# INSULIN-LIKE GROWTH FACTOR PEPTIDES AND MELATONIN AMONG ROTATING SHIFT NURSES

by

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# Abstract

**Background:** In 2007, the International Agency for Research on Cancer (IARC) classified long-term shift work as a probable human carcinogen; however, the mechanism through which shift work potentially increases cancer risk is not known. One hypothesis is that diminished melatonin production may be involved, possibly as a result of exposure to light during night work. Experimental studies suggest a link between melatonin and peptides in the insulin-like growth factor (IGF) family, also implicated in carcinogenesis. This research aimed to describe the distributions of circulating concentrations of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3) and their associations with urinary melatonin as possible intermediates in the pathway between work at night and breast cancer.

**Methods:** A cross-sectional study was conducted among 85 premenopausal nurses working a rotating shift pattern of two 12-hour days, two 12-hour nights, and five days off. Once during both the summer and winter seasons, melatonin metabolites were measured in urine samples and circulating concentrations of IGF-I and IGFBP-3 were determined from serum samples. Weight and height were measured by the study coordinator, while a questionnaire and study diaries were used to collect all other covariate information. Predictors of IGF levels were identified using multivariate mixed effects modeling and relationships between melatonin and the IGFs were investigated using Spearman's rank correlation and multivariate mixed effects modeling.

**Results:** Both age ($\beta = -3.6$, $p < 0.0001$) and current OC use ($\beta = -40.8$, $p = 0.003$) were associated with decreases in circulating IGF-I, while levels of IGF-I were increased in the winter months ($\beta = 26.3$, $p = 0.02$). A positive relationship between recent alcohol consumption and serum IGFBP-3 was also suggested ($\beta = 197.8$, $p = 0.05$). Neither

Spearman's rank correlations nor mixed effects modeling indicated that urinary melatonin was a determinant of serum IGFs.

**Conclusions:** Age, season, and current OC use were observed to predict circulating IGF-I, while recent alcohol consumption was a determinant of IGFBP-3 levels. A relationship between melatonin and IGFs, theorized as a component of the mechanism linking shift work and cancer, was not supported by the results of this project.

# Co-Authorship

This thesis contains the work of Kirstin Boehme in collaboration with her thesis supervisors, Drs Harriet Richardson and Kristan Aronson. The parent study of which this thesis was a part was designed by Drs Kristan Aronson, Charles Graham, Ian Janssen, Glenville Jones, Harriet Richardson, Joan Tranmer, and PhD student Anne Grundy. Funding for the parent study was received from the Workplace Safety and Insurance Board of Ontario (WSIB).

Measurement of serum insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3) was completed by Kirstin Boehme, while quantification of urinary creatinine and metabolites of melatonin was conducted by students Anne Grundy, Annie Langley, Mark McPherson, and Lindsay Kobayashi. All immunoassay assessments were completed in the laboratory of Dr. Graham. The statistical analyses, interpretation of results, and the writing of this thesis were completed by Kirstin Boehme, under the guidance of Drs Richardson and Aronson.

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# List of Acronyms and Abbreviations

| | |
|---|---|
| AADC | Aromatic aminoacid decarboxylase |
| AANAT | Arylalkylamine N-acetyltransferase |
| ALS | Acid labile subunit |
| aMT6s | 6-sulfatoxymelatonin |
| BMI | Body mass index |
| CI | 95% Confidence interval |
| CV | Coefficient of variation |
| ELISA | Enzyme-linked immunosorbent assay |
| EPIC | European Prospective Investigation into Cancer and Nutrition |
| GH | Growth hormone |
| HIOMT | Hydroxyindole-O-methyltransferase |
| HRP | Horseradish peroxidase |
| IARC | International Agency for Research on Cancer |
| ICC | Intra-class correlation coefficient |
| IGF | Insulin-like growth factor |
| IGF-I | Insulin-like growth factor-I |
| IGF-II | Insulin-like growth factor-II |
| IGFBP | Insulin-like growth factor binding protein |
| IGFBP-3 | Insulin-like growth factor binding protein-3 |
| IGF-I/IGFBP-3 | Molar ratio of IGF-I to IGFBP-3 |
| IGF-IR | Insulin-like growth factor receptor I |
| IGF-IIR | Insulin-like growth factor receptor II |
| KGH | Kingston General Hospital |
| KIRA | Kinase receptor activation assay |
| LAN | Light at night |
| MDE | Minimum detectable effect |
| MET | Metabolic equivalent score |
| mRNA | Messenger ribonucleic acid |
| MT1 | Melatonin receptor 1 |
| NHS | Nurses' Health Study |
| NHS I | Nurses' Health Study I |

| | |
|---|---|
| NHS II | Nurses' Health Study II |
| OR | Odds ratio |
| ORDET | Hormones and Diet in the Etiology of Breast Cancer Risk |
| OC | Oral contraceptive |
| $R^2$ | R-squared value |
| SAS | Statistical Analysis Software |
| Sirt1 | Sirtuin 1 |
| SCN | Suprachiasmatic nucleus |
| TMB | Tetramethylbenzidin |
| TRAMP | Transgenic adenocarcinoma of the mouse prostate |
| TRP | Tryptophan hydroxylase |
| WSIB | Workplace Safety and Insurance Board |

# Chapter 1
# Introduction

## 1.1 Breast cancer

Breast cancer continues to represent an important chronic disease worldwide. As the most common form of female cancer, breast cancer accounts for approximately 14% of all cancer-attributed deaths (1). In Canada, an estimated one in nine women will be diagnosed with breast cancer at some point within their lifetime (2). Although a multitude of factors have been determined or posited as predictors of breast cancer, such as age and exogenous estrogen exposure (3,4), established risk factors only seem to explain approximately half of the estimated probability of developing the disease (5).

## 1.2 Shift-work and breast cancer

In 2007, the International Agency for Research on Cancer (IARC) added "'shift-work that involves circadian disruption'" to their list of environmental risk factors, classifying the exposure as "'probably carcinogenic to humans'" (Group 2A) (6). This decision was based on the presence of "'sufficient evidence in experimental animals'" and "'limited evidence in humans'" (6). Epidemiologic research on nonstandard work schedules has demonstrated increases in risk for numerous cancers, with the plurality of work having pertained to breast cancer (7). Indeed, meta-analyses have documented statistically significant increases in breast cancer risk among shift working females, with increases ranging from 40 – 50% (8,9).

### 1.3 Light at night, melatonin, and the insulin-like growth factor family

Numerous theories regarding the pathway between shift work and increased breast cancer risk have been suggested (10), one of which implicates exposure to light at night (LAN) as a major contributing factor (11). Specifically, it has been hypothesized that exposure to irregular patterns of light alters the circadian production (i.e. daily cycle) of melatonin (11,12), a highly conserved hormone that aids in maintaining other important daily rhythms within the body by providing light:dark information (13,14). Circulating levels of melatonin have been shown to peak during hours of darkness, with the rhythmic production of the hormone being highly sensitive to exposures of LAN (14,15), as is commonly experienced by night shift workers.

Clinic-based studies regarding LAN and melatonin (16-18) and epidemiologic investigations pertaining to shift work, LAN, and urinary melatonin (19-21), as well as melatonin and breast cancer (22-27), have reported results that help to substantiate the melatonin hypothesis. Convincing evidence is also available from experimental studies. Rodents having been exposed to LAN experienced accelerated initiation and progression of carcinogenesis (28,29). Additional laboratory studies have documented melatonin's participation in scavenging free radicals and limiting vascular growth and cellular proliferation, properties associated with antioxidant, antiangiogenic, and antimitotic pathways, respectively (13,30).

The insulin-like growth factor (IGF) family has also been researched regarding cancer risk, initiation, and progression (31-33). Numerous reviews have been completed pertaining to IGFs and breast cancer specifically (34-38), with the most recent pooled analysis having found significantly increased risk among both pre- and postmenopausal women with elevated circulating concentrations of insulin-like growth factor-I (IGF-I) (38).

Furthermore, research involving animal models and various cancer cell lines have indicated that increased IGF-I heightens the risk of initial tumour development and that IGF-I acts as a powerful mitogen (cell transformer) for cancerous cells (31,32).

A link between melatonin and the IGF-axis may be an intermediary on the potential pathway between shift work and breast cancer. The most convincing evidence to support a relationship between melatonin and the IGF family has been provided by experimental studies among animals. Research pertaining to photoperiod, as well as endogenous and exogenous melatonin, have documented effects on levels of IGFs in a number of animal models (39-52); although, the direction of the association has been inconsistent. Additionally, a limited number of small epidemiologic studies have been completed; however, the results have not been conclusive (53-60).

### 1.4 Overview of study rationale

Evidence is mounting regarding the possible carcinogenic effect of nonstandard work schedules; nevertheless, a lack of knowledge pertaining to how this effect may be mediated still remains. One potential pathway involves melatonin and the IGF family. Experimental studies using animal models have assessed the relationship between LAN/melatonin and the IGF family and provided supporting evidence. However, the existence of a link between melatonin and IGF peptides among humans is still unclear. To date, limited epidemiologic research has been completed, with conflicting results reported. More observational studies, particularly conducted among healthy individuals, are required so that the presence of a possible association between melatonin and IGFs and its relevance to human health and disease may be discerned. In today's industrialized society, shift work remains a necessity and the prevalence is unlikely to decrease. A greater understanding of the mechanism through which shift work may

increase cancer risk is imperative, so that the development of interventions or the implementation of preventative measures may occur.

### 1.5 Study objectives and overview of design

Primarily, this thesis project aimed to examine the premenopausal relationship between urinary levels of melatonin and circulating concentrations of IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) among a group of healthy rotating shift nurses as a possible component in the pathway between exposure to shift-work and the development of breast cancer. Potential predictors of IGF-I and IGFBP-3 among the study population were also assessed.

Conducted cross-sectionally, this research was part of an observational study conducted by Dr. Kristan Aronson and collaborators, which aimed to investigate occupational and lifestyle factors as determinants of melatonin levels among female rotating shift nurses at Kingston General Hospital (KGH) (61). Participants, recruited over a two-year period, followed a rotating shift pattern that consisted of two 12-hour day shifts (7:00 am – 7:00 pm) and two 12-hour night shifts (7:00 pm – 7:00 am), accompanied by five days off. Nurses were asked to complete two data collection periods (summer and winter), with each including a night shift and a day shift component. Concentrations of melatonin and the IGFs were determined from overnight urine and fasting serum samples, respectively, while height and weight measurements were recorded by the study coordinator. Other covariate information was obtained from a self-administered questionnaire and 24-hour study diaries.

## 1.6 Thesis structure

This thesis is comprised of a review of the literature pertaining to shift work, LAN, melatonin, and the IGF family in the context of breast cancer risk; a detailed description of the methodological aspects of the study; a chapter of the main results obtained; as well as a general discussion of the study's findings, strengths and limitations, and final conclusions. Several appendices are also attached. Appendix A contains a brief overview of various possible biological mechanisms through which melatonin and the IGF family may be related. Important documents from the parent study, such as recruitment and data collection tools, may be found in Appendix B, while details regarding the laboratory procedures used to quantify levels of melatonin, creatinine, IGF-I, and IGFBP-3 are located in Appendix C. Documentation of ethics approval may be found in Appendix D. Finally, additional results are presented in Appendix E.

# Chapter 2
# Literature Review

## 2.1 Introduction

This chapter contains a summary of the current literature regarding breast cancer etiology, where shift work, melatonin, and the insulin-like growth factor (IGF) family are presented as components in a possible intermediary pathway.

## 2.2 Breast cancer etiology

Breast cancer continues to represent a major health issue globally. As the most common form of female cancer, breast cancer accounts for approximately 14% of cancer-attributed deaths worldwide (1). In 2011, 23 400 female incident cases and 5 100 female deaths due to breast cancer were expected to occur in Canada, and an estimated one in nine Canadian women will be diagnosed with breast cancer at some point within their lifetime (2). Numerous factors have been established or suggested as determinants of breast cancer, including age, anthropometry, geographic location of birth, family history, reproductive factors, and exogenous estrogen exposure (3,4). Unfortunately, research to date has yet to elucidate all major causes since established risk factors seem to explain only about half of the estimated probability of developing breast cancer (5).

## 2.3 Shift work and breast cancer

In 2007, the International Agency for Research on Cancer (IARC) added "'shift-work that involves circadian disruption'" to their list of environmental risk factors, classifying the exposure as "'probably carcinogenic to humans'" (Group 2A), a decision

driven by "'sufficient evidence in experimental animals'" and "'limited evidence in humans'" (6). Due to the 24-hour nature of today's industrialized society, shift work remains a necessity, especially in health care, and the prevalence is unlikely to decrease (7,62). It was estimated that in 2005, approximately 12% of female Canadian workers followed a work schedule other than a standard day shift, the most common of which included rotating and irregular shift schedules, two of the more difficult shift patterns with regard to physiological adjustments (63). Given this widespread exposure to shift work and the overall elevated risk of breast cancer, it is important to discern the mechanisms through which these two factors may be related in order that interventions may be successfully developed.

### 2.3.1 Epidemiologic studies

Epidemiologic studies concentrating on the association between shift work and various cancers, breast in particular, have provided limited yet compelling evidence for a relationship, with many having found statistically significant increases in risk (7-9,30,64). In 2005, a meta-analysis of seven airline cabin crew studies and six studies of other female shift workers found an aggregate risk estimate for breast cancer of 1.48 (95% CI 1.36-1.61) (9). Nonetheless, the total number of investigations completed and the variety of occupations evaluated have been relatively small, exposure classification has varied, and assessment of potential confounders has been limited (8-10,64). Furthermore, the particular components of shift work and the specific intermediary pathways through which these aspects may increase risk have yet to be elucidated (10).

### 2.3.2 Potential intermediate pathways

Determining the mechanism through which shift work and breast cancer may be linked is challenging. Multiple hypotheses have been put forth regarding potential intermediates, such as sleep disruption, decreased sunlight exposure and vitamin D production, and disrupted lifestyle behaviours (e.g. changes to diet, alcohol intake, or levels of exercise) (10). Consequently, a complex theoretical framework can be generated, given that several pathways may be responsible and mechanisms may be intertwined (10).

A common theory regarding nonstandard work schedules and increased breast cancer risk implicates exposure to light at night (LAN) as a major contributing factor. The original hypothesis regarding the carcinogenic role of LAN was put forth in 1987, with Stevens (11) citing correlated ecologic trends in breast cancer and electrical lighting, as well as animal laboratory studies as a basis for this supposition. Population studies focused on other markers for altered photoperiod, such as blindness, specifically those who do not experience any receptivity to light, longer sleep durations, and increased ambient light during hours of sleep, have also generally supported an association between LAN and breast cancer (7). Notably, experimental studies further reinforce this relationship as research has indicated that rodents having been exposed to altered light:dark cycles also experience an accelerated initiation and progression of carcinogenesis (28,29). The primary hypothesis regarding exposure to LAN and increased breast cancer risk suggests that the effects may be mediated through a disruption in the circadian secretion pattern (i.e. daily cycle) of the hormone melatonin (11,12).

