk have been reported directly[162-164]. However, the latter study, comparing proportions of menstrual cycle lengths shorter than 30 days among premenopausal cases to those in controls, produced modest effect sizes (ORs of 1.2) that were not quite statistically significant[164]. In a study where menstrual cycle length data was collected prospectively, a relative risk of 1.9 (95%CI: 0.9-4.1) for women with cycle lengths of 26 days or shorter was reported[163]. It should be noted, however, that this estimate applied only to median cycle lengths between 25 to 29 years of age. When the age range was extended to from 20 to 39 years there was little evidence of an association. Furthermore, women with extremely long cycles were also reported to be at increased risk. In light of null findings[165,166], it may be that observed associations are spurious, or alternatively, that this relationship exists only within certain windows of the reproductive life time. Nevertheless, shorter menstrual cycles have been reported

to be coincident with increased FSH output and elevated ovarian activity[70], implying a mechanism through which they might increase breast cancer risk. Shorter cycles also occurred more frequently in summer when photoperiod is longer, implicating involvement of melatonin signaling.

2.7.5 Circulating prolactin

Molecular mechanisms through which prolactin may promote tumour initiation and progression have been reviewed elsewhere[167,168] and will not be discussed in detail here. Briefly, prolactin has been observed to produce anti-apoptotic and mitogenic effects, as well as interfering with cell cycle regulators in human neoplastic tissue[169]. Early observations from rodent models indicated that increased levels of pituitary prolactin resulted in promotion of tumour growth (reviewed in Welsch and Nagasawa[170]). Subsequently, blockade of pituitary prolactin production by bromocriptine, a dopamine agonist, resulted in marked reduction of initiation and progression of mammary tumours in rats treated with a chemical carcinogen[171]. In addition to intervening corroborating lines of evidence from investigations in rodent models, it was more recently demonstrated that knocking out prolactin[172] or prolactin receptor[173] gene expression in these animals resulted in delayed tumourigenesis.

While the carcinogenic potential of elevated prolactin in humans has been debated, there has been growing evidence that it is as an important risk factor for both premenopausal and postmenopausal breast cancer[169]. The dubious link between prolactin and breast cancer toward the end of the twentieth century has been attributed to perceived physiological heterogeneity between rodent models, in which prolactin has long been shown to both initiate and promote mammary tumours[170], and humans. Findings from earlier cross-sectional, case-cohort and cohort studies were conflicting (reviewed in Bernstein and Ross[174]). Studies comparing families with hereditary disease to those without have been inconclusive, some indicating that familial disease is associated with higher prolactin concentrations[175,176], while others were null[177,178]. Some of this discrepancy may be due to confounding reproductive factors, implied by observations that daughters of breast cancer patients had significantly higher prolactin levels only at certain intervals of the menstrual cycle[179,180]. Comparisons between East Asians, known to have lower

incidence of breast cancer, and Caucasians[181,182] have also failed to uncover significant differences in circulating prolactin.

Despite this, recent prospective data is, overall, supportive of a positive association between circulating prolactin levels and breast cancer risk (reviewed in Tworoger and Hankinson[183]). The authors argue that case-control or cross-sectional designs are hampered by issues of temporality, chiefly arising from the observation that prolactin levels can change post-diagnosis due to multiple factors including treatment-induced psychological and physiological stress. There have been null prospective studies[184-186], but these have suffered from smaller case numbers raising issues of study power and potential selection bias. A somewhat larger prospective study among postmenopausal women combining two Swedish cohorts reported 30 percent increased odds of breast cancer comparing highest and lowest quartiles of circulating prolactin, though results were not statistically significant[187]. Data from the NHS I and II cohorts, together contributing case numbers almost an order of magnitude greater than the largest preceding prospective study, produced relative risks of 1.3 (95% CI: 0.9, 1.9)[188] and 1.5 (95% CI: 1.0, 2.5)[189], respectively, comparing incidence of breast cancer across lowest and highest prolactin quartiles. Pooling data from the NHS I and NHS II, including 78 and 79 percent of all published premenopausal and postmenopausal cases investigating this association prospectively, resulted in a 40 (p-trend=0.05) and 30 (p-trend=0.01) percent increased premenopausal and postmenopausal breast cancer risk, respectively, across top and bottom quartiles[183]. These recent prospective findings indicate that exposure to chronically elevated circulating prolactin may play a crucial role in breast cancer risk. Evidence supporting regulation of central prolactin secretion by melatonin provides an alternative means through which circadian disruption may contribute to this risk.

2.8 Rationale

While observational findings are supportive, overall, of an association between circadian disruption and breast cancer risk, key biological mechanisms remain elusive. Our hypothesis holds that circadian disruption, mediated by suppression and/or phase-shifting of melatonin signaling, may alter gonadal activity and central prolactin production. Chronic alteration of central reproductive signaling regulating gonadal activity may lead to changes in menstrual cycle duration and menopausal onset (Figure 2.1). Potentially resulting increased exposure to

endogenous steroid hormones, including estradiol and progesterone, and prolactin may exert proliferative pressure on breast tissue, increasing cancer risk. Later menopause is an established prognosticator of breast cancer risk, though to date the association with circadian disruption has yet to be assessed. Though there is evidence indicating that shift work is associated with variation in menstrual cycle duration, as far as we are aware, none have assessed the association between nocturnal melatonin production and this endpoint. Positive findings will enable future consideration of menstrual cycle metrics and menopausal timing as an indicator of susceptibility to circadian disruption-mediated breast cancer risk. While there has been some investigation of the association between nocturnal melatonin production and circulating hormone levels, only one or two studies have assessed the association among larger samples enabling frequentist analytical methods and consideration of potential confounding factors. Investigations of variation in the regulation of prolactin by melatonin by age or diurnal preference have not yet been reported.

This work aims to supplement the current evidence on circadian regulation of reproductive signaling, now recognized to be potentially important in the etiology of breast cancer. While it will not aid in the mechanistic characterization of melatonin control of gonadal activity, sex steroid hormones or prolactin secretion, or how alterations in these endogenous processes impact tumour initiation and progression, the demonstration of consistent associations between shift work exposure, nocturnal melatonin levels and relevant reproductive-related outcomes will help confirm circadian disruption as an important consideration for breast cancer risk. This research has aimed to alleviate these knowledge gaps using data from multiple study populations.

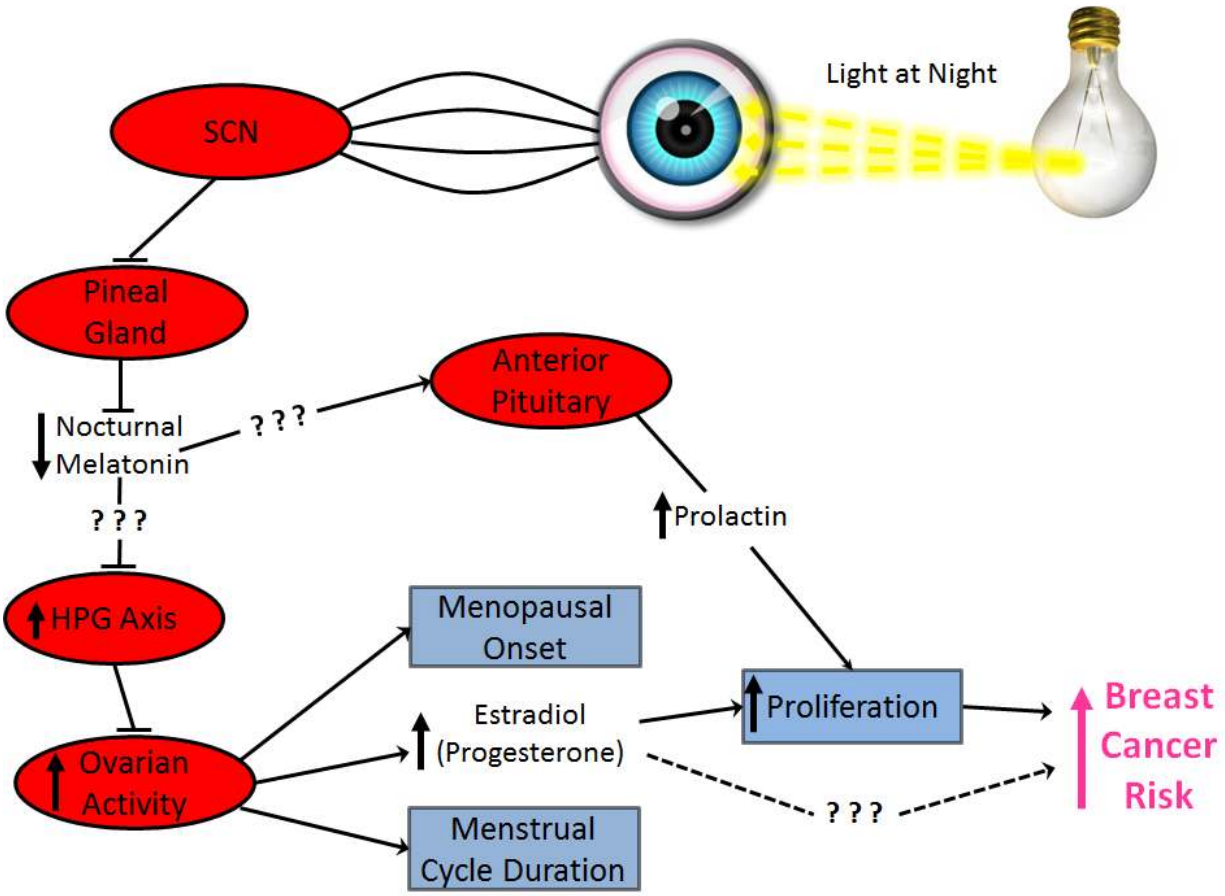

Figure 2.1. Overarching biological model: Circadian disruptive stimuli, predominant of which are photic cues which alter clock gene expression in the suprachiasmatic nucleus (SCN) via direct innervation from the retina, suppress and alter periodicity of the nocturnal melatonin signal. In turn, chronically disrupted melatonin signaling impacts reproductive function, particularly gonadal activity, leading to cumulative exposure to elevated circulated hormones which potential increase breast cancer risk through promotion of breast tissue proliferation. Circulating prolactin concentrations may additionally be altered by disrupted melatonin signaling and similarly contribute to increased breast cancer risk.

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Chapter 3 Methods

3.1 Study Population and Data Collection

3.1.1 Objective 1

Objective 1 of this thesis uses data from the Nurses' Health study (NHS) II cohort. The first NHS, established in 1976 with funding from the National Institutes of Health (NIH), was conceived to study long-term determinants of women's health, of which oral contraceptive exposure was of primary interest. This cohort was initially comprised of 122,000 married registered nurses from the 11 most populous states. A second cohort, the NHS II, (also funded by the NIH) was established in 1989 for the purpose of assessing the impact of other chronic disease determinants, in addition to oral contraceptives, beginning with a younger population of women relative to the NHS I. Recruitment targeted the states of California, Connecticut, Indiana, Iowa, Kentucky, Massachusetts, Michigan, Missouri, New York, North Carolina, Ohio, Pennsylvania, South Carolina and Texas. At inception, 517,000 invitation letters were mailed out and 124,000 women responded, resulting in a response rate of 24%. After exclusions for incomplete forms and eligibility, the initial NHS II cohort was comprised of 116,683 female (not-necessarily married) registered nurses who were between 25 and 42 years of age in 1989.

3.1.1.1 Data collection

Principal data collection for this cohort, as with the original NHS, is in the form of ongoing biennial, self-administered questionnaires. These query updated measures for core, in addition to select intermittent or one-time, determinants and indicators of women's health. Every four years, beginning in 1991, a detailed dietary component is administered as part of the questionnaire. A quality of life component was included with 1993 and 1997 questionnaires. Biological samples have been collected among subgroups; however, these were not used in the current research and will not be discussed further. Questionnaires were mailed out to participants, with the option of online completion beginning in 2001. As of 2009, the most recent questionnaire used in these analyses, loss to follow-up for the overall NHS II cohort was approximately 10 percent. For reference, the 1993 biennial questionnaire is included in Appendix 2.

3.1.1.2 Derivation of baseline study population

To assess risk of natural menopausal onset by rotating shift work exposure, a subsample of women meeting eligibility criteria were selected from the entire NHS II cohort at baseline and followed up until their 2009 questionnaire return. The derivation of this study sample is summarized in Figure 4.1(Chapter 4). Women were excluded if they did not return a 1991 questionnaire, from which baseline exposure information was captured, and additionally if they had missing or incomplete records for rotating shift work exposure at, or prior to, baseline (i.e., on the 1989 or 1991 questionnaire). Further exclusions included women who had undergone natural or induced menopause via surgery or radiation, had reported taking premenopausal hormone replacement therapy, were diagnosed with breast or other cancers or had died as of their 1993 questionnaire return date, the start of follow-up. Women were considered to have had unnatural menopause if they specifically reported menopause due to surgery or radiation or had undergone bilateral oophorectomy or hysterectomy. Those who had undergone unilateral oophorectomy and had not specifically reported unnatural menopause due to surgery or radiation were retained in the study sample. Deaths were reported by next-of kin and the postal service. They were also ascertained by searching the National Death Index for non-responders at each questionnaire cycle. Cancer diagnoses were ascertained via the questionnaires. Women who reported cancer diagnoses were contacted for permission to review medical records. Diagnoses were confirmed by a physician.

3.1.2 Objective 2

A cross sectional study design was used to assess the association between nocturnal melatonin output and subsequent daytime circulating steroid reproductive hormone levels. The study sample consisted of 137 young women between the ages of 18 and 22 living in the Toronto area, recruited for previous research. The target population was healthy, cycling, nulliparous women in early adulthood. Women were recruited for the purpose of assessing the relationships between various endogenous factors (e.g., melatonin, vitamin D) on circulating levels of reproductive hormones and other chemical messengers linked to breast development. The restricted age range was due to the hypothesis that the period of post-pubertal breast development may be of particular importance in the potentiation of future neoplastic onset. Women were recruited over

three years in approximately equal proportions in summer (June to September) and winter (December to March) from local hospitals, community colleges and universities. Eligibility for enrollment included not currently using oral contraceptives, never having been pregnant, no previous cancer diagnoses, not currently having highly irregular menstrual cycles and no night shift work or trans-meridian travel in the previous month.

3.1.2.1 Data collection

At initial contact, participants provided written consent and were provided with information about the study. At this time, they were asked to report the date of their last menses and were scheduled for a clinic visit on the 21st day of their menstrual cycle (i.e., coinciding with the luteal phase). Women were asked to fill out a short questionnaire capturing current medication use, cigarettes smoked, alcohol consumption, bed time, wake time and physical activity type, duration and intensity during the preceding 24 hours. Self-reported ethnicity was queried and height and weight were measured. A copy of the questionnaire is included in Appendix 3. Participants were supplied with a urine vessel and asked to return the following morning to turn in their urine sample and for blood draw, from which circulating serum reproductive hormone concentrations were assayed. Participants were instructed to collect voided urine from 8 PM until first morning void, inclusive.

3.1.3 Objective 3

A cross sectional study design was used to assess the association between nocturnal melatonin output and subsequent daytime circulating prolactin. The study sample of 213 women was initially recruited primarily to assess the effect of light exposure on nocturnal melatonin production in healthy women and how this relationship is impacted by secondary factors such as morning-evening preference (i.e., whether one feels more alert toward the beginning or end of the day), body composition, age and physical activity, among others. This sample of mostly premenopausal women was recruited through advertisements in local hospitals, universities, community colleges and community newspapers. Women were ineligible to participate if they had prior cancer diagnoses or kidney or liver disease. Working night shifts and trans-meridian

travel during the past month were also criteria for ineligibility. Eligibility was determined at initial contact. Willing participants were invited for an initial clinic visit during either summer (May through August) or winter (November through February) study sessions between November 2002 and August 2004. Women participating in the first session were asked to return for a second session during the opposite season.

3.1.3.1 Data collection

At the first clinic visit, women signed the consent form, received information about the study and completed a one-time questionnaire. The questionnaire queried information on age, self-reported height and weight, hormone use, oral contraceptive use, light exposure history, and included the 19 item Horne-Östberg morningness-eveningness scale[1]. Participants were instructed how to keep a diary over the 3-day study period. The questionnaire and sample 1-day diary collection form is included in Appendix 4. In addition to light exposure data, the diary was used to record alcohol use, sleep time, wake time, physical activity and medication use. Participants were given urine collection vessels with accompanying instructions for collection of three overnight samples (collected from 8pm through the first morning void), one for each consecutive night. Women were additionally instructed on the use of a light intensity measuring device; however, as light metrics do not pertain to this dissertation, this will not be discussed further. On the third morning, women were invited for a second visit for blood draw at which time urine vessels were returned.

3.2 Measurement

3.2.1 Main exposure variables

3.2.1.1 Objective 1: rotating shift work

Rotating shift work items on the NHS II questionnaires indicate shift schedules that include working day shifts concurrently with either evening or night shifts. The rationale for this classification is that working day and night shifts concurrently is likely more disruptive to the circadian system than working nights alone[2] presumably having a greater impact on associated

endocrine systems such as those regulating reproductive function. The 1989 NHS II questionnaire queried the number of years in which at least three nights, in addition to days and evenings, per month were worked. Women were asked to report if they worked no, one to two, three to five, six to nine, 10 to 14, 15 to 19 or 20 or more years of rotating shift work prior to 1989. Subsequent questionnaires in 1991, 1993, 1997, 2001, 2005 and 2007 queried the number of months worked rotating shifts since June of the previous questionnaire year (e.g., on the 1993 questionnaire, the relevant item is "Since June 1991, how many months have you worked ROTATING night shifts (at least 3 nights/month in addition to other days and evenings in that month)?"). The 2001 and 2005 questionnaires queried shift work exposure over multiple prior questionnaire intervals so that rotating shift work exposure for all nine intervals between 1989 and 2007 was reported. Number of months worked rotating shifts were recorded as ordinal categories consisting of none, one to four, five to nine, 10 to 14, 15 to 19, and 20 or more.

3.2.1.1.1 Classification of rotating shift work exposure for survival analyses

The availability of rotating shift work exposure prior to baseline (i.e., number of years worked rotating shifts before 1989) and longitudinal exposure queried over follow-up (together with baseline and updated data on potentially confounding factors) presented considerable flexibility for exposure classification. Though the use of changing (i.e., time-varying or time-dependent) independent variable data may not be useful for some of the more traditional applications of survival analyses - such as the prediction of future event probabilities from a set of prognosticators measured at, or by, some fixed time point - advantages for purposes of hypothesis testing exist. Longitudinal collection at multiple points over follow-up provides an opportunity for more informative exposure classification over baseline exposure data alone for certain disease models[3]. Related to this is the potential for reduced exposure misclassification, which enables parameter estimates from corresponding Cox proportional hazards models (or other regression methods used in the analysis of failure-time data) to more accurately reflect underlying biological processes. However, as Fisher and Lin demonstrate in their review of the topic, this increased flexibility can potentially induce bias if care is not taken to ensure that the use of updated variable information conforms to the etiology of the outcome of interest[4]. One particular caveat with time-dependent covariates is the potential for bias due to reverse causation.

If time-dependent variable values change as a result of decline in health or other factors associated with to the endpoint of interest, validity of modeled effects will likely be compromised[4]. An example is given with body weight and an endpoint of a disease process that itself induces wasting in subclinical stages. Using updated body weight as an independent variable would likely grossly exaggerate its effect if the objective was to evaluate or adjust for pre-disease states of this factor on a disease-related endpoint.

Our underlying hypothesis holds that increased lifelong exposure to elevated reproductive signaling attributed to chronic upregulation of the HPGA due to circadian disruption may delay menopause. The most accurate main exposure classification for rotating shift work was therefore conceived to be cumulative exposure over follow-up: complete history of exposure to rotating shift work would more accurately capture a disruptive effect on menopausal timing than a baseline measure alone. As we were unable to conceive how time to, or age at, menopause could influence prior shift work exposure, bias due to reverse causation was deemed unlikely. However, as the investigation of the association between night work and age at menopause is novel, we also decided to classify the exposure as an updated time-varying process restricted to that experienced during the questionnaire period prior to that in which the index event occurred. Further alternative classifications of time-varying rotating shift work exposure were not explored to avoid issues of multiple hypothesis testing.

Approximate cumulative number of months worked rotating shifts over follow-up was calculated by adding the midpoints of each ordinal category, or 20 months if reported working 20 or more months during the previous two-year interval on biennial questionnaires between 1991 and 2007, inclusive. Due to the availability of number of years worked shifts prior to 1989, two versions of the cumulative rotating shift variable were defined, differentiated by exclusion or inclusion of this historical exposure. The rationale for having two cumulative exposure definitions was due to the life-long exposure measure being less precise: though our biological model may dictate that it is appropriate to include exposure prior to 1989, we were uncertain whether or not this imprecision may mask a true effect. Having both cumulative classifications permitted exploration of this potential issue. For the version inclusive of life-long exposure prior to 1989, approximate cumulative months worked for this historical period was calculated by taking the midpoint of each ordinal category (in years) and multiplying by 12. Women who reported working 20 or more years were assigned 240 months of cumulative rotating shift work. These

quantities were added to cumulative exposure in months, updated biennially, over follow-up. For the recent rotating shift work exposure capturing the number of months in the two years prior to the previous questionnaire return date, a four-level ordinal variable was defined (0, 1 to 9, 10 to 19 and 20 or more months).

3.2.1.2 Objectives 2 and 3: urinary melatonin metabolite aMT6s

Overnight urinary creatinine standardized aMT6s was the exposure of interest for objectives 2 and 3 and is interpretable as a surrogate for overnight pineal melatonin production. Urinary aMT6s, the major melatonin metabolite excreted in urine, has been validated as an accurate marker of circulating melatonin[5]. Similar to plasma melatonin, urinary aMT6s concentrations exhibit a circadian pattern. Of the first to explore the relationship between nocturnal melatonin production and urinary aMT6s in humans, Wetterberg observed a positive correlation between plasma melatonin at 02:00 and levels of the metabolite in morning urine[6]. Other similar correlations have been noted since[7-11]. For both study populations (i.e., objective 2 and objective 3), women were instructed to include all voided urine between 20:00 and first morning void in the nightly sample. Overnight collection aimed to maximize the amount of aMT6s metabolized from overnight pineal melatonin production, inclusive of peak output that typically occurs around the middle of the night. The use of overnight urine collection instead of first morning void alone is validated by Lang *et al* who observed a higher Pearson correlation (0.74) between plasma melatonin in the middle of the night and overnight (21:00 to 07:00) urinary aMT6s compared to urinary aMT6s collected from 21:00 to 00:00 or 00:00 and 08:00 (Pearson correlations of 0.61 and 0.51, respectively)[8]. Urinary aMT6s is commonly standardized with urinary creatinine to minimize measurement error introduced by intra- and inter-individual variation in renal clearance[12] and has also been shown to correlate with both peak and total nocturnal plasma melatonin[11].

3.2.2 Main outcome variables

3.2.2.1 Objective 1: age at menopause

The outcome for objective 1 is self-reported age at menopause. Both night work[13,14] and age at menopause[15] have been associated with breast cancer risk, though the association between night work exposure and menopausal age had yet to be investigated. The NHS II maintains a cleaned age at menopause variable that reflects first reported age at menopause from each biennial questionnaire. These variables are coded with age at menopause, in years, once an age has been reported, or is otherwise assigned a missing value. Self-reported age at menopause was captured with the questionnaire item "Age natural periods ceased?". Menopausal status over follow-up was additionally queried on each biennial questionnaire. In the rare case (less than one percent) where age at menopause was missing, but women had reported transitioning from pre- to postmenopausal status on consecutive questionnaires, age at menopause was imputed as age at the midpoint between questionnaire return dates.

Age at menopause has never been used as an outcome variable in the NHS II. As such, there was question as to how to systematically obtain the most accurate age at natural menopause from one or more biennial queries. For women with a conflicting reported age at menopause on subsequent questionnaires, the first reported age was used. The decision to use first reported age, rather than a more sophisticated derivation incorporating multiple follow-ups, allowed for consistent classification over the entire follow-up as women who first reported becoming menopausal on the last questionnaire (i.e., 2009) would be treated the same as those who first reported previously. Most importantly, self-reported age at menopause in the NHS I has been validated as being at least moderately reliable[16]: over 40 percent of women who reported achieving natural menopause between 1976 and 1978 reported the same age at menopause on both 1978 and 1980 questionnaires and over 80 percent reported ages that were within one year of each other. Use of first-reported age is perhaps further supported by observed increased variability in this quantity with increasing number of years since menopausal onset, particularly after 10 or more, in the NHS I[16].

3.2.2.2 Objectives 2 and 3: menstrual cycle length and circulating estradiol, progesterone and prolactin

For objective 2, menstrual cycle length was calculated by subtracting most recent date of menses ascertained at clinical visit from following date of menses obtained by follow-up contact. Serum estradiol and progesterone concentrations were obtained the morning following the day of clinic visit when participants returned overnight urine vessels. For objective 3, morning serum prolactin was obtained at the end of the three day study period. The majority of participants contributed two samples, one each for summer and winter study sessions.

3.2.3 Potential confounding variables

3.2.3.1 Objective 1

Factors measured on the core biennial NHS II questionnaires reported or postulated to be potentially associated with age at menopause which could conceivably vary by rotating shift work status were selected as potential confounders. For objective 1, these factors consisted of smoking status, alcohol use, body mass index, physical activity, sleep duration and reproductive factors including oral contraceptive use, parity, total time spent breastfeeding and age at menarche. Smoking status, age at first birth, parity, body mass index (BMI) and oral contraceptive use information were updated every questionnaire cycle. Physical activity was assessed by querying number of minutes/hours per week spent on various activities, converted to metabolic equivalent tasks (MET)[17], on 1991, 1997, 2001 and 2005 questionnaires. Average hours of sleep per night were queried in 2001 and these values were considered reflective of sleep duration over the entire follow-up. Alcohol consumption was assessed by querying number of beverages by type consumed per week, converted to grams per week of alcohol, on 1991, 1995, 1999, 2003 and 2007 questionnaires. Updated total time spent breastfeeding information was available until 2003. Given that the youngest members of the NHS II cohort were around 40 years of age when completing the 2003 questionnaire, it is presumed that complete breastfeeding histories for most women would have been captured by this assessment. To maximize study power, the most recent values from previous survey cycles were substituted for those covariates that were not updated at a given cycle, or when encountering missing data.

3.2.3.1.1 Factors associated with menopausal timing

Smoking[18-20], parity[21], body size[22,23], oral contraceptive use[18], age at menarche[24], alcohol consumption[25,26] and physical activity[27] have previously been associated, to varying degrees, with timing of natural menopause. Briefly, smoking and alcohol use have been consistently linked to earlier natural menopause, the former having the largest impact on this outcome of any widely studied modifiable lifestyle factor. Physical activity has been inconsistently associated with age at natural menopause and it is likely that the direction of the association is dependent on frequency of intense activity as excessive exercise and low BMI have been linked to amenorrhea[28], while more moderate activity may moderately delay menopause due to maintaining a hormonal milieu associated with reproductive fitness. Older age at menarche has been mostly positively associated with later age at natural menopause, as is having had more children. BMI is positively associated with later menopause, attributed to elevated estrone production in adipose tissue which supplements estradiol signaling[23]. Oral contraceptives have been inconsistently linked to menopausal timing, though there is evidence that higher dose preparations may delay menopausal onset[29] which is in line with elevated steroid hormone mechanism thought to be behind the BMI-menopause association. Though direct evidence is lacking, breastfeeding has been speculated to impact this process due to effects of reduced circulating estrogens on follicular atresia[30]. While premenopausal daily sleep duration has not, as far as we know, been linked to timing of natural menopause, it is conceivable that sleep deprivation may negatively impact ovarian function.

3.2.3.1.2 Factors associated with rotating shift work

The prevalence of smoking has been consistently observed to be higher among night workers[31,32]. The existence of systematic differences in alcohol consumption by rotating or night shift work exposure is less certain. However there has been at least one report of higher proportions of binge drinking behavior among rotating shift workers[33] and rotating shift-working nurses have reported dependency on alcohol as a sleep aid in higher frequency relative to exclusive day and evening workers[34]. While duration and intensity of physical activity has not been conclusively demonstrated to vary between rotating shift and exclusive day workers, there is limited evidence that those working rotating or night shifts may have less opportunity to

engage in group leisure-time activities or sports[35]. Added to this is the speculation that shift work-induced fatigue and scheduling of such activities outside of the alert phase of the circadian rhythm may serve to demotivate the adoption of consistent exercise regimens[35]. There is a consensus of elevated BMI[36,37] and prevalence of obesity[38] among rotating shift workers, which may, at least in part, be due to reduced physical activity. While reasons for discrepancies in reproductive related factors including oral contraceptive use and parity are uncertain, these factors were observed to vary by duration of rotating shift work in years among participants of the NHS I cohort[39]. Sleep duration and sleep quality have also been observed to be reduced in rotating and night shift workers[40]. While there is a paucity of evidence supporting variation in menarcheal timing by rotating shift work exposure, this factor was included as a potential confounder as a precaution due to its expected strong association with the outcome.

3.2.3.2 Objectives 2 and 3

The rationale for inclusion of the potential confounders considered in the multivariable analyses testing the cross-sectional associations for objective 2 (i.e., nocturnal overnight urinary aMT6s and menstrual cycle length as well as daytime circulating estradiol and progesterone levels) and objective 3 (i.e., nocturnal overnight urinary aMT6s and daytime circulating prolactin) are discussed in this section. Factors were selected primarily based on evidence for potential association with the outcomes for each of the analyses in question. Justification for consideration of these factors as potential confounders follows.

3.2.3.2.1 Objective 2: data sources for potential confounders

Potential confounders consisted of age, physical activity, body size (i.e., BMI), alcohol consumption, smoking status, sleep duration and ethnicity. Number of sessions, type, duration and self-rated intensity of leisure-time physical activity during the past 24 hours were queried on the self-reported questionnaire. Intensity of physical activity was assessed using the Rating of Perceived Exertion (RPE) scale developed by Borg[41]. The 15-point scale, ranging from six to 20, is used to self-rate intensity of exertion and approximates number of heart beats per minute (i.e., 60 to 200). An average intensity score was derived from the ratio of perceived intensity and

reported duration of the activity. American College of Sports Medicine target heart rate guidelines for physical activity by age[42] were used to inform ordinal category bounds for exercise intensity. Physical activity was additionally classified by duration only, from total number of minutes of leisure-time activity reported in the past 24 hours. Body size was classified as BMI from height and weight measured at clinic visit. Alcohol consumption and smoking status were measured as number of drinks and cigarettes consumed, respectively, in the past 24 hours. Sleep duration was calculated from self-reported wake time on the current day and sleep time from the previous night. Ethnic background was categorized based on five broad clusters conforming to genetic population structure observed by Rosenberg *et al*[43,44] from a global sample of 52 populations (N=1,056). Luteal day, the number of days the blood sample was acquired prior to subsequent reported menses, was additionally adjusted for in estradiol and progesterone outcome analyses in attempt to account for variation in luteal phase progression and corresponding variation in ovarian output at blood draw.

3.2.3.2.2 Objective 3: data sources for potential confounders

For objective 3, age, alcohol use, body size, physical activity, oral contraceptive use, morning wake time, serotonin uptake inhibitor use, Horne-Östberg morningness-eveningness score[1] and approximate time of blood draw were considered as potential confounders. Body size was measured as BMI, calculated from height and weight measured at first clinic visit. Physical activity and alcohol use was derived from reported duration and type of leisure time exercise activities and number of drinks, respectively, recorded in the diary over the three-day study period. Only cardiovascular activities were included in the derivation of the physical activity variable as these were deemed to be reflective of higher intensity exertion and therefore more biologically relevant. The diary also contained daily morning wake time, which was averaged over the three days. Oral contraceptive use, queried as “Are you currently taking Oral Contraceptives?”, and morningness-eveningness score items were queried on the one-time questionnaire at first clinic visit. All other medication use, including that of SSRIs, were recorded in the study diary.

3.2.3.2.3 Factors associated with circulating melatonin

Recently, physical activity has been observed to be inversely associated with nocturnal melatonin secretion[45] among shift working nurses, while conversely, an analysis among women comprising the study sample for objective 3 demonstrated a positive association between exercise duration and nightly melatonin output[46]. A possible explanation for the discrepancy was the inclusion of sedentary activity in the tally of energy expenditure in the former. When restricted to moderate or vigorous physical activity, the imposed timing due to work schedules may have precluded the detection of a positive association. The work by Knight *et al* included leisure-time exercise only[46], likely to be of relatively high intensity, and the strongest positive associations between exercise and nocturnal melatonin was observed when these activities were performed later in the day. Indeed, high intensity physical activity has been associated with transiently increased circulating melatonin elsewhere[47]. Circulating levels of melatonin have been observed to decline with age in women[48,49]. Smoking[50] and alcohol[51] have also been observed to reduce endogenous melatonin, though moderate alcohol consumption (i.e., one to two drinks per day) appears to have a minimal[51] or negligible[52] effect on the urinary aMT6s. Current BMI has been observed to be moderately inversely correlated with urinary melatonin metabolite concentrations in the NHS I cohort[2], a finding that was upheld among that of the NHS II[53]. Increases in circulating melatonin have been noted in patients taking certain SSRI medications[54]. In the case of a particular SSRI, fluoxetine, the authors speculated that increases in endogenous melatonin may be due to decreased catabolism from competitive inhibition of the cytochrome P450-3A4 enzyme. To our knowledge, nocturnal melatonin output has not been shown to directly correlate with sleep duration, yet the rationale for a potential association might be made from observations of longer nocturnal melatonin secretion in response to shorter photoperiod[55] for those who sleep in light-controlled environments.

3.2.3.2.4 Factors associated with menstrual cycle length, estradiol and progesterone

Physical activity has been positively associated with menstrual cycle length[56], though the relationship was attenuated with increasing BMI and lower levels of circulating steroid reproductive hormones[57,58]. On the other hand, moderate exercise interventions among sedentary,

healthy cycling women have not been associated with meaningful changes in these endpoints[59]. This suggests that these effects depend on fitness level, adiposity and habitual activity intensity level. Smoking has been associated with shorter menstrual cycle length among a large sample of young women[60]. Menstrual cycle or follicular phase length has been reported to vary by age, alcohol consumption and ethnicity [61] and heavy alcohol consumption has been associated with menstrual irregularities[62]. Estradiol has also been observed to be significantly elevated among drinkers (i.e., more than one drink per day) relative to teetotalers[63], while progesterone levels have been negatively associated with alcohol intake[62]. Smoking has been associated with elevated circulating estrogens and progesterone among cycling women[64]. Menstrual cycle length has also been shown to vary with adiposity [65], prompting inclusion of BMI as a potential confounder. While BMI has been positively associated with circulating sex steroid hormone levels in postmenopausal women[66], the relationship prior to menopause is less certain. However, the inclusion of BMI as a potential confounder in estrogen and progesterone outcome regression models was considered warranted due to its association with menstrual cycle characteristics and other reproductive factors.

Levels of these hormones have been shown to vary by ethnicity. In a multivariable model, Asian Americans had significantly higher levels of both sex hormone binding globulin-bound and free circulating estradiol than Caucasians[67]. This is particularly relevant for objective 2 as a large proportion of the study sample was of Asian descent. Progesterone levels were also shown to be significantly higher in African Americans relative to Asian Americans. Sleep duration was considered in these analyses in attempt to reduce confounding attributed differences in circadian rhythm, observed to affect both timing of peak melatonin production, potentially impacting overall urinary excretion, and variation of reproductive hormone levels throughout the day. Luteal day, the number of days the blood sample was acquired prior to subsequent reported menses, was additionally adjusted for in estradiol and progesterone outcome analyses due to potential variation in luteal phase progression, and corresponding ovarian output, at blood draw.

3.2.3.2.5 Factors associated with prolactin

Circulating levels of prolactin have been observed to decline with age in women[68,69]. While inconsistent, there have also been observations of positive associations between BMI and

prolactin in both premenopausal and postmenopausal women[70]. Chronic alcohol use has been positively associated with circulating prolactin levels[71]. A similar relationship between intense cardiovascular exercise and circulating prolactin[70] has been noted. As participants reported specific activity performed, we were able to include only cardiovascular exercise duration as a potential confounder: reported cardiovascular activities were considered to be of higher exertion in most cases and therefore most likely to be associated with variation in circulating hormones. Circulating prolactin levels have been observed to be impacted by certain medications, including oral contraceptives and SSRIs[70]. Some SSRIs have been recently suspected of impacting dopamine signaling[72], while higher levels of dopamine have been observed to suppress central prolactin secretion[73]. While the impact of oral contraceptives on nocturnal melatonin production is uncertain, it has long been known that there is potential for these medications to elevate prolactin levels[74]. Considerable variation in timing of the early morning prolactin acrophase has been observed[75] and it is possible that late-morning nadir prolactin levels had not yet been reached at time of blood draw for some women. As such, morning wake time and Horne-Östberg morningness-eveningness score were included as potential confounders as a proxy for circadian phase, to adjust for variation in the serum prolactin outcome attributable to this factor. Approximate time of blood draw was included in an attempt to adjust for typical diurnal variation in prolactin levels.

3.3 Laboratory Assays

3.3.1 Objectives 2 and 3

3.3.1.1 Urinary melatonin metabolite aMT6s and creatinine

For both objectives 2 and 3, the volume of each overnight urine collection was measured. Two 1-ml aliquots were stored at -20°C for future assay. Melatonin has been observed to be stable in urine without preservatives for two years at this temperature[76]. Urinary creatinine concentration was determined with the automated Roche Cobas Integra 700 analyzer (F. Hoffmann- La Roche, Ltd., Basel, Switzerland), using an enzymatic creatinase-based method (COBAS INTEGRA® Creatinine plus ver.2, Cat. No. 03263991, Roche Diagnostics). Six-sulfatoxymelatonin (aMT6s) was assayed using a single-epitope competitive enzyme-linked immunoassay kit from IBL International GmbH (Hamburg, Germany; catalog number RE54031). All pipetting, incubation,

washing and reading steps of the assay protocol for aMT6s assays were robotized and completed over a 2-day period using the same lot of test kits to minimize sampling error. Urine specimens containing an aMT6s concentration in excess of the second highest standard (140 μg/L) were diluted further and assayed again. For objective 2, quality control samples from all assay batches were within manufacturer range, with coefficients of variation of 8.7 and 10.1 percent for high and low dilution test samples, respectively. For objective 3, the three urine samples from each consecutive night of each data collection session (provided at the end of the three day measurement period) were assayed sequentially and imprecision across all the assay runs, compared to controls, was 25 percent at a concentration of 13 μg/l and 17 percent at a concentration of 63 μg/l.

3.3.1.2 Steroid Hormones

For objective 2, serum estradiol and progesterone concentrations were measured using the Roche Diagnostics electrochemoluminescence immunoassay with intra-assay coefficient of variation (cv) of less than five percent and inter-assay cv ranging from six to 11 and four to 10 percent, respectively over the study term.