## 2.4 Melatonin

Produced primarily in humans by the pinealocytes of the pineal gland, melatonin is a highly conserved hormone involved in many biological processes; the most understood of which involves the regulation of other important circadian rhythms through the provision of light:dark information (13,14). Specifically, the majority of melatonin production is regulated through a phototransduction pathway linking photopigments within the retina, the suprachiasmatic nuclei (SCN) of the hypothalamus, termed the 'master circadian clock', and the pineal gland (14,65). Within the pinealocytes, the amino acid tryptophan undergoes a series of enzymatic processes, resulting in the formation of N-acetyl-5-methoxytryptamine, or melatonin (see Figure 2.1) (14). Melatonin is not stored in the pineal gland, but released into the blood stream, traveling throughout the body (14). Circulating levels of melatonin have been shown to peak during hours of darkness (approximately 2:00 am – 4:00 am) and decline during daylight hours, with the rhythmic production of the hormone being sensitive to exposures of light (14,15).

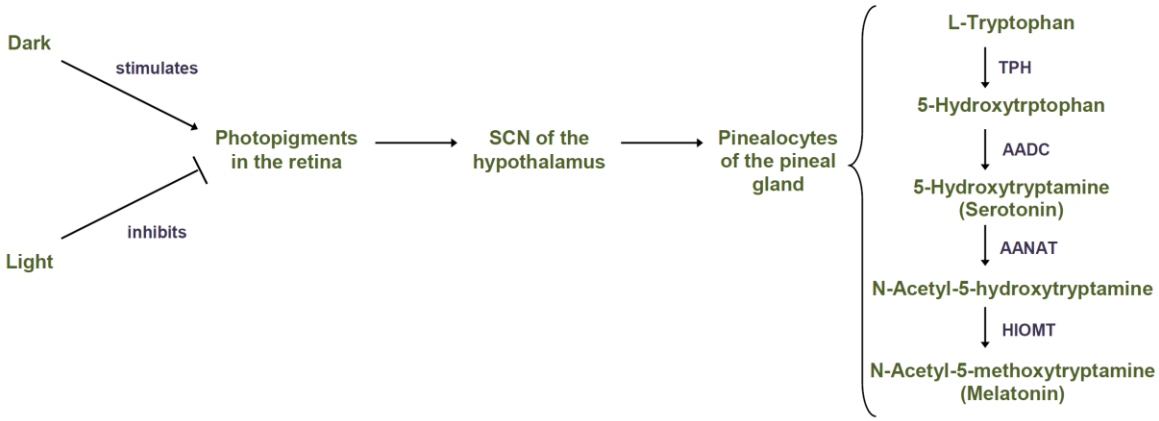


**Figure 2.1** Melatonin synthesis

(SCN = suprachiasmatic nuclei; TPH = tryptophan hydroxylase; AADC = aromatic aminoacid decarboxylase; AANAT = arylalkylamine N-acetyltransferase; HIOMT = hydroxyindole-O-methyltransferase) (14,15)

### 2.4.1 Light at night and melatonin

Given the role of light:dark cycles in regulating its rhythmic production, melatonin is a candidate for an intermediate in the theorized pathway between LAN and cancer (14,15,62). Indeed, controlled, clinic-based studies in humans have noted dose-response relationships between LAN exposure and reductions in melatonin levels (16-18). Several epidemiologic investigations regarding occupational LAN via rotating shift work also observed significant lowering of 24-hour urinary melatonin concentrations with night shifts, as compared to day shifts or days off (19,20). An inverse relationship between number of night shifts worked within the previous two weeks and morning urinary melatonin has been observed as well (21).

### 2.4.2 Determinants of melatonin

Additionally, several demographic, lifestyle, and environmental factors have been shown to influence melatonin concentrations.

*Age, sex, ethnicity, and body mass index*

Levels of melatonin appear to decrease with increasing age (14,66,67), as well as body mass index (BMI) (21,67,68). Some evidence also suggests that sex (69) and ethnicity (70,71) may affect melatonin concentrations.

*Hormonal and reproductive factors*

Hormonal and reproductive characteristics such as parity (67,68) and oral contraceptive (OC) use (67,72,73) may increase melatonin levels. The importance of menstrual cycle stage on melatonin secretion, however, remains unclear (74-76).

*Smoking status, alcohol and caffeine consumption, physical activity, and diet*

Lifestyle factors such as smoking (67,77), alcohol consumption (66,78), and caffeine intake (79-81) have all been observed to lower levels of melatonin. However, conflicting results have been reported regarding the effects of physical activity (66) on concentrations of melatonin, where positive, negative, and null associations have all been observed. Limited evidence exists for a relationship between nutritional status and melatonin synthesis or activity (82).

*Other potential predictors*

Related to light exposure, changes to day length, such as through season or sleep duration, may influence melatonin concentrations (14,15,83,84).

## 2.5 Melatonin and breast cancer

### 2.5.1 Epidemiologic studies

Epidemiologic studies concerning melatonin and breast cancer have contributed mixed evidence while attempting to substantiate the melatonin hypothesis. Although unable to provide temporal results, several case-control studies have noted decreased levels of urinary (22) and plasma (23) melatonin among breast cancer patients. However, investigations have also observed opposite effects, whereby increases in overnight circulating melatonin concentrations were seen among late-stage breast cancer patients (85,86). Of the five prospective studies completed, three have found significant protective associations between urinary melatonin and breast cancer (24-26), while one has not (68). Among a group of premenopausal women from the Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) cohort, a possible protective

effect was observed, but only when a lag time analysis was conducted (e.g. considering only those women diagnosed at least two years after urine sample collection) (27).

The observed inconsistencies in the literature may be the result of a more complex relationship than previously believed, as well as non-standardized biological sampling and analysis procedures, and short follow-up periods in the prospective investigations (27,30). For example, unlike the other prospective investigations, which measured overnight or morning urinary melatonin levels (to represent peak concentrations) (24-27), Travis et al. utilized 24-hour urine samples (to represent total melatonin levels) (68). Researchers have also speculated that melatonin may influence multiple stages within the cancer pathway, inhibiting tumour growth in the early stages of carcinogenesis, yet leading to re-stimulation of tumour growth and metastasis in the later stages (27,30).

### 2.5.2 Experimental studies

Convincing evidence to support melatonin's antineoplastic role in breast cancer stems from its various cellular and molecular oncostatic actions, as observed in numerous experimental studies. Via both receptor-dependant and receptor-independent processes, melatonin has been shown to partake in antioxidant, antiangiogenic, and antimitotic pathways, while also influencing fat metabolism and the immune system (13,30). For example, melatonin has been shown to scavenge free radicals, reduce estrogen synthesis through aromatase inhibition, and decrease the density of estrogen receptors in cancerous breast tissue (13).

## 2.6 Insulin-like growth factor family

IGFs are also of interest with respect to cancer risk, initiation, and progression because of their involvement in cellular proliferation and development, as well as their anti-apoptotic properties (31-33). The IGF family consists of eight proteins, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), and six IGF-binding proteins (IGFBP-1 through -6); two cell membrane receptors, insulin-like growth factor receptor I (IGF-IR) and insulin-like growth factor receptor II (IGF-IIR); and numerous IGFBP proteases (31,32). IGF-I and IGFBP-3 in particular have garnered a great deal of attention in cancer research.

### 2.6.1 Insulin-like growth factor-I

IGF-I is a peptide hormone synthesized primarily by the liver and is regulated mainly by growth hormone (GH), but also influenced by factors such as insulin (31,87). Additionally, many IGF-sensitive tissues are able to produce the peptide, such that it has not only endocrine, but also paracrine and autocrine signaling effects (31).

IGF-I seems to play a role in carbohydrate and peptide synthesis, by promoting the cellular uptake of glucose and amino acids, and is also involved in cellular proliferation and differentiation in various tissues, via processes such as the hastening of cell cycle progression and the stimulation of DNA replication (32,87). Moreover, IGF-I is able to prevent programmed cell death by inhibiting the initiation of the apoptotic signaling pathway (31,32). While IGF-I may circulate in a free state (less than 2%), most is bound to one of six binding proteins, with 75% – 90% of circulating IGF-I found in a ternary complex with IGFBP-3 and acid labile subunit (ALS) (32,88).

### 2.6.2 Insulin-like growth factor binding protein-3

Synthesized in the liver, as well as bone tissue, IGFBP-3 represents the binding protein with the greatest affinity for IGF-I (31,89). Important functions of IGFBP-3 include its involvement in transporting IGF-I in the blood stream and preserving IGF-I from degradation (32,89). When bound to IGFBP-3, the half-life of circulating IGF-I increases from several minutes to approximately half an hour; when bound in the ternary complex with IGFBP-3 and ALS, due to the inability of the complex to transverse capillary membranes, the half-life of IGF-I increases to 15 or more hours (89,90). While IGFBP-3 inhibits IGF-I activity by preventing an interaction between IGF-I and its receptor (IGF-IR), the binding protein has also been observed to increase the effects of IGF-I (31,32,89). Furthermore, research has shown IGFBP-3 to have IGF-independent effects, whereby the binding protein is capable of initiating cell death and restricting cell proliferation (32,89).

### 2.6.3 Molar ratio of IGF-I to IGFBP-3: IGF-I/IGFBP-3

Due to the high percentage of circulating IGF-I found bound to IGFBP-3, calculating the molar ratio of IGF-I to IGFBP-3 (i.e. IGF-I/IGFBP-3) emerged as a means of approximating levels of “bioactive”, or “free”, IGF-I, as well as adjusting for the binding protein in analyses (33,91). Albeit crude, as it fails to adjust for concentrations of other binding proteins or IGFBP proteases, this measure attempts to capture the amount of IGF-I truly active at the tissue (33,91).

### 2.6.4 Determinants of insulin-like growth factors

Numerous demographic, lifestyle, and environmental factors have been investigated as potential predictors of the inter-individual variation in levels of IGFs. However, concrete conclusions have not been made regarding many.

*Age, sex, ethnicity, and body mass index*

Age is an established determinant of IGF-I, where levels decline with increasing age (92-98). Similarly, concentrations of IGFBP-3 (92,95,96) and IGF-I/IGFBP-3 (92,94,96) also generally appear to decrease with increasing age. Evidence suggests that sex (99,100) and ethnicity (95,101) may influence IGFs as well. Conflicting reports have been published regarding BMI and IGFs. Although inverse relationships have been observed (92,95,102), several studies have also reported null findings pertaining to BMI and IGF-I (93,94,96,98) and IGF-I/IGFBP-3 (94,96). Positive (94), as well as null (92,95,96,98) associations have been found between BMI and IGFBP-3.

*Hormonal and reproductive factors*

Parity may influence IGF-I concentrations, though results are incongruous (92,94,95,97). OC use has been shown to lower circulating IGF-I levels (96,103,104), whereas plasma IGFBP-3 concentrations have been reported to increase with the use of OCs (96). Limited evidence has also suggested that concentrations of IGF-I may follow a cyclic pattern, whereby levels rise over the course of the menstrual cycle, peaking in the luteal phase (105,106). Although several studies have reported null associations between menopausal status and IGF-I, as well as IGFBP-3 (92,107), a larger, more recent study found menopausal status to modify relationships between IGFs and certain factors, including BMI, smoking, and alcohol consumption (108).

*Smoking status, alcohol and caffeine consumption, physical activity, and diet*

Smoking (94,95,99,109,110), alcohol consumption (93,95,96,98,100,105,111,112), and caffeine intake (109,112) have been reported to influence levels of IGFs, though heterogeneity exists among the associations observed. The relationship between physical activity and IGFs is believed to be intricate. Numerous studies have been completed, with a variety of activities, intensities, exercise durations, and follow-up times assessed; however, no conclusive evidence has been provided (32,88,113). Hypotheses put forth as possible explanations for the heterogeneity of results, beyond differences in study methodologies, include modification by age, where physical activity may increase blood IGF-I levels in adults, but decrease concentrations in children (32); differences in the baseline physical fitness of participants; and changes in plasma volume, which fluctuates with exercise (88,113). Threshold effects may also be a factor, where chronic training may initially reduce circulating IGF-I, yet increase levels when regiments are maintained for longer time periods, such as more than three months (88). Moreover, there is speculation that exercise may influence IGF-I synthesis and activity at the tissue level, leaving circulating concentrations relatively unchanged (113). Finally, components of one's diet, such as total caloric intake or protein levels, have been observed to be important predictors of IGFs (32,92,95,98,112,114).

*Other potential predictors*

Season (54,100) and time of day (53,115-121) may be associated with levels of IGFs, though evidence remains inconclusive. Additionally, concentrations of IGF peptides may be affected with certain illnesses. For example, IGF-I may be reduced with hypothyroidism (122). The IGF family has also been implicated in normal glucose

homeostasis, with altered levels of IGF-I having been associated with type II diabetes (87). Metformin, an anti-diabetic drug, has been linked to changes in the IGF-axis among women suffering from polycystic ovary syndrome (123,124). Furthermore, concentrations of IGF-I may be impacted by sleep disorders, such as obstructive sleep apnea (125,126).

## 2.7 Insulin-like growth factors and breast cancer

### 2.7.1 Epidemiologic studies

Several epidemiologic studies have assessed a potential role for IGFs in cancer development and progression. A number of exploratory studies have observed significant associations between increased levels of IGF-I or IGF-I/IGFBP-3 and cancers of the breast (127-129), colorectum (130,131), prostate (132-134), and lung (135). The strengths of association reported by these studies have been moderate to strong, ranging from 1.91 to 7.34. In addition, researchers have observed associations between IGFBP-3, but with heterogeneous results (129,130,132,134-140).

Specifically in relation to IGF-I, IGFBP-3, and breast cancer, there have been three meta-analyses (34-36) and one systematic review (using case-control and prospective studies) (37), as well as one pooled analysis (using prospective studies only) completed (38). In general, higher levels of IGF-I among premenopausal women were associated with a 1.21 (95% CI 1.00 – 1.45) fold increase in risk for breast cancer (38). The data are less consistent for IGF-I concentrations among postmenopausal women and IGFBP-3 in general, although the most recent pooled analysis suggested a 1.33 (95% CI 1.14 – 1.44) fold increase in risk for elevated IGF-I levels among postmenopausal women (38). No relationship between IGFBP-3 and breast cancer risk

was observed among premenopausal women (OR 1.00, 95% CI 0.82 – 1.22), but a positive association was noted for postmenopausal women (OR 1.23, 95% CI 1.04 – 1.45) (38).

A lack of standardization regarding biological sampling (i.e. fasting versus non-fasting, serum versus plasma), as well as different study populations and follow-up times have been suggested as possible explanations for incongruous results between studies regarding IGFs and cancer (34,38,141). Of particular interest is a large, nested case-control investigation completed by Rinaldi et al. (142), which, utilizing data from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (n of cases = 1081, n of controls = 2098), considered time-to-diagnosis. Women diagnosed with breast cancer two years or more after providing a blood sample were observed to have an increase in breast cancer risk associated with a doubling of IGF-I concentrations (OR = 1.51, 95% CI 1.19 – 1.91) (142). In contrast, an inverse relationship was noted among those women diagnosed within two years of the blood draw (OR = 0.76, 95% CI 0.57 – 1.03, with a doubling of IGF-I concentrations) (142). Similar, although weaker, heterogeneity was noted for IGFBP-3. Increased levels of IGFBP-3 were associated with an elevated risk for breast cancer in women diagnosed two or more years after serum collection (OR = 1.49, 95% CI 1.08 – 2.06, with a doubling of IGFBP-3 concentrations) (142). In contrast, a small, but protective effect was seen among those participants diagnosed within two years of blood sampling (OR = 0.93, 95% CI 0.60 – 1.45, with a doubling of IGFBP-3 concentrations) (142). Differences in quantification methods, for IGFBP-3 in particular, have also been posited as a potential limitation of investigations pertaining to IGFs and cancer (34,38,141,143-145). Whereas some quantification assays measure only the intact protein, others measure levels of total IGFBP-3

(fragments, created by IGFBP protease activity, and intact IGFBP-3) (141,143-145). Indeed, studies assessing the consequence of using different assays found that associations between IGFBP-3 and breast cancer varied by different assay method (143,145).

Importantly, increases in circulating IGF-I and IGFBP-3 of approximately 50 ng/ml (127,129) and 900 ng/ml (129), respectively, may be clinically relevant with respect to premenopausal women and the risk of developing breast cancer, according to research conducted among women of the NHS and the ORDET cohort.

### 2.7.2 Experimental studies

Experimental studies have provided evidence for both direct and indirect links between IGFs and cancer as well. Investigations using animal models and various cancer cell lines have indicated that increased IGF-I heightens the risk of initial tumour development and that the peptide acts as a powerful mitogen for cancerous cells, as mediated through IGF-IR or autocrine signaling (31,32). With respect to indirect pathways, IGF-I has been found to act synergistically with hormones and other growth factors also linked to carcinogenesis, such as estrogen and epidermal growth factor, while antiproliferative molecules and tumor suppressor genes seem to influence IGF signaling pathways as well (32). Regarding IGFBP-3, experimental evidence suggests that, similar to its role in normal physiology, IGFBP-3 possesses IGF-dependent and independent antimitotic and proapoptotic qualities in the context of cancer (32,141). However, due to fragmentation of the binding protein via IGFBP proteases, studies have also observed proliferative effects of IGFBP-3 on cancer cells (32,141).

## 2.8 Photoperiod/melatonin and insulin-like growth factors

### 2.8.1 Biological framework

The biological framework on which this study is based involves the theory that decreased melatonin will result in increased circulating concentrations of IGF-I, thus increasing the risk of breast cancer. Multiple biological mechanisms exist, which could potentially link melatonin and the IGF peptide family. Based on experimental evidence, the pathway may occur through the GH-IGF axis (42,48,52), through altered levels of sex hormones (46-49), via thyroid inhibition (46,47), through altered insulin secretion (87,146), via inhibition of sirtuin 1 (Sirt1), a histone deacetylase (147), or through melatonin receptor 1 (MT1)-mediated activation of IGF-IR (45). Greater detail regarding the several possibilities outlined above may be found in Appendix A, while a summary is illustrated in Figure 2.2. To date, no single mechanism has been proven unequivocally and given the complexity and incomplete knowledge of the elements involved, it is conceivable that more than one intermediary pathway may be involved.

The remainder of Section 2.8 contains a summary of the data from both animal and human studies that provide some evidence for a possible association between melatonin and the IGF family.

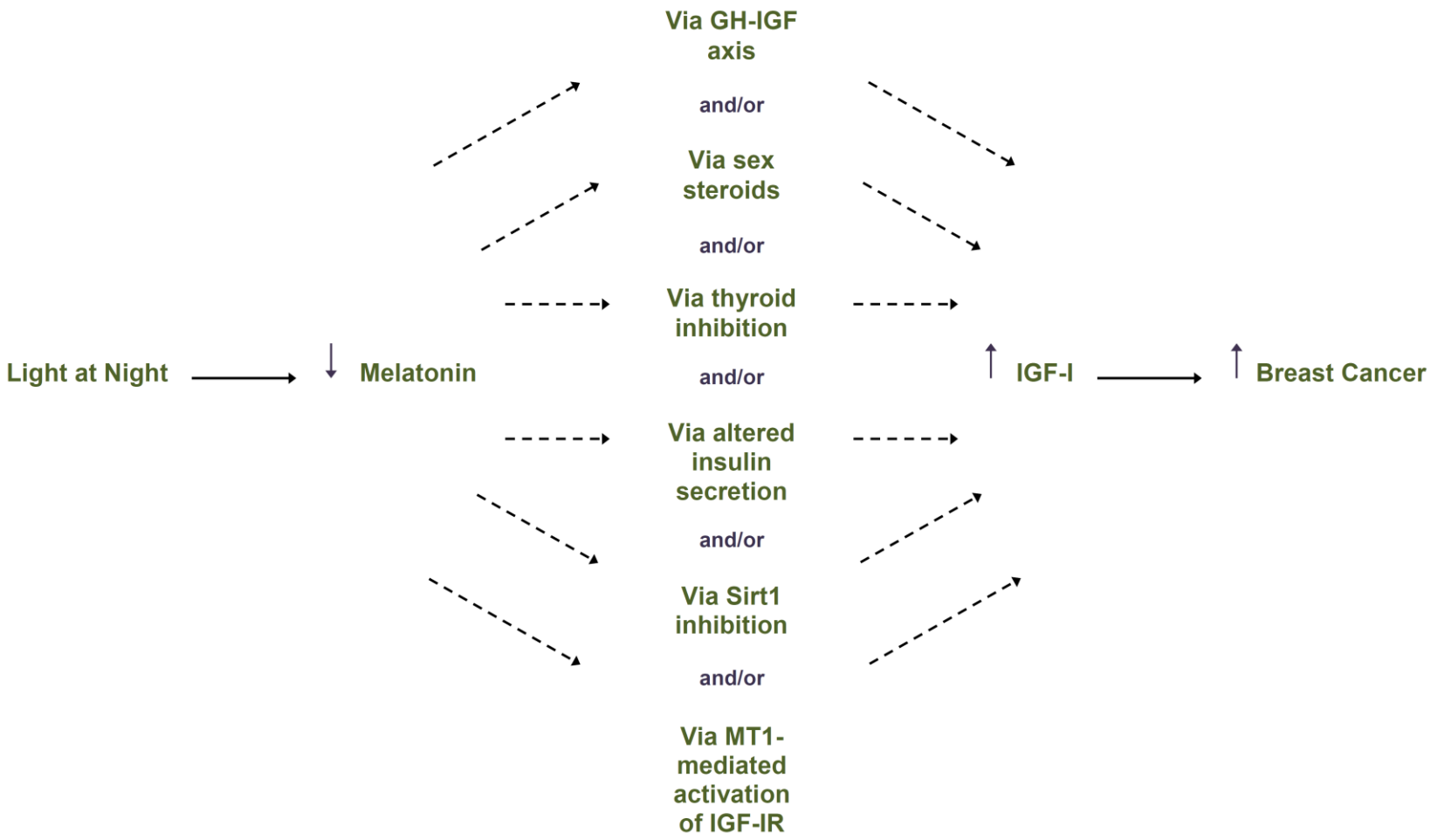


**Figure 2.2** Potential biological mechanisms linking melatonin and IGF-I

### 2.8.2 Experimental studies

*Animal models*

The majority of evidence for a relationship between melatonin and IGF peptides, or in a broad sense, between season or photoperiod and IGFs, has been provided by experimental studies conducted among animals. Research has been published from as early as 1974, when Smythe (39) observed the inhibitory effects of melatonin on IGFs in rats. In actuality, the majority of published research continues to involve the use of a variety of animal models. Interestingly, photoperiod manipulation in livestock cultivation as a means of increasing productivity and growth has been utilized for many years (40) and compelling data has emerged from several experiments in cattle. The documented effects of photoperiod, and in relation, season, on mammary tissue and galactopoietic yield, such as long days promoting growth during mammary development, as well as increases in milk production, have led researchers to hypothesize that melatonin and IGF-I may be the intermediaries responsible (40). Indeed, heifers exposed to short days were found to have continually lower circulating levels of IGF-I than those on a long-day schedule, while universal exposure to a long-day schedule resulted in increased levels of IGF-I; an effect which was attenuated in melatonin-fed heifers (40). Yet, not all investigations in cattle have found concordant results. It has been observed that exposure to constant light results in a decrease in circulating IGF-I (41), and at least one study has found that dim light did not affect IGF-I levels in cattle ($p = 0.89$), although levels of plasma melatonin and plasma IGF-I did tend towards an inverse linear relationship ($p = 0.10$) (41).

Numerous studies have been conducted in other animal models, including rats (42-45), Syrian hamsters (46-49), red deer (51), reindeer (50), as well as rhesus monkeys (52). Results from these studies have suggested a biological link between season, experimentally-altered light:dark patterning, and/or melatonin and IGF-I. However, similarly inconsistent associations have been observed. Notably, investigations involving Syrian hamsters have observed relationships between exogenous melatonin administration and serum levels of IGF-I, as well as photoperiod and IGF-I, which suggest that effects may differ by sex (46-49). Whereas short-day photoperiods and melatonin injections appeared to increase circulating concentrations of IGF-I in males, the opposite effect was observed in female hamsters (46-49). One must also consider the mixed use of nocturnal versus diurnal species, seasonal versus non-seasonal breeders, and long-day versus short-day seasonal breeders as animal models as a potential contributor to the lack of agreement across species. Nearly all species experience peak melatonin values during the night, regardless of their nighttime activity levels (14,15). Additionally, melatonin seems to inform certain seasonal physiological processes, such as reproduction, by relaying season and day length information; therefore indicating that physiological reactions to changes in melatonin concentrations are altered according to the activity and breeding patterns of species (14,15).

Overall, experimental studies completed to date have failed to provide definitive answers regarding melatonin and IGF-I. Possible contributing factors include differences in the age, sex, and activity/breeding patterns of animals studied; inconsistencies in experimental design and procedure, particularly regarding the concentration of melatonin administered or patterning and strength of light exposure studied; and the small sample sizes often employed. Nonetheless, experimental research has given insight regarding

the biological plausibility of a relationship between melatonin and IGF-I as numerous studies employing various species have found indication that the two hormones may be linked physiologically.

### 2.8.3 Epidemiologic studies

The potential relationship between melatonin, season, or artificially-altered photoperiod and IGFs has not been extensively studied in human populations. Of the epidemiologic studies completed, most have addressed patterns, associations, and treatment regimens involving melatonin and IGF-I following a cancer diagnosis, rather than mechanistic investigations in healthy individuals.

In terms of studies addressing potential etiology, in a study of 28 metastatic breast cancer patients, Haus and collaborators observed that serum melatonin showed a distinct diurnal circadian rhythm (peaking nocturnally), while serum IGFBP-3 seemed to follow an inverse, low amplitude circadian pattern (with a trough occurring nocturnally) (53). IGF-I levels did not appear to follow a definitive pattern; however, a significant peak was noted at 7:00 am, during the period of decline for melatonin ($p < 0.05$ when compared to IGF-I levels at 10:00 am, 4:00 pm, and 7:00 pm, and 3:00 am) (53). Circulating concentrations of IGF-I (118-120) and IGFBP-3 (116,121) are thought to remain relatively constant over 24-hour periods in healthy individuals. Nevertheless, diurnal variations have been noted in several studies, in which serum IGF-I levels have been seen to decrease in the evening or early morning (115-117). The circadian fluctuation of melatonin was not assessed among the participants of these studies, preventing a comparison of the hormonal profiles.

Seasonal variation also appeared to affect IGF-I levels in a small case-control study of normal women and premenopausal breast cancer survivors followed over the

course of approximately six months (54). Controls (n = 10), recruited from laboratory and clinical staff, as well as sisters or friends of cancer patients, were observed to have significantly lower serum concentrations of GH and IGF-I at winter solstice when compared to the summer solstice and higher serum levels of melatonin in the winter than in the summer (54). An altered profile was observed among the breast cancer survivors (n = 15), where seasonal differences were reversed for melatonin, non-existent for IGF-I, and identical for GH, suggesting that asynchronous and phase-shifted seasonal-hormone-patterning may result in or occur because of breast cancer development (54). Contradicting this suggestion are results from a study by Kajdaniuk and colleagues (55), who compared plasma melatonin and IGF-I levels among premenopausal stage II breast cancer patients (n = 24) and healthy volunteers (n = 16). The investigators observed a weakly negative correlation for melatonin and IGF-I ($r = -0.39$ $p = 0.06$) within only the breast cancer patients at baseline (55,56). This correlation disappeared following treatment with chemotherapy ($r = -0.02$ $p = 0.93$) (55).

Several investigations regarding the effects of exogenous melatonin have also been completed. In a small clinical trial of 14 cancer patients, which included both sexes and various cancer types, high pharmacological doses (20 mg) of melatonin were injected each afternoon, resulting in no change within the mean serum concentrations of GH and plasma levels of IGF-I measured prior to and following two months of treatment (57). In a second study, Lissoni et al. (58) evaluated the use of a combined tamoxifen and evening melatonin treatment (oral, 20 mg) among 14 women with metastatic breast cancer that had progressed or had not responded to tamoxifen therapy alone. Significant decreases in serum IGF-I were observed ($p < 0.05$ at one month, $p < 0.01$ at two and three months, compared to before treatment) (58); however, the combined use of

melatonin and tamoxifen, coupled with the lack of a control group, makes disentangling the effect attributable to the oral melatonin difficult. Conversely, in a small trial of elderly female volunteers (n = 14) investigating lower doses of melatonin (2 mg) and the aging process, a positive association between melatonin and IGF-I was reported (59). Following six months of daily nighttime melatonin treatment, participants' serum IGF-I concentrations were significantly increased compared to baseline (59). Most recently, a double-blind placebo trial among postmenopausal breast cancer survivors (n = 95) was conducted (60). In this last study, daily nighttime doses of melatonin (3 mg) over a four month period did not influence the circulating levels of IGF-I or IGFBP-3 of participants (Wilcoxon rank-sum p = 0.98, placebo versus treatment, at four months) (60).

Overall, the epidemiologic studies published to date have provided inconsistent results linking melatonin and IGF-I. The observation that circulating concentrations of IGF-I may vary seasonally in healthy individuals, the finding that low doses of melatonin seem to significantly increase circulating IGF-I in the elderly, and the ability of tamoxifen+melatonin treatment to significantly reduce IGF-I levels in metastatic cancer patients (who had progressed or failed to respond to tamoxifen treatment alone) provide some evidence for an association between the two hormones. Nonetheless, to date, only seven epidemiologic studies appear to have explored this question (one case study, two case-control studies, and four clinical trials). With the exception of the most recent trial by Schernhammer and colleagues (60), these investigations have been extremely small with low study power, limiting the confidence with which the results may be interpreted. A need for larger studies is evident. Moreover, the majority of subjects are those already diagnosed with breast cancer. Since the relationship between melatonin and IGF-I and/or IGFBP-3 may be altered during the carcinogenic process, these associations

should be studied for etiologic objectives in healthy populations. In many of these studies, assessment or control for potentially important confounders, such as age, was absent or limited, such that true effects of melatonin may be obscured or associations reported may be an artifact of uncontrolled confounding. Furthermore, of the three investigations considering endogenous levels of melatonin and IGF-I, two did not formally assess a relationship between melatonin and IGF-I. Regarding the trials completed to date, the studies have generally investigated different dosages, methods (injection versus orally) and timing of administration, as well as lengths of treatment and follow-up time. Thus, comparisons within the literature must be made with caution. For instance, exogenous treatment of melatonin may result in effects that differ from what occurs in normal human physiology, while different dosage strengths or treatment durations may yield dissimilar effects.