3.3.1.3 Prolactin

For objective 3, serum prolactin concentration was assayed by the multitest automated Immulite 2000 analyser using a two-site immunometric sandwich method with chemiluminescent detection commercially available from the manufacturer of the analyser (Siemens Medical Solutions Diagnostics). Inter-assay coefficient of variation was approximately 6 percent.

3.4 Statistical Analyses

All statistical analyses were performed using SAS statistical software, version 9.2 (SAS Institute Inc., Cary, North Carolina USA). For objective 1, effect estimates from proportional hazards regression models are presented as hazard ratios with 95 percent confidence intervals for

purposes of statistical inference. For objectives 2 and 3, effects from ordinary least squares linear regression and generalized estimating equation models are presented as coefficients of independent variables which can be interpreted as the change in predicted dependent variable value due to a single unit change in the independent variable. As generalized estimating equation approaches are based on the quasilikelihood and not maximum likelihood[77], techniques a modified Akaike's information criterion (QIC) was used as a quantitative means of assessing model fit[78]. Ninety-five percent confidence intervals are included for purposes of statistical inference. All presented p-values are two-tailed. Additional details specific to the analyses conducted for each objective are found in the corresponding manuscripts (Chapters 4, 5 and 6).

3.4.1 Model building

Statistical assessment of confounding in multivariable regression most commonly involves assessing either the statistical significance of the potentially confounding independent variable or change in the size of the main effect upon addition or removal of the variable from the model. Though either strategy can perform adequately, the change in effect size strategy has been observed to perform slightly better in some situations in simulation studies[79] and was the strategy chiefly used for model building herein. For all objectives, for purposes of data familiarization, all potential confounders were first modeled individually against the outcome. Next, these bivariable models were extended to include the main effect to rank which had the strongest potentially confounding effect. For objective 1, we were not overly concerned about achieving the most parsimonious model due to the ample study power encountered when assessing the non-rare outcome of menopause in the NHS II cohort: removal or addition of potential confounders that had a minimal impact on the main effect (i.e., rotating shift work exposure) did little to impact model fit statistics or standard errors of the main effect coefficients. As such, potential confounders selected a priori were retained in the multivariable proportional hazards models if they were not deemed to be potential mediators or collinear with the main effect (i.e., rotating shift work). For objectives 2 and 3, due to statistical power constraints, we attempted to achieve the most parsimonious models by evaluating change in main effect (i.e., creatinine-standardized urinary aMT6s) as described in the relevant manuscripts (Chapters 5 and 6).

3.4.2 Statistical analyses issues specific to objective 1

3.4.2.1 Statistical model and data structure

Cox proportional hazards models were used to assess the association between rotating shift work and time to menopause. The time metameter used was age in months: age at menopause or end of follow-up, in months, was subtracted from birth month and start time (t_0) was age in months at 1993 questionnaire return date. End of follow-up, for women who did not reach menopause or were not right-censored, was age in months at 2009 questionnaire return. There was a total of nine follow-up intervals, the lengths of which were defined by the number of months between consecutive return dates of biennial questionnaires between 1993 and 2009, inclusive. Due to variation in the time it took women to receive, complete and return biennial questionnaires, there was considerable variation in questionnaire return dates: follow-up intervals ranged from less than one year to 47 months, though the mode was 24 months and over half of the interval lengths were between 23 and 25 months, inclusive. Independent variable values were permitted to change with each questionnaire interval. To accommodate delayed entry (not all women were the same age at 1993 questionnaire return) and time-varying independent variable values, the counting process data structure was used, with intervals corresponding to the follow-up intervals described above.

3.4.2.2 Counting process data structure for proportional hazards models

The counting process data structure for proportional hazards models is characterized by the flexibility of allowing each member's contribution to the risk set to be divided into intervals, handled as individual observations, or data lines in the data structure[80]. This was used for objective 1 analyses regressing time to age at menopause on rotating shift work exposure to accommodate time-dependent covariates and delayed entry (i.e., left truncation); women did not enter the cohort at a common time point. Instead, start time (i.e., t_0) was the age at each individual's 1993 questionnaire return date. Time-dependent covariates were handled by allowing the values of each independent variable to change to a fixed constant for a specified interval. The statistical software created a corresponding step function with jumps at the beginning of each interval. The intervals were indexed by a start and stop time, which were used to align the correct independent variable values at the time of each event[80]. This data structure

was particularly suited for analyzing prospective cohort data based on regular recurring questionnaires. For objective 1, risk set membership was divided into intervals defined by time between biennial questionnaire return dates, the finest increments over which independent variables could change. The result was a maximum of nine observations per individual, representing possible risk set membership between 1993 and 2009 questionnaire return dates.

The proportional hazards model for censored data introduced by Cox spurred much interest due to its semiparametric nature; that estimation of covariate coefficients is not dependent on the specification of the baseline hazard function, treated as an infinite-dimensional nuisance parameter, under the proportional hazards assumption[81]. Of particular interest was the operationalization of this novel concept in form of the partial likelihood[82]. However, mathematical demonstration of the asymptotic properties of the partial likelihood has been called heuristic[81] and was only partially developed by Cox in his original publications on the topic[82,83]. In Cox's 1972 paper, the model was presented in the context of conditional probability, building on product limit methods introduced by Kaplan and Meier. While multiple mathematical validations of Cox's partial likelihood were presented following its introduction, these were all highly complex and specific to certain cases (e.g., Liu and Crowley[84]; Tsiatis[85,86]). Empirically, the Cox proportional hazards model has been demonstrated to be robust across a wide range of conditions and remains the most widely used multivariable regression model for estimating relative hazards from censored survival data. Yet mathematical proof of this robustness, both for parameter estimation and tests of statistical inference, was initially incomplete.

The use of the counting process approach for censored survival data was first presented by Aalen in the form of more general multiplicative intensity models[87]. Briefly, in its simplest form, the counting process approach to multiplicative hazards models can be conceptualized to be comprised of three basic components. First, there is the counting process itself (i.e., $N(t)$: $t \geq 0$) which is a left-bounded stochastic process that records the number of events that have occurred by a specific time, t. For instance, at t_0 N=0 and jumps to 1 at the time of the first event. Second, there is the intensity process, which loosely speaking, incorporates two processes, one describing risk set membership at time t and another, the instantaneous likelihood of N jumping from 0 to 1 at time t (analogous to a hazard function). The integration of the intensity process with respect to time gives the cumulative intensity process, $\Lambda(t)$. Finally, there is the counting process martingale, $M(t)$, a special form of stochastic process, which describes the delta between the

counting process and the cumulative intensity process at time *t* (i.e., $M(t)=N(t) - \Lambda(t)$). Due to properties of the martingale central limit theorem, the counting process martingale has an expectation value of zero between time *t* and the instantaneous interval following time *t*. From this, the counting process martingale can be considered analogous to a linear regression residual. These concepts form the superficial underpinnings of Aalens's approach to survival analysis that were adapted to provide a robust mathematical basis for Cox's proportional hazards model and partial likelihood estimation by Andersen and Gill[88]. Related theorems have been used to prove that covariate coefficients estimated by the partial likelihood are asymptotically normally distributed and that the associated covariance matrix can be computed directly from the observed information matrix[89]. Counting process theory has since motivated extensions of the Cox model and enabled model assessment techniques analogous to linear regression (e.g., martingale residual plots).

The above supports the use of the counting process data structure for the efficient handling of time-dependent covariates and accommodation of delayed entry for objective 1. Mathematical justification for usage of the counting process data structure and reliance on counting process theory to demonstrate the general robustness of the Cox proportional hazards model and its extensions require an understanding of probability measure theory. In addition, it requires knowledge of calculus methods required for the operationalization of related stochastic functions as components of regression models, maximum likelihood estimation and formulae for tests of statistical inference and, as such, is beyond the scope of this dissertation. The mathematical demonstration of the reformulation of the Cox model using counting process theory has already been provided elsewhere, first by Andersen and Gill[88], and more exhaustively in texts by Andersen and Gill[87] and Fleming and Harrington[90].

3.4.2.3 Censoring criteria

Women were censored due to loss to follow-up, at the end of follow-up, and in cases of competing outcomes (i.e., the observation is no longer at risk for the event of interest due to a condition that precludes the event from occurring). As such, women who developed a condition that precluded, or potentially substantially distorted the timing of, natural menopause were right censored.

In addition to loss to follow-up or end of follow-up interval, observations were right censored due to death, reports of overt unnatural menopause due to surgery or other factors, on initiation of premenopausal hormone replacement therapy (HRT) and cancer diagnosis. On each biennial NHS II questionnaire, participants were asked to report whether their periods had ceased permanently and if this was due to natural menopause, chemotherapy or radiation, or surgery. If surgery, women were asked to specify if they had undergone hysterectomy and/or unilateral or bilateral oophorectomy. Indications of bilateral oophorectomy, hysterectomy or otherwise unnatural menopause, HRT use and cancer diagnoses at sites other than breast resulted in women being censored at the return date of the questionnaire previous to the one on which these were first reported. Women were additionally censored due to breast cancer diagnosis, the most common cancer site in this study population, and death, at the month at which these events occurred.

Due to the ubiquity of HRT use during the 1990s and early 2000s, a substantial proportion of women in the NHS II reported taking these medications. To minimize the impact on study power, women remained eligible for the event if their first reported premenopausal HRT use and age at menopause occurred within the same questionnaire interval. The preceding of menopause by HRT initiation within the same questionnaire interval was deemed unlikely to significantly impact reported age at event in most cases given that sufficient use of these medications would be more likely to delay rather than advance reported menopause, as discussed in the next section. Despite this, approximately 20 percent of the baseline study population was censored due to HRT use over follow-up.

3.4.2.3.1 Rationale for censoring criteria

A competing event can be defined as one which occurs over follow-up that precludes those at risk from having the event of interest or fundamentally changes the probability of occurrence of the event[91]. Death and surgical or chemical menopause are clearly competing risks given that these women can no longer report onset of natural menopause, the outcome of interest. Onset of HRT use and having cancer were additionally considered competing events as these were likely to either directly or indirectly prevent, or substantially undermine the reliability of reported age of natural menopause. The decision to treat these as competing events, defining censoring

criteria, rather than as nuisance variables (i.e., potential confounders) to be included as covariates in the multivariable regression model, was based on the distinction that the event of interest is not age at which periods ceased, but rather age at which periods ceased naturally. While the onset of cancer and HRT use do not preclude the former, they may arguably the latter.

HRT is known to delay menopause[92] and use can itself induce menstrual bleeding and spotting[93]. Given that onset of HRT use in premenopausal women is typically temporally proximal to menopause, the conceptualization of HRT use as a competing risk was based on the rationale that it would potentially substantially undermine the ability to accurately report time of natural ovarian failure. Cancer treatments, such as steroid hormone-blocking chemotherapy, alkylating agents and pelvic radiation, have been associated with earlier menopausal onset[94-96]. Furthermore, some tumours have been observed to alter steroid hormone metabolism (e.g., via elevated aromatase[97] or estrogen alpha-hydroxylase[98] activity in breast tumours). Reports of variations in circulating reproductive hormones between breast cancer patients and healthy controls[99,100] suggests the potential for altered HPGA activity via hypothalamic feedback, perhaps leading to the masking of natural menopausal timing through delay of ovarian failure. Even if the cancer or related treatments do not significantly alter true natural menopausal timing, some (e.g., tamoxifen) may cause amenorrhea which may lead to substantial premature reporting of the event time[96].

3.4.2.3.2 Interpretation of effects in the presence of competing events

Due to the conception of HRT onset, cancer diagnosis, surgical or chemical menopause and death as potential competing events, consideration was given to how best assess the unbiased effect of rotating shift work on the outcome of interest, time to natural menopause. It should first be noted that irrespective of competing risks, it is not possible to directly infer relative cumulative incidence of natural menopause across rotating shift work exposure levels from corresponding hazard ratios estimated by our time-dependent covariate proportional hazards models (Chapter 4: Table 2). When using random (i.e., internal) time-dependent covariates, inference of an effect on the hazard function can still be estimated directly from the partial likelihood, conditional to the modelled covariates, obtained from parameter estimates of the corresponding proportional hazards model. Inference about the cumulative probability of an

event from covariate parameters based on the partial likelihood, however, is additionally dependent on parameters from a joint model of previously stopped covariate processes, which are not straightforward to identify[101].

As such, we were primarily interested in potential bias on relative cause-specific hazards of natural menopause across rotating shift work exposure due to the presence of potential competing events. In such a case, estimation of cause-specific hazards from classical proportional hazards models are considered valid if no significant dependence exists across competing events[102]. When dealing with competing events in the context of a competing risks analysis, current recommendations suggest the consideration of all cause-specific effects[103,104] to glean a more complete understanding of how removing observations from the risk set at the onset of one or multiple events can impact estimation of both cause-specific hazards and cumulative incidence.

In the analysis of right-censored data, an important assumption is that censoring is independent of the failure process. The literature on informative censoring contains a certain amount of terminological ambiguity[105]. Independent, random and non- or uninformative censoring are terms that describe closely related concepts that have been used interchangeably, though have slightly different theoretical origins. For covariate coefficient estimation using regression models for survival data, the important assumption of non-informative censoring can be said to be met if individuals with identical covariate information have an equal probability of being censored, at any time *t*, regardless of the reason for being censored[86]. As such, censoring women for any reason, including the conceptualized competing events of onset of HRT use or induced menopause, will potentially bias estimated effects if, on average over time, women who are censored have different modeled covariate structures, or differ by unmeasured factors related to modeled covariates (i.e., residual non-randomness). By extension, informative censoring is likely when the probability of being censored is associated with that of having the event of interest, by virtue of the modeled covariates or unmeasured factors also being associated with this outcome.

Discussion of evaluation of informative censoring is limited to that for competing events of HRT use and (surgical or chemically) induced menopause as these were the criteria for which the vast majority of women were censored. The proportion of women censored due to cancer diagnosis and death were relatively negligible compared to that reporting natural menopause. Investigation

of informative censoring by these competing outcomes is summarized in Appendix 1. First, the distribution of the exposure of interest, cumulative rotating shift work, by premenopausal HRT onset and induced menopause, was investigated. Substantial variation in cumulative rotating shift work by frequency of premenopausal HRT onset or induced menopause would likely indicate that the relative hazards of natural menopause across this exposure would be additionally dependent on variation in probability of being censored due to these criteria. Second, cause-specific relative hazards for premenopausal HRT and induced menopause onset as the events are presented. Third, sensitivity analyses were conducted after examples from survival analysis texts[86,106]. In classical cases of competing risks, there is usually no information available to indicate when or whether those affected would have been more or less likely to have had the event of interest (i.e., in cases where competing risks result in loss to follow-up). In such scenarios, some have recommended sensitivity analyses that compare "worst cases" [86,106]. Applied to our analysis of rotating shift work and time to natural menopause, women who were censored due to HRT onset or induced menopause would be treated as having had natural menopause at time of censorship in one analysis, and remain in the risk set for the maximum possible follow-up time in another. As women in the NHS II were still able to report an age at menopause after HRT onset, for the latter case, time of reported menopause after HRT onset, if available, was used. This was deemed to be more proximal to the true natural menopausal onset than allowing all women on HRT to remain in the risk set until end of follow-up. Finally, additional potential biases of the reported cumulative rotating shift work effects (Chapter 4: Table 2) are investigated. Most notable are the cohort effects of premenopausal HRT, menopausal onset, and the distribution of modelled cumulative rotating shift work, excluding exposure prior to 1989, over follow-up.

3.4.2.4 Evaluation of proportional hazards assumption

For objective 1, adherence to the proportional hazards assumption was evaluated both quantitatively and graphically. These methods have been discussed in recent applied texts on survival analysis[80,89]. Quantitative assessment consisted of adding product terms comprised of the logarithm of the outcome variable (i.e., time to age at menopause) and rotating shift work to the model and conducting likelihood ratio tests to determine if these terms improved model fit. Two graphical means were additionally employed. The first involved utilization of a SAS macro

provided by the Mayo Clinic which allows computation of cumulative probabilities of remaining event-free, as a function of time to age at menopause, by time-varying cumulative rotating shift work exposure levels. Graphing the negative logarithm of the logarithm of this cumulative probability function, i.e., -log(log(p)), against the logarithm of time to age at menopause, ensuring that the resulting lines for each exposure level remain roughly parallel and do not cross, has been recommended as a visual means to evaluate departures from proportional hazards[89]. Secondly, a pseudo-continuous variable representing cumulative rotating shift work exposure was modeled and the resulting Schoenfeld residuals were graphed as a function of time to age at menopause. A significant change in slope of residual values by time to age at menopause is indicative of violation of proportional hazards.

3.5 Ethics and Permissions

The University of Toronto's Health Sciences Research Ethics Board granted ethics approval for all research described in this dissertation (Protocol Reference # 27537). The formal letter of ethics approval is presented in Appendix 5. Additionally, permission to conduct all analyses using NHS II data was granted upon review of a research proposal presented at the NHS investigators' bi-weekly meeting.

3.6 References

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Chapter 4 Manuscript 1

Rotating shift work exposure and onset of natural menopause: Implications for postmenopausal breast cancer risk

This manuscript explores the association between rotating shift work exposure experienced over the female reproductive life time and age at natural menopause as a potential determinant of postmenopausal breast cancer risk within the Nurse's Health Study II cohort.

Abstract

Background: Circadian disruption has been hypothesized to impact reproductive signaling, which in turn has long been linked to breast cancer. Though results are conflicting, elevated exposure to night work, the most widely studied surrogate marker for exposure to circadian disruption in observational studies, has been associated with poorer reproductive outcomes including disruption of menstrual cycle patterns and increased risk of chronic diseases, the most prominent of which is breast cancer. Currently it has yet to be assessed whether night work exposure is associated with later menopausal onset, an established independent risk factor for breast cancer.

Methods: We assessed the association between both cumulative and current rotating shift work, and time to natural menopause, among 81,769 women of the Nurse's Health Study II cohort, over 16 years of follow-up (1993-2009). Cox proportional hazards models were adjusted for smoking status, body mass index, physical activity, alcohol consumption and endogenous and exogenous reproductive and hormonal factors.

Results: 30,191 women achieved natural menopausal over follow-up. A statistically significant 24% decreased risk of natural menopause (Hazard Ratio: 0.76, 95% Confidence Interval (CI): 0.69-0.85) was observed for women who experienced >10 years of cumulative rotating shift work since 1989, relative to non-rotating shift workers. When number of years of rotating shift work prior to 1989 was incorporated, there was indication of this effect only for women who had accumulated the most extreme exposure. Recent rotating shift work exposure was moderately associated with earlier natural menopause. Women who worked 20 or more months in the prior two-year interval had an 8% increased risk (95% HR: 1.08; 95% CI: 1.02-1.15) of becoming menopausal compared to those women who reported no rotating shift work during the same period.

Conclusion: If valid, these findings support a potential regulatory effect of circadian disruption on reproductive function and suggest that increased shift work exposure contributes to increased postmenopausal breast cancer risk via the same mechanisms that link later menopause to this outcome. However, further research incorporating more precise night work exposure measures is warranted.

Introduction

Wake and sleep schedules increasingly stray from entrainment to daily light and dark cycles. This desynchronization has become more common due to growth in round-the-clock labour requirements and night time social activities facilitated by the ubiquity of artificial light. The light at night hypothesis of breast cancer holds that increased exposure to electric light between sunset and sunrise may explain the rise of breast cancer incidence in developed countries[1]. While the mechanisms through which this is achieved have not yet been clearly delineated, the theory postulated that chronic exposure to electric light at night impacts reproductive signaling, in turn exerting oncogenic pressure via increased proliferatory stimulation of breast tissue. Light stimulates non-visual receptors in the retina that relay signals to the suprachiasmatic nucleus, known as the central pacemaker, which in turn, through direct innervation of the pineal gland, regulate melatonin production[2,3]. Normal production is characterized by peak pineal secretion toward the middle of the nightly sleep cycle. Under light at night exposure, this nocturnal acrophase is diminished and/or advanced or delayed depending upon the timing and recurrence of exposure[4]. Though the net effect of melatonin on human female reproduction has yet to be delineated, there is evidence to suggest that the hormone has an overall suppressive effect on the hypothalamic-pituitary-gonadal axis (HPGA) in humans[5,6]. On this basis, nocturnal suppression of melatonin may potentially increase breast cancer risk via increased lifetime exposure to elevated estrogenic signaling.

Night work, a common surrogate measure of circadian disruption in observational studies due to the high propensity for these workers to follow abnormal light and dark schedules, has been linked to abnormal reproductive function including adverse pregnancy outcomes[7]. Observations indicating variation in, or alteration of, menstrual cycle patterns by shift work exposure[8-10] are suggestive that circadian disruption affects ovulatory processes. Finally, an effect of circadian disruption on reproductive signaling in women is implicated by observed increased risk of reproduction-linked cancers, including those of the endometrium[11] and breast[12,13], among long-term shift workers.

To investigate the effect of circadian disruption on a reproductive outcome predictive of breast cancer risk, we assessed the novel association between rotating shift work and onset of natural menopause within the Nurses' Health Study (NHS) II cohort. We hypothesized that chronic

circadian disruption experienced in those working rotating shift schedules may delay menopause through diminished suppression of the HPGA and the resulting attenuation of reproductive senescence. Night work, through disruption of circadian signaling, may lead to higher life-long exposure to certain endogenous reproductive hormones, such as estrogens and progeterone, which have been implicated in the proliferation underlying the onset of reproductive-linked cancers. Such findings may help to elucidate the biological mechanisms behind the increased risk of postmenopausal breast cancer among shift workers, of which later menopause is an established independent prognosticator.

Methods

Study Population

The NHS II is a prospective cohort, initially comprised of 116,683 female registered nurses who were between 25 and 42 years of age in 1989[14]. Principal data collection for this cohort is comprised of ongoing biennial, self-administered questionnaires, beginning in 1989. Subsequent questionnaires query updated measures for core variables in addition to select novel indicators pertaining to women's health. Questionnaires were mailed to participants, with the option for online completion as of the 2001 cycle. The derivation of the study sample from the entire NHS II cohort is summarized in Figure 4.1. Briefly, women were excluded if they did not return a 1991 questionnaire or had missing or incomplete records for rotating shift work exposure at, or prior to, baseline. Further exclusions include women who had already achieved natural or induced menopause via surgical or other medical means, had reported taking premenopausal hormone replacement therapy (HRT), or were diagnosed with breast or other cancers prior to baseline. Women were considered to have had surgical menopause if they had undergone bilateral oophorectomy or hysterectomy.

Deaths were reported by next-of-kin and the postal service. They were also ascertained by searching the National Death Index for non-responders at each questionnaire cycle. Cancer diagnoses were ascertained via the questionnaires. Women who reported cancer diagnoses were contacted for permission to review medical records. Diagnoses were confirmed by a physician.

Rotating shift work exposure

Rotating shift work items on the NHS II questionnaires capture exposure to work schedules that include working days concurrently with either evening or night shifts. The rationale for this classification was that working day and night shifts concurrently is likely more disruptive than working nights alone[15]. The 1989 NHS II questionnaire queried number of years (one to two, three to five, six to nine, 10 to 14, 15 to 19 or 20 or more) in which at least three nights per month, in addition to days and evenings, were worked. Subsequent questionnaires in 1991, 1993, 1997, 2001, 2005 and 2007 queried the number of months (none, one to four, five to nine, 10 to 14, 15 to 19, and 20 or more) in which at least three nights per month, in addition to days and evenings, had been worked in the past two years. The 2001 and 2005 questionnaires queried multiple consecutive two-year intervals so that rotating shift work exposure over entire follow-up (i.e., 1989 to 2007) was captured.

Approximate number of cumulative months worked rotating shifts prior to 1989 was calculated by taking the midpoint of each category and multiplying by 12. Women who reported working 20 or more years were assigned 240 months of cumulative rotating shift work. Approximate cumulative months worked rotating shifts over follow-up was calculated by adding the midpoints of each category, or 20 months if reported working 20 or more months since June of the previous questionnaire year. Two time-dependent rotating shift work exposure definitions were investigated, differentiated by the inclusion and exclusion of number of years worked prior to 1989, due to concerns that rotating shift work exposure measured prior to 1989 may be more prone to misclassification resulting from recall and reduced precision inherent in the 1989 questionnaire item.

Age at natural menopause

Age at natural menopause was derived from self-reported age at menopause queried on each of the biennial NHS II questionnaires. Participants were asked whether their periods had ceased, at what age, and if this was due to natural menopause, chemotherapy or radiation, or surgery. Women were asked to report separately if they had undergone hysterectomy and unilateral or bilateral oophorectomy. Natural menopause is defined as first reported age at menopause among those who did not have induced menopause, hysterectomy or bilateral oophorectomy. Due to potential effects of the disease or the treatment thereof[16] on menstruation, those who were

diagnosed with cancer were considered ineligible for natural menopause. Women who reported premenopausal HRT were treated similarly as these medications have been observed to interfere with menopausal timing[17] and induce menstruation[18]. When reported age at menopause was inconsistent across questionnaires, the first reported age was used. For questionnaires from individuals who had not previously reported an age at menopause that were missing menopausal age, yet contained indication that periods had ceased permanently, age at menopause was assigned at the midpoint between the return date of the corresponding questionnaire and that of the previous.

Ascertainment of covariates

Potential confounders that may be associated with both shift work status and menopausal timing were selected a priori. Age, smoking [19-21], parity [22], body size [23], oral contraceptive use [19], age at menarche [22], alcohol consumption[24,25] and physical activity[26] have previously been associated, to varying degrees, with timing of natural menopause. Breastfeeding has been speculated to impact this process due to effects of reduced circulating estrogens on follicular atresia[27]. While premenopausal daily sleep duration has not, as far as we know, been linked to timing of natural menopause, it is conceivable that sleep deprivation may negatively impact fertility and was included as a potential confounder due to the presumed increased frequency among rotating shift workers.

Statistical Analyses

Cox proportional hazards models were used to assess the effect of cumulative and current rotating shift work on the hazard of natural menopause. The time metameter was age in months and cohort entry for all women was their age at the 1993 biennial questionnaire return date. A counting process data structure[28] was used to efficiently handle time-dependent covariates by permitting the creation of a new observation for each questionnaire period. Delayed entry, such that women were permitted to enter the cohort at differing start times, defined by their age at baseline, was additionally accommodated by this data structure. The main exposure, rotating shift work, was updated at each subsequent questionnaire return. Covariate values were also updated at each questionnaire return, given inclusion of corresponding items on subsequent questionnaires.

Three time-dependent definitions of rotating shift work exposure were investigated. The first two were cumulative number of months worked rotating shifts, differentiated by inclusion/exclusion of rotating shift work exposure prior to 1989. The third, number of months worked rotating shifts during the interval between the two preceding questionnaire returns, was assessed to determine whether intensity of recent rotating shift work exposure was associated with risk of later natural menopause.

All independent variables were modeled as categorical variables. Tests for linear trend were performed by entering rotating shift work indicators as a single pseudo-continuous variable in the model and noting the two-tailed p-value for the computed Wald chi-square test statistic of the corresponding model coefficient. All effect estimates are presented as hazard ratios with 95 percent confidence intervals. Reported p-values are two-tailed. All statistical analyses were performed using SAS statistical software, version 9.2 (SAS Institute Inc., Cary, North Carolina USA).

Women were right-censored at date of breast cancer diagnosis and death, and additionally, at the previous biennial questionnaire return date for other cancer diagnoses. They were censored at the date of the previous biennial questionnaire return for all indications of induced menopause including hysterectomy or bilateral oophorectomy, and for HRT use. Due to the cohort era, a large proportion of women reported taking premenopausal hormone replacement therapy. Additional analyses exploring potential bias from informative censoring due to premenopausal HRT onset and induced menopause were performed, discussed in Appendix 1.

Violations of the proportional hazards assumption were assessed visually[29]. Schoenfeld residuals from regression models were plotted against age in months to assess meaningful change in slope over time. Additionally, the negative natural logarithm of the natural logarithm of the cumulative probability of achieving menopausal age was plotted against the natural logarithm of age in months to determine whether the modeled hazards corresponding to rotating shift work exposure levels remained proportional over follow-up.

Assessment of effect modification by smoking status, oral contraceptive use and body size was undertaken as an exploratory analysis. We created indicator variables representing dichotomous categorizations of interest of the potential effect modifiers and performed omnibus likelihood ratio tests to determine whether the product terms created from these variables and cumulative

rotating shift work indicator variables added significantly to our multivariable Cox models. A corresponding p-value of 0.10 for the likelihood ratio tests was used as a threshold indicative of potential multiplicative effect modification.

Results

As of the 1991 questionnaire return, 81,238 women met eligibility criteria (Figure 4.1). Of these, the number of women who reported working any years of rotating shifts prior to 1989 and any months of rotating shifts over follow-up (1989 onward) was 49,829 (61.3%) and 30,005 (36.9%), respectively. Of women who reported working rotating shifts prior to 1989, the mean cumulative duration worked until end of follow-up (2009 questionnaire return date), was 67.8 (standard deviation (SD): 62.7) months. Of those who did not report working rotating shifts prior to 1989 but did so after baseline, the mean cumulative duration worked over follow-up was 43.3 (± 48.0 SD) months. Of those eligible at baseline, at any questionnaire cycle women were more likely to work rotating shifts during the past two years if they reported having done so on the previous questionnaire (all eight χ^2 tests: $p < 0.0001$). Mean age differences between those who reported working any compared to no rotating shift work were not markedly different at each of the nine follow-up intervals, ranging from 0.91 years (95% CI: 0.82-1.01) in 2001 to 1.31 years (95% CI: 1.24-1.39) in 1991. Yet these differences were statistically significant (all nine t-tests: $p < 0.0001$), indicating that women working rotating shifts were, on average, slightly younger.

The distribution of covariates by ever versus never rotating shift workers between 1989 and 1991 questionnaire return dates is shown in Table 4.1. The proportion of current smokers, those who were overweight (i.e., BMI ≥ 30 kg/m^2), and those who were nulliparous was statistically significantly higher among rotating shift workers (χ^2 test p-values <0.0001) during this interval. Rotating shift workers were also statistically significantly more likely to experience less sleep (χ^2 test p-value <0.0001).

Of the 81,238 eligible women at baseline, 55,137 (67.9%) reported an age at menopause between 1993 and 2009 biennial questionnaire return dates. There were 29,742 women who were right-censored prior to end of follow-up, as many as half of which may have been due to onset of HRT medication use. Of the 51,496 of the baseline study sample who were not right-sored, 30,306

women had natural menopause, the event of interest. Mean age of natural menopause for these 30,306 women was 50 (SD ± 3.9 years). There were 21,190 women remaining eligible for natural menopause at the end of study follow-up (i.e., the 2009 questionnaire return date).

Figure 4.2 depicts the temporal distribution of women who achieved natural menopause (i.e., had the event) over the 1993 through 2009 follow-up period. Months of rotating shifts worked during the previous follow-up interval, for 1991 through 2007, are included for women eligible to achieve natural menopause by the end of the subsequent questionnaire return date. This staggered representation is reflective of the lag between exposure and outcome modeled in our Cox proportional hazards models. While the number of women reaching natural menopause increases substantially over follow-up until the 2005 questionnaire return date, as expected due to the age range of the cohort, the proportion of women working rotating shifts declines moderately as the cohort ages, supporting that rotating shift work participation is less prevalent among older nurses.

Tables 4.2 shows the relative effects of rotating shift work exposure on the hazard of natural menopause over follow-up. Working 25 to 96 months of cumulative rotating shift work, including years accumulated prior to 1989, conveyed a slightly higher risk compared to never having worked rotating shifts, though none of the corresponding multivariable-adjusted effect estimates were statistically significant (Table 4.2; left). Conversely, women who worked more than 240 months had a moderately lower risk of natural menopause, though this effect was not conserved in the multivariable-adjusted model. More than 300 months of exposure was suggestive of a stronger protective effect (age-adjusted HR: 0.70, 95% CI: 0.56-0.87; multivariable-adjusted HR: 0.81, 95% CI: 0.64-1.02), though the multivariable-adjusted hazard ratio was only of borderline statistical significance as it was based on only 63 events.

When years of shiftwork prior to 1989 were omitted from the cumulative exposure tally (Table 4.2; right) there was a moderately increased risk of natural menopause across women who had worked 48 months compared to their non-rotating shift working counterparts. For women working more than 120 months of rotating shift work since their 1989 questionnaire, there was a 38 percent (HR: 0.62, 95% CI: 0.55-0.69) reduced risk of menopause compared to women who had not reported any rotating shift work exposure, reduced to 24% (HR: 0.76, 95% CI: 0.69-0.85) in the multivariable-adjusted model. While there was a statistically significant decreasing

linear trend in risk of natural menopause with increasing cumulative rotating shift work in the age-adjusted model ($p < 0.001$), this was not conserved across multivariable-adjusted effects.

The effects of recent rotating shift work exposure, experienced in the prior questionnaire cycle, on the hazard of natural menopause, are presented in Table 4.3. Both age and multivariable-adjusted models indicate an increasing risk of natural menopause with increasing rotating shift work exposure. For the age-adjusted model, all effect estimates fall below unity. Relative to non-rotating shift workers, only those who worked less than 20 months exhibited a statistically significant lower risk of menopause. Conversely, all of the multivariable-adjusted estimates are unity or greater, with women who worked 20 or more months within the prior questionnaire interval at a statistically significant 8% (HR: 1.08, 95% CI: 1.02-1.15) increased risk of natural menopause. We observed a statistically significant ($p=0.02$) increasing linear trend across increasing exposure levels for the multivariable-adjusted hazard ratios.

There was no indication of multiplicative effect modification of cumulative rotating shift work and hazard of natural menopause by BMI ($<$ versus ≥ 30 kg/m^2), smoking status (current versus past or never smokers) or oral contraceptive use (ever versus never users). Omnibus p-values from likelihood ratio tests evaluating the addition of modeled interactions between current smoking and cumulative rotating shift work, including and excluding exposure prior to 1989, were 0.20 and 0.16, respectively. Those for modeled interactions with BMI or oral contraceptive use were ≥ 0.48.

Discussion

This work denotes the first prospective investigation of the impact of shift work on menopausal timing. It was hypothesized that women working more rotating shifts over their reproductive lifetime would experience later menopausal onset, primarily due to chronic disruption of endogenous reproductive signaling, serving to delay perimenopausal onset and progression. Our results indicate that working low to moderate amounts of rotating shift work is not likely to materially affect menopausal onset. At most, it may predispose toward a slightly increased risk of earlier menopause, particularly if substantial exposure is experienced during perimenopause. Conversely, women who consistently work higher levels of rotating shifts over their reproductive

lifetime, may be significantly more likely to have later menopause, with potential implications of increased postmenopausal breast cancer risk. However, caution is warranted in the interpretation of this protective effect due to temporal alignment of competing causes of failure, menopause and cumulative rotating shift work exposure in this cohort.

While we did observe a statistically significant reduced risk of natural menopause for those who had more than 10 years of rotating shift work exposure since 1989, and suggestion of a similar effect among the small proportion of women who accumulated more than 25 years including exposure prior to 1989, there was little evidence of dose response. Given that our biological model is valid, this could be due to a threshold effect: perhaps, on average, women need to regularly work a minimum amount of rotating shifts over the long term in order to experience sufficient circadian disruption to meaningfully impact gonadal activity and ultimately, menopausal timing. In both NHS I and II cohorts, an association between rotating shift work and breast cancer risk was observed only among women who had worked 30[12] and 20[13] years or more of cumulative exposure, respectively. The finding in the NHS II was based on cumulative rotating shift work exposure including number of years worked prior to 1989. It should be declared, however, that at least some of the estimated protective effect of cumulative rotating shiftwork on menopausal onset may be artificial.

In the analysis of right censored data, censoring patterns that induce dependency between the probability of the event and exposure can lead to distorted cause-specific hazard ratios across the affected exposure levels in question. The competing event most concerning in the assessment of relative effects of rotating shift work on the hazard of natural menopause in our study sample is onset of HRT medication use. In summary, it is possible that women comprising the highest cumulative exposure level may have been less likely to have natural menopause by virtue of a portion of those more likely to have had this outcome being previously right censored, predominantly due to HRT onset, while occupying a lower cumulative exposure level.

Additional analyses performed to assess the impact of this potential bias (presented, for this thesis, in Appendix 1). Briefly, cause-specific hazard ratios for the effect of cumulative rotating shift work on HRT onset indicated a pronounced protective effect, potentially indicative of such a dependency. However much of this may have been driven by a decline in HRT onset due to changes in prescribing patterns juxtaposed with the highest cumulative rotating shift work

exposure only being achievable toward the end of the study period. Additionally, a pair of sensitivity analyses comprising proportional hazards models wherein women who would have been censored due to HRT were instead assigned the event time for natural menopause 1) at the censoring time or 2) either at their reported age of onset or at follow-up end did not reveal materially different effects, suggesting that this bias may have been of minimal impact. However, it is difficult to accurately assess biases arising from competing causes of failure due to the challenge of predicting probability densities of the event of interest, and future exposure statuses in the case of time-dependent covariates, among those censored had they not met the criteria.

The moderate, yet statistically significant increased likelihood of menopause observed among women working 25 to 48 months of rotating shifts since 1989 from the multivariable adjusted model is more difficult to explain Depression and stress are potential confounders for which we were unable to account. One explanation for an increased risk of earlier menopause among lower cumulative rotating shift work exposures may stem from experiencing increased depression or stress. There is indication of variation in ability to cope with night or rotating schedules and that some nurses may self-select night shift-heavy work schedules[30]. Those who reported less cumulative exposure may have had a mildly elevated risk of earlier menopause due to an increased propensity for rotating shift schedule-derived stress and depression, and the resulting disruptive impact on gonadal function, relative to the majority of women selecting more rotating shift work-intensive schedules.

We hypothesized that circadian disruption may delay menopause through chronic stimulation of the HPGA due to suppression and altered timing of nocturnal melatonin secretion. Though sites of action of melatonin on the HPGA have yet to be conclusively identified, the most likely ultimate targets are the GnRH-secreting neurons of the hypothalamus and/or gonadotrophs of the anterior pituitary. Limited support for the existence of melatonin targets within these central tissues comes from the discovery of melatonin-1 receptors in various regions of the human hypothalamus and pars distalis of the anterior pituitary[31,32]. Observations that depressed women experience hypothalamic amenorrhea[33], and that depression may impact the GnRH pulse signal via endogenous opioid signaling[34,35] are supportive of an opposing central regulatory effects on reproductive function. Self-reported depression has additionally been associated with early menopause, or more specifically, an increased likelihood of perimenopause[36].