Studies focused on the investigation of melatonin and IGF peptides among healthy humans are a subsequent step in the process of evaluating the possible relationship. Ideally, these studies would be much larger than the projects completed to date, therefore increasing the power to detect smaller effects and allowing for adjustment for potentially important confounders. Using a cohort of women, followed forward for some time, would allow for multiple cross-sectional assessments and provide the opportunity to research durations of exposure. The biologically relevant time window is unknown and it is possible that effects will only be evident after long periods of elevated or reduced melatonin levels. Several data collections, with an ample number of individuals, would also provide the opportunity to investigate a relationship between changes in concentrations of melatonin and changes in levels of IGFs. Finally, collecting multiple samples for both melatonin and IGF quantification at the time of each cross-

sectional evaluation would reduce the chance of missing an association due to latency. The effect of nighttime concentrations of melatonin on circulating levels of IGFs may be quite acute or could require many hours. Thus, multiple sampling may prove more effective than single specimens in this exploratory stage of research.

## 2.9 Summary of rationale

Breast cancer continues to be a highly prevalent disease, with less than half of cases attributed to known or suspected risk factors. Given the widespread exposure to nonstandard work schedules, investigations into the mechanisms through which these factors may increase cancer risk need to be completed, such that interventions can be developed and preventative measures implemented.

One potential pathway involves melatonin and the IGF family. Experimental and epidemiologic studies have assessed the relationship between LAN/melatonin and cancer, IGFs and cancer, as well as possible direct and indirect interactions between LAN/melatonin and the IGF axis, whereby the complexity and lack of complete knowledge regarding the relationships have been highlighted. Population studies regarding LAN/melatonin and the IGF family have used small sample sizes, have disregarded many potential confounders, and have failed to investigate fully the association between melatonin and IGF peptides in healthy females, with the majority focusing on hormone patterning and treatment options in individuals already diagnosed with cancer. This project aimed to address several of the gaps within the literature as it employed a larger sample size, as well as characterized and modeled the relationship between melatonin and IGFs in healthy individuals while controlling for potential confounders, thus contributing to our understanding of the causes of breast cancer.

Illustrated in Figure 2.3 is a conceptual model incorporating exposure, outcome, and covariate information for the potential relationship between melatonin and IGF-I.

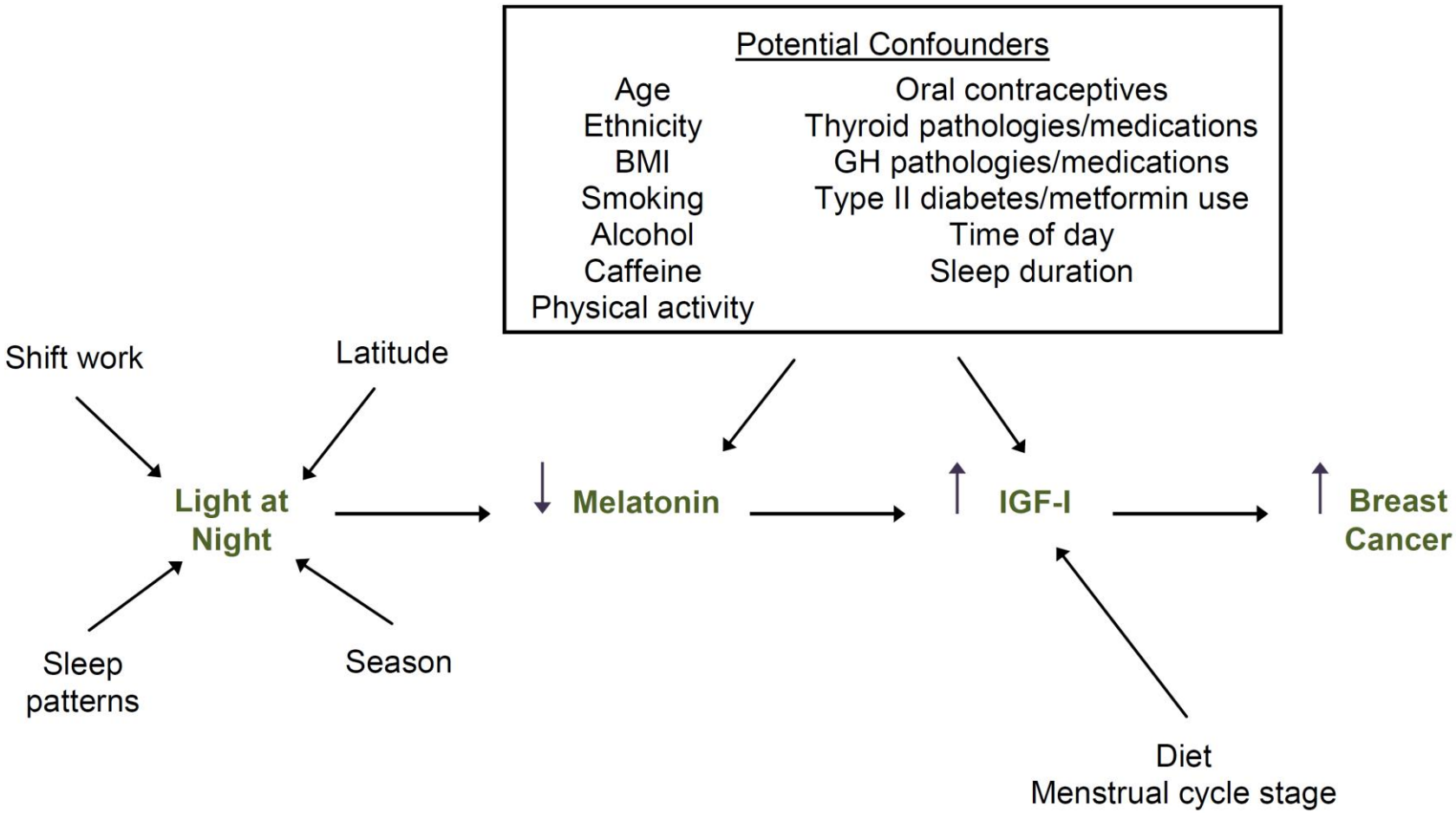


**Figure 2.3** Conceptual framework

# Chapter 3
# Methods

## 3.1 Study purpose and objectives

The main purpose of this project was to describe the premenopausal distribution of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3) and examine the premenopausal relationship between urinary levels of melatonin and circulating concentrations of IGF-I and IGFBP-3 as a possible component in the pathway between exposure to shift work and the development of breast cancer. Specifically, the objectives were to:

1) Determine the distribution and identify predictors of morning serum levels of IGF-I and IGFBP-3 among premenopausal rotating shift nurses.

2) Determine the relationship between overnight urinary levels of melatonin and morning circulating levels of IGF-I and IGFBP-3 among premenopausal rotating shift nurses.

   *Hypothesis*: Reduced levels of melatonin are associated with elevated circulating concentrations of IGF-I and reduced serum levels of IGFBP-3.

## 3.2 Overview of parent study

### 3.2.1 Introduction

This thesis project was part of a larger observational study conducted by Dr. Aronson et al. (61), entitled *Occupational and other factors as determinants of melatonin*

*levels among rotating shift nurses*. Funded by the Workplace Safety and Insurance Board of Ontario (WSIB) and conducted at Kingston General Hospital (KGH), Kingston, Ontario, the parent study ("WSIB study") aimed to assess occupational and lifestyle factors as potential determinants of melatonin levels among female rotating shift nurses with the purpose of generating further knowledge of cancer etiology.

### 3.2.2 WSIB study population

Sampling for the WSIB study followed a volunteer approach. Female nurses were recruited from the base population of all full-time registered practical nurses and registered nurses employed at KGH. Enrollment occurred over a two-year period, one cohort within 2008 (cohort A) and one within 2009 (cohort B), and was achieved through an advertisement campaign where brief study details and explicit eligibility criteria were provided to employees via posters, flyers, and oral presentations. Division of the study population into two cohorts was necessary as certain pieces of measurement equipment, specifically light meters and pedometers, were limited in number.

Women were asked to self-exclude based upon the information provided. In particular, participants had to be working the standard rotating shift pattern at KGH, which consisted of two 12-hour day shifts (7:00 am – 7:00 pm) and two 12-hour night shifts (7:00 pm – 7:00 am), accompanied by five days off. In addition, eligible nurses had to be female, could not be taking melatonin supplements, could not be pregnant, and could not have been pregnant or lactating within the preceding six months. These criteria were used in an attempt to limit the effect of known and possible predictors of melatonin concentrations that were not of direct interest in the parent study. Copies of the recruitment material employed may be found in Appendix B.

Interested parties were asked to contact the study coordinator to schedule a preliminary interview. Informed consent (Appendix B) was obtained from potential participants prior to study enrollment and a monetary honorarium of $200.00 was offered as compensation for the participants' time. The parent study population consisted of 123 female volunteers. Due to the enrollment approach, the number of eligible participants is unknown; thus an accurate recruitment rate could not be calculated.

### 3.2.3 Data collection

Nurses were asked to participate in two data collection periods – one during the summer season and one within the winter. Specifically, cohort A and cohort B completed their data collections during April – December 2008 and January – July 2009, respectively. Each data collection period included a 48-hour day shift and a 48-hour night shift component, scheduled approximately one month apart, with the order of the components being left to the preference of the participants.

Prior to the initial data collection segment, participants were asked to complete a detailed questionnaire from which demographics, employment history, and lifestyle information, as well as a pertinent medical history, were obtained. Further information on recent health status, including medication use; lifestyle habits, such as smoking, caffeinated and alcohol beverage consumption; lighting conditions, as well as sleep/wake patterns; and physical activity was captured via one-day study diaries completed during each data collection component. Furthermore, participants were asked to wear light meters and pedometers, in addition to completing the study diaries, to assess ambient light and physical activity, respectively. Vitamin D questionnaires were administered with each of the two day shift component diaries, as a means of collecting seasonal information on vitamin D exposure.

Biological samples were collected over the course of each 48-hour day shift and night shift component at pre-specified time points. All participants were to provide four saliva swabs and two urine samples per 48-hour window, which allowed researchers to measure melatonin at numerous instances, but in a fairly non-invasive manner. In addition, a single morning, eight-hour fasting serum sample was collected solely from premenopausal participants, during each of the two day shift components only, due to budgetary restrictions. Numerous hormones were to be quantified from the blood samples, in addition to the genotyping of various clock genes. A summary of the data collection schedule utilized by the WSIB study is shown in Figure 3.1. All study instruments used may be located in Appendix B.

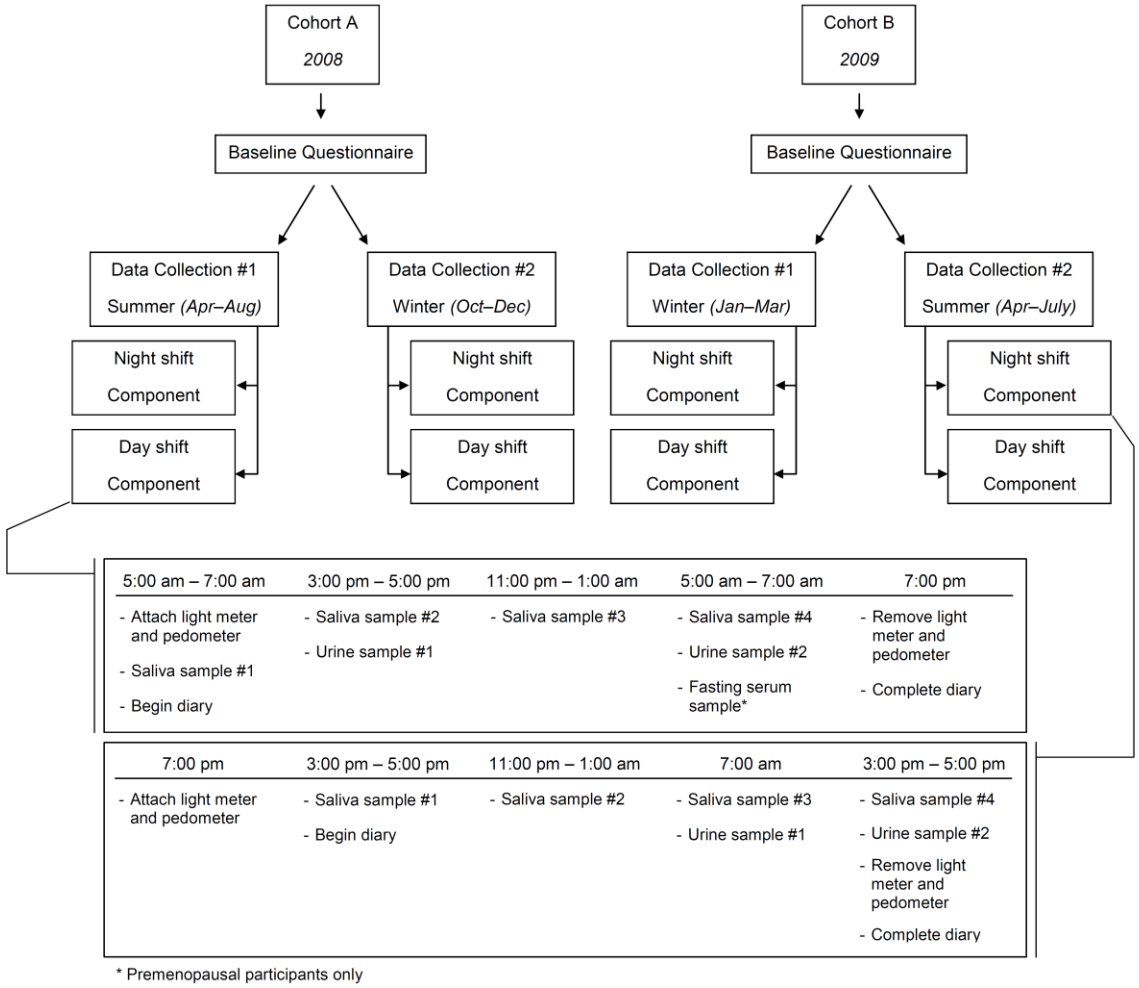


**Figure 3.1** WSIB study data collection summary

## 3.3 Overview of thesis project

### 3.3.1 Introduction

This specific project made use of those WSIB participants who were self-described as premenopausal, as serum samples available for insulin-like growth factor (IGF) analyses were limited to premenopausal individuals completing day shift collection windows only. This thesis focused on biological samples (urine sample #2 and the serum sample) and diary information acquired during the day shift data collections, with further covariate information obtained from the detailed baseline questionnaire. Specifically, the study design was such that the relationship between potential predictors and IGF-I, IGFBP-3, and IGF-I/IGFBP-3, as well as between melatonin and the IGF measures, were explored cross-sectionally, using two data collection periods: one per summer and winter season.

### 3.3.2 Study population

Within the WSIB study, investigators recruited 94 premenopausal women who self-identified as eligible. Prior to data collection, two women withdrew from the study and three were lost to follow-up, resulting in 89 study participants. Of this sample, two women failed to complete either day shift data collection period, thus resulting in their exclusion, while two nurses participated, but did not have serum samples available. Therefore, 85 women (90.4% of initial 94 nurses) provided at least one blood sample and constituted the final sample size relevant for this project. Of these 85 women, 64 nurses (68.1% of initial 94 nurses) gave both serum samples. For more information on the final study sample sizes, please see Section 3.7.1.

## 3.4 Melatonin

The primary exposure of interest for this study was melatonin. The measurement of 6-sulfatoxymelatonin (aMT6s), the primary metabolite of melatonin, in morning void urine samples served as a surrogate measure for the biologically relevant peak overnight circulating concentration of melatonin.