It may be that working nights or rotating shifts can be chronically stress-inducing, making it difficult to separate independent effects of circadian disruption and the stress response on menopausal onset, particularly when using shift work as a surrogate for circadian disruption exposure. Support for this can be found in studies that have observed alterations in cortisol profiles in night workers[37] or truncations of the cortisol quiescent period in experimentally circadian phase-advanced volunteers[38,39]. Animal models have indicated regulation of pituitary gonadotropin secretion in response to cortisol infusion[40]. Furthermore, a body of literature exists which is suggestive of extensive crosstalk between the hypothalamic-adrenal axis, of which cortisol is the major end product, and the HPGA[41]. In their investigation of factors affecting menopausal timing, Cassou *et al* observed that both currently working "high-strain jobs" and working prior jobs involving "difficult schedules" were associated with earlier menopause[42]. Given evidence that nurses are commonly called upon to work long shifts, it is plausible that a substantial proportion of participants in the NHS II may have met these criteria, though these were not clearly identifiable given the available data. In the above study, researchers also assessed the effect of self-reported shift work on menopausal timing and found no association. However, as there was no indication that the exposure queried pertained to night work or rotating night work, the relevance of these findings is uncertain. If working rotating shifts was associated with working stressful schedules or depression, any chronic increase in HPGA activity resulting from disruption of melatonin signaling could have been offset. This may have attenuated results, explaining observed cumulative rotating shift work effects that were null or weakly indicative of an association in the direction opposite to that hypothesized for women who worked 25 to 48 months of rotating shift schedules since 1989 (Table 4.2). However, the close proximity of effect estimates and overlapping confidence intervals with those of adjacent exposure levels dictates interpretation with caution.

Discrepancy across cumulative rotating shift work effects including and excluding exposure prior to 1989 (Table 4.2) is perhaps indicative that rotating shift work before 1989 (captured as number of years) and thereafter (captured as number of months worked in the past two years) are not equivalent. Contributing factors are relatively greater propensities for recall error and imprecision in the former metric, potentially attenuating our ability to detect an effect of cumulative exposure including years prior to 1989. That effects observed for the highest exposure levels were suggested to be stronger for the definition where exposure prior to 1989

was omitted, may, at least in part, be in testament to this. Conversely, treating women who reported exposure prior to 1989 as unexposed at baseline could itself be construed as contributing to misclassification of lifelong cumulative rotating shift work exposure. The evaluation of both cumulative exposure definitions, however, adds transparency to the true nature of this association.

The null effects from the multivariable-adjusted model for women working less than 20 months of rotating shift work during the prior questionnaire interval (Table 4.3) could indicate that once into perimenopause, the biological countdown to menopause has already been triggered. Therefore any recent circadian disruption potentially leading to chronically elevated gonadal activity may have little bearing on time to final cessation of menstrual cycling. The increased risk of menopause for women working more than 20 months of rotating shifts from the same model should be interpreted with caution due to substantial overlap of confidence intervals with that of the preceding exposure level. If the effect is true, it could be due to the increased stress of working continual rotating shift work schedules during perimenopausal progression.

Most of the direct evidence supporting a disruptive potential of night work on circadian rhythms has been limited to smaller quasi-experimental or observational studies demonstrating phase shifting[43-45] and/or suppression[46] of nocturnal melatonin among those working such schedules. While these observations support night work as a reasonably valid surrogate for circadian disruption exposure, lack of specificity in the NHS II definition may have further contributed the null or modest effect sizes across the majority of exposure levels. The items used to query rotating shift work on each biennial questionnaire may have inadequately captured meaningful variance among these schedules, limiting our ability to observe a resulting association with age at menopause. Though many nurses who reported working rotating shift schedules, defined as at least three nights in addition to days and evenings in a single month, likely worked significantly more than the three nights per month minimum, the inter-individual variation of this quantity is unknown. As such, it is possible for women with similar cumulative rotating shift work tallies to have experienced substantially different patterns of night work, and by extension, circadian disruptive stimuli.

A greater biological impact of night work with increased frequency of switching between days and nights underlies the rationale for a rotating, as opposed to exclusive, definition of night work

as a surrogate for circadian disruption[15]. Though the rate at which the circadian clock adapts to external cues has been observed to be highly variable, with those more resistant to this entrainment exhibiting greater difficulty functioning during night shifts[47], there is a characteristic lag observed when undergoing change in photoperiod[48]. Workers who have sufficient time and motivation to adapt to night work have demonstrated gradual phase shifts in melatonin peak secretory patterns and better overall acclimatization[49]. It stands to reason that those who fluctuate more rapidly between night and day shifts will be at greater risk of perpetual misalignment of their biological clock and external photic cues. Women who work night shifts more frequently, but do not live exclusive night schedules, may therefore be at greater risk of later menopausal onset. However, our rotating shift work exposure definition was unable to distinguish between women who worked less than one night shift per week, on average, and those who may have worked almost exclusively nights.

Self-selection of night work may have contributed, at least in part, to effect sizes of near unity observed for women comprising the lower cumulative exposure levels (Table 4.2). Nurses with lower cumulative rotating shift work exposure may have worked fewer nights and rotated between day and night schedules less frequently in a given period than their higher cumulatively exposed counterparts. Consequently, those who reported working more than 120 months of rotating shifts since 1989 may have may have experienced more pronounced and consistent circadian misalignment than nurses who contributed to the lower cumulative exposure categories.

Effect sizes may have been further attenuated due to our inability to account for effect modification by morning versus evening preference. Findings in humans have indicated that circadian rhythms of those with morning preference (i.e., feel more alert at the beginning, relative to the end, of the natural photoperiod) take more days to adapt to shifts in experienced photoperiod than do those with an evening preference[39,43,50] suggests that the same schedule can have varying biological effects. Unfortunately, we were unable to incorporate morning preference or any other factor quantifying innate susceptibility to circadian disruptive stimuli in our analyses.

Finally, exposure misclassification may have arisen as a result of the growing number of nurses who are called to work 12 or even 16 hour shifts in order to mitigate registered nurse shortages.

Of nurses surveyed in 2002, almost 40% reported working shifts longer than 12.5 hours while 14% reported working shifts 16 hours or longer at least once during month-long observation[51]. Working such lengthy shifts on a regular basis, even if not encompassing what respondents would have considered to be strictly "nights" may have caused sufficient circadian disruption, perhaps resulting in a significant number of women who experienced some degree of circadian disruption being classified as unexposed.

First-reported age at menopause in the NHS I has been validated as being reliable, at least insofar as remaining fairly consistent on the subsequent biennial questionnaire for most women[52]. In the NHS I, 82 percent of the 4,265 women achieving natural menopause (defined therein as menopause not induced by surgery or radiation) between 1976 and 1978 reported ages at menopause that were within one year of each other on 1978 and 1980 questionnaires. The method of capturing age at menopause at multiple biennial follow-ups was conserved in the NHS II. While our definition of natural menopause is more conservative, we observed a similar proportion of women (83.3 percent) who attained this outcome between 1997 and 1999 and reported ages at menopause that were within one year on 1999 and 2001 questionnaires (Figure 4.3). Though the women included in our comparison at 1999 and 2001 comprise only about six percent of all women who achieved natural menopause over follow-up, we have no reason to believe that these women are not representative of their peers, given the similarity in agreement at two years in our study compared to that performed in the NHS I. The decline in agreement with first-reported age on subsequent questionnaires is presumably due to increased recall error, in line with findings from another study of the reliability of self-reported menopausal age[53]. This further supports the use of first-reported age to define the timing of natural menopause.

Despite indications that self-reported menopausal age in the NHS II is of relatively high validity, some misclassification may have arisen due to the defining criteria. Unlike the widely accepted World Health Organization definition of menopause which requires periods to have ceased for 12 months[54], the NHS II definition is limited to querying whether a woman's periods had ceased at the time the biennial questionnaire was completed. Of women who reported ages at menopause that did not agree on subsequent questionnaires, more reported having later menopause than earlier (data not shown), which suggests that at least some of this disagreement may have stemmed from premature reporting of menopausal onset. The result of such misclassification, if significant, would be expected to have biased our results towards the null, since there is little

reason to suspect that premature reporting of menopause would be associated with rotating shift work exposure.

In addition to the novelty of this research, a key strength is the study power afforded by the NHS II, undoubtedly the largest cohort of female shift-workers of its kind. Furthermore, the NHS II remains one of the only sizable cohorts in existence that collects longitudinal data on night work-related exposures and potential confounding factors. In addition, the quality of the self-reported data has been previously validated for many of the items captured on the ongoing biennial questionnaires, such as self-reported menopausal status[52].

Conclusion

Recommendations for future studies assessing the effect of night shift work on reproductive outcomes or chronic disease risk include the collection of more detailed exposure information. It may be that rotating shift intensity, further qualifying accumulated exposure, may be a stronger predictor of adverse reproduction-related outcomes such as delayed menopause or breast cancer. However, we did not have the requisite work schedule data to thoroughly test this hypothesis. Collection of data on additional potentially confounding factors such as job stress and depression is warranted so that the independent effects of rotating shift work can be more effectively assessed. Finally, assessment of morning-evening preference in future related investigations that focus on determinants of chronic disease among populations that typically work nights, particularly among larger cohorts such as the NHS II, may be beneficial.

Our findings indicate that women accumulating the highest levels of rotating shift work may be at risk of delayed menopause, in line with those from the NHS I and II demonstrating increased risk of breast cancer among women working more than 30 and 20 years of this exposure. This suggests elevated exposure to endogenous reproductive signaling over the reproductive lifetime as an important mechanism through which circadian disruption impacts risk of reproduction-linked cancers. However, due to potential bias resulting from the high incidence of HRT medication use over the first half of follow-up, validation of this finding will be pending replication among future populations wherein prescribing of these medications prior to menopause is reduced.

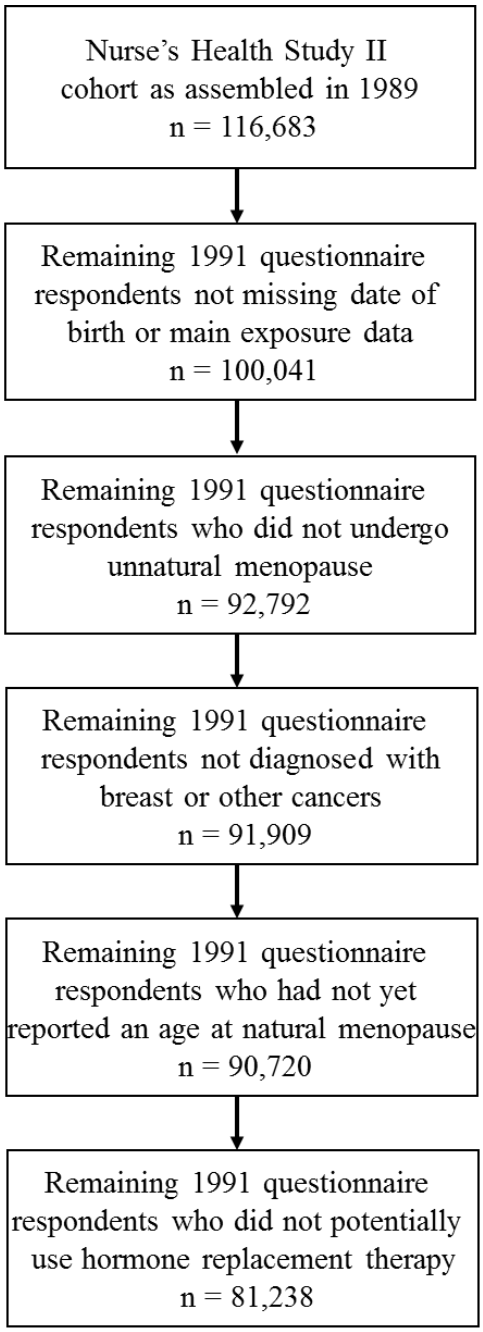

Figure 4.1. Summary of exclusions resulting in baseline study sample (81,238 women meeting eligibility criteria as of 1991 questionnaire return date).

Table 4.1. Distribution of covariates by rotating shift work within two years prior to 1991

	Never shift workers (n=62,508)	Ever shift workers (n=18,730)
Baseline Age (yrs ± SD)	36.5(4.5)†	35.2(4.7)
Alcohol (g/wk ± SD)	3.2(6.1)	3.0(5.6)
Smoking Status (%)		
never smoker	66.8	64.7
past smoker	22.2	20.6
current smoker	10.9	14.6
BMI (%)		
≤20 (kg/m^2)	14.0	11.6
21 to 25 (kg/m^2)	55.4	50.0
26 to 30 (kg/m^2)	19.8	22.1
> 30 (kg/m^2)	11.7	16.3
Lifelong Oral Contraceptive Use (%)		
1-23 mos	17.1	16.0
24-47 mos	41.6	41.6
48-95 mos	24.6	24.9
>96 mos	16.7	17.5
Parity (%)		
nulliparous	25.6	32.4
1 child	18.4	17.2
2 children	35.3	30.9
3 or more children	20.7	19.5
Age at First Birth(%)†		
≤20 yrs	7.7	13.0
21-25 yrs	37.2	41.4
26-39 yrs	41.1	36.5
≥30 yrs	14.1	9.2
Total time breast fed (%)†		
Never breast fed	18.8	19.1
Breast fed ≤ 1 yr	36.7	38.7
Breast fed > 1 yr	44.5	42.1
Physical Activity (%)		
<3 met/wk	15.7	14.5
3-9 met/wk	27.2	26.9
10-19 met/wk	22.6	22.1
20-30 met/wk	13.1	12.7
>30 met/wk	21.4	24.0
Sleep (%)		
≤ 5 hrs/night	4.8	7.8
6 hrs night	22.2	27.1
7 hrs night	43.2	39.9
8 hrs night	24.5	20.4
≥9 hrs night	5.3	4.7

	Never shift workers (n=62,508)	Ever shift workers (n=18,730)
Age at menarche (%)		
≤11 yrs old	23.7	24.5
12 yrs old	30.5	29.9
13 yrs old	28.1	27.2
≥ 14 yrs old	17.7	18.3

Values are means (SD) or percentages

[†]Parous women only

All covariate differences across binary baseline rotating shift work exposure were statistically significant with p-value < 0.0001, except where indicated where p<0.01

Menarche and occ use p<0.01

Everything else p<0.0001

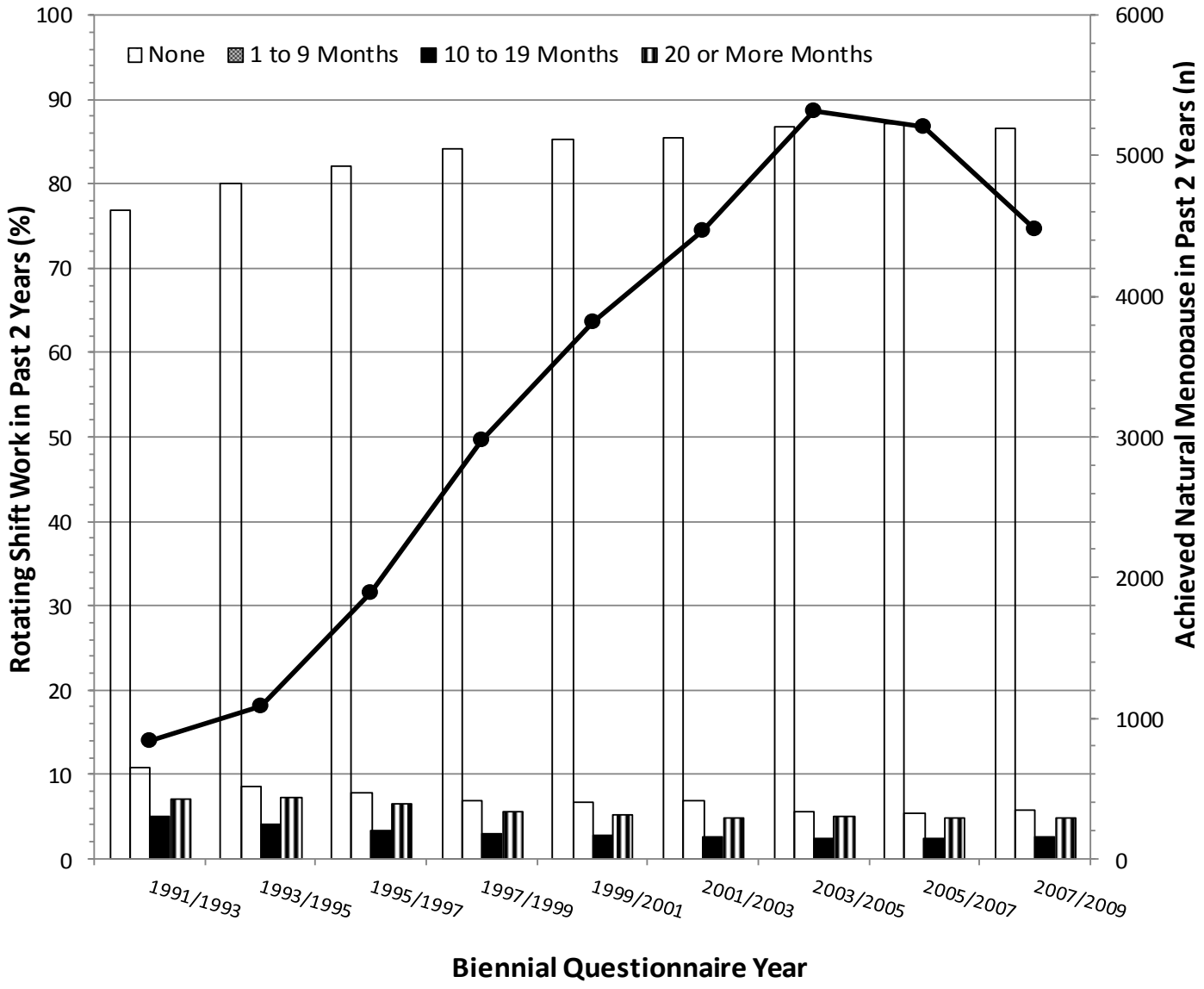

Figure 4.2. Natural menopause and rotating shift work over follow-up. Proportion of women working no, 1 to 9, 10 to 19, and 20 or more months of rotating shifts in past two years from 1991 to 2007. Number of women who achieved natural menopause in past two years from 1993 to 2009. Natural menopause is defined as first reported age at menopause among those who did not have induced menopause, hysterectomy, bilateral oophorectomy, those remaining cancer free and those who did not report premenopausal hormone replacement therapy.

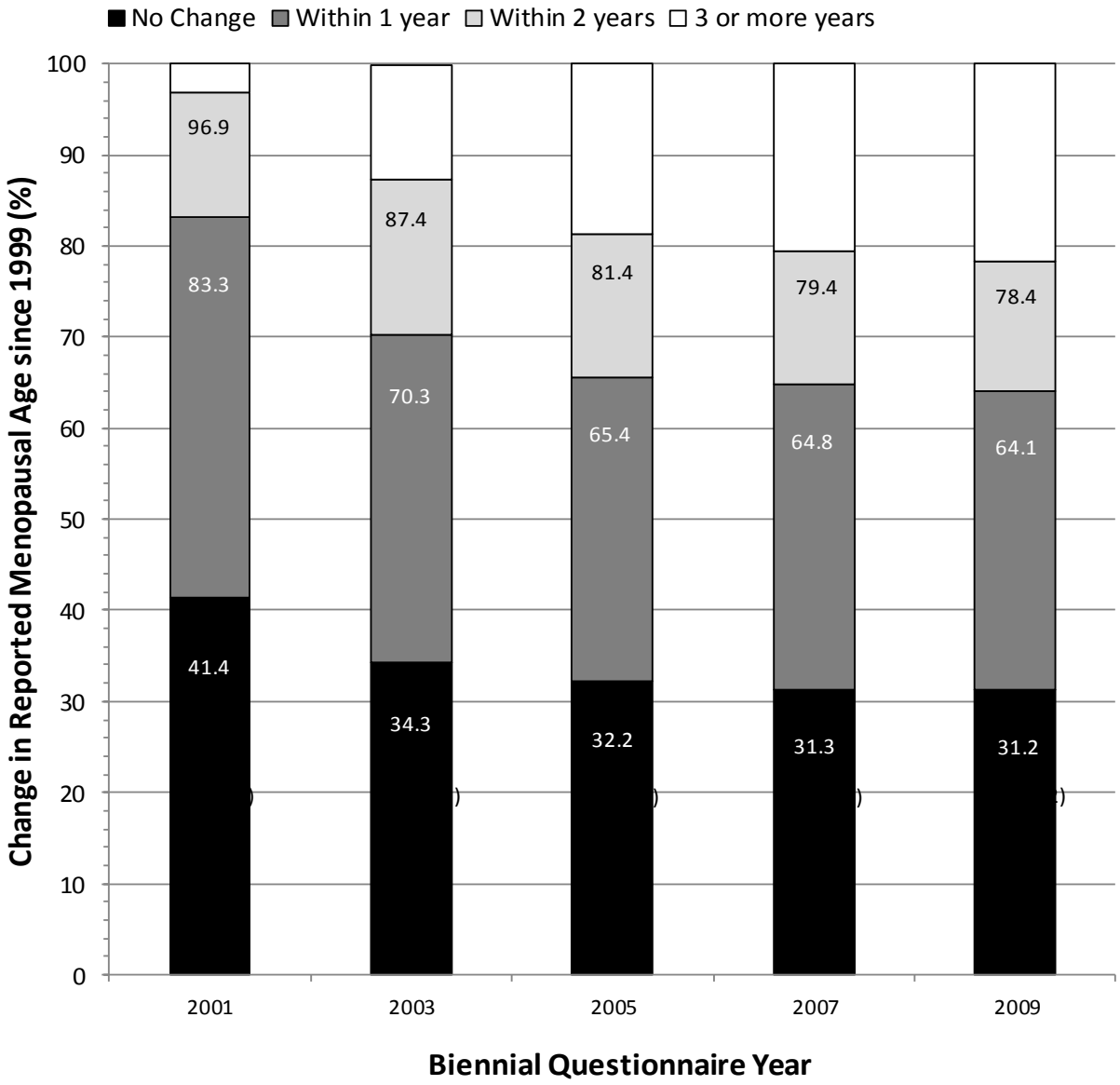

Figure 4.3. Cumulative percent change in reported age at menopause on the 1999 biennial questionnare at 2, 4, 6, 8 and 10 years among women who achieved natural menopause between 1997 and 1999 biennial questionnaire return dates. Natural menopause is defined as first reported age at menopause among those who did not have induced menopause, hysterectomy, bilateral oophorectomy, those remaining cancer free and those who did not report premenopausal hormone replacement therapy. Women with missing age at menopause on the 1999 and subsequent questionniares were excluded.

Table 4.2. Hazard ratios (HR) and 95% CIs (age and multivariable-adjusted) of time to natural menopause across cumulative rotating shift work exposure among 81,238 women of the Nurses' Health Study II, with prospective follow-up from 1993 through 2009.

	Including years of rotating shift work prior to 1989		Excluding years of rotating shift work prior to 1989	
Cumulative Rotating Shifts	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)
0 Months[¥]	1.0	1.0	1.0	1.0
1 to 24 Months	1.01 (0.98-1.08)	1.00 (0.98-1.07)	1.06 (1.02-1.09)	1.01 (0.98-1.04)
25 to 48 Months	1.02 (0.99-1.08)	1.02 (0.97-1.07)	1.13 (1.07-1.18)	1.06 (1.01-1.12)
49 to 72 Months	1.03 (0.98-1.16)	1.02 (0.98-1.13)	0.99 (0.93-1.06)	1.00 (0.93-1.07)
73 to 96 Months	1.05 (1.00-1.16)	1.04 (0.98-1.12)	1.04 (0.96-1.14)	1.04 (0.96-1.14)
97 to 120 Months	0.98 (0.91-1.05)	0.99 (0.92-1.06)	0.92 (0.85-1.01)	1.03 (0.94-1.12)
> 120 Months	N/A	N/A	0.62 (0.55-0.69)	0.76 (0.69-0.85)
121 to 240 Months	1.00 (0.96-1.05)	1.03 (0.99-1.08)	N/A	N/A
> 240 Months	0.90 (0.81-1.00)	0.99 (0.89-1.10)	N/A	N/A
P for trend[€]	1.00	0.18	<0.001	0.15

*Hazard ratios adjusted for age, smoking status (never, past or current smoker), age at first birth and parity combined (nulliparous; age at first birth <24, 1–2 children; age at first birth 24 to 29, 1 to 2 children; age at first birth >29, 1 to 2 children; age at first birth <23, >2 children; age at first birth 24 to 29, >3 children; age at first birth >29, >2 children), body mass index (<18.5, 18.5 to 20, >20 to 22.5, >22.5 to 25, >25 to 30, and >30 kg/m2), cumulative oral contraceptive use (0, 1 to 23, 24 to 47, 48 to 71, 72 to 95, 96 to 119 and >120 months), total time breast fed (never, <=1 yr, >1 yr), alcohol consumption (0, >0 to 1, >1 to 4, >4 to 8, >8 to 12, >12 g/wk), physical activity (<=3, >3 to 9,> 9 to 19, >19 to 27, >27 to 42, >42 METS/wk), age at menarche (<=9, 10, 11, 12, 13, 14, 15, >=16 yrs) and sleep in 24 hrs (<=4, 5, 6, 7, 8, >=9 hrs)

[¥]Reference category for all analyses

[€]P value for continuous linear term

Table 4.3. Hazard ratios (HR) and 95% CI's (age and multivariable-adjusted) of time to natural menopause across current, updated, rotating shift work exposure accumulated during the past two years among 81,238 women of the Nurses' Health Study II, with prospective follow-up from 1993 through 2009.

Updated Rotating shifts	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)
0 Months[¥]	1.0	1.0
1 to 9 Months	0.93 (0.88-0.98)	1.00 (0.95-1.05)
10 to 19 Months	0.92 (0.85-0.99)	1.02 (0.94-1.10)
≥ 20 Months	0.99 (0.94-1.04)	1.08 (1.02-1.15)
P for trend[€]	0.06	0.02

* Hazard ratios adjusted for age, smoking status (never, past or current smoker), age at first birth and parity combined (nulliparous; age at first birth <24, 1–2 children; age at first birth 24 to 29, 1 to 2 children; age at first birth >29, 1 to 2 children; age at first birth <23, >2 children; age at first birth 24 to 29, >3 children; age at first birth >29, >2 children), body mass index (<18.5, 18.5 to 20, >20 to 22.5, >22.5 to 25, >25 to 30, and >30 kg/m2), cumulative oral contraceptive use (0, 1 to 23, 24 to 47, 48 to 71, 72 to 95, 96 to 119 and >120 months), total time breast fed (never, <=1 yr, >1 yr), alcohol consumption (0, >0 to 1, >1 to 4, >4 to 8, >8 to 12, >12 g/wk), physical activity (<=3, >3 to 9,> 9 to 19, >19 to 27, >27 to 42, >42 METS/wk), age at menarche (<=9, 10, 11, 12, 13, 14, 15, >=16 yrs) and sleep in 24 hrs (<=4, 5, 6, 7, 8, >=9 hrs)

[¥]Reference category for all analyses

[€]P value for continuous linear term

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Chapter 5 Manuscript 2

Cross-sectional associations between nocturnal melatonin, circulating ovarian steroid hormones and menstrual cycle length

This manuscript assesses the cross-sectional association between nocturnal melatonin production and both subsequent daytime circulating estrogen and progesterone and current menstrual cycle length. The study sample is a population of young women who were not currently on oral contraceptives, recruited from hospitals and universities in Toronto.

Abstract

Background: Higher observed rates of cancers, particularly breast cancer, among night shift workers has spurred recent interest in circadian signaling and its potential impact on reproduction in humans. The melatonin hypothesis, that increased exposure to artificial light at night and resulting inhibition of nocturnal melatonin secretion may account, at least in part, for the increased breast cancer incidence in developed countries over the last century, has set much of this focus on this endocrine hormone. To assess the effect of nocturnal melatonin on reproductive signaling we examined associations between nocturnal melatonin production and circulating steroid reproductive hormones (estradiol and progesterone). The association between nocturnal melatonin production and menstrual cycle length was additionally investigated as a potentially more stable marker of the impact of circadian disruption on gonadal activity.

Methods: The study sample consisted of 137 women between the ages of 18 and 22 from the Toronto area. Cross-sectional associations between overnight urinary 6-sulfatoxymelatonin (aMT6s), a marker for overnight melatonin production, and circulating estradiol and progesterone concentrations measured via blood draw during the luteal phase were assessed using ordinary least squares regression. The association between aMT6s and menstrual cycle length was additionally investigated using these methods.

Results: We did not find evidence of a relationship between creatinine-standardized overnight urinary aMT6s and circulating luteal estradiol and progesterone. The association between this metabolite and menstrual cycle length was additionally null.

Conclusion: Our findings add to those of three recent studies that did not observe a relationship between aMT6s and circulating daytime serum estradiol or progesterone. Additionally, they do not support menstrual cycle length as a possible marker of variation in gonadal function attributable to circadian signaling mediated via endogenous nocturnal melatonin. Future investigation may benefit from longitudinal designs that assess variations in menstrual cycle patterns and reproductive hormones over multiple menstrual cycles, taking into account temporal variation in melatonin signaling.

Introduction

Recent interest in circadian regulation of reproductive function in humans has been stimulated by the hypothesis that higher rates of breast cancer in developed countries are at least partly attributable to exposure to light at night[1]. This has been supported by studies that have observed associations between night shift work, a commonly studied surrogate for circadian disruption exposure, and negative reproductive outcomes and chronic disease, the most prominent of which has been breast cancer[2,3]. Following this literature, in 2007, the International Agency for Research on Cancer identified shift work as a "probable" carcinogen[4].

Despite these findings, the biological mechanism linking shift work and breast cancer has remained uncertain. The light at night hypothesis focuses on suppression of nocturnal melatonin production[5], demonstrated in experimental settings in response to light stimuli[6], as the culprit. Light stimulates non-visual receptors in the retina that relay signals to the suprachiasmatic nucleus, which in turn, through innervation of the pineal gland, regulates central melatonin production[7,8]. Normal production is characterized by a peak in pineal secretion toward the middle of the nightly sleep cycle. Under exposure to light at night the nocturnal acrophase is diminished, and potentially advanced or delayed, depending upon the timing and frequency of exposure[9]. Animal models have demonstrated mechanisms through which interference in the endogenous melatonin signal may influence risk of reproductive-linked cancers via reproductive signaling[10]. Experimental findings in humans have been suggestive of a regulatory effect of melatonin on reproduction, impacting ovulation and circulating steroid hormones such estradiol and progesterone[11-13].

While individual reports of correlations between circulating melatonin and reproductive hormones are encouraging, the consistency of these findings in humans have been lacking. Small sample sizes, varying melatonin and reproductive hormone measurement methods and incomplete, or often no consideration of confounding, have likely contributed to this apparent inconsistency[14]. Observational studies that have assessed associations between circulating melatonin levels and reproductive hormones have been largely null[15-17], though Schernhammer *et al* did report a significant crude negative correlation between melatonin and bioavailable estradiol[15]. A possible explanation for the overall paucity of association is the challenge of reliably measuring circulating reproductive hormones in cycling women, suggesting that a more

stable marker of the impact of circadian disruption on reproductive function may be of value. Observational studies indicating variation in menstrual cycle patterns by shift work exposure[18-20] are suggestive that the circadian disruption experienced by these individuals may have an impact on ovulation and its underlying hormonal regulation, yet, to our knowledge, the association between nocturnal melatonin and menstrual cycle length has yet to be investigated.

The impact of circadian disruption on the hypothalamic-pituitary-gonadal-axis (HPGA) and downstream factors impacting circulating steroid hormone metabolism have been previously assessed, though within various disciplines and with varying methodology. While some reports suggest that the melatonin signal has a suppressive effect on the HPGA in humans (reviewed in Aleandri *et al*[21]), and therefore on downstream steroid hormones implicated in reproductive related diseases such as breast cancer[10], the evidence overall has been inconclusive. To test the hypothesis that variation in nocturnal melatonin production may be associated with reproductive factors linked with female breast cancer risk, we assessed the cross-sectional association between melatonin and the outcomes of menstrual cycle length, as well as circulating serum estradiol and progesterone levels in a sample of healthy, cycling young women.

Methods

Study Sample

The study sample consisted of 137 young women between the ages of 18 and 22 living in the Toronto area, recruited for previous research[22]. Women were recruited over three years in approximately equal proportions in summer (June to September) and winter (December to March) from local hospitals, community colleges and universities. Eligibility criteria for enrollment included not currently using oral contraceptives, never having been pregnant, no previous cancer diagnoses, not currently having highly irregular menstrual cycles and no night shift work or transmeridian travel in the previous month.

At initial contact, women were asked to report date of last menses and were scheduled for a clinic visit on the 21st day of their menstrual cycle, aimed to coincide with the luteal phase. Women were asked to fill out a short questionnaire capturing current medication use, cigarettes smoked, alcohol consumption, bed time, wake time and exercise type, duration and intensity during the preceding 24 hours. Self-reported ethnicity was queried, height and weight measured,

and overnight urine collected from 8pm through first morning void using a vessel supplied by the research team. Consenting participants were asked to return the following morning to turn in their urine sample and for blood draw from which circulating reproductive hormone concentrations were assessed.

Laboratory Assay

The volume of each overnight urine collection was measured. Two one-ml aliquots were stored at -20°C for assay. Urine creatinine was determined using the automated Roche Cobas Integra 700 analyzer (F. Hoffmann- La Roche, Ltd., Basel, Switzerland) using an enzymatic creatinase-based method. Six-sulfatoxymelatonin (aMT6s) was assayed using a single-epitope competitive enzyme-linked immunoassay kit from IBL International GmbH (Hamburg, Germany; catalog number RE54031). All aMT6s assays were robotized and completed over a 2-day period using the same lot of test kit to minimize sampling error. Urine specimens containing an aMT6s concentration in excess of the second highest standard (140 μg/L) were diluted further and assayed again. Estradiol and progesterone were measured using the Roche Diagnostics electrochemoluminescence immunoassay with intra-assay coefficient of variation (cv) of less than 5 percent and inter-assay cv ranging from 6 to 11 and 4 to 10 percent, respectively over the study term.

Main Exposure, Outcomes and Potential Confounders

Overnight urinary creatinine standardized aMT6s was the predictor of interest in all analyses, and was used as a marker for overnight melatonin production. Urinary aMT6s, the major melatonin metabolite excreted in urine, has been validated as an accurate marker of circulating melatonin. Nocturnal urinary aMT6s has been observed to accurately capture nocturnal melatonin production and has correlated well with plasma melatonin measures (reviewed in Mirick and Davis[23]). Urinary aMT6s is commonly standardized with urinary creatinine to minimize measurement error introduced by intra- and inter-individual variation in renal clearance[24].

Current menstrual cycle length was ascertained from subtracting self-reported most recent date of menses queried at first interview from subsequent reported date of menses attained at follow-up.

Several potential confounding factors were considered based on prior associations with circulating hormones. These included physical activity, age, alcohol consumption, smoking status and ethnicity. Women who exercise frequently tend to have higher circulating levels of melatonin[25] and vigorous physical activity has been associated with longer menstrual cycles[26] and lower levels of circulating reproductive hormones[27,28]. Intensity of physical activity was assessed using the Rating of Perceived Exertion (RPE) scale developed by Borg[29]. The 15-point scale, ranging from six to 20, is used to self-rate intensity of exertion and approximates heart beats per minute (60 to 200). American College of Sports Medicine target heart rate guidelines for physical activity were used to inform cutpoints used to categorize exercise intensity[30]. Menstrual cycle or follicular phase length has been reported to vary by age, alcohol consumption, smoking status and ethnicity[31]. Menstrual cycle length has also been shown to vary with adiposity[32], prompting inclusion of body mass index (BMI) as a potential confounder. Variations in circulating estradiol and progesterone have also been observed to be associated with these factors.

Ethnic background was categorized based on five broad clusters conforming to genetic population structure observed by Rosenberg *et al*[33,34] from a global sample of 52 populations. Sleep duration and morning wake time were considered in these analyses in attempt to reduce confounding attributed to differences in circadian rhythm, observed to affect both timing of peak melatonin production, potentially impacting overall urinary excretion, and variation of reproductive hormone levels throughout the day. Luteal day, the number of days the blood sample was acquired prior to subsequent reported menses, was additionally adjusted for in estradiol and progesterone outcome analyses in attempt to account for variation in luteal phase progression and corresponding ovarian output at blood draw.

Analytical Methods

Women who were missing creatinine-standardized aMT6s and the outcome variable of interest (menstrual cycle length serum estradiol or progesterone) were excluded from the relevant analyses. Women were also excluded from all analyses if missing data on potential confounders. Pearson correlations between natural logarithm-transformed creatinine-standardized aMT6s and serum levels of estrogen, progesterone, and unadjusted and multivariable ordinary least squares

(OLS) regression were used to assess the association between creatinine-standardized aMT6s and the three outcomes of interest.

Creatinine-standardized aMT6s was natural logarithm-transformed for all analyses as this produced a moderately more normal distribution over the commonly observed right-skewed linear form[23], which contributes to potentially overly influential observations in the upper range. Serum estradiol and progesterone were natural logarithm-transformed as this produced more normal-looking distributions of residuals from crude OLS regression models. The continuous menstrual cycle length outcome was left untransformed. Upon visual inspection, it was apparent that two extreme low range creatinine-standardized aMT6s values were having disproportionate influence on the crude linear slopes and were omitted from regression analyses. Luteal day was entered in the models as [luteal day + (luteal day)^2] as this variable was observed to have an approximate quadratic relationship ("n" shape) with the dependent hormone outcomes. Women were excluded from hormone outcome analyses if their luteal day was greater than 14 in attempt to avoid contamination from blood samples assayed outside of the luteal phase.

Multivariable model building was based on change in the effect of the main melatonin exposure[35]. Confounding of the crude relationship was assessed by first determining change in the creatinine-standardized aMT6s regression coefficient upon entry of each covariate individually into the crude model. Potential confounders were then entered in decreasing order of change produced in the creatinine-standardized aMT6s regression coefficient in the crude models. The adjusted model was attained when entry of covariates no longer effected a change in the main exposure coefficient of 10 percent or more.

Results

Derivation of the study populations used for all analyses, by menstrual cycle length, circulating estradiol, and progesterone outcomes, is summarized in Figure 5.1. There were 130 eligible women after those missing creatinine-standardized aMT6s and relevant menses dates were excluded. The study sample for the assessment of the menstrual cycle length outcome (n=124) was attained after further exclusion of four women missing covariate data and two more with low outlying creatinine-standardized aMT6s values of uncertain validity. The study sample sizes for

the estradiol and progesterone outcomes were n=99 and n=98, respectively. There were 19 women excluded due to blood draw occurring outside of the estimated 14-day luteal range. A further six women were excluded due to missing estradiol and seven were excluded due to missing progesterone data for analyses with these respective outcomes.

Characteristics of the overall study population (n=124) are summarized in Table 5.1. Mean creatinine-standardized aMT6s concentrations for summer and winter study participants were 35.7 and 34.9 ng/mg creatinine, respectively, the difference between which was not statistically significant (p for Cochran t-test for samples with unequal variances= 0.78). Menstrual cycle length values ranged from 21 to 59 days, with the middle 80 percent of values lying between 25 and 38 days. Menstrual cycle length, and estradiol and progesterone levels did not differ significantly by season (p for Cochran t-test for samples with unequal variances= 0.19, 0.38, 0.56, respectively).