Quantification of melatonin can be achieved through blood, saliva, or urine samples (15). Due to the invasive nature of drawing blood samples, the use of plasma for determining levels of melatonin is often not feasible or practical, and although saliva specimens represent a noninvasive method, overnight sampling requires the interruption of sleep (148). In contrast, urinary measurement of aMT6s is an alternative that is non-invasive and does not involve the disruption of sleep (148). First morning void urinary aMT6s levels have been found to be highly correlated with the total nocturnal concentration of circulating melatonin, as well as peak overnight melatonin levels (149,150), with 50-80% of the total aMT6s concentration being captured within an overnight urine sample (14). Moreover, despite melatonin's increased inter-individual variability, morning urinary aMT6s measurements appear to be relatively efficient predictors of longer-term melatonin levels in premenopausal women (intra-class correlation coefficient, ICC, of 0.72, 95% CI 0.65 – 0.82, three samples over three years) (21). As a result, measurement of aMT6s in urine is considered an accepted practice for research and clinical studies when attempting to noninvasively monitor melatonin levels (148).

### 3.4.1 Urine sample collection

Nurses were asked to collect their morning void urine in the standard 120 ml urine containers provided upon waking (5:00 am – 7:00 am) for their second shift of the

day shift data collection period. In an attempt to confirm adherence to protocol, participants were also asked to record the time of specimen collection in the study diary provided. The specimen jars, labeled with the participants' random identification numbers, were to be placed in the biohazard bags provided and brought to KGH, where the porter system would transport the samples from the nurses' floor to the KGH Core laboratory for storage at 2 – 8 $^{o}$C. Specimens were retrieved by study staff within 48 hours. Centrifugation at 2000 x g for five minutes was used to separate particulates and aliquots of the supernatant were placed in long-term storage at -80 $^{o}$C.

### 3.4.2 Laboratory analysis

*6-sulfatoxymelatonin assessment*

Concentrations of urinary aMT6s were measured using the Bühlmann aMT6s enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, Salem, NH, USA), a validated and commonly used technique. A competitive immunoassay, the ELISA operates through the capture antibody approach (151). Although not considered the gold standard for quantifying urinary aMT6s, the Bühlmann aMT6s ELISA does produce results highly comparable to the radioimmunoassay ($r = 0.97$; $p < 0.0001$), while also proving desirable given its shorter processing time and protocol that eliminates a reliance on radioactive materials and the associated equipment (151). The assay protocol followed, as well as quality control measures, may be found in Appendix C.

*Creatinine assessment*

In an attempt to control for inter-individual variability due to varying urine output volumes, aMT6s results were standardized using urinary creatinine concentrations, a practice often employed (21,26,27,66,152-154). The adjustment of urinary biomarker

concentrations based on creatinine excretion involves expressing levels of the biomarker of interest per mg/dl of urinary creatinine (21,26,27,66,152-154). Given that creatinine is formed and cleared at a somewhat constant daily rate, its urinary concentration can be used as a referent for the diluteness of an individual's sample (153). Urinary creatinine concentrations were measured via the Parameter™ Creatinine Assay (R&D Systems, Minneapolis, MN, USA), which uses the Jaffé reaction in its method of quantification (155); the procedure and quality control measures followed are summarized in Appendix C.

### 3.5 Insulin-like growth factors

Three outcomes were of interest for this research project: IGF-I, IGFBP-3, and IGF-I/IGFBP-3. The levels of total (bound and unbound) circulating IGF-I and intact IGFBP-3 measured from the eight-hour fasting serum samples were used to approximate the biologically relevant dose of each of these hormones. As the molecular ratio of circulating IGF-I/IGFBP-3 has been hypothesized to estimate the actual bioactivity of IGF-I within tissues (see Section 2.6.3) (33), these values were also calculated for each serum sample.

It is important to note that, although inter-individual variability is quite high, the intra-individual variation of repeat serum IGF-I samples has been shown to be fairly low in the short-term (100,156,157), as well as over periods ranging from several months to several years (129,158,159). In a study evaluating the effectiveness of various growth hormone (GH) deficiency screening tests, Biller et al. (156) reported a Pearson's correlation coefficient of 0.80 ($p < 0.001$) between serum IGF-I samples drawn five – 21 days apart in the group of healthy controls (n = 30; males = 20, females = 14). In several additional studies where the short-term reproducibility of serum IGF measures was

examined, comparisons of samples drawn between two and three weeks apart gave ICCs of 0.64 for IGF-I and 0.68 for IGFBP-3 (157), while between eight and 54 days apart gave a correlation of 0.94 ($p = 0.001$) for IGF-I (100). Assessments over longer periods report similar results. Findings for women specifically range from ICCs of 0.70 (age-adjusted; 95% CI 0.45 – 0.95) for IGF-I and 0.58 (95% CI 0.25 – 0.90) for IGFBP-3 measured over four months (158), to 0.81 (lower 95% CI limit 0.68) for IGF-I and 0.60 (lower 95% CI limit 0.33) for IGFBP-3 assessed over one year (129), and 0.69 (95% CI 0.46 – 0.93) and 0.71 (95% CI 0.36 – 1.0) for IGF-I assessed over one and five years, respectively (159). Therefore, serum samples have been shown to have at least a fair degree of reliability for IGF-I and IGFBP-3 and given the economic constraints of both the WSIB investigation and this thesis, their use was considered a reasonable means of determining IGF values for this particular study.

### 3.5.1 Serum sample collection

Nurses were asked to provide eight-hour fasting serum samples on the morning (5:00 am – 7:00 am) of their second shift of the day shift collection period. Blood was collected in 3.5 ml serum separator tubes, which contain a gel and clot activator that allows serum to be separated from the whole blood. Samples were labeled with the participants' random identification numbers, placed in biohazard bags, and retrieved via the KGH porter system to be processed and placed in refrigerated storage at the KGH Core laboratory. Serum samples were collected by the study staff and stored as 100 µl aliquots at -80 $^{o}$C until quantification for IGF measures could be completed.

### 3.5.2 Laboratory analysis

*Insulin-like growth factor-I assessment*

Serum levels of IGF-I were quantified using the R&D Systems Quantikine® assay (R&D Systems, Minneapolis, MN, USA). A non-competitive "sandwich" ELISA (160), the R&D Systems Quantikine® IGF-I kit is one of several commercially available assays commonly used and an alternative to the Nichols Advantage chemiluminescent immunometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), considered the definitive commercial kit, but discontinued in 2006 (161).

One common difficulty of IGF-I quantification methods is the interference of IGFBPs (161-163). Unfortunately, the ideal method for separating IGF-I from its binding proteins, acidic size-exclusion gel chromatography, involves a rigorous process not reasonable as a pre-treatment step for most measuring purposes (161-163). Consequently, commercially-available assays, such as the Quantikine® ELISA, rely on less labour-intensive methods that have been developed for addressing IGFBP interference (161,163). The assay protocol followed, as well as quality control measures are outlined in Appendix C.

*Insulin-like growth factor binding protein-3 assessment*

Serum concentrations of IGFBP-3 were assessed via the R&D Systems Quantikine® IGFBP-3 ELISA kit (R&D Systems, Minneapolis, MN, USA). Similar to the IGF-I assay used, the R&D Systems Quantikine® IGFBP-3 assay is a non-competitive "sandwich" ELISA (164). Notably, unlike IGF-I measurement procedures, IGFBP-3 assays do not require sample pretreatment to dissociate IGF-I-IGFBP-3 complexes (162). However, assessing the concentration of IGFBP-3 in biological samples is far from uncomplicated. Fragments of IGFBP-3, produced by the regulatory actions of

IGFBP proteases, have been speculated as one reason for the multifarious observations regarding the role of IGFBP-3 in cancer development and progression (141,143-145). Specifically, while some available biochemical tests measure total IGFBP-3 (fragments and intact IGFBP-3), others measure only the intact proteins (143,144). Moreover, fragments may interfere with assay detection, such that quantification tests commonly used may differ in the degree to which they measure levels of IGFBP-3 (143,144).

The R&D Systems Quantikine® IGFBP-3 ELISA was developed and has undergone manufacturer validation solely for intact IGFBP-3. While it is possible that the ELISA detects fragments containing regions of IGFBP-3 recognizable by the test kit antibodies, testing has not been completed to identify which fragments these would include or to what degree this would affect assay outcomes. The assay protocol followed, as well as the quality control measures adhered to may be found in Appendix C.

*Molar ratio (IGF-I/IGFBP-3) assessment*

The molar ratio of IGF-I to IGFBP-3 was calculated using the equation below.

| |
|---|
| IGF-I/IGFBP-3 = (0.130 x concentration of IGF-I in ng/ml) / (0.036 x concentration of IGFBP-3 in ng/ml)<br><br>Note: 1 ng/ml of IGF-I = 0.130 nM of IGF-I<br>1 ng/ml of IGFBP-3 = 0.036 nM of IGFBP-3 |

**Figure 3.2** Calculation of IGF-I/IGFBP-3

*Laboratory test batch*

As a means of assessing and adjusting for variation in levels of the IGF measures due to the required use of numerous ELISA test plates during quantification,

two variables were created (one for IGF-I and one for IGFBP-3) to reflect the batch in which each serum sample was tested. Laboratory test batch was conceptualized as a categorical variable (one, two, three, four, and five) for both IGF-I and IGFBP-3.

### 3.6 Confounders/predictors of IGF

Possible confounders, as well as potential predictors of IGF-I and IGFBP-3, were identified *a priori* from the literature (see Sections 2.4.2 and 2.6.4), and the pertinent data obtained from the baseline questionnaire and study diaries.

*Age, ethnicity, and body mass index*

Participants' birth dates and ethnicity were self-reported on the baseline questionnaire. For the purpose of this project, age (years) was treated as a time-independent, continuous variable. Due to decreased variability, ethnicity was dichotomized as "white" or "other" and used solely for descriptive purposes. Body mass index (BMI) ($kg/m^2$) values were calculated from weight (kg) and height (m) measurements captured at the time of enrollment, which were recorded by the study coordinator in an attempt to increase objectivity. BMI was then categorized based on quartiles ($\leq 22.7$ $kg/m^2$, $> 22.7 – 25.5$ $kg/m^2$, $> 25.5 – 29.4$ $kg/m^2$, and $> 29.4$ $kg/m^2$).

*Menopausal status, number of pregnancies, oral contraceptive use, and menstrual cycle stage*

Several reproductive characteristics were identified as potentially important covariates. Menopausal status was controlled through the design of the WSIB study, as only those women who self-described as premenopausal on the baseline questionnaire were asked to provide fasting serum samples, the means of assessing this project's main outcomes, IGF peptide levels. Menopausal status was reassessed in each study

diary. However, women who later indicated that they were no longer menstruating were considered likely to be perimenopausal (165) and kept in the study sample. Information regarding pregnancies was acquired from the baseline questionnaire, where participants were asked to indicate the number of times they had been pregnant, including not only live births, but also miscarriages and abortions. Number of pregnancies was treated as a categorical variable, using quartiles to determine intervals (none, one, two, and three or more pregnancies). Short-term effects of pregnancy were controlled through design, as the original exclusion criteria required eligible nurses not be pregnant or have been pregnant or lactating within the preceding six months.

Data on oral contraceptive (OC) use and menstrual cycle stage were available from the study diaries completed during each collection period, and thus treated as time-dependent variables. Specifically, nurses were asked to report if they had taken any prescribed birth control medication over the course of the applicable data collection period. While space was provided for participants to specify brand and dosing information, due to the poor quality of this data, recent OC use was treated dichotomously as either “yes” or “no”.

With respect to menstrual cycle stage, information the nurses provided regarding the date of the first day of their last menstrual cycle was used to determine, on the final day of the data collection period, how many days it had been since the start of each nurse’s previous menstrual cycle. A categorical variable was then created based upon the major hormonal events of a menstrual cycle. In particular, three categories were used: the follicular phase, when levels of both progesterone and estrogen tend to be low; the ovulatory or mid-cycle phase, when estrogen concentrations spike; and the luteal phase, when progesterone levels become elevated and estrogen concentrations decrease (though generally remain above levels observed in the follicular phase) (166).

Presuming a “standard” 28-day cycle, nurses were categorized as being in the follicular stage if zero – 10 days had passed since the start of their last menstrual cycle, mid-cycle if 11 – 16 days had passed, and luteal if 17 or more days had passed. Although ovulation generally occurs shortly after the actuating surge in luteinizing hormone, the “mid-cycle” window was expanded to six days in an attempt to properly capture the preovulatory estrogenic peak. The upper limit of the luteal stage category remained undefined to accommodate women who indicated that it had been longer than 28 days since the commencement of their last menstrual cycle.

Unfortunately, information regarding menstrual cycle length, which would have helped greatly in reducing the probability of misclassification, was not captured in the WSIB study. Knowing the length of the menstrual cycle would have allowed for more precise classification given that luteal phases seem to be fairly consistent in duration (approximately 14 days), regardless of the time span of the entire cycle (167,168). In an effort to limit the occurrence of misclassification, three broad categories were used, rather than numerous narrow divisions.

To preserve sample size, for those 10 participants who were missing menstrual cycle information in one of their day shift study diaries, but still indentified as premenopausal during that data collection, menstrual cycle stage was estimated using data obtained during the night shift collection period (completed approximately one month before/after the day shift collection). Using the assumed cycle length of 28 days and information regarding the last day of their previous menstrual cycle from their night shift study diary (exact dates of data collections also known), the current menstrual cycle stage could be approximated.

*Alcohol and caffeine consumption, smoking status, and physical activity*

For alcoholic drinking practices, both typical and recent consumption were evaluated. Specifically, typical consumption habits were acquired from the baseline questionnaire, where women were asked to report the average number of standard drinks of wine, beer, and spirits consumed per week for each decade of their lives. The current decade of life was believed to be the most biologically relevant, thus the average number of drinks of wine, beer, and spirits for the appropriate period were summed and expressed as a continuous variable (average number of drinks/week). Similarly, recent consumption, a time-dependent variable, represented a tally of the alcoholic beverages (wine, beer, and spirits) ingested over the 24-hour periods captured by the study diaries (number of drinks/day).

Data concerning recent caffeinated beverage consumption were also gathered from the one-day study diaries. Participants were instructed to record the quantity of coffee, tea, and other caffeinated beverages consumed during the 24-hour intervals evaluated by the diaries. Values were combined to generate a single continuous variable for all recent caffeinated beverage consumption (number of drinks/day). While space was provided for individuals to detail the size and type of beverage in question, as a result of the lack of compliance and general inconsistency regarding the manner of reporting, these data were not incorporated in the formation of the final variable. Information on usual caffeinated beverage consumption was not collected.

Information pertaining to smoking status was obtained from the study questionnaire, where nurses were asked if they had ever smoked more than 100 cigarettes in their lifetime (“yes” or “no”), as well as if they were currently smoking (“yes” or “no”). Smoking status was categorized as “current”, “past”, or “never” based on the

combination of responses to these two questions. Due to the small number of current smokers, further details regarding smoking habits were not considered.

Details concerning the nurses' usual levels of physical activity were acquired from extensive inquiries included in the one-day diaries and a summary variable constructed. For 15 common activities, participants were asked to indicate the number of times in the past month they had engaged in the exercise, the average duration of a session, and the typical intensity of the activity (three categories: light, requiring minimum effort; moderate, causing a slight increase in breathing and heart rate; and heavy, causing sweating and a substantial increase in breathing and heart rate). In addition, nurses were provided space to list up to three other activities in which they had participated in the past month and asked to likewise specify frequency, duration, and intensity. Occupational and household activity levels over the previous 30 days were incorporated in the summary score also. Women were instructed to report the average number of hours daily they spent performing light, moderate, and heavy chores (on working versus non-working days), as well as the amount of time spent on their feet and performing heavier activities while at work (such as bathing patients), via a set of closed-ended questions. Options provided ranged from "none at all" to "about 7 hours or more a day".