Figure 5.2 depicts the crude linear association between natural log-transformed creatinine-standardized aMT6s and all continuous outcomes: menstrual cycle length, natural log-transformed luteal estradiol and natural log-transformed luteal progesterone. Though the steroid hormone scatterplots indicate a potential modest inverse association, Pearson correlations were weak and not statistically significant (r for aMT6s-menstrual cycle length= 0.08, p= 0.41; r for aMT6s-estradiol= -0.09, p= of 0.32; r for aMT6s-progesterone= -0.07, p= 0.46).

Table 5.2 summarizes the results for all crude and adjusted OLS regression models. Moderate inverse associations between log-transformed creatinine-standardized aMT6s and log-transformed steroid hormones were not statistically significant in either crude or multivariable-adjusted models. Similarly, the estimated effects of melatonin on menstrual cycle length were also found to be not statistically significant. Our study sample contained four women with reported menstrual cycle lengths of greater than 45 days, observed to be rare for normal, cycling women[36](see Figure 5.2, top). Repeating the analyses omitting these women, while attenuating the multivariable estimate for aMT6s, did not meaningfully alter our results (data not shown).

In light of the recent finding that the urinary melatonin metabolite aMT6s was crudely associated with circulating estradiol in a sub-population of shift working nurses who participated during the winter[17], we assessed effect modification by season in our own dataset. T-statistics from corresponding modeled product terms did not indicate effect modification of the association

between creatinine-standardized aMT6s and menstrual cycle length (p = 0.71), serum estradiol (p = 0.53) or progesterone (p=0.36) by season.

Discussion

We did not find evidence to suggest a relationship between the creatinine-standardized overnight urinary melatonin metabolite aMT6s and menstrual cycle length, nor between aMT6s and circulating luteal estradiol and progesterone. Our findings neither support nor refute the use of menstrual cycle metrics as a reliable marker of the impact of circadian disruption, mediated via nocturnal melatonin suppression, on gonadal activity.

The strongest evidence for melatonin as a regulator of reproductive function is from seasonal breeders, animals who are reproductively active only at certain times of the year. At first, findings from long-day breeders, mammals who are fertile during long photoperiods (when daylight is longest), suggested that melatonin had an inhibitory effect on gonadal activity[37]. However, subsequent work in these animals[38,39], and in the short-day breeding ovine model[40], indicate that melatonin could also be gonadotropic. Further research gave rise to the idea that the duration of the nocturnal melatonin signal[41], which is longer during short photoperiods, and change in duration[42], is crucial for photoperiod-specific reproductive potentiation. While humans are not seasonal breeders, it is conceivable that more subtle changes in reproductive function may occur in response to changes in our photic environment through similar melatonin-mediated mechanisms. If so, the lack of associations observed herein may be due to the fact that the majority of the women in our study population had not been exposed to changing photoperiod trajectories, due to the propensity to maintain consistent effective photoperiod length with electric lighting at night.

In non-seasonally breeding mammals melatonin seems to exert an overall inhibitory effect on reproduction[43]. In humans, in vitro audioradiography[44] and immunocytochemical methods[45] have detected melatonin receptors in various regions of the hypothalamus and pituitary, yet it remains undetermined if, or how, these targets modify the HPGA and gonadal activity. Despite persisting uncertainty due to the complexity of central regulation of mammalian gonadal function, observational findings have shown seasonal variation in fertility in humans. Higher conception

rates have been observed during the summer in more seasonally photoperiod-diverse environments[46] or by latitude[47] corresponding to seasonal variation in circulating melatonin[48]. At least one study has directly concluded that women who live in northern climates experience enhanced hypothalamic-pituitary-ovarian-axis (HPGA) activity during longer photoperiods, suggestive of an overall inhibitory effect of melatonin on fertility, and in particular, ovulation[49]. Direct evidence of an inverse association between melatonin and gonadotropic potentiation are found from observations of women with menstrual disorders. Amenorrhea, anovulation and polycystic ovarian syndrome are coincident with abnormally high levels of melatonin[14]. Amenorrheic women exhibit reduced GnRH neuron activity in combination with higher peak levels and longer duration of nightly melatonin[12,50] and reduced circulating levels of estradiol[50]. Overall, such findings provide plausibility to the hypothesis that circadian disruption-linked cancer risk may be at least partially attributable to the upregulation of the HPGA and downstream reproductive hormones mediated via chronic suppression or phase-shifting of melatonin signaling. On the other hand, they perhaps suggest that in less photoperiod-diverse environments, such as those to which our study population would be conceivably exposed, any regulatory effect of melatonin on reproductive function in healthy, cycling women would be minimal.

This is the first observational study that we know of that has assessed the association between overnight endogenous melatonin production and menstrual cycle length. Shift workers have been observed to have more variable and irregular menstrual cycle lengths than their non-shift working counterparts[18-20]. The first two studies classified irregular cycles as <25 days or >35 days[18,19], while the latter[20] used more extreme cycle lengths of <21 and ≥40 days. It remains undetermined whether these more variable cycle lengths were attributable to melatonin signaling. While a small proportion reported longer menstrual cycles in our sample, as women with highly irregular cycles were avoided during recruitment, our ability to test this hypothesis was limited.

Statistically significant inverse correlations between melatonin and estradiol concentrations were reported by Tang *et al*[51] and Fernandez *et al*[52], though both had relatively small sample sizes (n=18, 40, respectively) and in the former, participants were anovulatory and infertile and had much higher levels of circulating melatonin than normal. In the latter, the urinary aMT6s measure was not reflective of total overnight excretion. Neither study attempted to adjust for

confounding. A more recent study, however, failed to show a significant association between these hormones[53].

There have been few studies that have investigated the relationship between nocturnal melatonin and serum estradiol and progesterone conducted among larger samples of premenopausal women while attempting to control for confounding due to behavioral and environmental factors. Findings have been largely null, in agreement with our study. In a sample of 80 premenopausal women from the Nurses' Health Study II cohort, Schernhammer *et al* found statistically significant correlations between urinary melatonin and progesterone and bioavailable estradiol, though after adjustment for age and BMI in multivariable models, the associations were no longer statistically significant[15]. In a larger follow-up study, Schernhammer *et al* reported that first-morning urinary aMT6s was not associated with any of the reproductive hormones assessed, including estradiol and progesterone sampled from both the luteal and follicular phases[16]. Recently, Langley *et al* similarly did not find statistically significant associations between first morning aMT6s and serum estradiol nor progesterone in multivariable regression models from a sample of 82 premenopausal shift-working nurses[17]. The authors did report a significant inverse crude relationship between aMT6s and estradiol in a sub-analyses restricted to winter participants, though this finding was not maintained in the multivariable adjusted model. The latter study did not attempt to restrict blood sample collection to the luteal phase, nor to women who were not on oral contraceptives, as we did, though they did control for menstrual cycle phase and oral contraceptive use in their multivariable analyses. While Fernandez *et al* reported a significant association between the urinary melatonin metabolite and estradiol, they used samples collected during the follicular phase, which may account for the discrepancy between findings[52].

Though the potential confounders considered in our multivariable analyses were comprehensive, a potential limitation is that behavioral factors (physical activity, smoking and alcohol use, wake time and number of hours sleep per night) were only queried for the past 24 hours. Physical activity among healthy younger women has been observed to correlate with immediate, short-term increase in melatonin production[54], rendering the immediately preceding 24 hour exposure window appropriate for a potential effect of physical activity on nocturnal melatonin production. However, it is likely that the impact of many of the included potential confounders on nocturnal melatonin output and following daytime steroid hormone concentrations would be less immediate. As such, interpretation of these results are made under the assumption that exposures

during the preceding 24 hours were representative of variation over the long term, which we were unable to validate. For the menstrual cycle length outcome, the validity of these factors as predictors of this outcome, measured over such a short term, is even less certain. In addition, we were unable to control for confounding due to caffeine, observed to be associated with both melatonin[14] and plasma estrogen levels[55].

It may be an unreasonable assumption that the duration of a single menstrual cycle is reflective of typical lengths for all participants: there is no way to be certain that the values reported herein were not outliers. An analyses of menstrual data from prospective menses diaries from more than 1,000 healthy, cycling, unmedicated women demonstrated that while menstrual cycle lengths tend to be consistent from one month to the next, 50 percent of women between the ages of 23 and 41 years exhibited a cycle length range of seven days or more[56], with the youngest women in this cohort exhibiting greater cycle length variability[57]. Measurement error due to this variability may have biased our results, attenuating our ability to detect an association.

Assuming nocturnal melatonin production meaningfully impacts circulating ovarian steroid hormones and menstrual cycle length, the restriction of only having access to data measured at a single time point could have severely limited our ability to detect such associations. As with the outcomes, the one-time aMT6s measure must be interpreted as being stable over the longer term for it to conceivably impact menstrual cycle length. Though Schernhammer *et al* noted reasonable correlation in intra-individual urinary melatonin metabolite readings over time[15], it is possible that some of the measures in the current study were non-representative, contributing to non-differential measurement error and null findings. This limitation also rendered us unable to monitor the association between nocturnal melatonin production and daytime circulating estradiol and progesterone across the menstrual cycle and across multiple cycles. For instance, it may be that nocturnal melatonin deficit has the most pronounced impact on circulating estradiol around ovulation when ovarian production of this hormone is highest.

Furthermore, the single aMT6s measure did not permit investigation of the effect of change in quantity or timing of the typical nocturnal peak in production on gonadal activity-related outcomes. There is indication that at least some of the adverse biological effects of shift work, such as increased risk of breast cancer, may be attributable to phase shifts in peak melatonin production caused by abrupt switching between day and night schedules and associated exposure

to inconsistent photoperiod. Limited evidence in humans comes from epidemiological studies showing increased incidence of breast cancer among rotating shift workers[58,59]. Mechanistically this is supported by animal models demonstrating increased tumour progression in response to phase advancement or complete decoupling of circadian signaling from the central clock in the suprachiasmatic nucleus[60,61].

A final limitation is study power. Given the observed linear effects of aMT6s on menstrual cycle length and circulating hormone outcomes, it is unlikely greater study power would have uncovered conclusive linear associations. However a larger sample size may have increased stability of our multivariable regression models, allowing for increased confidence in assessment of confounding and resulting adjusted aMT6s effects, particularly for the estradiol outcome.

Conclusion

Our study is one of the few to assess the relationship between nocturnal melatonin output and reproductive steroid hormone levels, and the only to assess the relationship between nocturnal melatonin and menstrual cycle length, while adjusting for potential confounders in a larger study population. If melatonin signaling does significantly regulate gonadal activity in humans, perhaps frequent phase shifts in nocturnal peak production, as presumed to be experienced by a large proportion of night workers, may result in detectable variation in reproductive outcomes such as those investigated here. Unfortunately, we were unable to assess this with our single overnight urinary aMT6s and one-time outcome measures. Due to the challenges associated with obtaining reliable steroid hormone measures among cycling women, future studies may benefit from longitudinal designs that assess changes across multiple menstrual cycles and include alternative reproductive outcomes such as menstrual cycle length or change therein. Employing these designs in populations most likely to experience the largest variation in circadian disruption and associated melatonin signalling may indicate whether increased incidence of breast cancer observed among these groups are associated with preceding detectable changes in reproductive function.

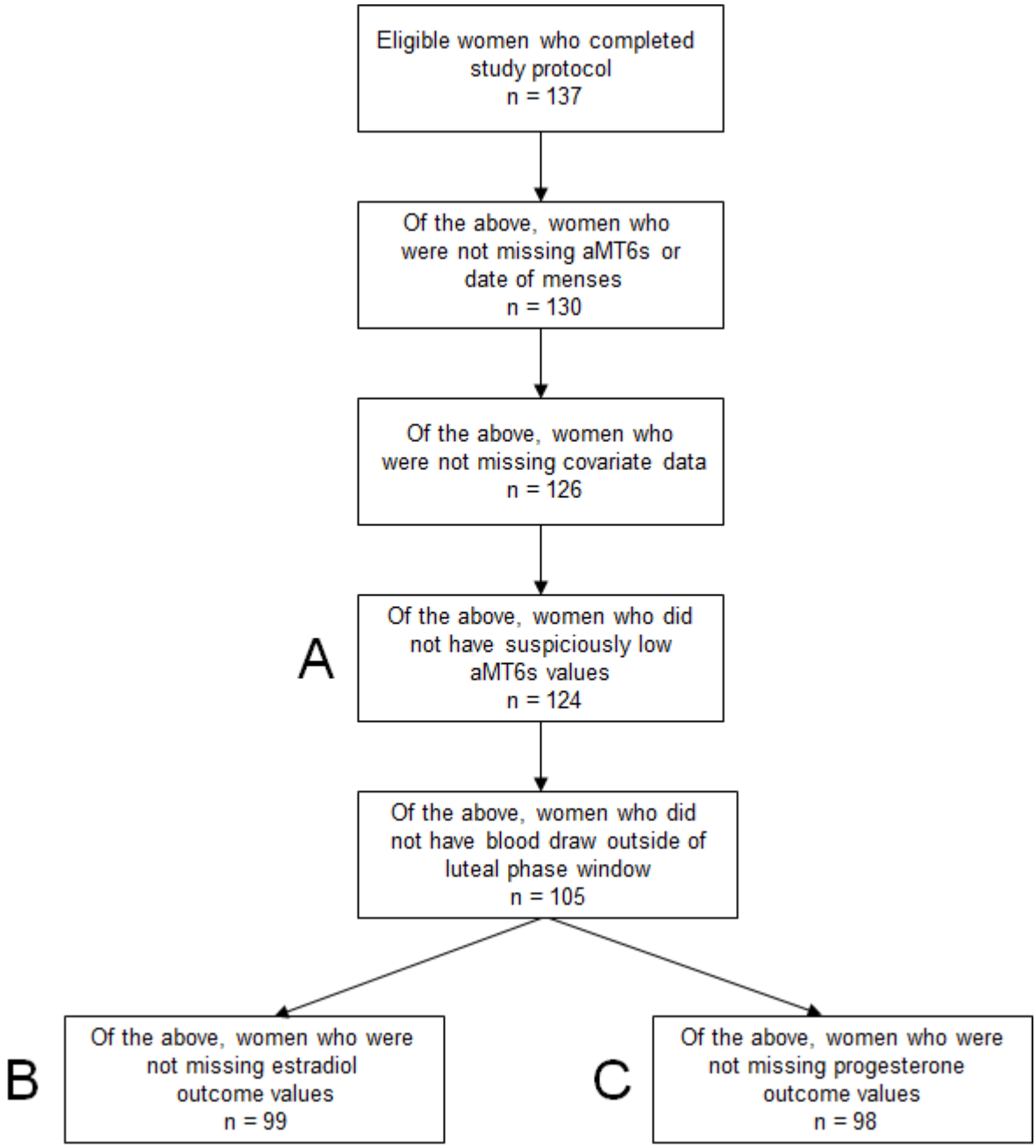


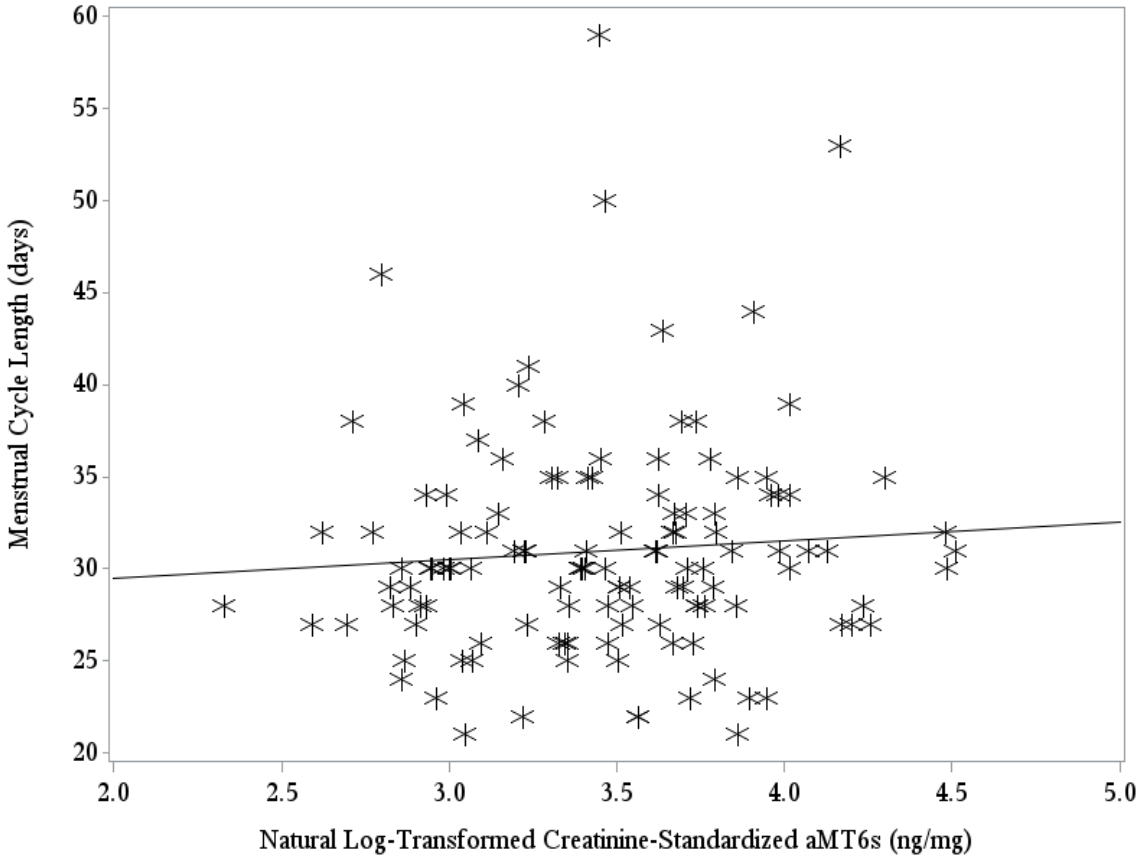
Figure 5.1. Derivation of study population as analyzed. Adjacent letters indicate study samples used to assess the associations between urinary aMT6s and A)menstrual cycle length, B)circulating estradiol and C) progesterone.

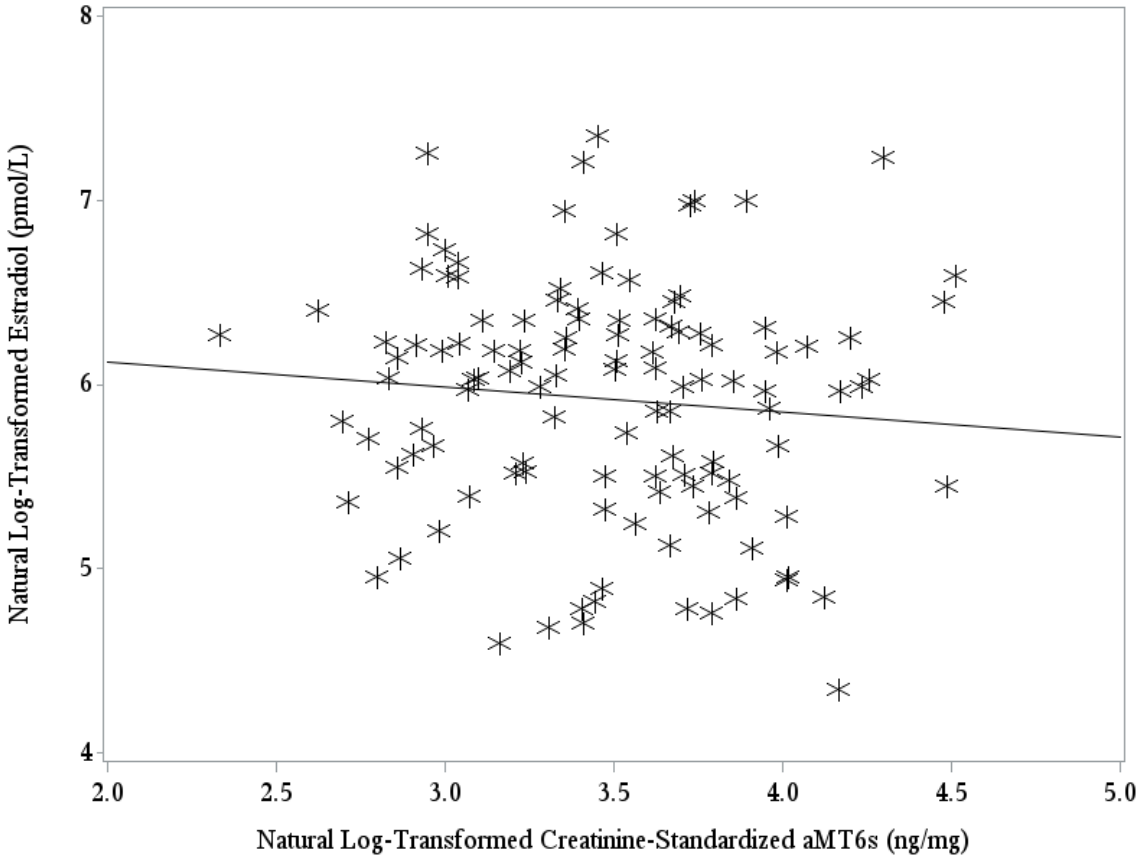
Table 5.1. Characteristics of study population (n=124)

Study Variables	Mean (SD)/[¥]Median (IQR) or Count
Menstrual cycle length (days ± SD)	31.0 (6.02)
Age (years ± SD)	20.3 (1.21)
Body mass index (kg/m^2 ± SD)	22.5 (4.30)
Study Season (%)	
Summer	63 (50.8)
Winter	61 (49.2)
Ethnicity (%)	
Caucasian	58 (46.8)
East Asian	45 (36.3)
Other	21 (16.9)
Circulating Hormones	
[†]aMT6s (ng/mg creatinine ± SD)	32.2 (22.0-43.0)[¥]
Estradiol (pmol/l ± SD)	416.0 (245.0-572.0)[¥]
Progesterone (nmol/l ± SD)	17.0 (4.00-36.0)[¥]
Blood draw time (hrs since 0:00)	10:29 (1.51 hrs)
Lifestyle Characteristics	
Nightly sleep duration (hrs ± SD)	7.25 (1.39)
Wake time (hrs 0:00 ± SD)	8.18 (1.40)
Alcohol (%)	
Users	6 (4.8)
Non-users	118 (95.2)
Smoking (%)	
Smoker	8 (6.5)
Non-smoker	116 (93.5)
Physical Activity[‡]	
Perceived Exertion (%)	
None (6)	79 (63.7)
Light (7 to 11)	28 (22.6)
Moderate (12 to 15)	11 (8.9)
Heavy (15 to 20)	6 (4.8)
Daily Exercise Duration (%)	
No physical activity	78 (62.9)
1 to 30 minutes	19 (15.3)
31 to 100 minutes	12 (9.7)
More than 100 minutes	15 (12.1)

[†]Creatinine standardize urinary 6-sulfatoxymelatonin

[‡]Borg scale of perceived physical exertion


Menstrual Cycle Length (days)
60
55
50
45
40
35
30
25
20
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Natural Log-Transformed Creatinine-Standardized aMT6s (ng/mg)


Natural Log-Transformed Estradiol (pmol/L)
8
7
6
5
4
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Natural Log-Transformed Creatinine-Standardized aMT6s (ng/mg)

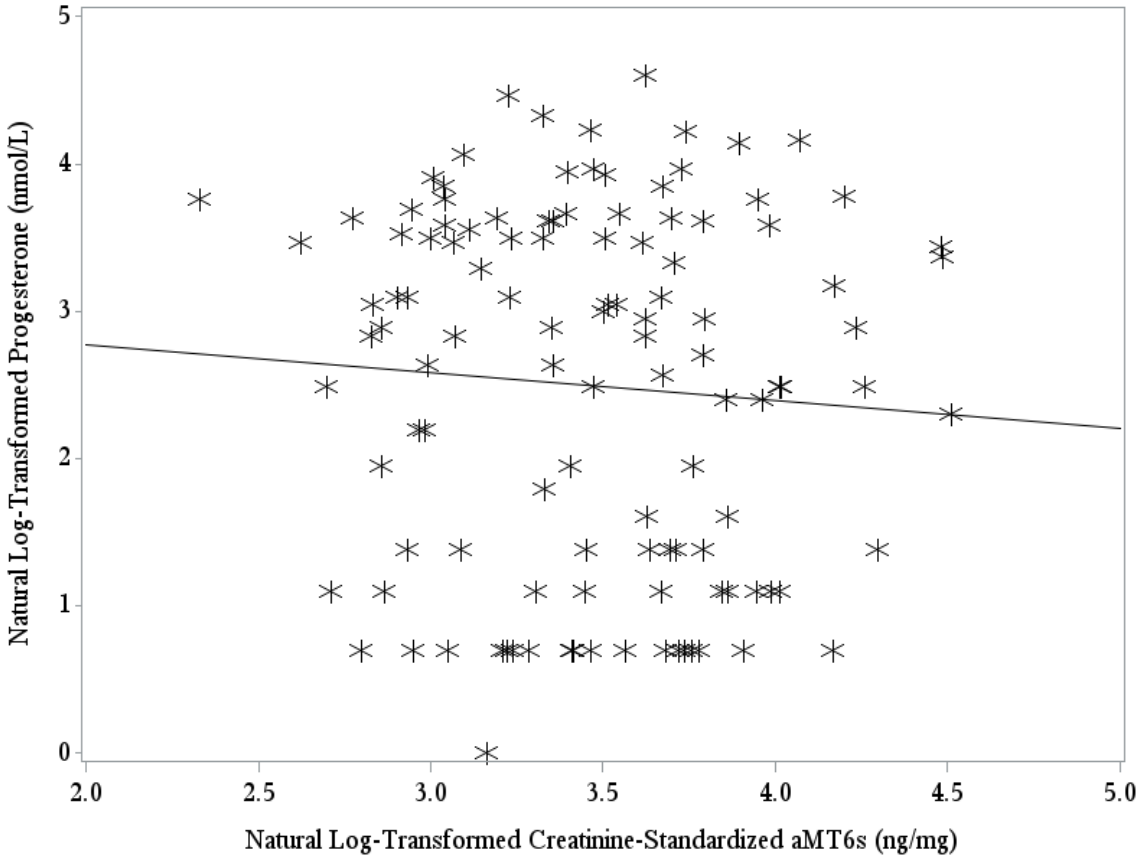


Figure 5.2. Linear relationship between overnight urinary natural log-transformed creatinine-standardized aMT6s and menstrual cycle length (top), natural logarithm-transformed circulating serum estradiol (middle) and progesterone (bottom). Pearson correlation coefficients and corresponding p-values: r= 0.08 , p-value= 0.41 (top); r= -0.09, p-value= 0.32 (middle) and r= -0.07, p-value= 0.46 (bottom).

Table 5.2. Regression models summarizing natural log-transformed, creatinine standardized urinary aMT6s effects on menstrual cycle length, natural log-transformed circulating estradiol and natural log-transformed progesterone

	Regression Models	aMT6s Parameter Estimate	95% CI
Menstrual Cycle	Crude model	1.02	(-1.40, 3.43)
Length (days)	†Adjusted Model	0.391	(-2.16, 2.94)
Circulating	Crude Model	-0.0687	(-0.330, 0.192)
Estradiol (pmol/l)	‡Adjusted Model	-0.00124	(-0.292, 0.289)
Circulating	Crude Model	-0.101	(-0.554, 0.352)
Progesterone (nmol/l)	¥Adjusted Model	-0.174	(-0.633, 0.284)

† Adjusted for age, BMI, ethnicity, alcohol use and exercise duration, wake time.
‡ Adjusted for age , BMI, ethnicity, alcohol use, exercise duration, wake time, luteal day (luteal day + (luteal day)^2), blood draw time, smoking, and season
¥ Adjusted for age, ethnicity, alcohol use, luteal day (luteal day + (luteal day)^2), blood draw time and exercise intensity

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Chapter 6 Manuscript 3

Cross-sectional association between nocturnal melatonin and daytime serum prolactin

This manuscript assesses the cross-sectional association between nocturnal melatonin and subsequent daytime circulating serum prolactin. The study sample is a population of mostly young premenopausal women recruited from hospitals and universities in Toronto.

Abstract

Background: The light at night hypothesis holds that the moderately higher rates of breast cancer observed among night shift workers is due to suppression of nocturnal melatonin production leading to chronically elevated circulating levels of estrogens. Studies assessing the association between nocturnal melatonin output and circulating steroid reproductive hormones have been largely null. The melatonin signal has additionally been observed to regulate mammalian central prolactin secretion. In this study the association between nocturnal melatonin production and subsequent daytime circulating prolactin levels in a sample of mostly premenopausal is investigated. Effect modification by age and diurnal preference are additionally considered.

Methods: The study sample was comprised of overlapping women participating in summer and winter data collection sessions. Mean values of overnight, creatinine-standardized 6-sulfatoxymelatonin (aMT6s) were computed over three consecutive daily samples. A single serum prolactin measure was obtained on the final day of data collection for each session. Ordinary least squares and generalized estimating equations regression models were used to model crude and multivariable-adjusted linear associations between the two hormones among individual summer and winter sessions and combined samples, respectively.

Results: Our final study sample was comprised of 212 volunteer women recruited from the community comprised of women who participated in winter (n=192) and summer (n=168) sessions, with overlap. We did not observe an overall association between nocturnal aMT6s production and daytime serum prolactin concentrations nor within either summer or winter data collection sessions. We did, however, observe both a crude and adjusted statistically significant inverse association among younger (age <25 years) women. No indication of effect modification by diurnal preference (Horne-Östberg morningness-eveningness score) was noted.

Conclusion: With the exception of women under 25 years, we did not observe any association between overnight creatinine-standardized aMT6s and daytime serum prolactin concentrations. Longitudinal studies with repeated measures capturing full circadian profiles of melatonin and prolactin among sufficiently large samples is the next logical step in the characterization of this relationship.

Introduction

The observation of moderately higher incidence of breast and other cancers among night shift workers have led to shift work being recognized as a potential carcinogen by the International Agency of Research on Cancer[1]. Though the underlying mechanisms are not well understood, a popular theory has been the light at night hypothesis[2] which suggests that exposure to artificial light during habitually dark periods conveys breast cancer risk mediated via endogenous melatonin signalling dysfunction. Out of photoperiod (i.e., night) light stimuli inhibits nightly pineal function via direct innervation from non-visual retinal receptors[3], the degree to which is dependent on duration, intensity and wavelength[4]. The result is diminished[5] or at least phase-shifted[6] nocturnal melatonin production.

In addition to limiting potential innate oncostatic actions of the pineal hormone[7,8], suppression or disruption of the nocturnal melatonin signal is thought to increase lifelong exposure to endogenous estrogens, identified as an important risk factor for breast cancer [2]. Night shift work, a widely used surrogate for circadian disruption exposure in observational studies[9,10], has been associated with lower[11] or atypical[12] nocturnal melatonin. Melatonin levels have also been inversely associated with breast cancer risk in at least two cohorts[13,14], though in one study[13] the relationship was only statistically significant after excluding smokers and allowing for a sufficient induction period. Despite these findings, the mechanisms behind a relationship between chronic exposure to lower nocturnal endogenous melatonin and breast cancer remain uncertain and cross-sectional associations between nocturnal melatonin levels and daytime steroid hormone levels have been largely null[15-17]. Though there is evidence of melatonin having regulatory control over the hypothalamic-pituitary-gonadal-axis (HPGA) in animal models[18], and limited indication of a suppressive effect on ovarian function and ovulation in humans in populations residing in northern regions that experience more diverse photoperiods[19,20], a conclusive link between the nightly melatonin surge and ovarian steroid hormone output remains elusive. An alternative endocrine mediator linking chronic disruption in melatonin signaling and increased breast cancer risk may lie in prolactin[21].

Though the role of prolactin in human breast cancer may still be debated, there is a growing body of literature implicating the hormone in at least some subtypes of the disease[22]. Increased serum prolactin levels have been linked to both premenopausal[23,24] and postmenopausal[25,26] breast

cancer risk in the Nurses' Health Study cohorts, the strength of the effect appearing to increase with age of diagnosis. The increased risk in postmenopausal women has been mirrored in other study populations, though effects were not statistically significant[27-29], presumably due to smaller sample sizes.

The overall regulatory effect of endogenous melatonin on pituitary prolactin secretion is uncertain. It has been posited that melatonin is chiefly responsible for the observed circadian pattern of prolactin in humans and a daily rise in prolactin corresponding to sleep cycles has long been recognized[30], suggestive of an acute stimulatory effect. In healthy cycling women, night time melatonin supplementation has been observed to potentiate prolactin secretion[31]. Other observations of increased pituitary prolactin secretion following administration of melatonin or melatonin agonists have been reported[32,33].

Conversely, evidence from murine models has been suggestive of an opposite effect, accounting for a potentially inverse relationship between nocturnal melatonin output and circulating prolactin. It has been demonstrated that melatonin can activate tuberoinfundibular dopaminergic neurons and in turn inhibit prolactin gene expression[34] and lower circulating prolactin levels [34,35]. A potential direct mechanism of prolactin inhibition by melatonin involving melatonin (MT1) receptors on the pars distalis of the pituitary has been described[36]. If such a mechanism persists in humans, chronic circadian disruptive stimuli, as potentially experienced by night workers, could alter and perhaps elevate circulating levels of prolactin, particularly following shorter photoperiods when the corresponding nocturnal melatonin signal is longest. In this study, we attempt to better characterize the association between nocturnal melatonin and central prolactin production in a population of mostly premenopausal women at photoperiodically-diverse times of the year, while accounting for potentially confounding factors. Effect modification by age and diurnal preference are additionally investigated.

Methods

Study Population

A sample of mostly premenopausal women was recruited through advertisements in local hospitals, universities, community colleges and community newspapers from Toronto, Canada

and has been described previously[37]. Women were eligible to participate if they had no prior cancer diagnoses or kidney or liver disease. Shift workers and those who had travelled across time zones during the past month were also ineligible. Two hundred thirteen eligible women consented to participate in at least one of two three-day data collection sessions between November 2002 and August 2004 in either summer or winter. All potentially confounding variables for regression analyses were derived from self-reported data. Women were scheduled for an initial clinic visit in winter (November through February) or summer (May through August). During this visit, participants completed a questionnaire querying anthropometry (height and weight) and diurnal preference – whether habitually more alert in the morning or evening – using the Horne-Östberg morningness-eveningness (HOME) questionnaire[38]. A diary was provided in which women kept track of medication use, alcohol consumption, physical activity and morning wake time over the three- day data collection session.

Main Exposure and Outcome Measures

Overnight urinary creatinine-standardized 6-sulfatoxymelatonin (aMT6s), a marker for nocturnal melatonin production, was the main exposure in all analyses. Urinary aMT6s, the major melatonin metabolite excreted in urine, has been validated as an accurate marker of circulating melatonin. Nocturnal urinary aMT6s has been observed to accurately capture nocturnal melatonin production and observed to correlate with plasma melatonin measures[39]. Urinary aMT6s is commonly standardized with urinary creatinine to minimize measurement error introduced by intra-individual and inter-individual variation in renal clearance[40]. The mean of three consecutive overnight aMT6s concentrations, measured from self-collected complete overnight urine samples (from 8pm up to and including first morning void), was used in all analyses in an attempt to provide a reliable measure of characteristic nocturnal melatonin output. The outcome of interest was serum prolactin concentration, measured once during the morning, at the end of each of the three day data collection sessions from blood samples drawn in clinic.

Potential Confounders

To minimize bias in the investigation of the association between nocturnal urinary aMT6s excretion and circulating prolactin levels, age, body mass index (BMI), current alcohol intake, physical activity, oral contraceptive and selective serotonin reuptake inhibitor (SSRI) use, morning wake time, HOME score and approximate time of blood draw were considered as

potential confounders in our multivariable analyses. Briefly, circulating levels of both melatonin[41,4241,42] and prolactin[43,4443,44] have been observed to decline with age and increase after physical activity in women[22,45]. Blood prolactin concentrations have been observed to be impacted by certain medications, including oral contraceptives and SSRIs[22,46]. As prolactin and melatonin exhibit distinct diurnal excretion patterns, morning wake time, HOME score and time of blood draw were considered to adjust for confounding due to inter-individual variation in circadian rhythm and time at which the blood draw for prolactin assay occurred.

Laboratory Assay

Overnight urine collection volume was measured and creatinine concentration was assayed with an automated Roche Cobas Integra 700 analyzer (F. Hoffmann- La Roche, Ltd., Basel, Switzerland) using a manufacturer-provided enzymatic method (COBAS INTEGRA® Creatinine plus ver.2, Cat. No. 03263991, Roche Diagnostics). Aliquots were stored at -20°C for future analysis. Following data collection between November 2002 and August 2004, one of the frozen urine aliquots was thawed, manually diluted and assayed for aMT6s using a single epitope competitive enzyme-linked immunoassay kit (IBL Gesellschaft für Immunochimie und Immunobiologie mbH, Hamburg, Germany; catalog number RE54031). The kit was automated using robotics to perform all pipetting, incubation, washing and reading steps of the assay protocol. All assays were performed over a two day interval using assay kits from the same lot. The three urine samples provided by each study participant (during a single three day measurement period) were assayed sequentially. Standards and controls provided with each kit were included on the titer plate. Imprecision across all the assay runs, compared to controls, was 25 percent at a concentration of 13 μg/l and 17 percent at a concentration of 63 μg/l. Urine samples with aMT6s concentrations higher than the second highest standard (140 μg/l) were further diluted and re-assayed. Prolactin was assayed by the multitest automated Immulite 2000 analyser using a two-site immunometric sandwich method with chemiluminescent detection commercially available from the manufacturer of the analyser. Inter-assay coefficient of variation was approximately 6 percent.

Analysis

The cross-sectional, linear association between overnight urinary creatinine-standardized aMT6s excretion and subsequent morning serum prolactin concentrations was assessed using ordinary least squares regression among both summer and winter study groups, providing the opportunity to assess effect modification by maximum variation in natural photoperiod. Creatinine-standardized aMT6s was natural logarithm-transformed for all analyses as this produced a more normal distribution over the commonly observed right-skewed linear form[47,48]. Serum prolactin was also natural logarithm-transformed as this produced more normal-looking distributions of residuals from crude OLS regression models.

In the event of no meaningful effect modification by season, we employed a repeated measures model, combining data from both summer and winter study groups to maximize study power. Generalized estimating equations (GEE) using a canonical link function with an exchangeable correlation structure was used[49]. For comparability, multivariable OLS regression models by seasonal data collection session were adjusted with the same independent variable specification achieved for the GEE models.

Confounding was assessed in all GEE models by first determining change in the creatinine-standardized aMT6s regression coefficient upon entry of each covariate individually into the crude, bivariable models[50]. Potential confounders were then entered in decreasing order of magnitude of change in the creatinine-standardized aMT6s regression coefficient assessed in bivariable models. The final adjusted model was attained when entry of covariates no longer effected a change of 10 percent or more in the creatinine-standardized aMT6s coefficient.