Through the determination of metabolic equivalent scores (METs) and METmin values, the extracted information was used to create a single continuous physical activity variable. A MET score represents the ratio of the metabolic rate of a given exertion to the accepted resting metabolic rate (4.184 $kJ \cdot kg^{-1} \cdot hr^{-1}$) (169). For instance, while sitting quietly is considered to have a MET score of 1.0, a MET value of 8.0 is given to activities that require eight times the energy expenditure required when at rest. MET values were assigned to the nurses' reported activities using the *Compendium of Physical Activities*,

a document often used when attempting to quantify and standardize self-reported physical activity data (169). The compendium is comprised of over 600 activities, for which detailed energy expenditure information is provided (169). Namely, MET values, as determined using calorimetric data obtained in a laboratory setting, are listed for each activity, completed at various levels of effort (169). MET scores were determined for each participant's activities based on the individual's perceived levels of intensity, the compendium, and the recognized cut-points of 1.5 to < 3.0 METs for low intensity, 3.0 – 6.0 METs for moderate intensity, and > 6.0 METs for high intensity (169-171). For exercise reported, but not listed in the compendium, peer-reviewed scientific articles were consulted. MET scores were then multiplied by the average daily duration (minutes) for each activity and summed, giving a total physical activity variable presented in the units of average METmin/day.

For individuals who reported a value for at least one of the three attributes assessed in the usual physical activity portion of the study diary (i.e. number of times, average duration, or typical intensity), missing values were estimated based on information provided by women who had completed the given activity. This approach was employed in the interest of preserving an already small sample size. Estimated values were calculated separately for the first and the second data collection periods, with median values substituted for continuous variables and mode values substituted for categorical variables.

*History of shift work, season, and sleep duration*

History of night shift work was determined from the study questionnaire, which included an employment history portion, by summing the number of years employed where at least 50% of the hours worked per week involved night shifts (years of night

shift work). Season was treated as a dichotomous variable, “summer” versus “winter”, based on recruitment cohort (A or B) and data collection number (1 or 2) (see Figure 3.1). Sleep duration (hours) was calculated from information provided in the one-day diaries; women were asked to indicate the times at which they went to sleep and woke up.

*Other potential covariates not investigated*

Several potentially important covariates were controlled via the exclusion criteria employed in the WSIB study, including sex, as participants had to be female, and exogenous melatonin, as the women could not be taking melatonin supplements. While diet is considered an important predictor of IGF measures, food consumption was not evaluated in this thesis. Two short food frequency questionnaires were administered as part of the WSIB study; however, these assessments were tailored to investigate dietary vitamin D levels. Thus, the food items included did not fully lend themselves to an investigation of the IGF family. Instructing participants to provide fasting serum samples at a consistent time of day presumably controlled for recent food intake, although confirmation of fasting was not obtained. Lastly, thyroid and GH-related pathologies and their associated medications, as well as metformin use were identified *a priori* as possible confounders of the investigated relationship between melatonin and IGF measures. Due to the extremely low prevalence of thyroid medications (two of 85 women; 2.3%), GH-related medications (zero of 85 women; 0%), and metformin use (two of 85 women; 2.3%) in the study population, these variables were excluded from the analysis.

## 3.7 Statistical analysis

A majority of statistical analyses were completed using SAS® (Version 9.2, SAS Institute, Cary, North Carolina, USA). Measures of intra- and inter-plate variability for the IGF-I and IGFBP-3 ELISAs were calculated using Microsoft Excel® (2007 Version, Microsoft Corporation, Redmond, Washington, USA), while power calculations were completed using PS Power and Sample Size Calculations (Version 3.0.43). All analyses were completed separately for each IGF outcome.

### 3.7.1 Subject participation and final sample sizes

As previously mentioned, the final sample size for this project was comprised of those 85 premenopausal women (90.4% of initial premenopausal 94 nurses recruited) who provided at least one serum sample as part of the original WSIB study (see Section 3.3.2). Participants were enrolled and completed the study in one of two cohorts (see Figure 3.3). Specifically, 48 women constituted cohort A, while cohort B contained 37 women. All 48 women of cohort A participated in the first data collection; however, only 44 of these nurses provided the required serum sample. Between the first and second day shift collection periods for cohort A, four women withdrew, two nurses became ineligible (one switched to a part time schedule, one became pregnant), and one woman was lost to follow-up. Of the 41 women who also took part in the second data collection, 40 gave a blood sample. During the initial day shift portion for cohort B, 36 women participated, 35 of whom provided serum samples. Three nurses did not partake in their second collection window; one woman withdrew, one switched to a day shift only schedule, and one was lost to follow-up. Conversely, one individual failed to complete the first day shift study portion, but did participate in the second (due to late recruitment).

Blood samples were obtained from 30 of the 34 second data collection participants of cohort B.

Overall, 149 samples were available for laboratory analysis, with 21 nurses (22.3% of initial 94 nurses) having provided only one serum samples and 64 having given both (68.1% of initial 94 nurses). However, several samples were excluded or missing for the different IGF outcomes considered. Two samples were removed as outliers from further IGF-I analysis (see Section 3.7.2), while two samples contained IGFBP-3 levels that could not be quantified. In addition, 11 missing urine samples further limited sample sizes for the aMT6s and IGF analyses. Final samples sizes are summarized in Table 3.1.

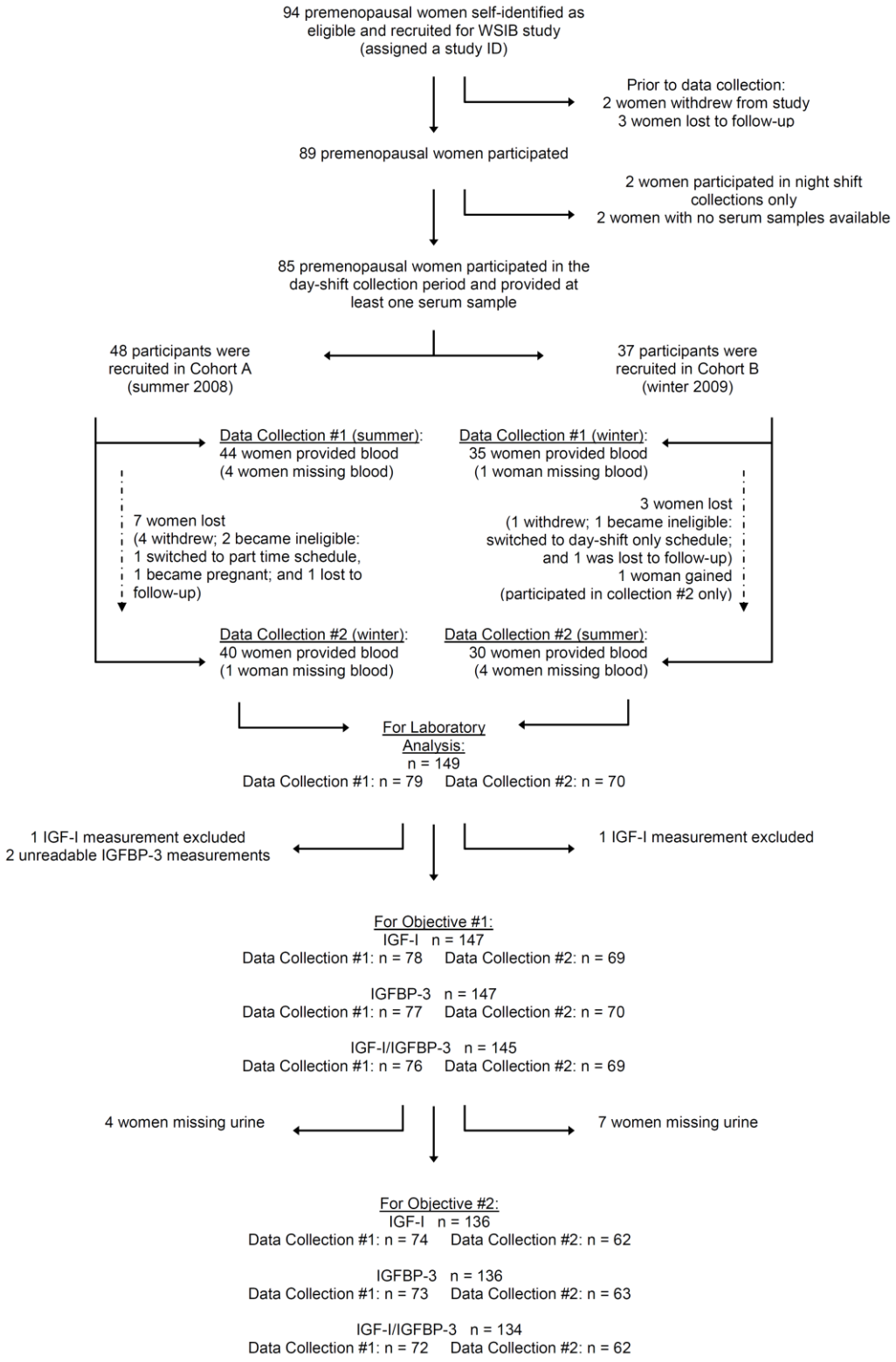


**Figure 3.3** Sampling diagram

**Table 3.1** Final sample sizes

| | IGF-I (N) | IGFBP-3 (N) | IGF-I/IGFBP-3 (N) |
|---|---|---|---|
| *Objective 1* | | | |
| Data collection #1 | 78 | 77 | 76 |
| Data collection #2 | 69 | 70 | 69 |
| Pooled | 147 | 147 | 145 |
| *Objective 2* | | | |
| Data collection #1 | 74 | 73 | 72 |
| Data collection #2 | 62 | 63 | 62 |
| Pooled | 136 | 136 | 134 |

The majority of analyses were completed using the pooled data sets (data collection #1 + data collection #2) to increase statistical power.

### 3.7.2 Data cleaning

Prior to completing any statistical analysis, issues regarding errors in data entry, such as spelling mistakes, main exposure and outcome distributions, and missing data were addressed. Errors in data entry were corrected by consulting the original database, as well as hard copies of the participants' study material. The main exposure variable, urinary aMT6s, was log-transformed to improve the normality of the variable's distribution; a practice commonly employed (21,24,26,68,152,172). The distributions of the IGF-I and IGFBP-3 variables were assessed for outliers, whereby values larger than the 75$^{th}$ percentile plus three times the interquartile range were excluded from further analysis (94,112), including the calculation of IGF-I/IGFBP-3. One individual's IGF-I values (two observations) exceeded the cutoff criteria and were removed from the data set and excluded from IGF-I/IGFBP-3 calculations. None of the IGFBP-3 values met the outlier criteria.

To preserve the already limited study sample, single imputations were used to manage incomplete data. Median and mode substitution, for continuous and categorical variables, respectively, were chosen for their simple and conservative approach (173).

Variables were imputed based on information gathered from those 85 women who provided at least one serum sample. Notably, imputation for time-dependent covariates was completed separately for each of the two day shift components, using information from only the applicable collection period, not the pooled data. When possible, qualifying information was taken into consideration in an effort to impute a more accurate estimate. For example, regarding number of pregnancies, for women who selected “yes” when asked if they had ever been pregnant, but did not indicate the total number of times, the median value for imputation was calculated using those women who indicated at least one pregnancy (i.e. women who had never been pregnant were excluded). For women who did not indicate if they had ever been pregnant or report a number, imputation was completed using the median value from the entire sample. Missing data for continuous variables that were conceptualized as categorical (BMI and number of pregnancies) were addressed prior to categorization. Age, season, smoking status, and chronic alcohol consumption had no missing values and did not require imputations. A summary of the number of imputations and values used may be found in Table 3.2.

**Table 3.2** Summary of imputations

| Predictor | Number of missing values | Value imputed |
|---|---|---|
| BMI ($kg/m^2$)* | 1 | 25.50761 |
| Ethnicity (white, non-white)* | 2 | no imputations (used descriptively only) |
| History of night shift work (years)* | 1 | 9.0 |
| Sleep duration (hour) | day shift #1: 2<br>day shift #2: 3 | day shift #1: 7.0<br>day shift #2: 7.04 |
| Menstrual cycle stage | day shift #1: 12<br>day shift #2: 6 | day shift #1: follicular<br>day shift #2: luteal |
| Current oral contraceptive use (yes, no)† | day shift #1: 7<br>day shift #2: 5 | day shift #1: no<br>day shift #2: no |
| Number of pregnancies* | 5 | 1.0 (1 individual; median for those missing a response for ever pregnant, "yes" vs. "no")<br>2.0 (4 individuals; median for those who answered "yes" for ever pregnant) |
| Recent alcohol consumption (# drinks/day)† | day shift #1: 6<br>day shift #2: 4 | day shift #1: 0<br>day shift #2: 0 |
| Recent caffeine consumption (# drinks/day)† | day shift #1: 11<br>day shift #2: 8 | day shift #1:<br>2.0 (median for those missing a response for recent caffeine, "yes" vs. "no")<br>2.5 (median for those who answered "yes" for recent caffeine)<br>day shift #2:<br>3.0 (median for those missing a response for recent caffeine, "yes" vs. "no")<br>3.0 (median for those who answered "yes" for recent caffeine) |
| Total physical activity (avg. METmin/day)Ψ | day shift #1: 6<br>day shift #2: 4 | day shift #1: 1446.0<br>day shift #2: 1407.667 |

* measured at baseline only
† last 24 hours
Ψ last 30 days

### 3.7.3 Descriptive analysis

To characterize the distribution of the baseline and time-dependent attributes of the study sample, univariate analyses were conducted, namely frequency distributions

for categorical variables, as well as measures of central tendency and dispersion (medians and ranges) for continuous variables.

To better understand the distributions of IGF-I, IGFBP-3, and IGF-I/IGFBP-3, and to preliminarily explore the relationships between these IGF outcomes and their potential predictors, concentrations of the outcome variables were assessed stratified by the various covariates. Generally, strata for continuous variables were created based on quartiles, using all (pooled) data for time-dependent variables and baseline data (n = 85) for variables only assessed upon study enrollment, and kept consistent for all analyses. Exceptions included history of shift work, which was categorized using five-year intervals, and recent alcohol consumption, which, due to the extreme lack of variability in the nurses' responses, was categorized by collapsing quantiles until three intervals could be constructed. The presence of linear trends across the strata was also tested (presented as p-trend) by regressing the continuous IGF outcomes on the potential predictors, treated as ordinal variables. Stratification by covariates and linear tests for trend were completed for the pooled data, using mixed effects modeling to account for correlation between repeated measures (see Section 3.7.5), as well as for each data collection period, using simple linear regression (latter results not shown in thesis).

### 3.7.4 Spearman's rank correlations

Spearman's rank correlations were used as a means of quantifying the relationship between overnight urinary levels of melatonin and morning circulating levels of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 at first day shift, such that comparisons with existing literature could be made. The correlation between IGF-I and IGFBP-3 was also assessed, mainly as a quality control measure.