Effect modification of creatinine-standardized aMT6s by age and HOME score was assessed in the adjusted GEE model. Both age and Horne-Östberg score were defined as three-category variables. Age category cutpoints (<25 years, 25-39 years and ≥40 years) were based on observations of significantly higher circulating prolactin levels in 15 to 25 year olds compared to 45-65 year olds[44] and dips in endogenous melatonin production around menopause[51]. Categories for HOME score were based on cutpoints defining a morning (16-41), neutral (42-58) or evening (>58) preference. A p-value of <0.05 for the score test assessing the overall addition of both corresponding product terms for each model was deemed indicative of statistically significant effect modification.

Results

There were 201 and 175 participants in the winter and summer data collection sessions, respectively, that met our eligibility criteria. Of those, six women from the winter, and five from the summer sessions were excluded due to missing aMT6s, creatinine, or prolactin data. A further three women from the winter and two from the summer sessions were excluded due to missing covariate data. Our final study sample was comprised of 212 women, with 192 and 168 women completing winter and summer data collection sessions, respectively, and 148 women who participated in both. The vast majority of women contributed three urine samples, one for each morning of each data collection session, of which the mean creatinine-standardized aMT6s value was used in all analyses. The exceptions were three women who contributed only two samples (second measure missing) in the summer data collection session. These women were retained and readings for the two samples were used to generate a mean creatinine-standardized aMT6s value.

The study population is characterized by season in Table 6.1. Mean creatinine-standardized aMT6s concentrations were unexpectedly higher in summer (45.9 ng/mg creatinine) than in winter (39.4 ng/mg creatinine) sessions. However, a paired t-test including the 148 women participating in both summer and winter sessions on the natural logarithm-transformed variable did not reveal a statistically significant difference (mean difference= 0.06; p= 0.35). Conversely, serum prolactin concentrations were higher in winter (13.0 μg/l) than in the summer (12.4 μg/l) group, though again, the mean difference between the natural logarithm-transformed means among women participating in both seasons (mean difference= 0.05) was not statistically significant (p= 0.38). Intraclass correlation coefficients for the serial creatinine-standardized aMT6s measures within summer and winter sessions were 0.49 and 0.60, respectively, indicating only moderate, though unexplainably higher in summer, variability in melatonin output across consecutive nights.

Figure 6.1 depicts crude linear relationships between logarithm-transformed creatinine-standardized aMT6s and prolactin by summer and winter study sessions. There were no notable correlations (r=0.03, p=0.61 and r=0.01, p=0.91, for summer and winter, respectively). Table 6.2 summarizes crude and adjusted associations between nocturnal urinary aMT6s secretion and daytime serum prolactin levels for winter and summer study groups. No statistically significant

associations were observed. As we did not find evidence of meaningful effect modification by season (data not shown), we combined data from both summer and winter study groups. Crude and multivariable GEE models are shown in Table 6.3. There was no evidence that nocturnal urinary aMT6s was associated with subsequent daytime serum prolactin concentrations from either the crude or adjusted models. Substituting the three-night mean urinary aMT6s measure with that from the night prior to serum prolactin assay as the main exposure did not materially change the magnitude or the precision of the parameter estimates (data not shown).

The addition of product terms comprised of urinary aMT6s and age in the multivariable GEE model was suggestive of statistically significant effect modification (p= 0.02), while there was no indication of effect modification by HOME score (p= 0.60). The estimated combined aMT6s and interaction effects are presented in Table 6.3 and graphically in Figure 6.2. In women less than 25 years, there was indication of statistically significant inverse association between nocturnal melatonin output and subsequent morning prolactin (multivariable-adjusted p-value= 0.002), interpretable as an approximate 2.2% decrease in circulating daytime prolactin per 10% increase in nocturnal urinary aMT6s excretion. The multivariable GEE estimated aMT6s effects in women 25 to 39 inclusive and 40 years and older were weaker and not statistically significant.

Discussion

Overall, we did not find that overnight urinary aMT6s concentrations were associated with subsequent day time serum prolactin levels in our cross-sectional analyses. While our GEE models produced effects that were suggestive of an inverse association, they were of moderate magnitude and not statistically significant. We did not observe any indication of effect modification by season or diurnal preference as quantified by the overall score from the self-administered HOME questionnaire. We did, however, observe a statistically significant inverse association between overnight urinary aMT6s and subsequent day time serum prolactin concentrations in women under the age of 25.

Superficial evidence of a role of endogenous melatonin in the regulation of prolactin comes from observations of seasonal variation in prolactin profiles in mammals, particularly seasonal breeders[52], that correspond to seasonal change in the nocturnal melatonin signal which in turn

correspond to change in photoperiod. The mechanism behind a regulatory effect of melatonin on prolactin may lie with dopamine. Melatonin has been observed to inhibit dopaminergic neuron activity[53], attenuating dopaminergic negative regulation of the high basal secretory tone of pituitary lactotrophs[54], suggestive of an overall stimulatory effect. The distinct diurnal pattern of prolactin secretion, independent of sleep[55], with an early morning peak coincident with or following the nightly melatonin acrophase[56], can perhaps be viewed to support a positive regulatory pressure of melatonin on prolactin.

Conversely, melatonin has been shown, in vivo, to upregulate dopaminergic neuron activity resulting in a corresponding reduction in circulating prolactin in the non-seasonally breeding rat[35], suggestive of a negative regulatory effect on pituitary prolactin. Recently, the discovery of melatonin (MT1) receptors on cells within the mammalian pars tuberlalis, including that of humans[57], has identified targets by which melatonin could exert direct regulatory control on prolactin secretion from lactotrophs in the adjacent pars distalis, thought to be mediated by a yet unidentified "tuberlin"[36]. Compelling evidence has come from a series of *in vivo* experiments in hypothalamo-pituitary disconnected rams (innervations between the hypothalamus and pituitary surgically severed)[58,59]. It has been demonstrated in this model that while noradrenaline and dopamine may be responsible for acute suppression of prolactin, long term photoperiod-driven variation in prolactin secretion is likely mediated via MT1 receptors of the pars distalis[60]. This pathway suggests negative control of melatonin on prolactin, resulting in elevated levels during long photoperiods when the nocturnal melatonin signal is weakest. This direct regulatory pathway, if persistent in humans, could result in increased circulating prolactin in response to the artificially lengthened and/or fluctuating photoperiods associated with circadian disruption, thereby potentially increasing risk of breast cancer.

Our results, however, do not support a direct, or inverse, association between nocturnal melatonin output and daytime serum prolactin levels in humans overall. This may be due to competing regulatory mechanisms masking an overall relationship. However, though a mechanism by which melatonin acts directly on the pars tuberalis has been corroborated in other seasonal breeders[61], the prominence of such pathways in non-seasonally breeding humans, exhibiting negligible seasonal variation in prolactin secretion as observed in our study sample and in agreement with other findings[17,20,62], is uncertain. While there are reports of seasonal variation in female fertility in some populations, particularly those at more extreme latitudes

exposed to more seasonally diverse photoperiods[63], it could be that seasonal variation in prolactin levels, corresponding to changes in photoperiod length, has waned in recent human evolution. Whether the tendency to modify photoperiod with artificial light has hastened such a transition, if present to begin with, is unknown.

Experimental studies within humans have presented somewhat conflicting results, though overall seem supportive of an acute stimulatory effect of nocturnal melatonin on serum prolactin. Hyperprolactinemia has been observed to be coincident with higher levels of melatonin[64,65]. Exposure to light at night, known to suppress the nocturnal melatonin acrophase, has been correlated with subsequent lower levels of circulating prolactin in a sample of cycling women[66]. Another study, however, while observing nocturnal melatonin suppression in response to 3:00 AM light exposure, did not observe a significant accompanying change in prolactin, though sample size was small (n=6) and only two participants were women[67]. Perhaps the most direct evidence supporting an acute positive regulatory control of melatonin on central prolactin output in women are experiments reporting increased secretion in response to exogenous melatonin[31,33].

Working night shifts has corresponded with suppression of prolactin secretion[68], though Schernhammer *et al* did not observe a significant association between number of years working rotating shifts and serum prolactin in the Nurses' Health Study Cohort[16], failing to support a cumulative effect of chronic nocturnal melatonin disruption on prolactin production. As far as we are aware, the only epidemiologic study to examine the cross-sectional relationship between nocturnal urinary melatonin and daytime prolactin while rigorously controlling for confounding, did not find any association[17] in either summer or winter study samples and are therefore in agreement with our overall findings. However these researchers did not investigate effect modification by age or diurnal preference.

Aging has been observed to modify circadian regulation of various endocrine functions[69]. Our decision to assess effect modification was based on reports that both melatonin[42] and prolactin[44] production in women decline, overall, until menopause. The decline in melatonin production may be attenuated or even reversed around then, though it resumes thereafter[41], while serum prolactin gradually increases after menopause[43], potentially indicative of a changes in the coregulation of these hormones with age. Pituitary prolactin production has been observed to be induced in response to gonadotropin releasing hormone (GnRH), the extent of which varies by

menstrual cycle phase[70,71]. Young women, particularly those not taking oral contraceptives, are known to have more irregular menstrual cycles[72]. This increased irregularity could have altered the gonadotropic potentiation of pituitary prolactin relative to women of mid reproductive age corresponding to our middle age category of 25-40 years. Though the characterization of the relationship between melatonin and reproductive endocrinology has proven challenging in humans, melatonin has been observed to suppress GnRH gene expression *in vitro*[73]. Additionally, the localization of sex hormone receptors on the pineal gland and abnormal melatonin levels associated with reproductive disorders[74] suggests that the hormonal underpinnings responsible for the increased variability in menstrual cycle length may also impact melatonin signaling and its impact on pituitary prolactin.

Our rationale for investigating effect modification by HOME score is based on observations that genetics may account for variation in processes culminating in the endogenous melatonin signal[75]. It has been postulated that morning types may be more entrained to the circadian signal than their evening counterparts. Though nocturnal melatonin acrophase amplitude has been observed to vary inconsistently by diurnal preference, morning types typically exhibit an earlier nightly melatonin acrophase and find it more difficult to adjust to night shift work[76,77]. A stronger association among morning types may identify those at greater risk for the potentially harmful effects of circadian disruption and hint at the underlying biological mechanism. However, we did not find any evidence of effect modification by HOME score.

A possible limitation of our study is that time of blood draw may have varied more than what could be considered optimal in light of the observation that serum prolactin seems to exhibit a distinct circadian pattern, with higher morning levels dropping to nadir for late morning or early afternoon[56]. Measurement error or poor model specification may have led to residual confounding by this factor, particularly since there is likely considerable intra-individual variation in diurnal patterns of circulating prolactin[78]. We attempted to investigate the extent to which this may have skewed our results by examining how prolactin measurements changed with blood draw time. Though we did see a slight overall decline in mean serum prolactin concentrations with increasing blood draw time, the linear trend was not statistically significant (data not shown).

Though we were able to assess confounding by previously unconsidered factors, it is possible that some important confounders were not included. First, we were unable to consider the effect of menstrual cycle phase. While prolactin output has been observed to vary minimally over the menstrual cycle[20,79-81], there is some evidence of levels being moderately elevated around ovulation[81,82]. Nocturnal melatonin output has also been observed to vary negligibly across the menstrual cycle[83-85], though a handful of inconsistent reports of changes in melatonin secretion across cycle phases exist[86]. Second, we were unable to control for tobacco use. There have been indications that both melatonin[15] and prolactin[79,87] levels may differ among smokers. While it is likely that the proportion of smokers in our study population is small after comparison with a similar locally-recruited study sample[88], we cannot rule out residual confounding due to smoking.

One explanation for our null main findings may be that our study sample did not experience sufficient variation in circadian disruption and resulting nocturnal melatonin production and excretion. However, the single study investigating this association among rotating shift workers, a sample that would presumably exhibit more variation in nocturnal melatonin production, supports the lack of a cross-sectional linear association between nocturnal melatonin output and daytime prolactin [17].

Conclusion

Future attempts to model the association between melatonin and central prolactin output require the consideration of individual circadian hormonal profiles. Both of these hormones exhibit marked variation throughout the day which can vary considerably by individual. Longitudinal observation with repeated measures capturing full circadian profiles of melatonin and hormones such as prolactin over consecutive circadian cycles in a sufficiently large study sample with appropriate consideration of confounding is the next logical step in the characterization of this relationship. Unfortunately the feasibility of such endeavors is undermined by the associated high costs and increased demands on study participants. Further investigation of effect modification by diurnal preference, latitude, and, due to the results presented herein, age, may also be warranted.

If the observed effect modification is true, differences in prolactin regulation by melatonin across age may indicate that women may be more susceptible to deleterious effects of circadian

disruption at the early stages of their reproductive lifetime. Given increased circulating prolactin does increase risk of breast cancer precursors, this could be notable for younger women experiencing circadian disruption as their cumulative lifelong risk of breast cancer due to this oncogenic pressure may be elevated.

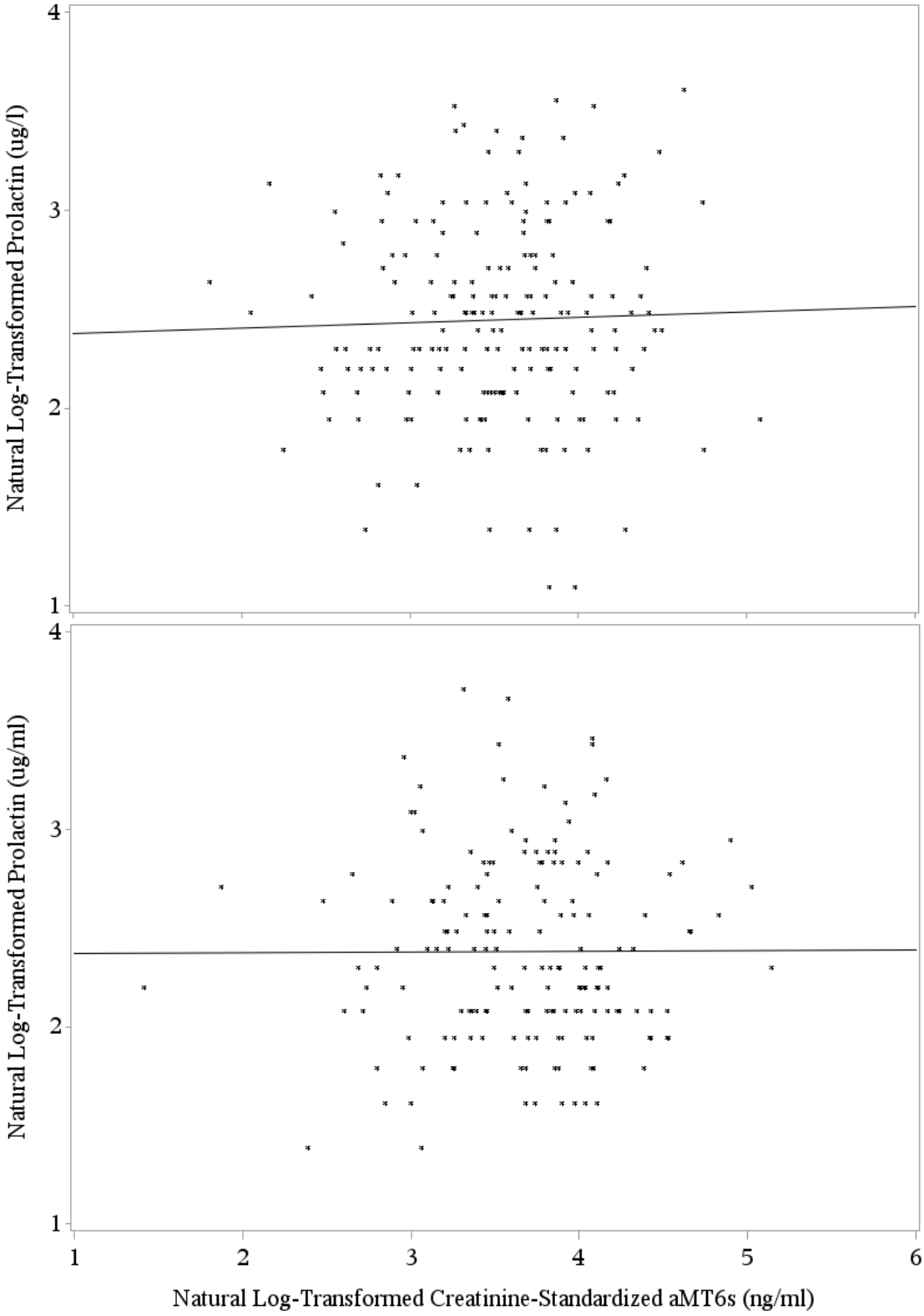

Figure 6.1. Linear relationship between natural logarithm transformed, creatinine-standardized aMT6s, by winter (top) and summer (bottom) data collection sessions. Pearson correlation coefficients and corresponding p-values: r= 0.03 , p-value=0.67 (winter); r= 0.01 , p-value=0.91 (summer).

Table 6.1. Characteristics of study population

Study Variables	Mean (SD)/¥Median (IQR) or Count Winter (n=192)*	Summer (n=168)*
Prolactin (μg/l ± SD)	11.5 (8.0-16.0)¥	10.0 (8.0-16.0)¥
†aMT6s (ng/mg creatinine ± SD)	34.6 (24.3-48.3)¥	42.6 (28.6-57.0)¥
Age (years ± SD)	30.4 (10.5)	30.4 (10.2)
BMI (kg/m^2 ± SD)	22.4 (4.1)	22.3 (3.8)
Wake time (hrs from 0:00)	8.0 (1.4)	7.9 (1.4)
Time of blood draw (hrs from 0:00)	10.1 (1.4)	10.5 (1.8)
‡Alcohol (%)		
Never drinkers	149 (77.6)	119 (70.8)
Less than 3 drinks	29 (15.1)	38 (22.6)
More than 3 drinks	14 (7.3)	11 (6.6)
‡Cardio Exercise Duration (%)		
Never exercised	135 (47.9)	109 (47.3)
Less than 108 minutes	22 (16.2)	20 (18.0)
108 to 225 minutes	17 (19.3)	22 (16.2)
More than 225 minutes	15 (12.5)	21 (18.6)
Horne-Östberg M.E. Score (%)		
Less than 42 (evening type)	28 (14.6)	28 (16.7)
42 to 58 (neither)	120 (62.5)	108 (64.31)
Greater than 58 (morning type)	44 (22.9)	32 (19.0)
Oral contraceptive use (%)		
Non-users	144 (75.0)	125 (74.4)
Users	48 (25.0)	43 (25.6)
£SSRI use (%)		
Non-users	185 (96.4)	161 (95.8)
Users	7 (3.6)	7 (4.2)

*N= 212 (combining summer and winter study sessions)
†Average of overnight urinary 6-sulfatoxymelatonin over three consecutive nights
‡Cumulative over the 3-day data collection interval
£Selective serotonin reuptake inhibitor; any recorded over the 3-day data collection interval

Table 6.2. Crude and multivariable-adjusted parameter estimates for creatinine-adjusted, natural logarithm transformed overnight urinary melatonin metabolite aMT6s from ordinary least squares linear regression models of natural logarithm-transformed daytime prolactin

Models	Parameter Estimate (aMT6s)	95% CI
Winter		
Crude model	0.028	-0.101, 0.156
†Adjusted Model	-0.007	-0.129, 0.115
Summer		
Crude model	0.008	-0.126, 0.142
†Adjusted Model	-0.033	-0.162, 0.097

† Adjusted for age, BMI, wake time, blood draw time, HOME score.

Table 6.3. Crude and multivariable-adjusted parameter estimates for creatinine-adjusted, natural logarithm transformed overnight urinary melatonin metabolite aMT6s from general estimating equation models of natural logarithm-transformed daytime prolactin

Models	Parameter Estimate (aMT6s)	95% CI
Crude model	-0.027	-0.111, 0.057
†Multivariable-adjusted model	-0.057	-0.140, 0.025
Effect modification by age		
Crude model		
Less than 25 years (‡n=82)	-0.240	-0.373, -0.106
25 to 39 years, inclusive (n=95)	0.085	-0.044, 0.215
Greater than 39 years (n=35)	-0.089	-0.241, 0.062
†Multivariable-adjusted model		
Less than 25 years (n=82)	-0.216	-0.351, -0.080
25 to 39 years, inclusive (n=95)	0.054	-0.080, 0.189
Greater than 39 years (n=35)	-0.062	-0.204, 0.080

† Adjusted for BMI, wake time, blood draw time, HOME score.
‡Total measures for each age category were: <25 years=135; 25-40 years=168; >40 years=57

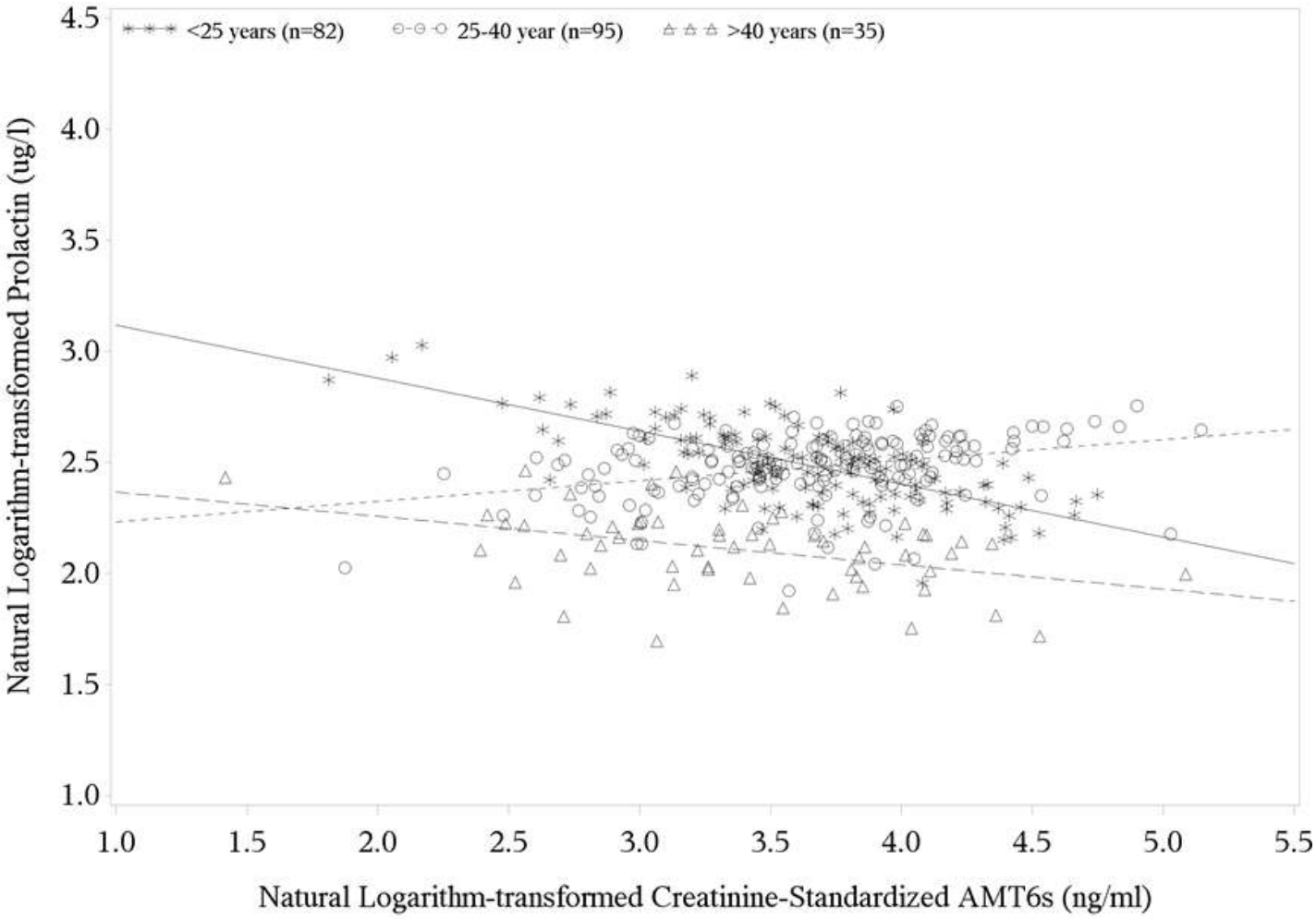

Figure 6.2. Effect modification of cross-sectional association between nocturnal aMT6s and prolactin by age. Data points are observed prolactin values. Total measures for each age category were: <25 years=135; 25-40 years=168; >40 years=57

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Chapter 7 Discussion

7.1 Summary of Findings

This dissertation investigates novel associations characterizing circadian regulation of human female reproductive signaling, focusing on precursors of breast cancer risk. Additionally, it supplements current sparse observational literature describing cross-sectional relationships between the endogenous nocturnal melatonin signal and circulating reproduction-related hormones. Of note is the finding, from objective 1, that women exposed to the highest levels of cumulative rotating shift work may be at risk of delayed menopausal onset. Secondly, we observed a moderately increased risk of earlier menopause among women who worked 20 or more months of rotating shifts during the preceding two-year interval. Findings from objective 3 indicate there was an inverse association between nocturnal melatonin output and subsequent daytime circulating prolactin among women less than 25 years of age. Main null results were the paucity of observed cross-sectional associations between nocturnal melatonin production and daytime circulating estrogen, progesterone (objective 2) and prolactin (objective 3) in the overall population. For the latter, there was no evidence of effect modification by diurnal preference. Similarly, we did not observe evidence of a cross-sectional association between nocturnal melatonin production and menstrual cycle length (objective 2).

Based on self-reported data ascertained at approximate two-year intervals in the NHS II, women who work the highest levels of cumulative rotating shifts have a greater risk of delayed menopause (adjusted HR excluding exposure prior to 1989: 0.76, 95% CI: 0.69-0.85). This is in line with findings from both NHS cohorts demonstrating a statistically significant increased risk of breast cancer among rotating shift workers with the highest levels of cumulative exposure[1,2]. Women working 30 years or more of rotating shifts from the NHS I cohort had a 34 percent increased risk (RR: 1.34, 95% CI: 1.04-1.78) of breast cancer, relative to non-rotating shift workers, while those from the NHS II who worked 20 or more years were observed to have an 79 percent increased risk (RR: 1.79, 95% CI: 1.06-3.01) of this outcome. In both cohorts, lower cumulative exposure was not statistically significantly associated with breast cancer, nor were corresponding effect sizes demonstrative of a dose-response, similar to that observed in our findings (Chapter 4; Table 4.2). This is perhaps indicative of a threshold effect or that women who work the highest levels of rotating shift work may be systematically different. These results,

together with our findings, suggest that delayed menopause could be on the causal pathway between lifelong rotating shift work exposure and breast cancer. However, this effect may be material only for select women who work the most intensive lifelong rotating shift schedules.

The overall approximate three percent increased risk of breast cancer observed with each additional year of menopausal onset[3], appears to support delayed menopause as a mediator between sufficient rotating shift work, and by extension, circadian disruption, and breast cancer risk. In accordance with our biological model, increased breast cancer risk is likely incurred via prolonged exposure to a hormonal environment conducive to unchecked proliferation of breast tissue, for which delayed menopause is a marker. The biological plausibility of later menopause, as well as other reproductive-related prognosticators of breast cancer such as parity[4], age at first birth[5] and breast feeding[4], have previously been attributed to increased lifelong exposure to endogenous reproductive hormones, particularly estrogens, producing a net increase in proliferatory pressure in breast tissue[6]. This is in line with multivariable-adjusted risk estimates for breast cancer among highest cumulative rotating shift work exposure levels for both premenopausal (RR: 1.34) and postmenopausal (RR: 1.36) women in the NHS I being similar to each other and that of the overall cohort. However, due to the small number of breast cancer cases among premenopausal women (n=14), the finding among this group was not statistically significant[1].

Some uncertainty stems from variation in cumulative rotating shift work definitions. Both of the above NHS breast cancer risk studies used number of years worked rotating shifts (i.e., number of years in which at least 3 nights, in addition to days and/or evenings, per month were worked). For the NHS I study, cumulative rotating shift work was ascertained from a one-time questionnaire item[1], while the number of years worked prior to 1989 was added to months worked in prior two-year intervals thereafter for the NHS II[2], a definition analogous to that used in objective 1. While superficially similar, unlike the effects of highest exposure to cumulative rotating shift work and breast cancer risk observed in NHS cohorts, we failed to observe as strong an indication of an association with menopausal timing when incorporating number of years worked prior to 1989 into our cumulative exposure measure. This indicates that the single measure of prior rotating shift years may not capture the same construct as the biennially updated months of rotating shift work measure (discussed further in 7.3.3.1).

Alternatively, at least some of the protective effect of working more than 10 years of cumulative rotating shift work excluding exposure prior to 1989, or more than 25 years including exposure prior to 1989, may be artifactual. This is attributable to informative censoring, predominantly due to HRT onset, juxtaposed with the temporal distribution of the highest cumulative rotating shift work exposure level membership and temporal trends in incidence of premenopausal HRT and natural menopause over follow-up. It is possible that a portion of women comprising this highest cumulative exposure group may have been less likely to have natural menopause by virtue of women who would have been more likely to have had this outcome being previously censored while occupying a lower cumulative exposure level. However, the impact of this potential bias is difficult to estimate due to the challenge of predicting future exposure and outcome status among those censored. See Appendix 1 for a more detailed discussion of this potential source of bias.

The moderate, yet statistically significant multivariable adjusted 8% increased risk of menopause (HR: 1.08; 95% CI: 1.01-1.15) for women working 20 or more months in the preceding two years is more difficult to interpret. It could be that once the perimenopausal progression has started, the circadian disruptive component of shift work exposure no longer has material bearing on menopausal onset. The statistically significant risk estimate may be explained by residual confounding, discussed below (7.3.2). Together, this suggests that while long-term chronic circadian disruption may moderately increase breast cancer risk via reproductive signaling involved in delaying reproductive senescence, intermittent or shorter intervals of intensive rotating shift work may not, particularly when this exposure is temporally proximal to menopause.

A finding of note from objective 3 was the negative linear association between nocturnal melatonin output and daytime circulating prolactin observed in women under the age of 25. There is evidence that aging modifies the circadian regulation of various endocrine functions[7] and both endogenous melatonin[8] and prolactin[9] production has been observed to decline over the reproductive lifetime. In the production of pineal melatonin, this is perhaps partially due to the decreasing impact external cues may have on the master clock in the suprachiasmatic nucleus (SCN)[10]. In addition to other factors, pituitary prolactin is positively regulated by gonadotropin releasing hormone (GnRH) and this potentiation is smallest during the follicular phase[11,12]. The effects of melatonin on hypothalamic targets have not been fully elucidated in humans, yet

exogenous melatonin has been reported to suppress GnRH gene expression *in vitro*[13]. Expression of sex steroid hormone receptors on the pineal gland and abnormal melatonin levels associated with reproductive disorders[14] suggests that the mechanisms leading to increased variability in menstrual cycle length may also impact melatonin secretion and any downstream regulatory effect of this hormone on pituitary prolactin. There is indication that mean menstrual cycle length increases with variability at both extremes of the reproductive lifetime[15]. Had younger women had longer cycles on average, they would have spent proportionately more time in the follicular phase and therefore been more likely to have had reduced prolactin attributable to the GnRH signal. If melatonin truly negatively regulates prolactin, be it through GnRH gene expression, other targets on the HPG axis, or directly via the anterior pituitary, systematically longer menstrual cycle lengths experienced by the youngest age group is one possible explanation for the observed effect modification. Alternatively, there could be a shift in the regulation of prolactin by the melatonin signal through yet undiscovered morphological changes in central targets (i.e., MT1 receptor expression) with age. However the complexity of these central processes renders the validation of such hypotheses challenging.

Null findings from objective 2 are supported by a few prior quasi-experimental[16,17] and observational[18-20] studies that have largely failed to uncover evidence of variation in day time circulating steroid hormone levels by nocturnal melatonin production. The validity of these findings is, however, contingent on further research leveraging more precise, unbiased exposure and outcome measures and larger, representative study samples. Furthermore, important temporal relationships, such as overall change in the nocturnal melatonin signal in response to patterns of rotating shift work or other circadian disruptive stimuli, are ignored in cross-sectional designs. Confounding due to the periodic nature of mammalian endocrine signaling exemplified by circulating sex steroids over the menstrual cycle, or the circadian rhythmicity of circulating melatonin or prolactin, are potential sources of bias. While considerations were made for some of these factors, such as measuring day time sex steroid hormones during the luteal phase in objective 2, or attempting to measure day time prolactin from morning blood draw in objective 3, we were unable to take into account all, underscoring improvements that can be made in future research.

The remainder of this chapter explores the largest potential threats to the validity of findings discussed in chapters 4 through 6, highlights the impact that this and future related research may

have on policy changes impacting women's health, and concludes with recommendations for further investigation of these topics.

7.2 Biological Model

We acknowledge that the biological model underpinning the hypotheses tested in this dissertation may be incorrect, or, more likely as some literature suggests, does not encompass all mechanisms by which circadian disruption, via melatonin, may impact breast cancer risk. Our model was based predominantly on the Light at Night (LAN) hypothesis. Initially, biological plausibility focused on central prolactin and ovarian estrogen production: recurrent suppression of nocturnal melatonin by light at night was postulated to contribute to a chronic hormonal milieu conducive to unchecked proliferation of breast tissue resulting in oncogenesis[21]. Later revisions focused increasingly on direct mechanisms of the pineal hormone, presumably due to a lack of evidence in humans showing inverse relationships between melatonin and circulating endogenous reproductive hormones[22]. These included the innate anti-oxidant action of melatonin and, most notably, its role as an estrogen antagonist in target tissues[22-24]. Foremost among the latter was evidence of melatonin reducing invasiveness of estrogen-responsive human breast cancer cell lines through estrogen receptor potentiation and as a negative regulator of aromatase activity, potentially limiting local estrogen production[25]. Added to this was melatonin's potential role in the synchronization of the master clock of the SCN with peripheral clock genes involved in cell cycle control, and in turn, regulation of apoptosis in breast tissue[22-24]. No anti-cancer mechanisms of melatonin have been validated in vivo in humans and the results from this dissertation cannot conclusively support or refute a role for melatonin as a modulator of the hypothalamic pituitary gonadal axis (HPGA) leading to circadian regulation of central prolactin or ovarian hormone production. As such, increased breast cancer risk attributable to circadian disruption mediated through melatonin signaling may be largely independent of the central reproductive targets delineated in our proposed biological model (Chapter 2; Figure 2.1), despite evidence from animal models, particularly that from seasonal breeders, to the contrary. This may account for null findings in this work, although it does not explain the positive findings.

7.3 Limitations

7.3.1 Measurement validity

A measurement-related issue potentially obscuring interpretation of findings, therefore leading to information bias, is the validity of proxy measures representing an underlying construct. This is analogous to the idea of construct validity, which has been defined as "the degree to which a test measures what it claims, or purports, to be measuring"[26]. While construct validity has traditionally been applied to the evaluation of psychometrics, it is used here similarly to denote the extent to which a proxy measure captures an exposure (or outcome) it is intended to capture.

7.3.1.1 Objective 1: Rotating shift work as a marker for circadian disruption

Rotating shift work has been conceptualized as a proxy or marker for circadian disruption exposure. For quantitative applications, a "representation is optimal when its covariations with optimal representations of other constructs provide a reasonable estimate of parametric values"[27]. While it has become a popular proxy for circadian disruption, the extent to which circadian disruption exposure covaries with rotating shift work exposure is not well documented. Chiefly responsible is the complexity encountered in defining, detecting and quantifying circadian disruption exposure. If circadian disruption is to be defined, as declared in Chapter 2, as "the alteration of function of biological systems due to interruption of governing regulatory processes exhibiting 24-hour periodicity" then direct quantification of this change of function, or immediate precursor thereto, would serve as a measurement paradigm. One such measure may be the extent to which the periodicity of pacemaker neurons of the SCN deviates in response to disruptive stimuli, which, from a measurement perspective, is perhaps most easily approximated by corresponding change in timing and duration of the nocturnal melatonin acrophase. However, because local clock-regulated processes, entrained to the SCN, do not all operate at the same time, or may receive moderating entrainment from other circadian cues (e.g., timing of food intake and the gut), disruption that results in a phase advance of SCN neuron activity, rather than a phase delay, may be differential across peripheral targets. Or it may be that the threshold for meaningful circadian disruption exposure varies across these targets. In the context of breast cancer, as touched on above, given the predication that circadian disruption does increase risk,

the mechanism by which this occurs has yet to be delineated. It may be that measures of downstream quantities, such as change in timing of clock gene expression in breast tissue, are a better marker of the impact of circadian disruption on breast cancer risk than those capturing changes in melatonin secretory patterns. Regardless, epidemiologic studies rarely have access to such biological data, and it is likely for this reason more than any other that shift work has become the de facto proxy measure for circadian disruption for such research.

In 2007, the International Agency for Research on Cancer (IARC) declared shift work to a "probable carcinogen"[28]. Since that time, the responsible working group has developed guidelines with the aim of improving the validity of shift work exposure as a proxy for circadian disruption in cancer epidemiologic studies. Key "domains" are:

- *Shift system:* Start time of shift, number of hours per day, rotating or permanent, speed and direction of rotating system, regular or irregular.
- Years on particular non-day shift schedule and cumulative exposure to the shift system over working life
- *Shift intensity:* time between successive work days on the shift schedule

The above are based on biological plausibility given the available evidence. For example, capturing "speed and direction of rotating system" was recommended based on evidence that changing shift schedules resulting in a phase advance rather than a phase delay, and changing shift patterns more frequently, is more disruptive. For objective 1, while Nurses' Health Study data enabled the derivation of exposure measures based on cumulative rotating shift work, arguably satisfying the second criterion, we were unable to incorporate data pertaining to "shift system" or "shift intensity". Empirically, it has been demonstrated that rotating shift work is more strongly associated with adverse reproductive cancer-related outcomes than exclusive night shifts alone[29], suggesting that the former exposure, used for objective 1, is of greater relevance on gonadal activity and menopausal timing.

In quantitative analyses, a proxy measure with poor construct validity can lead to bias through misclassification, discussed below (7.3.3). That is, if rotating shift work exposure does not covary sufficiently with effective circadian disruption, women with higher levels of cumulative rotating shift work may have experienced less chronic circadian disruption exposure than some

of their counterparts, and vice versa, leading to non-differential misclassification. As the proxy measure is defined identically and measured prospectively for the entire study sample, differential misclassification across outcome status is deemed unlikely, given there is no reason to suspect that women achieving and reporting cessation of menstruation would be more or less likely to erroneously report cumulative rotating shift work exposure than those who did not. Additionally, obfuscation of the main exposure construct may introduce bias indirectly. An example is given using the potential mediator/confounder of stress discussed below (7.3.2.1). Given data availability, it may have been inappropriate to include a variable(s) representing stress in our multivariable regression model, based on the justification that stress could act as mediator between circadian disruption and menopausal timing. Women who work more rotating shifts may experience increased stress, potentially contributing to premature ovarian failure. The increased stress, however, may be due to elevated psychological and physical fatigue associated with working regular rotating shifts, rather than circadian disruption or its resulting biological effects. In this scenario, if we are only interested in the effect of the circadian disruption component of rotating shift work on menopausal timing, it could be argued that stress may be appropriately conceptualized as a potential confounder, validating its candidacy for inclusion in the multivariable model. Construct validity of an exposure measure leading to ambiguity between confounders and mediators has been encountered elsewhere, such as the example of the impact of self-rated health, accounting for negativity or neuroticism, on physiological outcomes.[30,31]

7.3.2 Residual confounding

While there has been considerable debate regarding how to detect and adjust for confounding in epidemiologic research over the evolution of the field[32-34], first principles dictate that a confounder is a factor that is associated with both an exposure of interest and, additionally, is an independent predictor of the outcome[35]. That is, confounding occurs when an additional unmeasured, or inadequately measured or modeled, factor, or set of factors, covary with both the exposure and outcome within the study sample, where these additional variables are not on the causal pathway between them (i.e., a mediator). In the following section, potential sources of residual confounding that may have biased results presented in the preceding manuscript chapters are discussed.