### 3.7.5 Multivariate analysis

To determine important predictors of IGFs and estimate more accurate measures of effect for the relationship between melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3, multivariate linear models were constructed. As the majority of participants contributed two sets of exposure and outcome measurements to the pooled data sets used for these analyses, one per collection period, correlation between the repeated measures was a concern. Consequently, mixed effects modeling with a random subject effect was used for the multivariate analyses. This extension of the simple linear regression model accounts for the dependence of observations within individuals by adjusting for the underestimation of standard errors that occurs when observations are correlated (174-176). Moreover, mixed effects modeling does not require that each individual have equal numbers of measurements, thus allowing those participants who only provided information for one data collection to remain within the model (175,176).

*Objective 1: Predictive models*

Initially, unadjusted and partially adjusted (forcing age, BMI, and laboratory test batch; identified *a priori*) models were constructed for each potential predictor and IGF outcome. The partially adjusted models were subsequently used as part of a screening procedure, whereby variables meeting the pre-established liberal cutoff of $p \leq 0.20$ were selected for further assessment. After fitting the full models with those variables meeting the screening criteria, final models were built using manual backwards selection and a significance level of $p \leq 0.10$.

*Objective 2: Melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3 models*

Similar to the predictive models, unadjusted and partially adjusted models (forcing age, BMI, and laboratory test batch; identified *a priori*) were used to evaluate the relationship between melatonin and the IGF measures. As a means of assessing confounding, a systematic approach incorporating a backwards change-in-estimate method, similar to that described by Rothman & Greenland (177), was employed. Those covariates previously identified in the Objective 1 screening step as being associated with the IGF measure ($p \leq 0.20$, partially adjusted model) were selected to be placed in a full model. Using stepwise deletion, those covariates that were found to alter the exposure parameter estimate by 10% or greater (the *a priori* inclusion limit) upon removal were included as confounders in the final model. To assess possible effect modification, partially adjusted associations between melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3 were stratified by season (using multivariate linear modeling) and the exposure effect estimates compared.

Additional analyses were completed based on the final adjusted models, but using melatonin quartiles. Modeling melatonin as a categorical variable was conducted as a means of producing more intuitive and easily interpreted results, but also to investigate possible threshold or non-linear effects. Quartiles were computed using the pooled data and held constant for all analyses.

### 3.7.6 Sensitivity analysis and regression diagnostics

To assess the impact of imputation on the results obtained, multivariate analyses were repeated with the imputations removed, using pairwise deletion to manage incomplete data, and the observations compared. Residual analysis and influence diagnostics (restricted likelihood distance and Cook's D, MDFFITS, COVRATIO, and

COVTRACE for both the fixed effects and the covariance parameters) were used to aid in evaluating the assumptions of linear regression.

### 3.7.7 Minimal detectable effect

Using the method described by Dupont & Plummer (178), minimum detectable effect (MDE) calculations for the relationships between melatonin and the IGF measures were conducted in place of sample size or power calculations.

*A priori* calculations were completed using a power of 80% and a significance level (α) of 0.05, as is consistent with general epidemiological practice. Standard deviations for both exposure and outcome variables of 1.0 were employed, as corresponds to the standard normal distribution, given that information on "normal" variation in melatonin and IGF-I is limited. Estimated sample size values of 86 (number of individuals with at least one blood sample) and 134 (total number of blood samples with a corresponding urine sample) were assessed. Accordingly, *a priori* estimations indicated that the study was adequately powered to detect effects (parameter estimates, β) as low as +/- 0.244 (n = 134) and +/- 0.371 (n = 86). *Post-hoc* MDEs were calculated as well, using the final sample sizes and observed distributions for melatonin and the IGFs (see Table 3.3). Notably, as the MDE calculations do not take into account the dependence of observations in repeated measures data, the computed MDE values therefore overestimate the minimum alternative hypothesis that could be detected.

**Table 3.3** *Post hoc* minimum detectable effect

| **IGF-I** MDE (N) | **IGFBP-3** MDE (N) | **IGF-I/IGFBP-3** MDE (N) |
|---|---|---|
| +/- 12.5 (136) | +/- 208.6 (136) | +/- 0.015 (134) |

## 3.8 Ethical considerations

Approval through the Queen's University Health Sciences Human Research Ethics Board was achieved for the parent study, as well as this project specifically (Appendix D). All questionnaires are stored in a locked file cabinet at the Queen's University Cancer Research Institute, where only study personnel may access it, and de-identified information was entered into a secure database on a password-protected computer at the Queen's University Cancer Research Institute. All data, including biological samples, were analyzed using numerical codes, without the use of any subject identifiers, to ensure that the anonymity of all participants was maintained. A study identification number key, available to only three members of the study team, including the study coordinator, was used exclusively for collection scheduling purposes. It is also important to note that all study participants gave informed consent prior to study enrollment and were provided with copies of their signed consent forms (Appendix B). An honorarium of $200.00 was provided as compensation due to the time-consuming nature of the study.

## 3.9 Student contributions

The topics for the thesis project were put forth by Drs Harriet Richardson and Kristan Aronson. The candidate completed all required laboratory analyses for the IGF outcome measures assessed; other members of the research team conducted the quantification of urinary aMT6s and creatinine. Under the guidance of Drs Richardson and Aronson, the candidate completed all statistical analyses and wrote all chapters of this thesis.

# Chapter 4
# Results

## 4.1 Characteristics of study population

Characteristics of the study population are presented in Tables 4.1 and 4.2. The average age of these premenopausal nurses was 37 years and half (49.4%) had a body mass index (BMI) measurement of greater than 25.5 kg/m$^2$. Half (49.4%) of the women had been pregnant at least once and the median number of years of previous night shift work was nine. Over the course of their first day shift collection period, the nurses slept an average of seven hours, nearly a quarter reported recent oral contraception (OC) use (22.4%), and the median number of alcoholic beverages consumed in the last 24 hours was zero.

The participants had an average urinary melatonin concentration of 29.6 aMT6s ng/mg creatinine at their first day shift collection. Untransformed, the overall distribution of melatonin was heavily skewed. Post-log-transformation, values of melatonin were more normally distributed (Appendix E, Figure E.1). Median values for serum insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3) were 141.9 ng/ml and 4315.2 ng/ml, respectively. The distribution of IGF-I values, from both day shift periods, was positively skewed; upon the deletion of two outliers (two observations from same individual), the distribution improved slightly, becoming more normal (Appendix E, Figure E.2). In contrast, the overall distribution for IGFBP-3 levels was fairly normal and no outliers were identified (Appendix E, Figure E.3). Finally, the participants had a median IGF-I/IGFBP-3 value of 0.118 at their first day shift collection. The dispersion of the molar ratio was quite positively skewed, which did not improve following the removal of the two IGF-I outliers (Appendix E, Figure E.4). Average values

of melatonin, IGF-I, IGFBP-3, and IGF-I/IGFBP-3 stratified by age (quartiles) indicated that older women tended to have reduced levels of IGF-I and IGF-I/IGFBP-3, whereas concentrations of melatonin and IGFBP-3 did not appear to vary by age category (Table 4.3).

**Table 4.1** Baseline characteristics of study population

| Characteristic | n = 85 |
|---|---|
| Age (years) | |
| median (range); N | 37.0 (23.0 – 54.0); 85 |
| BMI (kg/m$^2$) (N, %) | |
| ≤ 22.7 | 22 (25.9) |
| > 22.7 – 25.5 | 20 (23.5) |
| > 25.5 – 29.4 | 21 (24.7) |
| > 29.4 | 21 (24.7) |
| *Missing* | 1 (1.2) |
| Ethnicity (N, %) | |
| White | 81 (95.3) |
| Non-white | 2 (2.4) |
| *Missing* | 2 (2.4) |
| Number of pregnancies (N, %) | |
| None | 38 (44.7) |
| 1 | 7 (8.2) |
| 2 | 20 (23.5) |
| ≥ 3 | 15 (17.6) |
| *Missing* | 5 (5.9) |
| Smoking status (N, %) | |
| Never | 53 (62.4) |
| Past | 20 (23.5) |
| Current | 12 (14.1) |
| Alcohol consumption (avg. # drinks/week)* | |
| median (range); N | 2.0 (0 – 12.5); 85 |
| History of night shift work (years) | |
| median (range); N | 9.0 (0 – 38.0); 84 |

* Average value for current decade of life

**Table 4.2** Time-dependent characteristics of study population, at first day shift of participation

| Characteristic | n = 85* |
|---|---|
| *Study diary measures* | |
| Sleep duration (hours) | |
| median (range); N | 7.0 (3.5 – 13.0); 83 |
| Menstrual cycle stage (N, %) | |
| Follicular | 33 (38.8) |
| Mid-cycle | 14 (16.5) |
| Luteal | 25 (29.4) |
| *Missing* | 13 (15.3) |
| Current oral contraceptive use (N, %)‡ | |
| No | 58 (68.2) |
| Yes | 19 (22.4) |
| *Missing* | 8 (9.4) |
| Recent alcohol consumption (# drinks/day)‡ | |
| median (range); N | 0 (0 – 3.0); 78 |
| Recent caffeine consumption (# drinks/day)‡ | |
| median (range); N | 2.0 (0 – 14.0); 73 |
| Total physical activity (avg. METmin/day)† | |
| median (range); N | 1440.9 (421.7 – 3314.8); 78 |
| *Biological samples* | |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | |
| geometric mean (back-transformed range); N | 29.6 (0.8 – 1195.6); 81 |
| IGF-I (ng/ml) | |
| median (range); N | 141.9 (56.0 – 333.4); 79 |
| IGFBP-3 (ng/ml) | |
| median (range); N | 4315.2 (1476.8 – 7269.1); 78 |
| IGF-I/IGFBP-3 | |
| median (range); N | 0.118 (0.049 – 0.670); 77 |

*84 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)
‡ last 24 hours
† last 30 days

**Table 4.3** Median melatonin, IGF-I, IGFBP-3, and IGF-I/IGFBP-3 concentrations stratified by age

| Characteristic | Log-transformed melatonin (n = 138)* Geometric mean (back-transformed range); N (aMT6s ng/mg creatinine) | IGF-I (n = 147)‡ Median (range); N (ng/ml) | IGFBP-3 (n = 147)† Median (range); N (ng/ml) | IGF-I/IGFBP-3 (n = 145)Ψ Median (range); N |
|---|---|---|---|---|
| Age (years) ‡‡ | | | | |
| ≤ 30 | 27.7 (0.9 – 93.7); 35 | 182.7 (109.5 – 337.1); 37 | 4051.4 (2436.9 – 6164.1); 38 | 0.172 (0.086 – 0.331); 36 |
| > 30 – 37 | 23.9 (0.9 – 1195.6); 32 | 152.4 (57.1 – 344.1); 37 | 3897.9 (1476.8 – 6176.3); 37 | 0.141 (0.060 – 0.670); 37 |
| > 37 – 43 | 31.3 (0.8 – 114.3); 37 | 136.5 (56.0 – 290.8); 39 | 3827.1 (2124.6 – 7269.1); 39 | 0.129 (0.049 – 0.400); 39 |
| > 43 | 33.6 (1.0 – 717.2); 34 | 143.4 (65.3 – 287.6); 34 | 4167.8 (2310.1 – 6452.7); 33 | 0.119 (0.052 – 0.374); 33 |

* n = 138; 138 observations, 83 individuals; overlap of 55 individuals across day shift collection periods
‡ n=147; 147 observations, 84 individuals; overlap of 63 individuals across day shift collection periods
† n=147; 147 observations, 85 individuals; overlap of 62 individuals across day shift collection periods
Ψ n=145; 145 observations, 84 individuals; overlap of 61 individuals across day shift collection periods
‡‡ measured at baseline only

Measures of central tendency and dispersion for melatonin, IGF-I, and IGFBP-3 were compared to manufacturer-provided reference ranges and examples from the literature (Table 4.4). Average morning urinary levels of melatonin were slightly higher and the range wider than those observed among a group (n = 459) of Nurses' Health Study II (NHS II) participants of a marginally greater age (range = 33 – 50 years) (67). Similarly, serum IGF-I and IGFBP-3 concentrations were slightly elevated as compared

to the laboratory reference ranges provided (160,164). When considered against average values among older individuals ($10^{th}$ – $90^{th}$ percentile: 45 – 66) from the Nurses' Health Study I (NHS I), IGF-I values observed in this study were decreased, whereas levels of IGFBP-3 were marginally increased (94). The ranges for both IGF-I and IGFBP-3 were larger than those reported among the participants NHS I (94). It is important to note, however, that while insulin-like growth factor (IGF) peptides were quantified via serum samples, using Quantikine® enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems for this study, values for the NHS I participants were obtained from plasma samples, via ELISA kits from Diagnostic Systems Laboratory (94).

**Table 4.4** Melatonin, IGF-I, and IGFBP-3 values with laboratory/population reference ranges

| Biological sample | Observed values | | | Laboratory references | Population values |
|---|---|---|---|---|---|
| | Mean | Median | Range; N | Mean (range); N | Median (range: 10th – 90th percentile); N |
| Log-transformed melatonin (aMT6s ng/mg creatinine)* | 23.6 | 28.9 | 0.8 – 1195.6; 138‡ | -- | 19.6 (5.5 – 43.1); 459[b] |
| IGF-I (ng/ml) | 167.6 | 156.2 | 56.0 – 344.1; 147† | 105 (40 – 258); 61[a] | 180 (102 – 289); 1037[c] |
| IGFBP-3 (ng/ml) | 4094.7 | 4040.6 | 1476.8 – 7269.1; 147Ψ | 2375 (835 – 3778); 70[a] | 3980 (2946 – 51129); 1037[c] |

* geometric mean, back-transformed median and range
‡ n = 138; 138 observations, 83 individuals; overlap of 55 individuals across day shift collection periods
† n=147; 147 observations, 84 individuals; overlap of 63 individuals across day shift collection periods
Ψ n=147; 147 observations, 85 individuals; overlap of 62 individuals across day shift collection periods
a: from R&D Systems Quantikine® ELISA kit inserts, based on samples of seemingly healthy volunteers (no medical histories obtained) (160,164)
b: from study of aMT6s and lifestyle factors and endogenous hormones, based on samples from the Nurses' Health Study II (age range: 33 – 50 years); Bühlmann aMT6s ELISA kit (67)
c: from study of IGFs and lifestyle factors, based on sample from the Nurses' Health Study I (age range, 10th – 90th percentile: 45 – 66 years); Diagnostic Systems Laboratory ELISA kits (94)

Baseline characteristics and IGF outcome measures were also considered based on level of participation. Specifically, characteristics of women with blood available for both day shifts, those who provided a single serum sample, and those who failed to provide any blood specimens are presented in Appendix E, Tables E.1 and E.2. Time-dependent characteristics of the nurses compared by data collection period may be found in Appendix E, Table E.3. Mean concentrations of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 stratified by covariates were also determined and tests for trend completed (see Appendix E, Table E.4).

## 4.2 Objective 1: Predictive models

### 4.2.1 Insulin-like growth factor-I: unadjusted and adjusted mixed effects models

Assessments of potential predictors revealed that age, season, and current OC use were associated with serum IGF-I (Table 4.5). In the final multivariate model, an inverse relationship was observed between serum IGF-I and age ($\beta = -3.6$, $p < 0.0001$) and current OC use ($\beta = -40.8$, $p = 0.003$), while winter was associated with an increase in IGF-I levels ($\beta = 26.3$, $p = 0.02$).