7.3.2.1 Objective 1: stress and depression

While the NHS collects a wide range of self-reported data on reproductive-related factors that could be accounted for, unmeasured stress and/or depression may have confounded the association between rotating shift work and menopausal timing. Chronic stress and depression could potentially be independent predictors of menopausal timing via exerting opposing pressure on central reproductive processes regulating ovarian activity, thus both potentially fulfilling one of the key criteria of a potential confounder in a longitudinal observational study[33]. Depressed women may exhibit altered central regulation of ovarian function mediated via opioid signaling, resulting in hypothalamic amenorrhea[36,37] in severe cases. Chronic stress may affect this outcome through crosstalk between the hypothalamic-adrenal axis and the HPGA[38]. The candidacy of these factors as potential confounders is dependent on whether women who work more cumulative rotating shift work are more likely to be afflicted by depression or stress, that is, whether there is an imbalance of women with these conditions across exposure levels. This could not be verified in our study sample for objective 1, but it is plausible that shift work is associated with depression and stress. Circadian disruption, and particularly night work, has been associated with altered cortisol profiles, a characteristic of both stress and depressive disorders[39].

It is possible, however, that either stress or depression may have mediated the association between rotating shift work, as an indicator of circadian disruption, and menopause. As such, had these factors been measured, it may have been inappropriate to adjust for them in our multivariable models[40]. In the context of mediation, an exposure can be causally related to the outcome either directly, or indirectly by acting through one or more mediating factors. In quantitative analysis, the sum of direct and indirect pathways represents the overall effect of the exposure on the outcome. As such, adjusting for a factor comprising an indirect pathway will risk attributing some or all of the dependent variable variance to this mediating factor, limiting the ability to make inference about the overall exposure effect from the corresponding multivariable-adjusted estimate. Had working more cumulative rotating shifts resulted in elevated stress or depression through a mechanism related to circadian disruption, neither of these variables should have been included in our multivariable models.

7.3.2.2 Objective 2: menstrual cycle and periodic hormone measures

Though we attempted to restrict all steroid hormone measures to the luteal phase, the least variable of menstrual phases, and considered luteal day as a potential confounder, previously reported variation in luteal phase length allows for the potential for significant variation in our study sample. While most observations suggest an average luteal phase length of approximately 13 days, the luteal phase has been observed to range from 8[41] to as many as 20[42] days in normal cycling women. Both estradiol and progesterone secretion rises until around the mid-luteal phase and declines thereafter[43]. As such, variation in circulating estradiol or progesterone due to variation in luteal phase length may have been insufficiently accounted for by inclusion of luteal day alone, contributing to residual confounding. Observations that menstrual cycle length[41,44] is more variable among younger women increase the likelihood of this being relevant to our objective 2 study sample, the age range of which was 18-22 years.

7.3.3 Measurement error

Information bias in epidemiologic studies commonly arises due to measurement error. Measurement error has been labeled a source of "misclassification bias" where true exposure or outcome status has been misclassified[45]. Misclassification can result from numerous causes and commonly arises from recall error in self-reported data or imperfection of the measurement tool in the quantitative classification of biological specimens. Misclassification can either be differential or non-differential, the former case occurring when the probability, direction or magnitude of measurement inaccuracy of the exposure is dependent on outcome status, or vice versa. In the case of non-differential misclassification, measurement inaccuracy is equivalent across the entire study sample. In the most straightforward case of a dichotomous exposure and outcome, differential misclassification can bias effect estimates in either direction, while non-differential misclassification will always bias results toward the null. Beyond dichotomous categorizations, non-differential misclassification can lead to bias in either direction[46]. While we have no reason to suspect differential misclassification was a significant source of bias in any of the results discussed in this dissertation, it is plausible that non-differential measurement error in our primary factors of interest impacted some of our findings, possibly accounting for null or smaller than expected effect sizes. It should also be noted that measurement error in the

classification of confounding variables may have contributed to residual confounding in adjusted estimates.

7.3.3.1 Objective 1: rotating shift work (primary exposure) and menopause (outcome)

Rotating shift work: Though number of years worked rotating shifts was found to be associated with breast cancer risk for women among the highest exposure categories in the NHS I, and when incorporated with biennially updated measures for the NHS II, we failed to observe as strong an indication of an inverse association with onset of natural menopause when years of rotating shift work prior to 1989 was included in our cumulative exposure definition. While at face value, this suggests that delayed menopause does not mediate increased breast cancer risk attributable to rotating shift work, another explanation may be attenuation of effect size due to misclassification. The longer recall and the increased potential for ambiguity inherent in the questionnaire item querying number of years worked rotating shifts prior to1989 (i.e., number of prior years in which at least three nights per months in addition to days or evenings), relative to rotating shift work exposure measured thereafter (i.e., number of months in which at least three nights in addition to days or evenings since last questionnaire), may have resulted in a higher degree of misclassification. Differential measurement error is unlikely as the one-time assessment of number of years worked rotating shifts was ascertained prior to breast cancer diagnosis in the NHS I, or menopause and breast cancer diagnosis in the NHS II. It is more plausible that non-differential misclassification of number of years worked rotating shifts prior to 1989 across the menopause outcome impacted our results, potentially contributing to attenuated and null findings across the highest cumulative exposure levels when incorporated into the cumulative rotating shift work measure. If non-differential misclassification was less prevalent across breast cancer outcomes in prior NHS I and II studies, this may explain the discrepancy between findings. However, it is not clear why non-differential misclassification would have occurred across menopause, yet not breast cancer, outcomes. As discussed directly below, there is a greater likelihood of measurement error for the menopause outcome than for that of breast cancer, the latter of which was confirmed pathologically. Thus, differences in the strength and certainty of effects of cumulative rotating shift work, including years of exposure prior to 1989, on hazards of menopause and breast cancer, might be attributable to increased non-differential

misclassification of menopause. As many more women achieved menopause in the NHS II than did breast cancer, another explanation for the discrepancy in the association between cumulative rotating shift work and these two outcomes is that the smaller number of women achieving the latter were less representative of women eligible for these outcomes in the underlying target population. That is, perhaps women who were diagnosed with breast cancer after 20 or 30 years of rotating shift work are not generalizable to the larger group of women achieving menopause.

Menopause: In Chapter 4, evidence was presented supporting self-reported age at menopause in the NHS II as at least a moderately reliable indicator of timing of menopausal onset. It is conceivable, however, that the NHS criterion for achieving menopause ("Age natural periods ceased") used in data collection may have led to misclassification. Unlike the widely accepted World Health Organization (WHO) definition of menopause which requires periods to have ceased for 12 months[47], the NHS II definition is limited to querying whether a woman's periods had ceased at the time the biennial questionnaire was completed. This may have contributed to premature reporting of menopause in cases where the perimenopausal progression resulted in the introduction of cycle irregularity marked by intermittent, uncharacteristically long cycles. As shift work exposure has been associated with having both shorter and longer menstrual cycle lengths in the NHS[48], it is possible that this misclassification may have been differential. If women working more rotating shifts were prone to premature reporting of menopause, this could have attenuated reported effect estimates.

7.3.3.2 Objectives 2 and 3

The validity of reproductive hormone measures comprising the exposures and outcomes for objectives 2 and 3 is uncertain. For example, several studies that have addressed the reproducibility of serum estradiol measurements made over time in premenopausal women have found that a single measurement of estradiol does not accurately reflect a woman's long-term average blood concentration, unlike single measurements of androgens[49-51]. While we were interested only in quantifying associations at a single time point, this is perhaps indicative that our hormone outcome measures, particularly estrogen, may have been prone to random variability that may have been more transparent given repeated measures over a short time interval. Intraclass correlation has been observed to be particularly low for repeated measures of

estradiol[52], which may partly explain why we did not observe an association with overnight urinary melatonin, and why model fit was observed to be particularly poor for this association (data not shown). Related measurement error for steroid hormone outcomes may have stemmed from lack of standardization of menstrual cycle phase[50]. Obtaining all measures of estradiol and progesterone during the luteal phase and treating luteal day as a potential confounder may have mitigated this variability at least somewhat, though perhaps not completely.

7.4 Statistical Power

Overall, it is unlikely that the interpretation of the findings reported in this dissertation would be materially affected by Type II error (failing to reject the null hypothesis given it is false).

7.4.1 Objective 1

It is unlikely that objective 1 was affected by insufficient study power. After baseline exclusions, our study sample comprised over 80,000 women, over 25 percent of whom had the outcome by the end of follow-up. Ninety-five percent confidence intervals about hazard ratio estimates were narrow, allowing us to detect a statistically significant eight percent increased risk of menopause among women who had worked 20 or more months of rotating shifts in the previous questionnaire period. A larger study sample would not have materially changed reported results, or their interpretation. It could be argued that an abundance of study power might lead to over-interpretation of small, statistically significant effect sizes at an alpha level of 0.05. Alternatively it may facilitate due consideration of smaller, though potentially interesting, effects that may have been biased towards the null due to misclassification or other sources of bias.

7.4.2 Objectives 2 and 3

While the study sample for objective 2 was relatively small, it is unlikely that our findings would have been altered had it been larger, given observed estimated effects. Weak crude linear correlations (Chapter 5: Figure 5.2) and respective multivariable-adjusted parameter estimates

characterizing linear change in outcomes of menstrual cycle length, circulating estradiol and progesterone from regression models indicates that a single measures of overnight nocturnal melatonin metabolite, aMT6s, is not likely to be associated with these outcomes. A possible exception, the strongest estimated multivariable-adjusted effect from objective 2, was that of the aMT6s effect on progesterone. The parameter estimate indicated that a 10 percent increase in aMT6s would be associated with less than a two percent change in following daytime steroid hormone levels. However a larger sample size may have increased stability of our multivariable regression models, allowing for increased confidence in assessment of confounding and resulting adjusted aMT6s effects, particularly for the estradiol outcome.

For objective 3, the estimated overall multivariable-adjusted effect of nocturnal melatonin indicated that a 10 percent change in aMT6s would have been associated with only about a half a percent change in daytime prolactin, should the null hypothesis be rejected. Though the probability of a type I error (rejecting the null hypothesis, given it is true) for this association was the lowest out of all reported non-statistically significant effects across objectives 2 and 3, it is unlikely that increased sample size alone would be sufficient to evince a meaningful association. Prior related literature[18-20] reporting similar null cross-sectional associations between nocturnal melatonin and day time reproductive hormone levels further supports that greater statistical power alone would likely have not substantially changed our interpretation. Our study sample did appear to be sufficiently powered to detect the reported effect modification by age: 95 percent confidence intervals bounding melatonin parameter estimates among those under 25 years did not overlap with confidence intervals for the corresponding melatonin parameter estimate for the middle age category. It is possible, however, that our sample was insufficiently powered to estimate the effects of nocturnal aMT6s on prolactin in the oldest age group (i.e., ≥ 40 years).

7.5 External Validity

7.5.1 Objective 1

The NHS II cohort, a large national cohort of women recruited from the 11 most populous states between the ages of 25 and 42 in 1989, could be considered a relatively representative sample of

women who would be susceptible to chronic changes in reproductive biology associated with circadian disruption attributable to night work. Though NHS participation is limited to nurses, "health care and social assistance" was reported to comprise the largest number of night-working women of all labour sectors according to Statistics Canada's 2005 Survey of Labour and Income Dynamics[53]. Despite this, departures from generalizability could have occurred due to cohort effects potentially obscuring the associations under study, predominantly the waxing and waning of premenopausal HRT incidence due to radical changes in the prescribing of these medications around the beginning of the millennium. As discussed previously, this may have contributed to biased estimation of relative cause-specific hazards of natural menopause across cumulative rotating shift work exposure. Other departures may stem from limited representation of age and ethnicity. If circadian disruption experienced with shift work was particularly harmful for younger women, then the biennially assessed cumulative exposure captured among a sample with a mean age of approximately 36 years at baseline would not reflect this hypothetical crucial exposure window. While number of years of rotating shift work prior to the 1989 enrollment was captured, the age at which this exposure occurred was not recorded. There is insufficient evidence to support such a crucial exposure window for the impact of circadian disruption on menopausal timing and breast cancer risk. That stated, younger rotating shift workers are more likely to be nulliparous. First full-term pregnancy is known to be protective against breast cancer[54]. In addition to lifelong exposure to steroid hormones such as estrogens, this has been attributed to changes in lobular composition during pregnancy that potentially pinpoints a window of general heightened susceptibility for younger nulliparous women[54,55]. If circadian disruption does increase breast cancer risk via estrogenic signaling, this suggests the possibility of a synergistic impact on breast cancer risk with other reproductive factors in younger women. In addition, findings from objective 3 indicate that the regulation of reproductive function by melatonin, and in turn, the circadian system, may not be entirely homogenous across age. As such, the youngest women exposed to routine circadian disruption may have been underrepresented in our study sample. Secondly, the NHS II cohort is predominantly white (>90%)[56], therefore, our findings may not be generalizable to more ethnically diverse populations. While non-western countries have historically exhibited lower breast cancer risk[57], conclusive evidence indicating that biological differences due to ethnicity-associated genetic admixture, or behavioral heterogeneity due to culture, would significantly modify the association between rotating shift work and menopause, is lacking.

7.5.2 Objectives 2 and 3

It should be acknowledged that study populations for objectives 2 and 3 were initially recruited for research that, although related, resulted in samples perhaps not completely generalizable to all healthy cycling women. The largest potential threat to external validity for objective 2 is the narrow age range of our study sample. The study sample used for the cross-sectional investigation of the linear association between nocturnal melatonin production and menstrual cycle length, and day time circulating estradiol and progesterone comprised women between the ages of 18 and 22 years. While it has been previously speculated that ovarian activity may be more susceptible to disruptive circadian stimuli earlier on in the reproductive lifetime, it could be that, for the majority of women, this exposure window does not fall within this narrow age range. This may partly explain why we saw no evidence of an association between nocturnal melatonin and ovarian activity-related endpoints among these younger women. It could simply be that the women comprising the study sample for objectives 2 and 3 did not experience sufficient circadian disruption for associations between nocturnal melatonin and daytime reproductive hormone levels, or menstrual cycle metrics, to have been evident. Null findings from similar cross-sectional associations among nurses[18-20], however, question the validity of this hypothesis. Alternatively, it may be the variability in change in the nocturnal melatonin signal over consecutive nights, rather than of output from a single night (or average output from three nights as in objective 3), that may materially impact reproductive function.

7.6 Strengths

Key strengths of this research include the large study sample of women with a high prevalence of shift work contributed by the NHS II cohort, affording ample study power for the investigation of the association between rotating shift work exposure and menopausal onset.

For objectives 2 and 3, overarching strengths are the comprehensive data collection facilitating adjustment for potentially confounding factors. Together with the larger sample size relative to prior quasi-experimental designs, this facilitated the validation of previous findings on the association between nocturnal urinary melatonin and reproduction-related hormones. A specific strength was the use of overnight, as opposed to first-morning void, urine collection that has been

suggested to potentially better capture complete nocturnal central melatonin production[58]. For objective 2, the measurement of steroid hormone concentrations from the luteal phase, and the ability to adjust for luteal phase progression by the incorporation of luteal day, could be considered a key strength. While there may be a margin of error in how well luteal day accounts for variation in ovarian steroid hormone production attributable to menstrual phase progression, resulting in residual confounding, it is an improvement over prior studies that did not collect such information[20]. A strength for objective 3 was the variation in participant age across the premenopausal range, allowing the novel investigation of effect modification by this factor. The use of repeated urinary melatonin measures, over the course of three consecutive nights, potentially minimized bias contributed by spurious outliers in the measurement of this quantity. Although we did not find any evidence of effect modification by Horne-Östberg morningness-eveningness score, the collection of these data allowed for the investigation of the impact of diurnal preference on the association between the melatonin signal and reproductive function, which is of growing interest in related epidemiologic research[59].

7.7 Public Health Significance

Findings from this research will be of interest in the study of female reproductive disorders and fertility related to gonadal function and in identifying higher risk groups for circadian-disruption related cancers and potentially other diseases. There is evidence that a substantial proportion of Canada's work force is comprised of women who work rotating or exclusive night shifts[53]. Shift work-related descriptive statistics from Statistics Canada's 2005 Survey of Labour and Income Dynamics indicated that approximately 12 percent of employed Canadians worked rotating shifts, while six and 2.3 percent worked exclusive evenings and night shifts, respectively. Labour sectors with the most night shift-working Canadian women include "health care and social assistance", followed by "trade" and "accommodation and food services", together comprising almost 900,000 women. With the modest overall increase in night shifts worked between the mid 1990s and 2000s[53], it is likely that these three sectors alone currently account for in excess of one million night shift working Canadian women. If a large component of these women are at greater risk for breast cancer or other reproductive-related adverse events it is crucial that the etiology of

such health outcomes among these women be further delineated for more effective prevention and management. This and future-related work are important steps in attaining that goal.

It is arguable that there is insufficient evidence on which to base policy restricting night shift work for the prevention of adverse reproductive outcomes and related diseases such as breast cancer. Yet at least one country has based public policy decision making based on findings in this field. In 2008, largely based on the IARC monograph identifying shift work as a "probable carcinogen", Denmark decided to award compensation to women contracting breast cancer after a long history of shift work exposure[60]. Though such a decision may be considered premature given the overall evidence, one policy recommendation could be mandatory provisioning of information by employers summarizing current evidence on risks of adverse reproductive related outcomes in women who have worked rotating shifts, particularly that for breast cancer among women working many years of cumulative exposure. Policy recommending abstainment from, or active restriction of, night work is contingent on future research identifying high risk groups that are particularly susceptible.

7.8 Future Directions

In light of our overall findings, a few recommendations for future research may be offered.

First, in line with IARC recommendations discussed above[28], future observational studies assessing associations between markers of circadian disruption and various health outcomes are best served employing as accurate and detailed exposure information as possible. For night work, validation of previous prospective studies suggesting a significant increased risk of breast cancer, and our findings of an association between rotating shift work and delayed natural menopause, require additional information such as those covered under the domains of "shift system" and "shift intensity". Due to the lack of precision resulting from variability of perimenopausal progression, it is recommended that some degree of retrospective verification be incorporated in prospective ascertainment of menopausal timing, as exemplified by the WHO definition.

It may be prudent to capture variation in night shift work intensity by age. There is evidence that women become less sensitive to circadian disruptive stimuli with age. This is supported by consistent observations of declining pineal melatonin output that may be linked to reduced

photoreception leading to impaired entrainment by photoperiod, and accumulation of deficits in neurotransmission between the SCN and pineal gland[10]. Specific gene products involved in the attenuation and reduction in plasticity of the central circadian signal with age have already been identified in animal models[61]. It could be that a large portion of what predisposes women who have worked ten or more years of rotating shifts to later menopause, or 20 and 30 or more years of this exposure to increased breast cancer risk, in the NHS, is not simply the amount of accumulated exposure, but that sufficient levels occurred before age-related factors attenuate the ability of disruptive circadian stimuli to sufficiently compromise biological function. The observation from objective 3 that nocturnal melatonin was inversely related to daytime serum prolactin among women 24 years and younger might be interpreted as being in tenuous support of a window of increased susceptibility to circadian disruptive stimuli earlier on in the reproductive lifetime.

Analogous to age of exposure, other modifying factors may explain more moderate observed effect sizes for cumulative rotating shift work on outcomes such as menopausal onset and breast cancer. It has been speculated that certain women are innately more susceptible to circadian disruption imparted by night work than others. Related mechanisms currently under investigation include genetic variation of clock genes, the expression of which follow a circadian pattern that can be impacted by circadian stimuli (i.e., chronodisruptors), and epigenetics moderating the degree to which these genes impact function[62]. Clock genes have been implicated in key cellular processes such as the cell cycle and metabolism, both of which have implications in reproduction and cancer etiology. The identification of sufficiently penetrant clock gene variants or distinct, repeatable patterns of epigenetic modification of molecular mechanisms involved in modifying or relaying the central circadian signal may uncover novel markers by which gene-environment interaction hypotheses can be tested in the identification of subpopulations at elevated risk from circadian disruption-inducing exposures such as night work.

In the meantime, observational studies may include existing factors that potentially mark susceptibility to circadian disruption-inducing exposures. One example is diurnal preference, likely underpinned by genetic variation and epigenetics. Though we did not observe effect modification of the cross sectional association between nocturnal melatonin production and daytime prolactin in objective 3, this factor is starting to be investigated as an effect modifier for cancer risk associated with night work exposure, largely based on observations that a morning

preference is associated with more difficulty adjusting to night work schedules. There has been at least one study, conducted among the Danish military that found that increased breast cancer risk associated with working nights was elevated among those with a morning preference[59]. The further study of these factors may identify high risk groups that would be effectively targeted by policy restricting the most disruptive night work schedules among these women. Querying diurnal preference in future data collection for new and existing large occupational cohorts that also assess night work exposure, such as the 2009 update to the NHS II questionnaire, is recommended. Validation of self-reported diurnal preference queried with tools such as the Horne-Östberg morningness-eveningness questionnaire, with biological data such as the onset of nocturnal peak melatonin production[63], within a subsample of future epidemiologic study samples, is additionally recommended.

Future investigation of the relationship between the circadian signal and reproduction-linked hormones should employ repeated measures over time where possible. Our findings from objectives 2 and 3, along with those from previous work assessing associations between nocturnal melatonin and day time circulating reproduction-linked hormones suggest that cross-sectional designs using single exposure and outcome measures are of limited utility in characterizing circadian regulation of reproductive function. It may be that women who exhibit elevated temporal variability in nocturnal melatonin production, either representing increased exposure, or sensitivity to circadian disruptive stimuli, might demonstrate the greatest variability in these reproductive endpoints. While objective 3 used multiple measures of nocturnal melatonin over consecutive nights, and for both summer and winter data collection sessions for a large proportion of the study sample, the intervals at which these measures were taken did not allow for the investigation of this potentially more interesting hypothesis. Longitudinal designs, attempting to characterize the association between nocturnal melatonin production and steroid hormone outcomes might be more informative if incorporating multiple measures across menstrual cycle phases, and across multiple cycles. For prolactin, the production of which demonstrates circadian periodicity, multiple measures over the course of a few days may be sufficient as it does not appear to vary materially over the menstrual cycle. Obtaining multiple hormone measures will, however, increase study time, resource requirements and participant contact, limiting feasibility.

Some of these feasibility issues may be mitigated by measuring hormone levels from biological markers, as was done for melatonin with the urinary metabolite, aMT6s, for objectives 2 and 3. For these objectives, all outcome hormone measures were obtained from blood draw, yet it has been suggested that assaying estrogen from urinary metabolites may provide more stable, representative values[64]. The procurement of urinary metabolite measures would be less invasive, perhaps augmenting participation. It has been noted that employing multiple measures of reproductive hormones that take into account natural temporal variability should significantly improve accuracy[52], and bolster study power through reduction of non-differential misclassification arising from measurement error.

7.9 References

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Appendices

Appendix 1: Investigation of informative censoring by HRT onset and induced menopause

As stated in Chapter 3, section 2.4.3.2, cause-specific hazards are considered unbiased by competing outcomes when the probability of these outcomes are independent of one another. This chapter explores potential dependency across the event of interest, natural menopause, and competing outcomes of premenopausal hormone replacement therapy (HRT) onset and induced menopause, which account for the majority of right censored observations over follow-up in our main effect cause-specific hazard models summarized in Chapter 4: Table 4.2. Additionally, it attempts to provide an alternative explanation that may account, at least in part, for the magnitude of the protective cause-specific effect observed for the highest cumulative rotating shift work exposure level. The following discussion focuses on cumulative rotating shift work exposure excluding years worked prior to 1989 as this definition produced the strongest observed protective effect against menopausal onset, potentially indicative of greater susceptibility to this source of bias.

Distribution of induced menopause and cumulative rotating shift work over follow-up

Dependency across outcomes exists when censoring is informative, that is, when individuals with identical covariate information have unequal probabilities of being censored, at any time *t*, regardless of the reason for being censored[1]. By extrapolation, such dependency across outcomes can arise when a covariate that is associated with the event of interest (i.e., natural menopause) is additionally associated with the probability of being censored. Unequal distribution of censoring across cumulative rotating shift work categories can thereby be indicative of dependency across competing outcomes, potentially leading to bias of corresponding cause-specific effects on the hazard of natural menopause (Chapter 4:Table 4.2; Table A.1a below). Figure A.1 depicts the distribution of the major competing outcomes of premenopausal HRT onset over follow-up. For the cumulative rotating shift work definition including years prior to 1989, for which we observed a weaker multivariable-adjusted effect of

shift work on time to natural menopause, there is minimal variation in distribution of either HRT onset or induced menopause across exposure levels. The largest proportion delta is observed between consecutive 73-96 and 97-120 month categories for premenopausal HRT. However, this only constitutes a maximum approximate 10% change across these exposure categories. The largest percent change between extreme proportions across the rotating shift work excluding years prior to 1989, HRT across lowest (referent) and highest exposure levels, is almost two-fold (approximately 19%). This is perhaps suggestive of an implicit dependency between HRT onset and natural menopause.

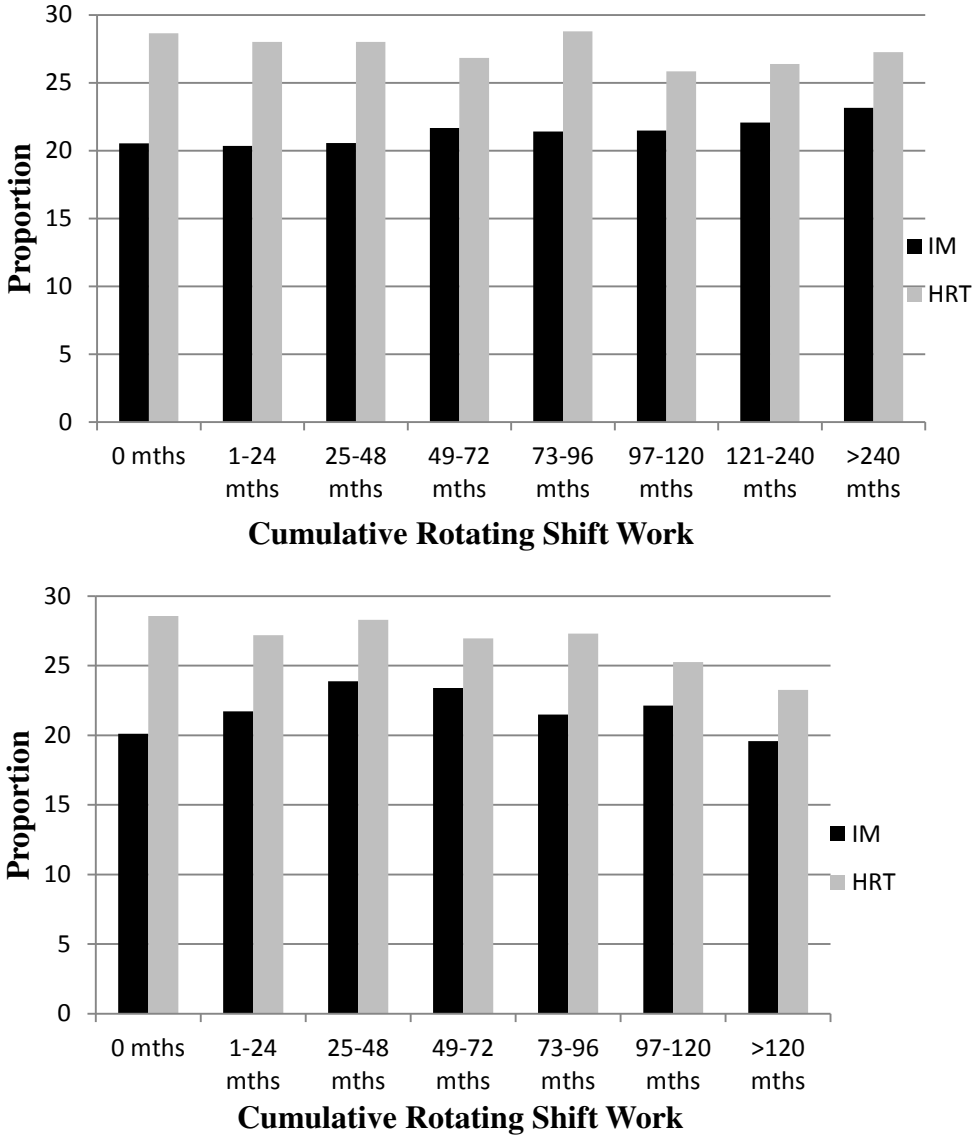


Figure A.1. Proportion of induced menopause (IM) and hormone replacement therapy (HRT) onset by cumulative rotating shift work (top: including years worked prior to 1989; bottom: excluding years worked prior to 1989) over entire follow-up (1993-2009).

Cause-specific effects of cumulative rotating shift work on competing outcomes

The distributions depicted in Figure A.1 do not take into account timing of competing events over follow-up, nor observations that would have been censored at co-competing events (e.g., HRT onset that occurred prior to induced menopause). As such, their ability to represent the impact of censoring for the competing outcomes of induced menopause and premenopausal HRT onset on the partial likelihood estimation producing cause-specific effects summarized in Chapter 4: Table 4.2 is limited. To address this further, cause-specific proportional hazards models, modeling time to HRT onset and induced menopause, are presented in Table A.1.

Table A.1. Relative effects of cumulative rotating shift work on the cause-specific hazards of a) natural menopause (events: 29,175); b) induced menopause (events: 11,556); c) premenopausal HRT (events: 15,339); and d) composite outcome of induced menopause or HRT (events:17,376)

Table 1a. Event: Natural menopause (Table 4.2)

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months¥	1.0	1.0	1.0	1.0
1 to 24 Months	1.01 (0.98-1.08)	1.00 (0.98-1.07)	1.06 (1.02-1.09)	1.01 (0.98-1.04)
25 to 48 Months	1.02 (0.99-1.08)	1.02 (0.97-1.07)	1.13 (1.07-1.18)	1.06 (1.01-1.12)
49 to 72 Months	1.03 (0.98-1.16)	1.02 (0.98-1.13)	0.99 (0.93-1.06)	1.00 (0.93-1.07)
73 to 96 Months	1.05 (1.00-1.16)	1.04 (0.98-1.12)	1.04 (0.96-1.14)	1.04 (0.96-1.14)
97 to 120 Months	0.98 (0.91-1.05)	0.99 (0.92-1.06)	0.92 (0.85-1.01)	1.03 (0.94-1.12)
> 120 Months	N/A	N/A	0.62 (0.55-0.69)	0.76 (0.69-0.85)
121 to 240 Months	1.00 (0.96-1.05)	1.03 (0.99-1.08)	N/A	N/A
> 240 Months	0.90 (0.81-1.00)	0.99 (0.89-1.10)	N/A	N/A

Table 1b. Event: Induced menopause

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months¥	1.0	1.0	1.0	1.0
1 to 24 Months	0.99 (0.94-1.03)	1.00 (0.95-1.05)	1.18 (1.12-1.23)	1.11 (1.06-1.16)
25 to 48 Months	1.00 (0.95-1.06)	1.04 (0.98-1.09)	1.31 (1.22-1.14)	1.21 (1.13-1.30)
49 to 72 Months	1.13 (1.05-1.22)	1.12 (1.03-1.20)	1.13 (1.01-1.26)	1.08 (0.97-1.21)
73 to 96 Months	1.01 (0.94-1.09)	1.05 (0.97-1.13)	1.20 (1.04-1.38)	1.16 (1.06-1.34)
97 to 120 Months	1.04 (0.94-1.16)	1.03 (0.93-1.15)	0.93 (0.79-1.09)	0.96 (0.82-1.12)
> 120 Months	N/A	N/A	0.59 (0.47-0.74)	0.66 (0.52-0.82)
121 to 240 Months	1.04 (0.97-1.12)	1.03 (0.96-1.12)	N/A	N/A
> 240 Months	0.92 (0.74-1.14)	0.93 (0.75-1.16)	N/A	N/A

Table 1c. Event: Hormone replacement therapy

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months[¥]	1.0	1.0	1.0	1.0
1 to 24 Months	1.00 (0.96-1.04)	1.00 (0.96-1.04)	1.01 (0.97-1.06)	0.97 (0.94-1.01)
25 to 48 Months	0.97 (0.92-1.02)	1.00 (0.95-1.04)	0.99 (0.93-1.06)	0.95 (0.89-1.02)
49 to 72 Months	1.00 (0.93-1.07)	1.01 (0.94-1.08)	0.83 (0.75-0.92)	0.84 (0.76-0.94)
73 to 96 Months	0.96 (0.90-1.02)	0.98 (0.91-1.04)	0.86 (0.75-1.00)	0.90 (0.78-1.04)
97 to 120 Months	0.83 (0.75-0.91)	0.85 (0.77-0.94)	0.46 (0.38-0.56)	0.54 (0.45-0.66)
> 120 Months	N/A	N/A	0.28 (0.21-0.37)	0.37 (0.28-0.49)
121 to 240 Months	0.88 (0.82-0.94)	0.92 (0.86-0.99)	N/A	N/A
> 240 Months	0.55 (0.43-0.70)	0.62 (0.49-0.79)	N/A	N/A

Table 1d. Event: Hormone replacement therapy or induced menopause

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months[¥]	1.0	1.0	1.0	1.0
1 to 24 Months	1.00 (0.96-1.04)	1.01 (0.97-1.05)	1.04 (1.00-1.08)	1.00 (0.96-1.04)
25 to 48 Months	0.98 (0.94-1.03)	1.01 (0.96-1.05)	1.07 (1.00-1.14)	1.02 (0.96-1.08)
49 to 72 Months	1.04 (0.97-1.10)	1.04 (0.98-1.11)	0.87 (0.79-0.96)	0.88 (0.80-0.97)
73 to 96 Months	0.98 (0.92-1.04)	0.99 (0.93-1.06)	0.88 (0.77-1.01)	0.91 (0.80-1.04)
97 to 120 Months	0.85 (0.78-0.94)	0.88 (0.80-0.96)	0.53 (0.45-0.63)	0.61 (0.52-0.72)
>120 Months/	N/A	N/A	0.36 (0.28-0.46)	0.46 (0.37-0.59)
121 to 240 Months	0.90 (0.84-0.96)	0.93 (0.88-1.00)	N/A	N/A
>240 Months	0.60 (0.48-0.74)	0.66 (0.53-0.82)	N/A	N/A

*Hazard ratios adjusted for age, smoking status (never, past or current smoker), age at first birth and parity combined (nulliparous; age at first birth <24, 1–2 children; age at first birth 24 to 29, 1 to 2 children; age at first birth >29, 1 to 2 children; age at first birth <23, >2 children; age at first birth 24 to 29, >3 children; age at first birth >29, >2 children), body mass index (<18.5, 18.5 to 20, 20 to 22.5, 22.5 to 25, 25 to 30, and >30 kg/m2), cumulative oral contraceptive use (0, 1 to 23, 24 to 47, 48 to 71, 72 to 95, 96 to 119 and >120 months), total time breast fed (never, <=1 yr, >1 yr), alcohol consumption (0, 0 to 1, 1 to 4, 4 to 8, 8 to 12, >12 g/wk), physical activity (<=3, 3 to 9, 9 to 19, 19 to 27, 27 to 42, >42 METS/wk), age at menarche (<=9, 10, 11, 12, 13, 14, 15, >=16 yrs) and sleep in 24 hrs (<=4, 5, 6, 7, 8, >=9 hrs)

[¥]Reference category for all analyses

The discussion of each of the above presented effects on competing outcomes, as summarized in Table A.1, is avoided in the interest of brevity. For the competing outcome of induced menopause (Table A.1b), despite possible indication of a moderate positive effect in the 49-72 month exposure level, cumulative rotating shift work including years prior to 1989 does not appear to have a meaningful effect on the hazard of this outcome. For cumulative rotating shift work excluding years prior to 1989, while a similar positive association is suggested in the

second and third highest exposure levels, the strongest effect is protective, observed in the highest exposure level, similar to the cause-specific effect on natural menopause comprising our main results (Table A.1a).

For the competing event of HRT onset (Table A.1c), the protective effect in the highest exposure categories is apparent for both cumulative rotating shift work definitions. Both exhibit increasing protective trends on this hazard across the highest exposure levels, though the effects are more pronounced for cumulative rotating shift work excluding years prior to 1989. A similar pattern is observed with the induced menopause-HRT onset composite outcome (Table A.1d), though the effects, as expected, are moderately attenuated compared to those for the hazard of HRT onset alone (Table A.1c). In isolation, the cause-specific hazards summarized in Table A.1 can be suggestive of a dependency across the major competing events in our data. That is, censoring women at the end of the questionnaire period prior to that in which they reported induced menopause and onset of premenopausal HRT, may be informative, thereby distorting the cause-specific effects presented in our main result. Specifically, the protective effect on natural menopause inferred by the statistically significant multivariable-adjusted hazard ratio of 0.76 for the highest cumulative rotating shift work exposure level excluding years prior to 1989 may be artifactual, or at least inflated, due to informative censoring for induced menopause, and in particular, premenopausal HRT onset. However while the results from Table A.1 may suggest the possibility of informative censoring, they are unable to confirm it. It is difficult to assess dependency between competing outcomes directly as it is impossible to determine with certainty the probability of having the event of interest (i.e., natural menopause) among those having a competing event, had that competing event not occurred.

The next section employs a sensitivity analysis that, while crude, provides further insight on the likelihood that informative censoring may have significantly biased the effect of cumulative rotating shift work on the hazard of natural menopause.

Sensitivity analysis: outcome either induced menopause or HRT

To further assess whether our main results presented in Chapter 4, particularly that for the effect of cumulative rotating shift excluding exposure prior to 1989 on the hazard of natural

menopause, may be biased due to informative censoring, we conducted a sensitivity analysis modeled after examples from survival analysis texts[1,2]. Briefly, these analyses involve comparisons of two cause-specific models of the natural menopause outcome that have been termed "worst case". In the first model (Table A.2a), women who were censored due to HRT onset or induced menopause are treated as having the event at time of censorship. In a second model (Table A.2b), women are allowed to remain in the risk set for the maximum possible follow-up time. Normally, in the case of competing risks, this means individuals who were censored would remain in the risk set, to be censored with the residual risk set at end of follow-up. As women in the NHS II were still able to report an age at menopause after HRT onset, we assigned event times for women censored due to this criterion to be the time of reported menopause. As HRT medications are perceived to delay the reporting of natural menopause, this was deemed to provide an event time more proximal to the true timing than allowing these women to remain in the risk set event free.

Table A.2. Sensitivity analysis comparing "worst case" scenarios. a)Women who are censored for induced menopause and HRT onset are assigned the event times for natural menopause at the time of censorship for these competing events. b) Women are allowed to remain in the risk set until follow-up end, or are assigned the event time at reported age of menopause following HRT onset.

Table 2a

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months¥	1.0	1.0	1.0	1.0
1 to 24 Months	0.99 (0.97-1.02)	1.00 (0.98-1.02)	1.04 (1.01-1.06)	0.99 (0.97-1.02)
25 to 48 Months	1.00 (0.97-1.02)	1.01 (0.99-1.04)	1.07 (1.03-1.11)	1.02 (0.98-1.06)
49 to 72 Months	1.02 (0.98-1.06)	1.01 (0.98-1.05)	0.94 (0.89-1.00)	0.93 (0.88-0.98)
73 to 96 Months	1.00 (0.97-1.04)	1.01 (0.98-1.05)	0.91 (0.91-1.05)	0.97 (0.90-1.04)
97 to 120 Months	0.92 (0.88-0.97)	0.93 (0.88-0.98)	0.72 (0.72-0.84)	0.84 (0.77-0.90)
> 120 Months	N/A	N/A	0.44 (0.44-0.54)	0.56 (0.50-0.63)
121 to 240 Months	0.98 (0.94-1.01)	0.99 (0.95-1.02)	N/A	N/A
> 240 Months	0.85 (0.77-0.93)	0.88 (0.80-0.97)	N/A	N/A

Table 2b

	Including Years of Rotating Shift Work Prior to 1989		Excluding Years of Rotating Shift Work Prior to 1989	
Cumulative Rotating Shifts	Age-adjusted HR (95% CI)	Adjusted HR* (95% CI)	Age-adjusted (95% CI)	Adjusted HR* (95% CI)
0 Months[¥]	1.0	1.0	1.0	1.0
1 to 24 Months	1.01 (0.99-1.04)	1.00 (0.98-1.03)	1.04 (1.02-1.07)	1.01 (0.98-1.03)
25 to 48 Months	1.02 (0.99-1.06)	1.02 (0.99-1.05)	1.11 (1.06-1.16)	1.06 (1.01-1.10)
49 to 72 Months	1.03 (0.98-1.08)	1.01 (0.97-1.06)	0.99 (0.94-1.06)	0.99 (0.93-1.05)
73 to 96 Months	1.03 (0.99-1.07)	1.02 (0.97-1.06)	1.03 (0.95-1.11)	1.03 (0.95-1.11)
97 to 120 Months	0.98 (0.92-1.04)	0.99 (0.93-1.05)	0.92 (0.84-0.99)	1.01 (0.93-1.09)
> 120 Months	N/A	N/A	0.64 (0.58-0.70)	0.77 (0.70-0.85)
121 to 240 Months	0.99 (0.95-1.03)	1.01 (0.97-1.05)	N/A	N/A
> 240 Months	0.92 (0.84-1.02)	0.99 (0.90-1.08)	N/A	N/A

*Hazard ratios adjusted for age, smoking status (never, past or current smoker), age at first birth and parity combined (nulliparous; age at first birth <24, 1–2 children; age at first birth 24 to 29, 1 to 2 children; age at first birth >29, 1 to 2 children; age at first birth <23, >2 children; age at first birth 24 to 29, >3 children; age at first birth >29, >2 children), body mass index (<18.5, 18.5 to 20, 20 to 22.5, 22.5 to 25, 25 to 30, and >30 kg/m2), cumulative oral contraceptive use (0, 1 to 23, 24 to 47, 48 to 71, 72 to 95, 96 to 119 and >120 months), total time breast fed (never, <=1 yr, >1 yr), alcohol consumption (0, 0 to 1, 1 to 4, 4 to 8, 8 to 12, >12 g/wk), physical activity (<=3, 3 to 9, 9 to 19, 19 to 27, 27 to 42, >42 METS/wk), age at menarche (<=9, 10, 11, 12, 13, 14, 15, >=16 yrs) and sleep in 24 hrs (<=4, 5, 6, 7, 8, >=9 hrs)

[¥]Reference category for all analyses

The results from the sensitivity analysis indicate that, given all women who were censored due to a competing event were to have had natural menopause at the earliest possible time, our main results would have potentially underestimated the protective effect of cumulative rotating shift work on natural menopause (Table A.2a). In the other extreme, should these women not have had natural menopause until follow-up end, or until reported after onset of premenopausal HRT (Table A.2b), results would have not changed materially to those presented as our main findings (Table A.1a). This crude approach is therefore suggestive that should informative censoring have plagued cause-specific effects of cumulative rotating shift work on the hazard of natural menopause, our main results as presented in Chapter 4: Table 4.2 are conservative.

Distribution of main competing outcomes and cumulative rotating shift work over follow-up

This section further explores the apparent protective effect of more than 120 months of cumulative rotating shift work, excluding years prior to 1989, on hazards of natural menopause,

accounting for the cause-specific effects on competing outcomes as shown in Table A.1. Figure A.2, depicts the approximate timing, at 2-year intervals, of the distribution of these events over follow-up. Plots of outcomes over follow-up do not take into account censoring as modelled in respective cause-specific proportional hazards models (e.g., reported natural menopause is counted following premenopausal HRT onset). Due to cohort effects of premenopausal HRT use onset and menopausal timing as a result of the discontinuation of wide-spread prescribing of HRT at the beginning of the last decade, and additionally cohort age, the distribution of these events are not uniform over follow-up. Premenopausal HRT onset ramps up as the cohort ages, and then declines as prescribing of these medications suddenly diminished, largely due to concerns of postmenopausal breast cancer risk during that calendar period. As menopause is highly dependent on age, there is a left-tailed "n"-shaped distribution for reported menopause, mirroring the increasing age of the cohort. Additionally, cumulative rotating shift work exposure, excluding years prior to 1989, demonstrates uneven distribution over follow-up, with larger relative membership in the referent category (no exposure) toward the beginning, and the highest exposure level only non-zero during the last three questionnaire periods. This suggests that at least some of the protective effects across the highest exposure levels on the hazard of premenopausal HRT onset may be explained by the temporal alignment of the covariate vectors contributing to the partial likelihood estimation as these events occur over follow-up. That is, the strong protective effect of cumulative rotating shift work on the hazard of premenopausal HRT onset (Table A.1c) is likely inflated by the decrease in incidence of this outcome over the latter half of follow-up, together with the clustering of non-zero values for the rotating shift work exposure at follow-up end juxtaposed by higher membership of the reference category toward the beginning of follow-up.

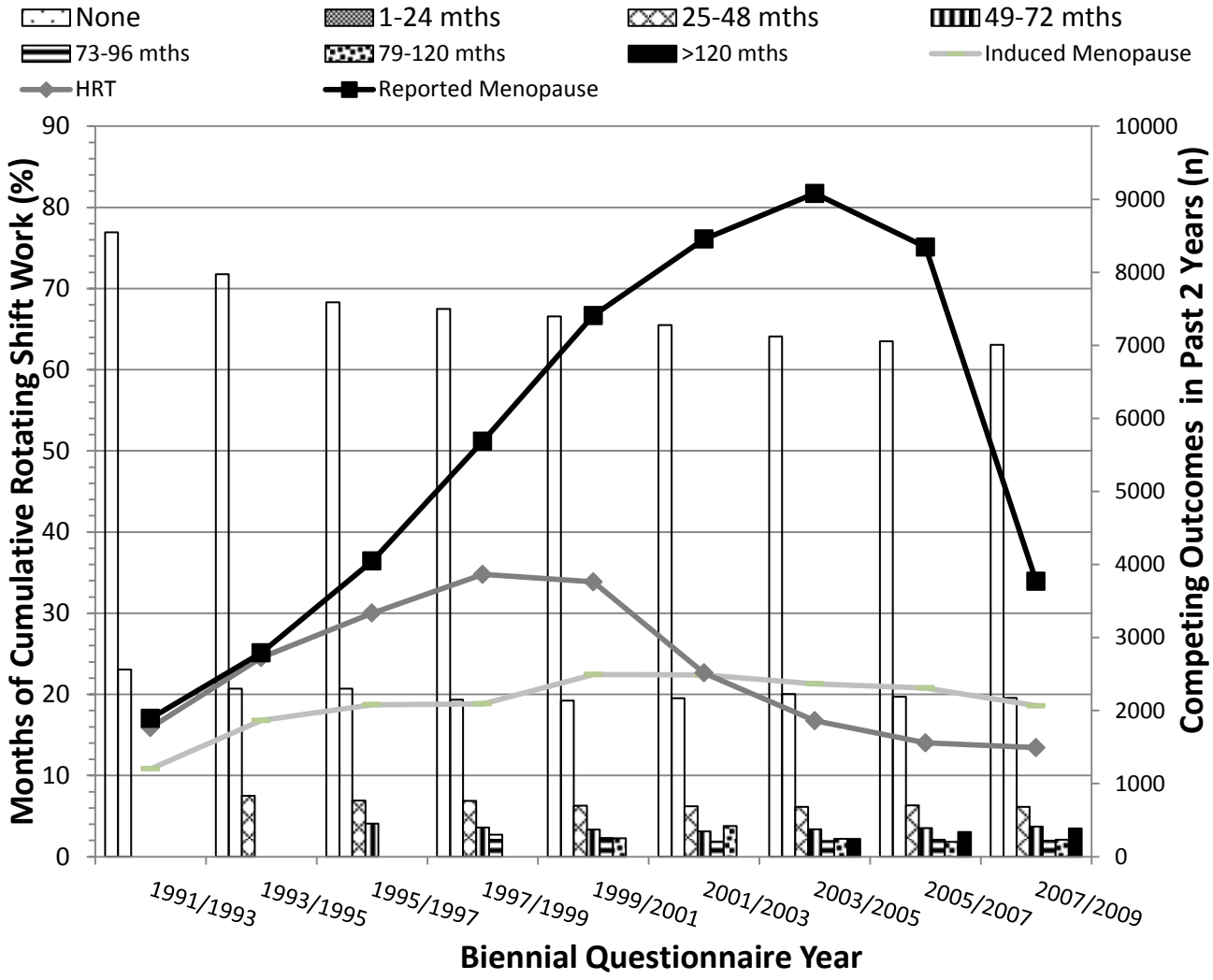


Figure A.2. Cumulative rotating shift work exposure (excluding years worked prior to 1989) and incident competing outcomes of induced menopause, hormone replacement therapy initiation and natural reported age at menopause (cause-specific event of interest for objective 1).

The protective effect of the highest exposure level of cumulative rotating shift work, excluding years prior to 1989, on hazard of natural menopause, does not appear to be explained by distribution of this exposure and outcome over follow-up. Based on Figure A.2 alone the higher incidence of menopause is coincident toward the end of follow-up with non-zero values for the highest exposure level. As such, women achieving natural menopause would have a higher probability of having covariate structures comprising the highest rotating shift work exposure level. Figure A.3 plots crude incidence of natural menopause, by rotating shift work categories (middle exposure categories collapsed for clarity), at two year intervals, over follow-up.

Denominators do not include women that would have been censored during prior intervals in the corresponding proportional hazards models.

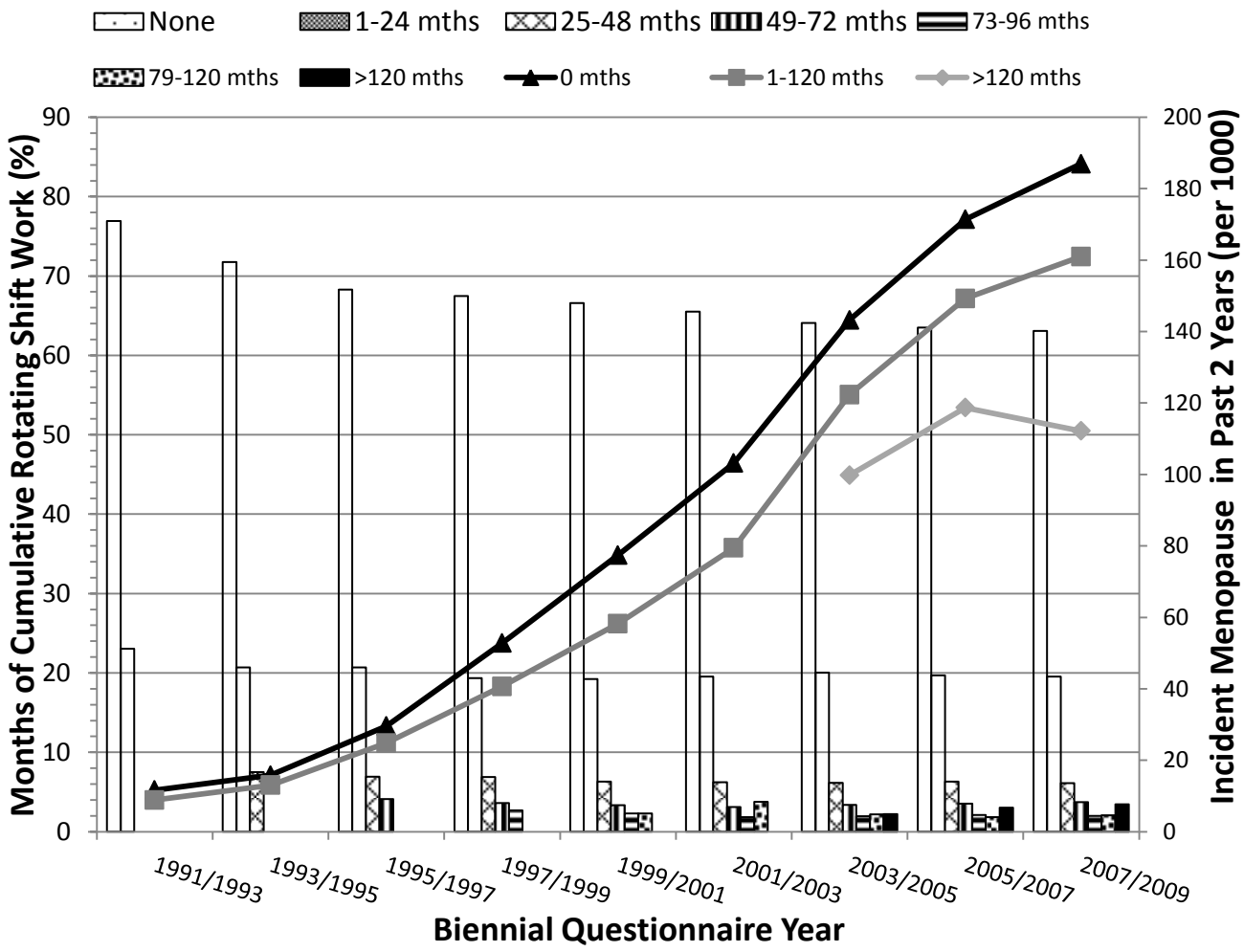


Figure A.3. Cumulative rotating shift work exposure (excluding years worked prior to 1989) and incident natural reported age at menopause (cause-specific event of interest for objective 1) among women not censored for HRT or induced menopause (or other prior censoring criteria).

Figure A.3 indicates that women comprising the highest cumulative rotating shift work exposure level do in fact have a lower crude incidence of natural menopause. This appears to support a true protective effect for the highest exposure category of rotating shift work, excluding work prior to 1989. However it is important to note that this representation does not take into account the temporal distribution in the cumulative rotating shift work exposure and the potential for bias

due to informative censoring when combined with cohort effects of premenopausal HRT onset and natural menopause over follow-up illustrated in Figure 2.

Age-stratified distribution of natural menopause over follow-up

Figure A.4 supports a cohort effect in the temporal distribution of natural menopause due to the majority of the cohort reaching menopausal age toward the end of follow-up. As with Figure A.3, denominators exclude women that would have been censored at the end of previous intervals in the corresponding proportional hazards model. Due to the co-distribution of premenopausal HRT onset and reported menopause over follow-up, if censoring at the former precluded the latter, a lower incidence of natural menopause among a given age group would be expected at an earlier calendar time. From Figure A.4, incident menopause remains fairly flat among those 50 years or younger, suggesting no overall effect of censoring on the cause-specific hazard of natural menopause. The steep increase between the first and second plotted point for those achieving menopause having started the 2-year interval at 51 to 54 years of age might ordinary be indicative of a change in the probability of natural menopause due to variation in censoring density over time. However it should be noted that the first plot point is based on relatively small counts, orders of magnitude lower than for those generating the remainder of data points comprising the plotted line, and only 0.53% of events, for this age group. As such, this jump in incidence may be spurious. Finally, the decline in incident natural menopause among the oldest age group, over the remainder of follow-up, is likely predominantly attributed to most women achieving menopause proximal to the lower age limit (i.e., 55 years), the proportion of which declines with time. Overall, Figure A.4 fails to provide strong indication that competing events precluded natural menopause. However, it does not rule out informative censoring due to premenopausal HRT onset across cumulative rotating shift work exposure.

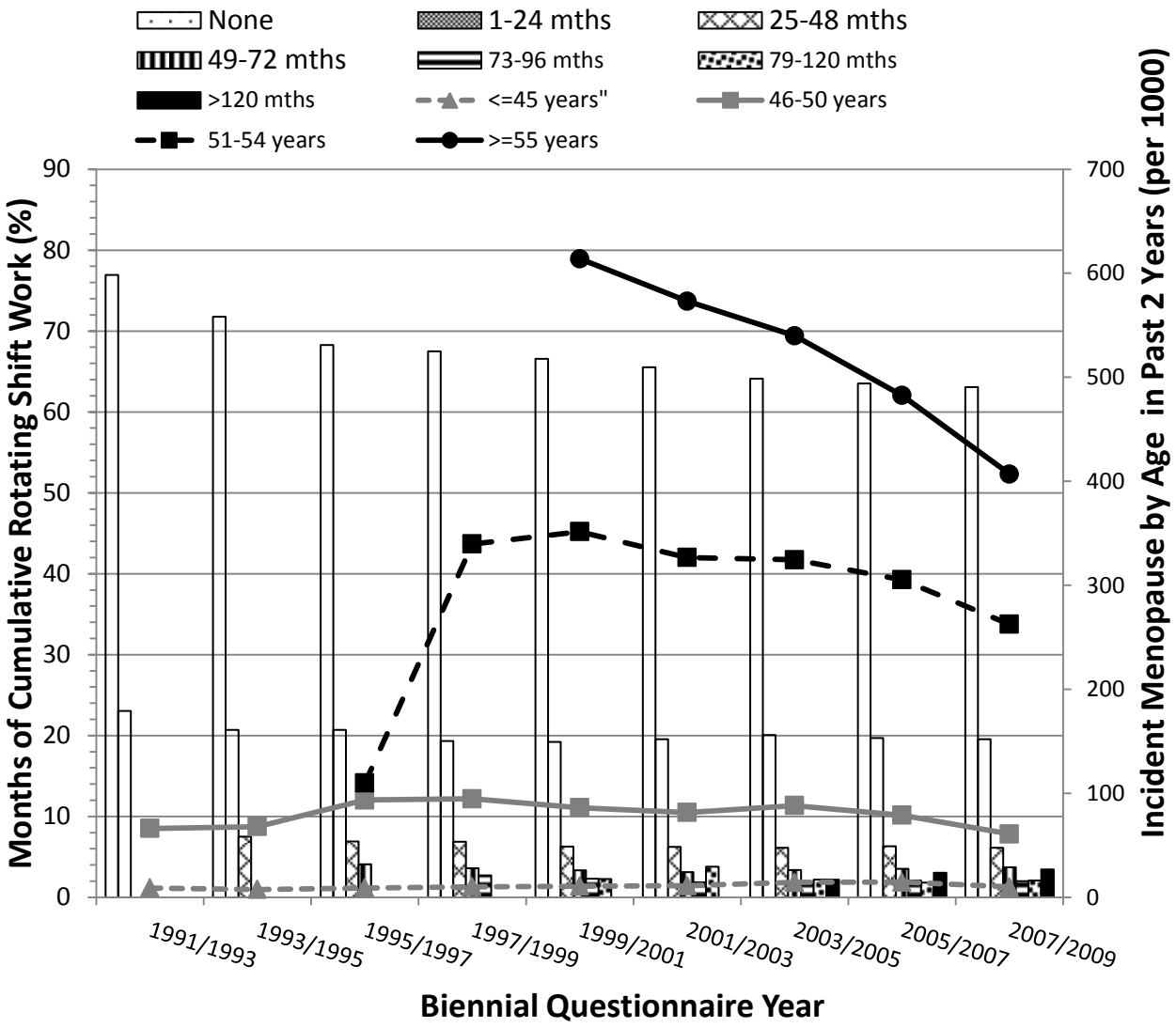


Figure A.4. Cumulative rotating shift work exposure (excluding years worked prior to 1989) and incident natural reported age at menopause (cause-specific event of interest for objective 1) by age group, among women not censored for HRT or induced menopause (or other prior censoring criteria). Number of events by age group over follow-up: <=45 years = 3,148; 46-50 years: 10,165; 51-55: 13,009; >=55 years: 4,079.

Cumulative rotating shift work exposure

While it is generally difficult, if not impossible, to be certain that there is no dependency between competing outcomes across covariate structures in a given survival analysis, the above additional analyses discussed so far might suggest that the statistically significant protective effect of working >120 months of rotating shift work since 1989 is not substantially biased due

to informative censoring. However the cumulative nature of the exposure, exacerbated by the uneven distribution of the higher exposure levels over follow-up, in particular that non-zero values for the highest exposure levels only occur during the last third of the study period, in addition to only being able to arrive at this exposure level by progressing through lower exposure membership earlier in follow-up, may have obscured assessment of this bias. Had this rotating shift work exposure been fixed, or less temporally volatile as with the definition including work prior to 1989 when defining the upper exposure category as >240 months, the sensitivity analysis summarized in Table A.2 may have been more likely to uncover indication of such a bias had one been present. If a substantial proportion of women had been more likely to have had earlier menopause after progressing to the highest exposure level at the end of the study period, but were instead censored due to HRT onset while occupying a lower cumulative exposure membership earlier in the study period when HRT onset was more prevalent, at least some of the protective effect indicated by the statistically significant hazard ratio of 0.76 may be artificial. However, unlike with time-fixed exposure constructs, the likelihood of such a bias is even more difficult to assess with time-dependent definitions. Not only is it difficult to accurately assess the true hazard of natural menopause after women begin premenopausal HRT, there is, in addition, the uncertainty of predicting which future cumulative rotating shift work exposure levels women would have attained.

Under the assumption that, of those who did not undergo induced menopause, women would have typically began premenopausal HRT after beginning the perimenopausal transition due to onset of related symptoms, it is perhaps unlikely that a substantial proportion of those censored at premenopausal HRT onset would have been eligible for natural menopause when cumulative rotating shift work, excluding exposure prior to 1989, was non-zero. While definitions of perimenopause have been ambiguous, the average length of this transitional phase has been estimated to be approximately four years, based on data from two studies comprising almost 1,500 women combined[3]. Given that this average duration is generalizable to the NHS II cohort, it is possible that the majority of women at risk of premenopausal HRT during the first half of the study period would be unlikely to still have been at risk for natural menopause when the highest level of the cumulative rotating shift work exposure, excluding work prior to 1989, was non-zero and incidence of natural menopause was highest, had they not began premenopausal HRT. In the NHS II, women still reported an age of menopause after premenopausal HRT. In our

cause-specific analyses of the hazard of natural menopause, women were censored for HRT at the beginning of the questionnaire period on which use was first reported and time of menopause was assigned at the midpoint of the year corresponding to reported menopausal age. Given these conditions, of just over 6,000 women who reported both beginning premenopausal HRT and an age at menopause, the median interval between HRT onset and menopause was estimated to be 4.5 years. The hypothesis that HRT could inflate the reported age of menopause due to prolonged intermittent menstruation and spotting, as discussed in Chapter 3; section 4.2.3.1, perhaps indicates that this estimate is higher than the true median value, had women not began HRT. This is supports that, given the temporal trends in HRT and menopause over the study period, a large proportion of women who would have achieved the highest cumulative rotating shift work exposure level in question, that were instead censored for premenopausal HRT, would not still have been at risk for natural menopause toward the end of the study period. As such, it may have been that the majority of women would not have been in a position to contribute to this potential informative censoring-related bias.

Conclusion

In summary, while it should be reiterated that it is impossible to definitively rule out bias due to informative censoring, these additional analyses provide some additional transparency to the observed relative cause-specific effects on the hazard of natural menopause across highest and no cumulative rotating shift work exposure levels, comprising main findings for objective 1 (Chapter 4). Though we are unable to entirely discount that the effect may be inflated, it perhaps provides some credibility to the interpretation that women who accumulate the highest levels of cumulative rotating shift work exposure may, in actuality, have a greater risk of delayed menopause.

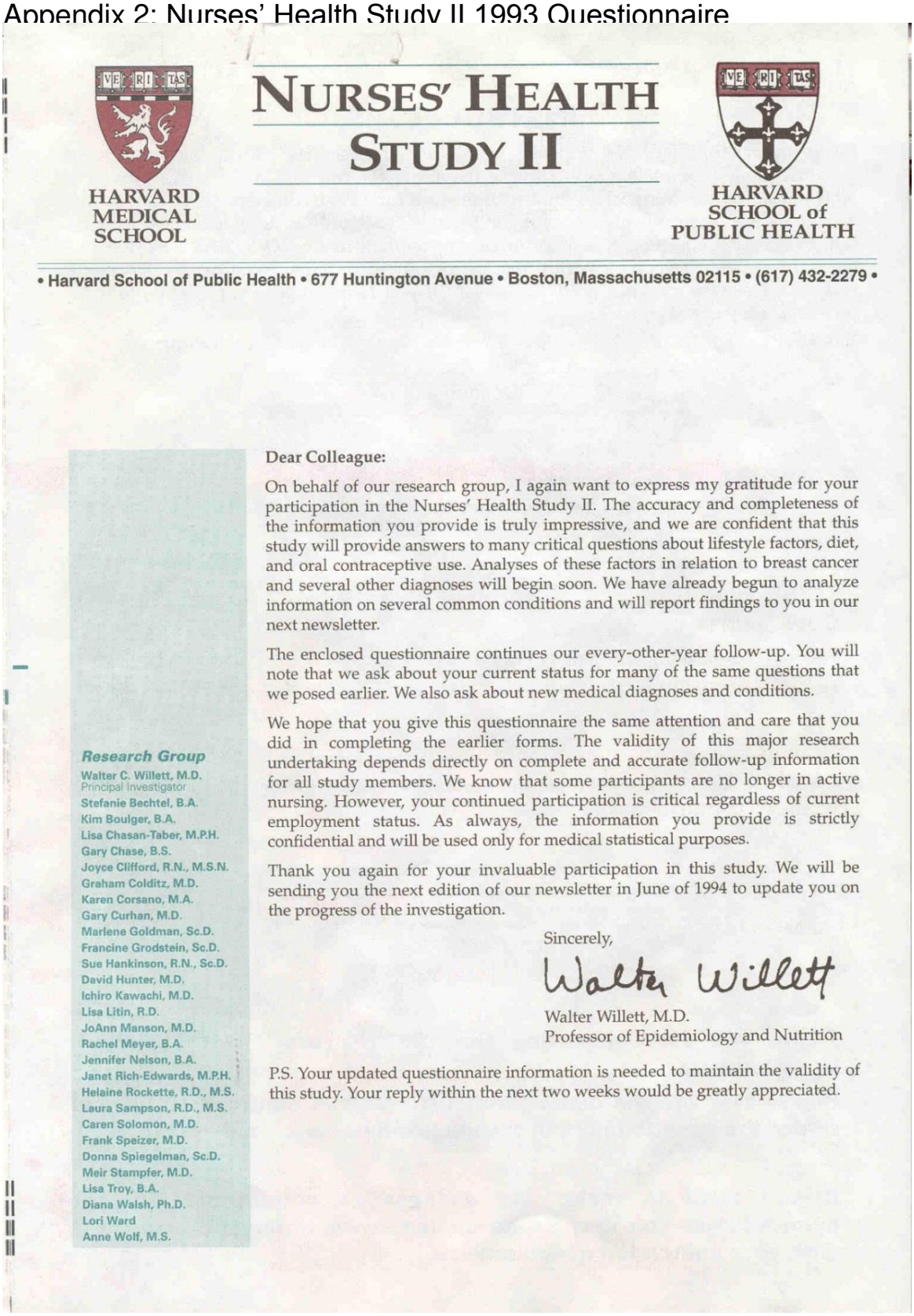
References

1. Kleinbaum DGK, M. *Survival Analysis: A Self-Learning Text*. 2nd ed. New York: Springer; 2005.

2. Allison PD. *Survival Analysis Using SAS: A Practical Guide*. Cary, North Carolina: SAS Institute Inc.; 1995.

3. Li S, Lanuza D, Gulanick M, Penckofer S, Holm K. Perimenopause: the transition into menopause. *Health Care Women Int.* Jul-Aug 1996;17(4):293-306.

Appendix 2: Nurses' Health Study II 1993 Questionnaire



HARVARD MEDICAL SCHOOL

NURSES' HEALTH STUDY II

HARVARD SCHOOL of PUBLIC HEALTH

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Dear Colleague:

On behalf of our research group, I again want to express my gratitude for your participation in the Nurses' Health Study II. The accuracy and completeness of the information you provide is truly impressive, and we are confident that this study will provide answers to many critical questions about lifestyle factors, diet, and oral contraceptive use. Analyses of these factors in relation to breast cancer and several other diagnoses will begin soon. We have already begun to analyze information on several common conditions and will report findings to you in our next newsletter.

The enclosed questionnaire continues our every-other-year follow-up. You will note that we ask about your current status for many of the same questions that we posed earlier. We also ask about new medical diagnoses and conditions.

We hope that you give this questionnaire the same attention and care that you did in completing the earlier forms. The validity of this major research undertaking depends directly on complete and accurate follow-up information for all study members. We know that some participants are no longer in active nursing. However, your continued participation is critical regardless of current employment status. As always, the information you provide is strictly confidential and will be used only for medical statistical purposes.

Thank you again for your invaluable participation in this study. We will be sending you the next edition of our newsletter in June of 1994 to update you on the progress of the investigation.

Sincerely,

Walter Willett

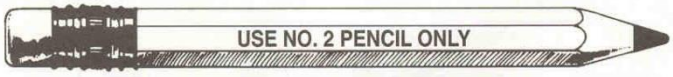
Walter Willett, M.D.
Professor of Epidemiology and Nutrition

P.S. Your updated questionnaire information is needed to maintain the validity of this study. Your reply within the next two weeks would be greatly appreciated.

HARVARD UNIVERSITY — NURSES' HEALTH STUDY II

INSTRUCTIONS

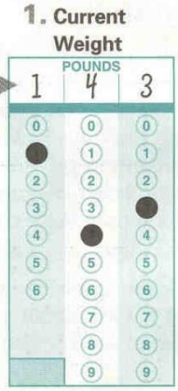
Please use an ordinary No. 2 pencil to answer all questions. Fill in the appropriate response circles completely, or write the requested information in the boxes provided. Note that some questions ask for information **since June 1991**, some ask for **current status**, and some ask about events over **longer periods**. The form is designed to be read by optical-scanning equipment, so it is important that you make **NO STRAY MARKS** and keep any write-in responses **within** the spaces provided. Should you need to change a response, erase the incorrect mark completely. If you have comments, please write them on a separate piece of paper.



EXAMPLE 1: **Write in your weight in the boxes...**

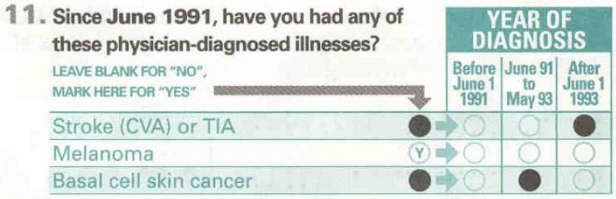
...and fill in the circle corresponding to the figure at the head of each column.

Please fill in the circle completely, do not mark this way: ✓ ✗ ◒



NOTE: It is important that you write in your weight in addition to completing the corresponding circles. This allows us to confirm that the correct circles have been darkened.

EXAMPLE 2: **Mark "Yes" circle and Year of Diagnosis circle for each illness you have had diagnosed.**



Thank you for completing the 1993 Nurses' Health Study II Questionnaire.

Please tear off the cover letter (to preserve confidentiality) and return the questionnaire in the enclosed postage paid envelope.

If you need to make any changes or corrections to your name/address you may do so on the cover letter and enclose it with your completed questionnaire.

NURSES' HEALTH STUDY II — PAGE 1 — HARVARD UNIVERSITY

1. PLEASE USE PENCIL!

CURRENT WEIGHT (POUNDS)

2. We would like to update your pregnancy history from the time of the first questionnaire in 1989 to the present.

a) Since September 1, 1989 have you been pregnant?
○ No - go to question 3 ○ Yes

b) Are you currently pregnant?
○ No ○ Yes – continue with part c, but do not fill in a bubble for current pregnancy

c) For each pregnancy *ending* after September 1, 1989, fill in a response bubble for the year during which each pregnancy *ended*.

Calendar Year	Pregnancies lasting 6 months or more: Single Birth	Pregnancies lasting 6 months or more: Twins/Triplets	Pregnancies lasting less than 6 months (include miscarriages/ induced abortions)
9/1/89 - 12/31/89	○	○	○
1990	○	○	○
1991	○	○	○
1992	○	○	○
1993	○	○	○
1994	○	○	○

3. Do you CURRENTLY use any of these forms of contraception? (Mark all that apply.)
○ None ○ Oral contraceptive ○ Norplant ○ Diaphragm/Cervical cap ○ Tubal ligation ○ Foam/Jelly/Sponge
○ Condom ○ Intrauterine device ○ Rhythm/NFP ○ Vasectomy ○ Depo Provera ○ Other

4. SINCE JUNE 1991, have you used oral contraceptives (OC's)?
○ Yes → a) How many months have you used OC's since June 1991?
○ 1 or less months ○ 2 - 4 ○ 5 - 9 ○ 10 - 14 ○ 15 - 19 ○ 20 or more months
○ No
b) Please indicate the brand and type of OC used longest during this time period. Refer to the OC Brand Code Sheet enclosed with this questionnaire and write the code in this box. →

5. What is the current usual length of your menstrual cycle (interval from first day of period to first day of next period)?
○ < 21 days ○ 21-25 ○ 26-31 ○ 32-39 ○ 40-50 ○ 51+ days or too irregular to estimate

6. What is the current usual pattern of your menstrual cycles (when not pregnant or lactating)?
○ Extremely regular (no more than 1-2 days before or after expected) ○ Very regular (within 3-4 days)
○ Regular (within 5-7 days) ○ Usually irregular ○ Always irregular ○ No periods

7. Have your menstrual periods ceased PERMANENTLY?
○ No: Premenopausal
○ Yes: No menstrual periods → a) Age natural periods ceased? AGE
○ Yes: Had menopause but now have periods induced by hormones
○ Not sure

b) For what reason did your periods cease?
○ SURGERY: If due to surgery, were your ovaries removed?
○ Yes, both ○ Only uterus removed ○ One only
○ RADIATION or CHEMOTHERAPY
○ NATURAL: If natural (non-surgical) menopause, have you had subsequent surgery to remove ovaries or uterus?
○ No ○ One ovary removed ○ Uterus removed ○ Both ovaries removed

8. SINCE JUNE 1991, have you used female replacement hormones (other than oral contraceptives)?
○ No
○ Yes, currently → a) How many months have you used them since JUNE 1991?
○ Yes, discontinued
○ 1-4 mo. ○ 5-9 ○ 10-14 ○ 15-19 ○ 20+ months

b) Mark the types of hormones you have used the longest during this period.
Estrogen: ○ Oral Premarin ○ Estrace ○ Ogen ○ Patch Estrogen ○ Vaginal Estrogen ○ Other Estrogen
Progesterone/Progestin (e.g., Provera): ○ Oral ○ Vaginal ○ Other (specify below)
Other type of hormones used, please specify: →

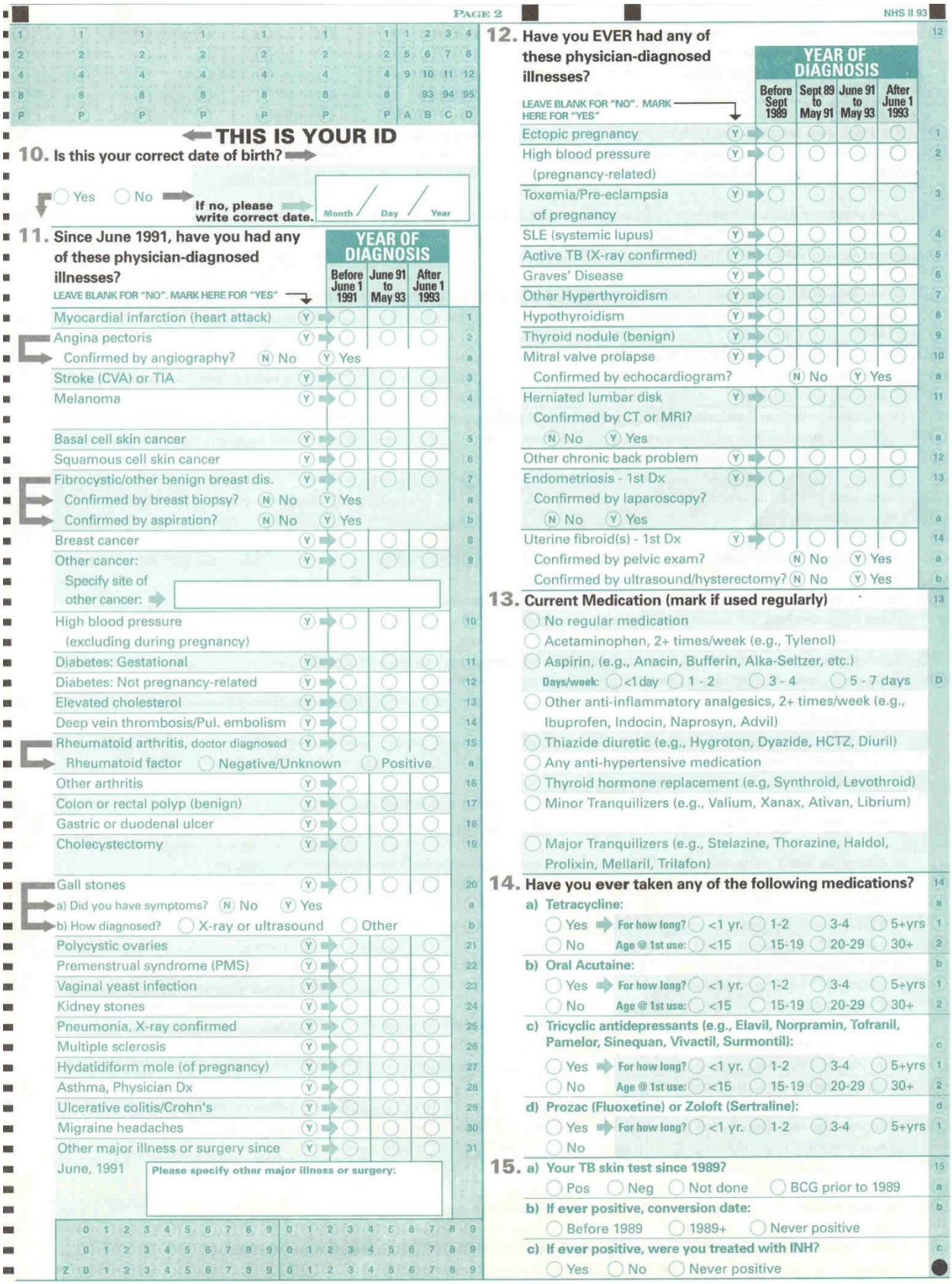
c) If you used oral conjugated estrogen (e.g., Premarin) what dose did you usually take?
○ .30 mg/day or less (Green) ○ .625 mg/day (Brown) ○ .9 mg/day (White) ○ 1.25 mg/day (Yellow)
○ More than 1.25 mg/day ○ Dose unknown ○ Did not take oral conjugated estrogen

d) If you used oral Medroxy Progesterone (e.g., Provera, Cycrin), what dose did you usually take?
○ < 5 mg ○ 5-9 mg ○ 10 mg ○ More than 10 mg ○ Dose unknown ○ Not used

e) What was your pattern of hormone use (Days per Month)?

Oral or Patch Estrogen: Days per Month	○ Not used	○ <1 day/mo	○ 1-8 days	○ 9-18	○ 19-26	○ 27+ days/mo	
Progesterone: Days per Month	○ Not used	○ <1 day/mo	○ 1-8 days	○ 9-18	○ 19-26	○ 27+ days/mo	

9. Have you had a tubal ligation?
○ No
○ Yes → At what age? ○ <25 ○ 25-29 ○ 30-34 ○ 35-39 ○ 40-44 ○ 45+



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THIS IS YOUR ID

10. Is this your correct date of birth? ○ Yes ○ No → If no, please write correct date. Month / Day / Year

11. Since June 1991, have you had any of these physician-diagnosed illnesses? LEAVE BLANK FOR "NO". MARK HERE FOR "YES"

		YEAR OF DIAGNOSIS: Before June 1 1991	June 91 to May 93	After June 1 1993
Myocardial infarction (heart attack)	Y	○	○	○
Angina pectoris	Y	○	○	○
Confirmed by angiography?	N No Y Yes			
Stroke (CVA) or TIA	Y	○	○	○
Melanoma	Y	○	○	○
Basal cell skin cancer	Y	○	○	○
Squamous cell skin cancer	Y	○	○	○
Fibrocystic/other benign breast dis.	Y	○	○	○
Confirmed by breast biopsy?	N No Y Yes			
Confirmed by aspiration?	N No Y Yes			
Breast cancer	Y	○	○	○
Other cancer:	Y	○	○	○
Specify site of other cancer:				
High blood pressure (excluding during pregnancy)	Y	○	○	○
Diabetes: Gestational	Y	○	○	○
Diabetes: Not pregnancy-related	Y	○	○	○
Elevated cholesterol	Y	○	○	○
Deep vein thrombosis/Pul. embolism	Y	○	○	○
Rheumatoid arthritis, doctor diagnosed	Y	○	○	○
Rheumatoid factor	○ Negative/Unknown ○ Positive			
Other arthritis	Y	○	○	○
Colon or rectal polyp (benign)	Y	○	○	○
Gastric or duodenal ulcer	Y	○	○	○
Cholecystectomy	Y	○	○	○
Gall stones	Y	○	○	○
a) Did you have symptoms?	N No Y Yes			
b) How diagnosed?	○ X-ray or ultrasound ○ Other			
Polycystic ovaries	Y	○	○	○
Premenstrual syndrome (PMS)	Y	○	○	○
Vaginal yeast infection	Y	○	○	○
Kidney stones	Y	○	○	○
Pneumonia, X-ray confirmed	Y	○	○	○
Multiple sclerosis	Y	○	○	○
Hydatidiform mole (of pregnancy)	Y	○	○	○
Asthma, Physician Dx	Y	○	○	○
Ulcerative colitis/Crohn's	Y	○	○	○
Migraine headaches	Y	○	○	○
Other major illness or surgery since June, 1991	Y	○	○	○

Please specify other major illness or surgery:

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12. Have you EVER had any of these physician-diagnosed illnesses? LEAVE BLANK FOR "NO". MARK HERE FOR "YES"

		YEAR OF DIAGNOSIS: Before Sept 1989	Sept 89 to May 91	June 91 to May 93	After June 1 1993
Ectopic pregnancy	Y	○	○	○	○
High blood pressure (pregnancy-related)	Y	○	○	○	○
Toxemia/Pre-eclampsia of pregnancy	Y	○	○	○	○
SLE (systemic lupus)	Y	○	○	○	○
Active TB (X-ray confirmed)	Y	○	○	○	○
Graves' Disease	Y	○	○	○	○
Other Hyperthyroidism	Y	○	○	○	○
Hypothyroidism	Y	○	○	○	○
Thyroid nodule (benign)	Y	○	○	○	○
Mitral valve prolapse	Y	○	○	○	○
Confirmed by echocardiogram?	N No Y Yes				
Herniated lumbar disk	Y	○	○	○	○
Confirmed by CT or MRI?	N No Y Yes				
Other chronic back problem	Y	○	○	○	○
Endometriosis - 1st Dx	Y	○	○	○	○
Confirmed by laparoscopy?	N No Y Yes				
Uterine fibroid(s) - 1st Dx	Y	○	○	○	○
Confirmed by pelvic exam?	N No Y Yes				
Confirmed by ultrasound/hysterectomy?	N No Y Yes				

13. Current Medication (mark if used regularly)

- ○ No regular medication
- ○ Acetaminophen, 2+ times/week (e.g., Tylenol)
- ○ Aspirin, (e.g., Anacin, Bufferin, Alka-Seltzer, etc.)
  - Days/week: ○ <1 day ○ 1 - 2 ○ 3 - 4 ○ 5 - 7 days
- ○ Other anti-inflammatory analgesics, 2+ times/week (e.g., Ibuprofen, Indocin, Naprosyn, Advil)
- ○ Thiazide diuretic (e.g., Hygroton, Dyazide, HCTZ, Diuril)
- ○ Any anti-hypertensive medication
- ○ Thyroid hormone replacement (e.g, Synthroid, Levothroid)
- ○ Minor Tranquilizers (e.g., Valium, Xanax, Ativan, Librium)
- ○ Major Tranquilizers (e.g., Stelazine, Thorazine, Haldol, Prolixin, Mellaril, Trilafon)

14. Have you ever taken any of the following medications?

a) Tetracycline:
○ Yes → For how long? ○ <1 yr. ○ 1-2 ○ 3-4 ○ 5+yrs
○ No → Age @ 1st use: ○ <15 ○ 15-19 ○ 20-29 ○ 30+

b) Oral Acutaine:
○ Yes → For how long? ○ <1 yr. ○ 1-2 ○ 3-4 ○ 5+yrs
○ No → Age @ 1st use: ○ <15 ○ 15-19 ○ 20-29 ○ 30+

c) Tricyclic antidepressants (e.g., Elavil, Norpramin, Tofranil, Pamelor, Sinequan, Vivactil, Surmontil):
○ Yes → For how long? ○ <1 yr. ○ 1-2 ○ 3-4 ○ 5+yrs
○ No → Age @ 1st use: ○ <15 ○ 15-19 ○ 20-29 ○ 30+

d) Prozac (Fluoxetine) or Zoloft (Sertraline):
○ Yes → For how long? ○ <1 yr. ○ 1-2 ○ 3-4 ○ 5+yrs
○ No

15. a) Your TB skin test since 1989?
○ Pos ○ Neg ○ Not done ○ BCG prior to 1989

b) If ever positive, conversion date:
○ Before 1989 ○ 1989+ ○ Never positive

c) If ever positive, were you treated with INH?
○ Yes ○ No ○ Never positive

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16. Since June 1991, have you tried to become pregnant for more than one year without success? 16
- ○ Yes → What was the cause? (Mark all that apply.) a
- ○ No

○ Tubal blockage ○ Ovulatory disorder ○ Endometriosis ○ Cervical mucous factors
○ Spouse/Partner ○ Not investigated ○ Not found ○ Other

17. Have you ever taken Clomid (Clomiphene) or Pergonal to induce ovulation? 17
- ○ Yes → a) In how many months was Clomid used: ○ 0 months ○ 1 ○ 2-3 ○ 4-5 ○ 6-11 ○ 12+ months a
- ○ No b) In how many months was Pergonal used: ○ 0 months ○ 1 ○ 2-3 ○ 4-5 ○ 6-11 ○ 12+ months b

18. Have you ever had a miscarriage or induced abortion before the sixth month of pregnancy? 18

Miscarriage:	○ No	○ Yes: at what age(s)	○ <18	○ 18-20	○ 21-23	○ 24-26	○ 27-29	○ 30-34	○ 35+ a
Induced Abortion:	○ No	○ Yes: at what age(s)	○ <18	○ 18-20	○ 21-23	○ 24-26	○ 27-29	○ 30-34	○ 35+ b

19. Since June 1991, how many months have you worked ROTATING night shifts (at least 3 nights/month in addition to other days and evenings in that month)? 19
○ None ○ 1-4 months ○ 5-9 ○ 10-14 ○ 15-19 ○ 20+ months

20. Which best describes your current employment status? 20
○ Inpatient or ER Nurse ○ Outpatient/Community ○ OR Nurse ○ Nursing Education ○ Student
○ Nursing Administration ○ Other Nursing ○ Non-nursing employment ○ Fulltime Homemaker ○ Disabled

21. How many times per week do you engage in physical activity long enough to perspire heavily (including swimming)? 21
○ Less than once/week ○ Once/week ○ 2-3 times/week ○ 4-6 times/week ○ 7 or more times/week

22. In how many months did you practice breast self-examination in the past year? 22
○ None ○ One month ○ 2-3 ○ 4-6 ○ 7-11 ○ 12 months

23. Since June 1991, have you had: 23

	No	Yes, for screening	Yes, for symptoms
Mammogram	○	○	○
Breast exam by clinician	○	○	○
Colonoscopy/Sigmoidoscopy	○	○	○
Pap smear	○	○	○

24. How many months <u>in total</u> (all births combined) did you breast feed? 24
○ Did not breast feed ○ <1 month ○ 1-3 mo. ○ 4-6 mo. ○ 7-11 mo. ○ 12-17 mo.
○ No children ○ 18-23 mo. ○ 24-35 mo. ○ 36-47 mo. ○ 48+ mo. ○ Cannot remember

25. Between the ages of 18 and 30 (excluding illness and pregnancy-related changes): 25
a) What was your: Minimum weight _____ lbs. Maximum weight _____ lbs. a
b) Between the ages of 18 and 30, how many times did you lose each of the following amounts of weight on purpose? MN

5-9 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times MX
10-19 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times b
20-49 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times
50+ pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times

26. Within the last 4 years (excluding illness and pregnancy-related changes): 26
a) What was your: Minimum weight _____ lbs. Maximum weight _____ lbs. a
b) Within the last 4 years, how many times did you lose each of the following amounts of weight on purpose? MN

5-9 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times MX
10-19 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times b
20-49 pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times
50+ pounds:	○ 0 times	○ 1-2 times	○ 3-4 times	○ 5-6 times	○ 7+ times

c) What primary method(s) did you use for your most recent weight loss of 10 or more pounds? (Mark all that apply) c
○ Did not lose 10 or more pounds ○ Diet pills ○ Increased exercise
○ Low calorie diet ○ Commercial weight loss program ○ Decreased alcohol intake
○ Low fat diet ○ Gastric surgery/intestinal bypass ○ Resumed/increased smoking
○ Skipped meals/fasted ○ Other
○ Weight loss was unintentional (e.g., illness, unusual stress, depression)

27. Do you currently smoke cigarettes? 27
○ No ○ Yes → How many per day? ○ 1-4 ○ 5-14 ○ 15-24 ○ 25-34 ○ 35-44 ○ 45 or more a

28. What was the cup size of your bra when you were 20 years old? (Estimate if you did not wear a bra.) 28
○ A or smaller ○ B ○ C ○ D or larger

29. How many biological sisters do you have? 29
○ 0 ○ 1 ○ 2 ○ 3 ○ 4 ○ 5 or more

30. Did your mother or any of your sisters have ovarian cancer? 30
○ No ○ Yes → ○ Mother ○ Sister ○ Both e

31. When your mother was pregnant with you, did she take DES (Diethylstilbestrol) or other hormones? 31
○ Don't know ○ No ○ Yes → ○ DES ○ Other hormones a

Please Continue on Page 4

PAGE 4

32. In which state were you born?
In which state did you live at age 15?
In which state did you live at age 30?

33. During summers how many times per week were you outdoors in a swimsuit:
a) as a teenager? ◯ <1/week ◯ 1/week ◯ 2/week ◯ Several/week ◯ Daily
b) in the past summer? ◯ <1/week ◯ 1/week ◯ 2/week ◯ Several/week ◯ Daily

34. When you were outside at the pool or beach, what percent of the time did you wear sunscreen:
a) as a teenager? ◯ Not in sun ◯ 0% ◯ 25% ◯ 50% ◯ 75% ◯ 100%
b) in the past summer? ◯ Not in sun ◯ 0% ◯ 25% ◯ 50% ◯ 75% ◯ 100%

35. Is your biological mother still living?
◯ Yes ◯ No → a) At what age did she die? ◯ <50 ◯ 50-59 ◯ 60-69 ◯ 70-79 ◯ 80+
b) Was this due to: ◯ Heart Disease ◯ Cancer ◯ Trauma/Accident/Suicide ◯ Other

36. Is your biological father still living?
◯ Yes ◯ No → a) At what age did he die? ◯ <50 ◯ 50-59 ◯ 60-69 ◯ 70-79 ◯ 80+
b) Was this due to: ◯ Heart Disease ◯ Cancer ◯ Trauma/Accident/Suicide ◯ Other

37. What is your current marital status?
◯ Married ◯ Divorced/Separated ◯ Widowed ◯ Never Married

38. What is your current living arrangement?
◯ Alone ◯ With husband/partner ◯ With other family ◯ Other

39. Do you currently take a multi-vitamin? (Please report additional individual vitamins in question 40.)
◯ No ◯ Yes
a) How many do you take per week? ◯ 2 or fewer ◯ 3-5 ◯ 6-9 ◯ 10 or more
b) What specific brand do you usually use? (Please specify exact Brand and Type.)

40. Not counting multi-vitamins, do you regularly take any of the following preparations:

		AMOUNT PER DAY
a) Beta-carotene?	◯ No ◯ Yes	◯ Less than 8,000 IU per day ◯ 8,000 to 12,000 IU ◯ 13,000 to 22,000 IU ◯ 23,000 IU or more ◯ Amount unknown
b) Vitamin A? (excluding carotene)	◯ No ◯ Yes	◯ Less than 8,000 IU per day ◯ 8,000 to 12,000 IU ◯ 13,000 to 22,000 IU ◯ 23,000 IU or more ◯ Amount unknown
c) Vitamin C?	◯ No ◯ Yes, seasonal only ◯ Yes, most months	◯ Less than 400 mg. ◯ 400 to 700 mg. ◯ 750 to 1,250 mg. ◯ 1300 mg. or more ◯ Amount unknown
d) Vitamin E?	◯ No ◯ Yes	◯ Less than 100 IU ◯ 100 to 250 IU ◯ 300 to 500 IU ◯ 600 IU or more ◯ Amount unknown
e) Calcium? (elemental)	◯ No ◯ Yes	◯ Less than 400 mg per day ◯ 400 to 800 mg. ◯ 900-1,200 mg. ◯ 1,300 mg. or more ◯ Amount unknown
f) Folic acid?	◯ No ◯ Yes	◯ Less than 100 mg. ◯ 100 to 300 mg. ◯ 301-500 mg. ◯ 501 mg. or more ◯ Amount unknown

41. Please indicate the name of someone at a DIFFERENT PERMANENT ADDRESS to whom we might write, in the event we are unable to contact you:

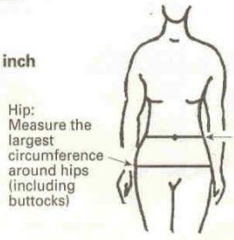
Name: _____

Address: _____

42. Question 42, which should only be answered if a tape measure is convenient, asks about body measurements. This information will be more accurate if you follow these suggestions:

- Make measurements while standing
- Avoid measuring over bulky clothing
- Try to record answers to the nearest 1/4 inch (do not estimate)

If a tape measure is not available, please leave blank.



WAIST		HIP	
Inches	Fraction	Inches	Fraction
0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 8 9	4: 0, 1/4, 2/4, 3/4	0 0 1 1 2 2 3 3 4 4 5 5 6 6 7 8 9	4: 0, 1/4, 2/4, 3/4

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NURSES' HEALTH STUDY II — PAGE 5 — HARVARD UNIVERSITY

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Many participants have pointed out that stress, personal and family relationships, and other aspects of quality of life are important factors relating to health. We have added the following questions to learn more about these areas. (As always, all of your responses will remain strictly confidential.)

43. **These questions are about how you feel and how things have been with you *during the past 4 weeks*.**
For each question, please give the one answer that comes closest to the way you have been feeling.
How much of the time during the *past 4 weeks* ... *(Mark one response on each line.)*

	All of the time	Most of the time	A Good Bit of the time	Some of the time	A Little of the time	None of the time
Did you feel full of pep?	○	○	○	○	○	○
Have you been a very nervous person?	○	○	○	○	○	○
Have you felt so down in the dumps nothing could cheer you up?	○	○	○	○	○	○
Have you felt calm and peaceful?	○	○	○	○	○	○
Did you have a lot of energy?	○	○	○	○	○	○
Have you felt downhearted and blue?	○	○	○	○	○	○
Did you feel worn out?	○	○	○	○	○	○
Have you been a happy person?	○	○	○	○	○	○
Did you feel tired?	○	○	○	○	○	○

44. **During the *past 4 weeks*, how much of the time has your *physical health or have emotional problems* interfered with your social activities (like visiting with friends, relatives, etc.)?**
○ All of the time ○ Most of the time ○ Some of the time ○ A little of the time ○ None of the time

45. **Please choose the answer that best describes how true or false each of the following statements is for you.** *(Mark one response on each line.)*

	Definitely True	Mostly True	Not Sure	Mostly False	Definitely False
Over the past 4 weeks, I have felt about the same as I have felt during the past year	○	○	○	○	○
I seem to get sick a little easier than other people	○	○	○	○	○
I am as healthy as anybody I know	○	○	○	○	○
I expect my health to get worse	○	○	○	○	○
My health is excellent	○	○	○	○	○

46. **The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?** *(Mark one response on each line.)*

	Yes, Limited A Lot	Yes, Limited A Little	No, Not Limited At All
Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	○	○	○
Moderate activities, such as moving a table, pushing a vacuum, bowling, or golfing	○	○	○
Lifting or carrying groceries	○	○	○
Climbing *several* flights of stairs	○	○	○
Climbing *one* flight of stairs	○	○	○
Bending, kneeling, or stooping	○	○	○
Walking *more than a mile*	○	○	○
Walking *several blocks*	○	○	○
Walking *one block*	○	○	○
Bathing or dressing yourself	○	○	○

47. **During the *past 4 weeks*, have you had any of the following problems with your work or other regular daily activities as a result of any *emotional problems* (such as feeling depressed or anxious)?** *(Mark one response on each line.)*
a) Cut down the amount of time you spent on work or other activities ○ Yes ○ No
b) Accomplished less than you would like ○ Yes ○ No
c) Didn't do work or other activities as carefully as usual ○ Yes ○ No

48. **During the *past 4 weeks*, to what extent has your physical health or have emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?**
○ Not at all ○ Slightly ○ Moderately ○ Quite a bit ○ Extremely

49. **How much *bodily* pain have you had during the *past 4 weeks*?**
○ None ○ Very mild ○ Mild ○ Moderate ○ Severe ○ Very severe

50. **During the *past 4 weeks*, how much did *bodily pain* interfere with your normal work (including both work outside the home and housework)?**
○ Not at all ○ A little bit ○ Moderately ○ Quite a bit ○ Extremely

51. **During the *past 4 weeks*, have you had any of the following problems with your work or other regular daily activities as a result of your *physical health*?** *(Mark one response on each line.)*
a) Cut down the amount of time you spent on work or other activities ○ Yes ○ No
b) Accomplished less than you would like ○ Yes ○ No
c) Were limited in the kind of work or other activities ○ Yes ○ No
d) Had difficulty performing the work or other activities (for example, it took extra effort) ○ Yes ○ No

PLEASE CONTINUE ON PAGE 6

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52. **In general, would you say your health is:** ○ Excellent ○ Very Good ○ Good ○ Fair ○ Poor

53. **Do you have an unreasonable fear of being in enclosed spaces such as stores, elevators, etc.?**
○ Often ○ Sometimes ○ Never

54. **Do you find yourself worrying about getting some incurable illness?** ○ Often ○ Sometimes ○ Never

55. **Are you scared of heights?** ○ Very ○ Moderately ○ Not at all

56. **Do you feel panicky in crowds?** ○ Always ○ Sometimes ○ Never

57. **Do you worry unduly when relatives are late coming home?** ○ Yes ○ No

58. **Do you feel more relaxed indoors?** ○ Definitely ○ Sometimes ○ Not particularly

59. **Do you dislike going out alone?** ○ Yes ○ No

60. **Do you feel uneasy traveling on buses or trains even when they are not crowded?**
○ Very ○ A little ○ Not at all

61. **If you are retired or stopped working due to illness/injury, at what age were you last in paid employment?**
○ Still working ○ < Age 25 ○ 25 - 29 ○ 30 - 34 ○ 35 - 39 ○ Age 40 or older

62. **If you have been employed within the past 2 years, the following questions relate to your current or most recent job:**
○ Not employed in last 2 years

Please choose the answer which best describes the degree to which you agree or disagree with each of the following statements.	Strongly Disagree	Disagree	Agree	Strongly Agree
My job requires that I learn new things	○	○	○	○
My job involves a lot of repetitive work	○	○	○	○
My job requires me to be creative	○	○	○	○
My job allows me to make a lot of decisions on my own	○	○	○	○
My job requires a high level of skill	○	○	○	○
On my job, I have very little freedom to decide how I do my work	○	○	○	○
I get to do a variety of different things on my job	○	○	○	○
I have a lot of say about what happens on my job	○	○	○	○
I have an opportunity to develop my own special abilities	○	○	○	○
My job requires working very fast	○	○	○	○
My job requires working very hard	○	○	○	○
My job requires lots of physical effort	○	○	○	○
I am not asked to do an excessive amount of work	○	○	○	○
I have enough time to get the job done	○	○	○	○
My job security is good	○	○	○	○
I am free from conflicting demands that others make	○	○	○	○
People I work with are competent in doing their jobs	○	○	○	○
People I work with take a personal interest in me	○	○	○	○
People I work with are friendly	○	○	○	○
People I work with are helpful in getting the job done	○	○	○	○

	Strongly Disagree	Disagree	Agree	Strongly Agree	Not Applicable
a) My supervisor is concerned about the welfare of those under her	○	○	○	○	○
My supervisor pays attention to what I am saying	○	○	○	○	○
My supervisor is helpful in getting the job done	○	○	○	○	○
My supervisor is successful in getting people to work together	○	○	○	○	○

b) **How long have you worked in the job you described above?**
○ < 6 months ○ 6 months - 11 months ○ 1 - 2 years ○ 3 - 4 years ○ 5 - 9 years ○ 10 or more years

c) **How many hours per week do you work, on average, in your job?**
○ < 15 hours ○ 15 - 20 ○ 21 - 40 ○ 41 - 60 ○ 61 - 80 ○ More than 80 hours per week

63. **How many hours per week do you spend in housework (including cooking, cleaning, shopping for food, doing laundry and dishes, doing repairs, paying bills, making arrangements and caring for children)?**
○ 0 - 19 hours ○ 20 - 39 ○ 40 - 59 ○ 60 - 79 ○ 80 - 100 hours

64. **Thinking of all the things that are done in your household, what percentage do you personally do?**
○ 0 - 25 percent ○ 26 - 39 ○ 40 - 60 ○ 61 - 74 ○ 75 - 99 ○ 100 percent

65. **Is there any one special person you know that you feel very close to; someone you feel you can share confidences and feelings with?**
○ Yes → a) **How often do you see or talk with this person?**
○ No ○ Daily ○ Weekly ○ Monthly ○ Several times/year ○ Once/year or less

THANK YOU! Please return the questionnaire in the enclosed postage-paid envelope to: Walter Willett, M.D. Nurses' Health Study II 677 Huntington Avenue Boston, MA 02115

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Appendix 3: Objective 2 Data Collection Tools

Light Environment Study

Participant Questionnaire

First Name_____ ID_____

1. Current Weight _____kg or _____lbs.

2. Height_____ cm or _____ feet _____ inches

3. Are you currently taking any medications, hormones or supplements? Yes No

   a. If yes, please tell me all the medications you are currently taking: **Please include all vitamins, supplements, prescription and non-prescription medications**:

Medication	Dosage	Time taken

4. In the past 24 hours have you had any alcoholic beverages? Yes No

   a. If yes, how many drinks did you have in the past 24 hours? #_____

5. In the past 24 hours have you smoked any cigarettes? Yes No

   a. If yes, how many cigarettes did you smoke in the past 24 hours? #_____

6. In the past 24 hours have you done any exercise? Yes No

If yes, please tell me what exercise you did in the past 24 hours:

Type of Exercise	Time of Exercise	Duration in Minutes	Intensity Level

7. Approximately what time did you go to bed last night? _____

8. Approximately what time did you get up this morning?_____

9. Looking at this list, which category or categories best describe your ethnic background? (which most closely describes the part of the world you or your ancestors came from) You can pick as many as apply:

❍ British (such as England, Scotland, Ireland, Wales)

❍Northern European (such as, Austria, Latvia, Lithuania, Estonia, Denmark, France Germany, Luxemburg, Netherlands/Holland, Sweden, Norway, Finland, Switzerland)

❍Southern European (such as, Greece, Italy, Portugal, Spain, Former Yugoslavia, Malta, Cyprus, other Southern European country)

❍Eastern European (such as, Bulgaria, Former Czechoslovakia, Hungary, Poland, Romania, Russia)

❍North-East Asian (such as, China, Hong Kong, Japan, Korea, Macao, Taiwan)

❍South-East Asian (such as, Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar/Burma, Philippines, Singapore, Thailand, Vietnam)

❍South Asian (such as, Afghanistan, Bangladesh, India, Nepal, Pakistan, Sri Lanka)

❍Middle Eastern (such as, Israel, Iran, Iraq, Lebanon, Turkey, Egypt)

❍African (such as, North Africa, Sub-Saharan Africa, Zimbabwe, South Africa)

❍First Nations (Native Canadian/American, Inuit)

❍Latin American (such as, Mexico, Central or South America)

❍Caribbean (such as, Jamaica, Trinidad & Tobago, Haiti, Guadeloupe, Dominica, Anguilla, Dominican Republic)

❍Other (please specify) _____

Appendix 4: Objective 3 Data Collection Tools

Appendix 4.1: Sample Day 1 from 3-Day Diet and Lifestyle

DAY 1 Date _____

SLEEP:
Went to Bed _____ a.m. / p.m. Went to Sleep _____ a.m./ p.m. Woke Up _____ a.m./ p.m. (stop urine collection after morning void)

Notes
_____

Time	Exercise	Alcohol	Medications	Notes
12:00 a.m. *midnight*				
1:00 a.m.				
2:00 a.m.				
3:00 a.m.				
4:00 a.m.				
5:00 a.m.				
6:00 a.m.				
7:00 a.m.				
8:00 a.m.				
9:00 a.m.				
10:00 a.m.				
11:00 a.m.				
12:00 p.m. *noon*				
1:00 p.m.				
2:00 p.m.				
3:00 p.m.				
4:00 p.m.				

5:00 p.m.				
6:00 p.m.				
7:00 p.m.				
8:00 p.m. START URINE COLLECTION				
9:00 p.m.				
10:00 p.m.				
11:00 p.m.				

Appendix 4.2: Light Study Questionnaire

1. How old are you? _____yrs

2. How tall are you? _____cm **or** _____ft _____in

3. How much do you currently weigh? _____kgs or _____lbs

4. Considering only your own "feeling best" rhythm, at what time would you get up if you were entirely free to plan your day?

   - O 5:00-6:30 a.m. (5)
   - O 6:30-7:45 a.m. (4)
   - O 7:45-9:45 a.m. (3)
   - O 9:45-11:00 a.m. (2)
   - O 11:00 a.m.-12:00 (noon) (1)

5. Considering only your own "feeling best" rhythm, at what time would you go to bed if you were entirely free to plan your evening?

   - O 8:00-9:00 p.m. (5)
   - O 9:00-10:15 p.m. (4)
   - O 10:15 p.m.- 12:30 a.m. (3)
   - O 12:30-1:45 a.m. (2)
   - O 1:45-3:00 a.m. (1)

6. If there is a specific time at which you have to get up in the morning, to what extent are you dependent on being woken up by an alarm clock?

- O Not at all dependent (4)
- O Slightly dependent (3)
- O Fairly dependent (2)
- O Very dependent (1)

7. Assuming adequate environmental conditions, how easy do you find getting up in the mornings?

- O Not at all easy (1)
- O Not very easy (2)
- O Fairly easy (3)
- O Very easy (4)

8. How alert do you feel during the first half-hour after having woken in the mornings?

- O Not at all alert (1)
- O Slightly alert (2)
- O Fairly alert (3)
- O Very alert (4)

9. How is your appetite during the first half-hour after having woken in the mornings?

- O Very poor (1)
- O Fairly poor (2)
- O Fairly good (3)
- O Very good (4)

10. During the first half-hour after having woken in the morning, how tired do you feel?

- O Very tired (1)
- O Fairly tired (2)
- O Fairly refreshed (3)
- O Very refreshed (4)

11. When you have no commitments the next day, at what time do you go to bed compared to your usual bedtime?

- O Seldom or never later (4)
- O Less than one hour later (3)
- O 1 – 2 hours later (2)
- O More than two hours later (1)

12. You have decided to engage in some physical exercise. A friend suggests that you do this one hour twice a week and the best time for him is between 7:00 – 8:00 a.m. Bearing in mind nothing else but your own "feeling best" rhythm, how do you think you would perform?

- O Would be in good form (4)
- O Would be in reasonable form (3)
- O Would find it difficult (2)
- O Would find it very difficult (1)

13. At what time in the evening do you feel tired, and as a result, in need of sleep?

- O 8:00-9:00 p.m. (5)
- O 9:00-10:15 p.m. (4)
- O 10:15 p.m.- 12:45 a.m. (3)
- O 12:45-2:00 a.m. (2)
- O 2:00-3:00 a.m. (1)

14. You wish to be at your peak performance for a test which you know is going to be mentally exhausting and lasting for two hours. You are entirely free to plan your day and condsidering only your own "feeling best" rhythm which ONE of the four testing times would you choose?

- O 8:00-10:00 a.m. (6)
- O 11:00 a.m.-1:00 p.m. (4)
- O 3:00-5:00 p.m. (2)
- O 7:00-9:00 p.m. (0)

15. If you went to bed at 11:00 p.m. at what level of tiredness would you be?

- O Not at all tired (0)
- O A little tired (2)
- O Fairly tired (3)
- O Very tired (5)

16. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which ONE of the following events are you most likely to experience?

- O Will wake up at usual time and will NOT fall asleep (4)
- O Will wake up at usual time and will doze thereafter (3)
- O Will wake up at usual time but will fall asleep again (2)
- O Will NOT wake up until later than usual (1)

17. One night you have to remain awake between 4:00-6:00 a.m. in order to carry out a night watch. You have no commitments the next day. Which ONE of the following alternatives will suit you best?

- O Would NOT go to bed until watch was over (1)
- O Would take a nap before and sleep after (2)
- O Would take a good sleep before and nap after (3)
- O Would take ALL sleep before watch (4)

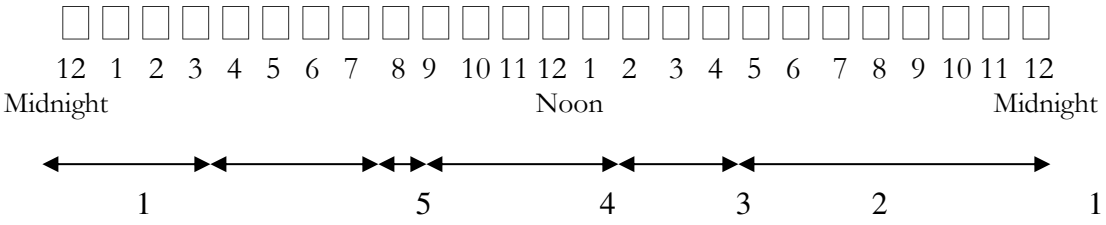
18. You have to do two hours of hard physical work. You are entirely free to plan your day and considering only your own "feeling best" rhythm which ONE of the following times would you choose?

- O 8:00-10:00 a.m. (4)
- O 11:00 a.m.-1:00 p.m. (3)
- O 3:00-5:00 p.m. (2)
- O 7:00-9:00 p.m. (1)

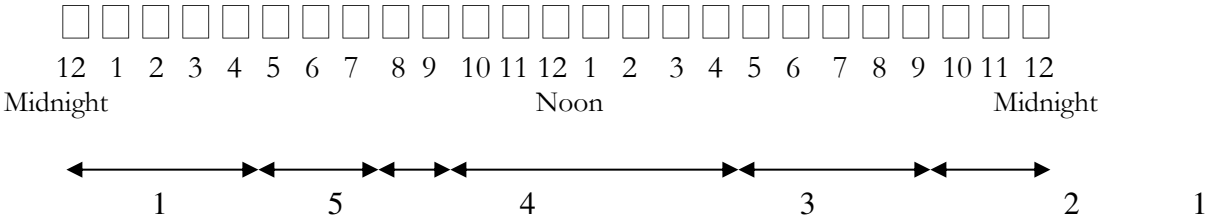
19. You have decided to engage in hard physical exercise. A friend suggests that you do this for one hour twice a week and the best time for him is between 10:00 – 11:00 p.m. Bearing in mind nothing else but your own "feeling best" rhythm how well do you think you would perform?

- O Would be in good form (1)
- O Would be in reasonable form (2)
- O Would find it difficult (3)
- O Would find it very difficult (4)

20. Suppose that you can choose your own work hours. Assume that you worked a FIVE hour day (including breaks) and that your job was interesting and paid by results. Which FIVE CONSECUTIVE HOURS would you select?



21. At what time of the day do you think that you reach your "feeling best" peak?



22. One hears about "morning" and "evening" types of people. Which ONE of these types do you consider yourself to be?

- O Definitely a morning type (6)
- O More a morning than an evening type (4)
- O More an evening than a morning type (2)
- O Definitely an evening type (0)

23. Where do you usually spend your days?

- O Home
- O At the home of someone else
- O Work or School → please specify e.g. office, restaurant, classroom, outdoors

  _____

- O Other → please specify _____

24. Where do you spend most of your time during the day?

- O Outdoors
- O Indoors, in very bright light (either artificial or from windows)
- O Indoors, average office brightness
- O Indoors, dimmer than an average office
- O Indoors, in dim light
- O Don't know

25. Where do you spend most of your time during the evening?

- O Outdoors
- O Indoors, in very bright light (either artificial or from windows)
- O Indoors, average room brightness
- O Indoors, dimmer than an average room
- O Indoors, in dim light
- O Don't know

26. At night, do you have a light on in the bedroom while sleeping?

- O Yes, regular light
- O Yes, nightlight
- O No

27. During your childhood (12 yrs and under), how would you describe your usual environment during the day?

- O Very bright (e.g., outdoors)
- O Brighter than average (bright lights and/or lots of window light)
- O Average brightness (average office brightness)
- O Dimmer than average (average home brightness without a lot of window light)
- O Very dim
- O Don't know / Don't remember

28. During your childhood (12 years and under), how would you describe your environment during the evening?

- O Very bright (bright as day)
- O Brighter than average
- O Average home brightness
- O Dimmer than average
- O Very dim
- O Don't know / Don't remember

29. During your childhood did you have a light on while sleeping?

- O Yes, regular light
- O Yes, nightlight
- O No

30. During your teenage years, how would you describe your environment during the day?

- O Very bright (e.g., outdoors)
- O Brighter than average (bright lights and/or lots of window light)
- O Average brightness (average office brightness)
- O Dimmer than average (average home brightness without a lot of window light)
- O Very dim
- O Don't know / Don't remember

31. During your teenage years, how would you describe your environment during the evening?

- O Very bright (bright as day)
- O Brighter than average
- O Average home brightness
- O Dimmer than average
- O Very dim
- O Don't know / Don't remember

32. During, your teenage years, did you have a light on while sleeping?

- O Yes, regular light
- O Yes, nightlight
- O No

Appendix 5: Ethics Approval Letter

UNIVERSITY OF TORONTO

OFFICE OF THE VICE PRESIDENT, RESEARCH

PROTOCOL REFERENCE # 27537

April 11, 2012

Dr. Julia A Knight
DALLA LANA SCHOOL OF PUBLIC HEALTH
FACULTY OF MEDICINE

Mr. David Stock
DALLA LANA SCHOOL OF PUBLIC HEALTH
FACULTY OF MEDICINE

Dear Dr. Knight and Mr. David Stock,

Re: Your research protocol entitled, "Reproductive markers of circadian disruption and breast cancer risk"

ETHICS APPROVAL	**Original Approval Date: April 11, 2012** **Expiry Date: April 10, 2013** **Continuing Review Level: 1**

We are writing to advise you that the Health Sciences Research Ethics Board (REB) has granted approval to the above-named research protocol under the REB's delegated review process. Your protocol has been approved for a period of **one year** and ongoing research under this protocol must be renewed prior to the expiry date.

Any changes to the approved protocol or consent materials must be reviewed and approved through the amendment process prior to its implementation. Any adverse or unanticipated events in the research should be reported to the Office of Research Ethics as soon as possible.

Please ensure that you submit an Annual Renewal Form or a Study Completion Report 15 to 30 days prior to the expiry date of your current ethics approval. Note that annual renewals for studies cannot be accepted more than 30 days prior to the date of expiry.

If your research is funded by a third party, please contact the assigned Research Funding Officer in Research Services to ensure that your funds are released.

Best wishes for the successful completion of your research.

Yours sincerely,

Judith Friedland, Ph.D.
REB Chair

Daniel Gyewu
REB Manager

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