Unadjusted regressions (Table 4.5) and descriptive evaluations (Appendix E, Table E.4) also indicated an inverse relationship between history of night shift work and IGF-I (Table 4.5: $\beta = -2.0$, $p = 0.004$; Table E.4: p-trend = 0.002). However, this relationship was attenuated after adjusting for age, BMI, and laboratory test batch, and not included in further assessments. Partially adjusted linear models suggested that the luteal phase of the menstrual cycle may be negatively associated with IGF-I concentrations ($\beta = -15.4$, $p = 0.08$) and that three or more pregnancies were associated with increased IGF-I levels ($\beta = 38.9$, $p = 0.03$). These relationships did not persist in the

fully adjusted model, resulting in the removal of the covariates during the backwards selection process. Finally, a positive linear trend was observed when stratifying mean IGF-I levels by quartiles of usual physical activity (Table E.4: p-trend 0.02); nevertheless, physical activity did not significantly predict levels of IGF-I when assessed using mixed effects models (unadjusted or partially adjusted).

**Table 4.5** Predictive models for IGF-I

| Predictor | **Unadjusted** Parameter estimate[a] (p-value) | **Partially Adjusted** Parameter estimate[b] (p-value) | **Final Model** Parameter estimate[c] (p-value) |
|---|---|---|---|
| *Forced covariates* ('Partially Adjusted' = mutual adjustment for age BMI and test batch predictors) | | | |
| Age (years)* | -3.1 (<0.0001) | -2.4 (0.0009) | -3.6 (<0.0001) |
| BMI (kg/m$^2$)* | | | |
| ≤ 22.7 | Ref | Ref | Ref |
| > 22.7 – 25.5 | 0.7 (0.97) | 4.5 (0.76) | 5.5 (0.70) |
| > 25.5 – 29.4 | -22.5 (0.19) | -4.4 (0.76) | -9.4 (0.52) |
| > 29.4 | -2.0 (0.91) | 1.8 (0.90) | -5.9 (0.69) |
| Test batch | | | |
| 1 | Ref | Ref | Ref |
| 2 | -34.9 (0.007) | -29.3 (0.02) | -5.7 (0.71) |
| 3 | 55.0 (<0.0001) | 55.6 (<0.0001) | 50.4 (0.0001) |
| 4 | 28.0 (0.04) | 25.0 (0.06) | 36.0 (0.01) |
| 5 | 21.5 (0.49) | 23.7 (0.44) | 39.1 (0.21) |
| *Potential covariates* | | | |
| History of night shift work (years)* | -2.0 (0.004) | -0.4 (0.63) | -- |
| Season | | | |
| Summer | Ref | Ref | Ref |
| Winter | 43.3 (<0.0001) | 23.4 (0.05) | 26.3 (0.02) |

Con't

| | | | |
|---|---|---|---|
| Sleep duration (hour) | 1.6 (0.75) | 3.4 (0.40) | -- |
| Menstrual cycle stage | | | |
| Follicular | Ref | Ref | |
| Mid-cycle | -20.4 (0.19) | -6.8 (0.59) | -- |
| Luteal | -3.0 (0.79) | -15.4 (0.08) | -- |
| Current oral contraceptive use† | | | |
| No | Ref | Ref | Ref |
| Yes | -3.2 (0.83) | -37.7 (0.006) | -40.8 (0.003) |
| Number of pregnancies* | | | |
| None | Ref | Ref | |
| 1 | -8.7 (0.69) | 7.5 (0.69) | -- |
| 2 | -22.8 (0.12) | 14.1 (0.33) | -- |
| ≥ 3 | -5.3 (0.76) | 38.9 (0.03) | -- |
| Smoking status* | | | |
| Never | Ref | Ref | |
| Past | -6.9 (0.65) | 1.9 (0.88) | -- |
| Current | -3.6 (0.84) | -12.2 (0.44) | -- |
| Chronic alcohol consumption (avg. # drinks/week)* ‡ | 1.9 (0.35) | -0.5 (0.77) | -- |
| Recent alcohol consumption (# drinks/day)† | 0.5 (0.95) | 1.5 (0.82) | -- |
| Recent caffeine consumption (# drinks/day)† | -1.7 (0.53) | -0.2 (0.94) | -- |
| Total physical activity (avg. METmin/day)Ψ | 0.02 (0.17) | 0.006 (0.55) | -- |

n=147; 147 observations, 84 individuals; overlap of 63 individuals across day shift collection periods
* measured at baseline only
‡ average value for current decade of life
† last 24 hours
Ψ last 30 days
a: unadjusted
b: adjusted for age, BMI, and test batch
c: variables mutually adjusted; all variables remaining after backwards selection, inclusion cutoff of $p \leq 0.10$

### 4.2.2 Insulin-like growth factor binding protein-3: unadjusted and adjusted mixed effects models

Recent alcohol consumption was the only covariate observed to predict serum IGFBP-3 concentrations (Table 4.6). There was a borderline positive association between recent alcohol consumption and IGFBP-3 ($\beta = 197.8$, $p = 0.05$). The magnitude of this relationship persisted from that observed prior to the removal of a highly influential individual from the model (data not shown).

Unadjusted and partially adjusted regressions (Table 4.6), as well as descriptive assessments (Appendix E, Table E.4), suggested associations between season and IGFBP-3 (Table 4.6: partially adjusted $\beta = 321.4$, $p = 0.08$) and menstrual cycle stage and IGFBP-3 (Table 4.6: partially adjusted, luteal phase $\beta = -260.3$, $p = 0.05$; Table E.4: p-trend = 0.003). These covariates, however, did not remain in the final model following the backwards selection process. Similarly, number of previous pregnancies, while meeting the predetermined cutoff of $p \leq 0.20$ for inclusion in the selection method (Table 4.6: partially adjusted, single pregnancy $\beta = -646.2$, $p = 0.09$), was not found to be associated with IGFBP-3 when controlling for other predictors.

**Table 4.6** Predictive models for IGFBP-3

| Predictor | Unadjusted<br>Parameter estimate[a]<br>(p-value) | Partially Adjusted<br>Parameter estimate[b]<br>(p-value) | Final Model**<br>Parameter estimate[c]<br>(p-value) |
|---|---|---|---|
| *Forced covariates*<br>('Partially Adjusted' = mutual adjustment for age BMI and test batch predictors) | | | |
| Age (years)* | -13.2 (0.31) | -17.9 (0.18) | -19.0 (0.12) |
| BMI (kg/m$^2$)* | | | |
| ≤ 22.7 | Ref | Ref | Ref |
| > 22.7 – 25.5 | 166.0 (0.58) | 62.2 (0.83) | 166.5 (0.54) |
| > 25.5 – 29.4 | -212.9 (0.46) | -134.8 (0.64) | -201.9 (0.45) |
| > 29.4 | 97.2 (0.74) | -31.7 (0.91) | -0.6 (1.0) |
| Test batch | | | |
| 1 | Ref | Ref | Ref |
| 2 | 1300.7 (<0.0001) | 1309.3 (<0.0001) | 1327.6 (<0.0001) |
| 3 | 817.5 (0.0001) | 864.3 (<0.0001) | 831.1 (0.0002) |
| 4 | 390.6 (0.05) | 427.6 (0.04) | 458.3 (0.02) |
| 5 | 1425.6 (<0.0001) | 1471.7 (<0.0001) | 1429.9 (<0.0001) |
| *Potential covariates* | | | |
| History of night shift work (years)* | -14.0 (0.25) | -9.5 (0.51) | -- |
| Season | | | |
| Summer | Ref | Ref | |
| Winter | 518.4 (0.0002) | 321.4 (0.08) | -- |
| Sleep duration (hour) | 68.1 (0.38) | 46.0 (0.46) | -- |
| Menstrual cycle stage | | | |
| Follicular | Ref | Ref | |
| Mid-cycle | -118.8 (0.62) | -148.9 (0.43) | -- |
| Luteal | -500.6 (0.003) | -260.3 (0.05) | -- |
| Current oral contraceptive use[†] | | | |
| No | Ref | Ref | |
| Yes | 402.1 (0.09) | 100.2 (0.64) | -- |

Con't

| | | | |
|---|---|---|---|
| Number of pregnancies* | | | |
| None | Ref | Ref | |
| 1 | -568.9 (0.13) | -646.2 (0.09) | -- |
| 2 | -169.1 (0.50) | -114.6 (0.69) | -- |
| ≥ 3 | -14.4 (0.96) | 43.6 (0.90) | -- |
| Smoking status* | | | |
| Never | Ref | Ref | |
| Past | -306.7 (0.23) | -229.3 (0.37) | -- |
| Current | -219.0 (0.48) | -351.1 (0.27) | -- |
| Chronic alcohol consumption (avg. # drinks/week)* ‡ | -23.6 (0.50) | -21.9 (0.54) | -- |
| Recent alcohol consumption (# drinks/day)† | 289.6 (0.02) | 212.9 (0.04) | 197.8 (0.05) |
| Recent caffeine consumption (# drinks/day)† | 10.1 (0.82) | -28.0 (0.46) | -- |
| Total physical activity (avg. METmin/day)Ψ | 0.3 (0.14) | 0.08 (0.64) | -- |

n=147; 147 observations, 85 individuals; overlap of 62 individuals across day shift collection periods
* measured at baseline only
** n = 145; 145 observations, 84 individuals (one influential individual deleted)
‡ average value for current decade of life
† last 24 hours
Ψ last 30 days
a: unadjusted
b: adjusted for age, BMI, and test batch
c: variables mutually adjusted; all variables remaining after backwards selection, inclusion cutoff of $p \leq 0.10$

### 4.2.3 IGF-I/IGFBP-3: unadjusted and adjusted mixed effects models

Similar to IGF-I, multivariate mixed modeling suggested that both age and current OC use were negatively associated with IGF-I/IGFBP-3 levels ($\beta = -0.003$, $p = 0.0004$ and $\beta = -0.037$, $p = 0.003$, respectively) (see Appendix E, Table E.6 for detailed analysis). These parameter estimates did not differ from those observed prior to removing three influential individuals from the analyses (data not shown). In contrast to IGF-I, season was not a predictor of IGF-I/IGFBP-3.

## 4.3 Objective 2: Associations between melatonin and the insulin-like growth factor family

### 4.3.1 Melatonin and insulin-like growth factor-I: correlations, unadjusted and adjusted mixed effects models

Neither Spearman's rank correlation nor multivariate-adjusted mixed modeling indicated a relationship between melatonin and IGF-I (Tables 4.7 and 4.8). The Spearman's rank correlation coefficient for the relationship between melatonin and IGF-I at the participants' first day shift was -0.1 (p = 0.32). While a small negative association between melatonin and IGF-I was initially indicated by mixed effects modeling, it did not reach significance (unadjusted β = -2.6, p = 0.55). The relationship was further attenuated when adjusted for confounders (β = -1.2, p = 0.74).

**Table 4.7** Spearman's rank correlation between log-transformed melatonin and IGF-I at first day shift of participation

| | **IGF-I** |
|---|---|
| | Correlation coefficient (p-value); N |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -0.1 (0.32); 75* |

* 74 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)

**Table 4.8** Associations between log-transformed melatonin and IGF-I

| Predictor | Unadjusted | Partially Adjusted | Final Model |
|---|---|---|---|
| | Parameter estimate[a] (p-value) | Parameter estimate[b] (p-value) | Parameter estimate[c] (p-value) |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -2.6 (0.55) | -1.3 (0.74) | -1.2 (0.74) |

n = 136; 136 observations, 82 individuals; overlap of 54 individuals across day shift collection periods
a: unadjusted
b: adjusted for age, BMI, and test batch
c: adjusted for age, BMI, test batch, season, menstrual cycle stage, current OC use, and number of pregnancies

Figure 4.1 reflects the relationship between melatonin and IGF-I, adjusted for confounders. A high degree of variability in the values of IGF-I is evident, while the slope of the regression line illustrates the lack of an association between the two hormones.

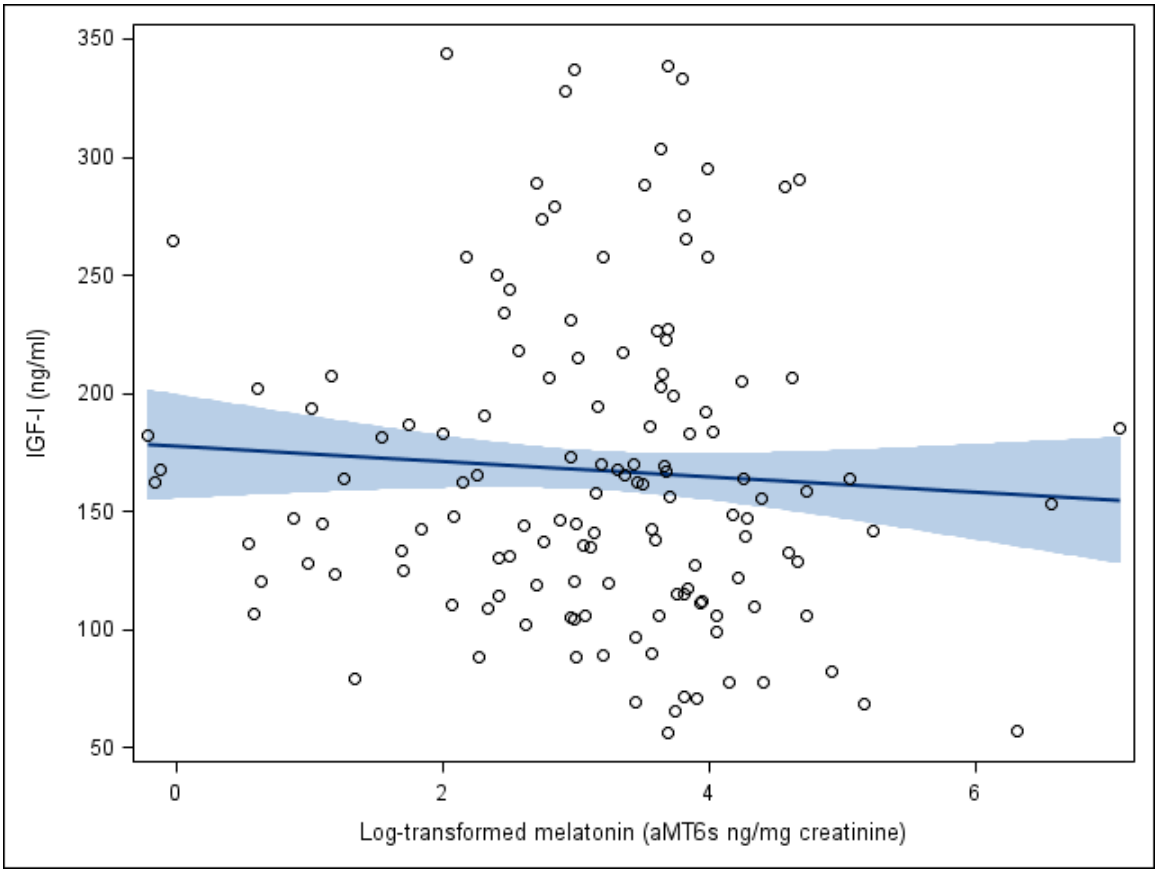


**Figure 4.1** Graph of log-transformed melatonin (aMT6s ng/mg creatinine) vs. IGF-I (ng/ml)

### 4.3.2 Melatonin and insulin-like growth factor bind protein-3: correlations, unadjusted and adjusted mixed effects models

Similarly, levels of urinary melatonin did not predict serum concentrations of IGFBP-3 (Table 4.9 and 4.10). Melatonin and IGFBP-3 did not appear to be correlated at the nurses' first day shift collection period ($r = 0.06$, $p = 0.61$). A small positive association between melatonin and the IGF peptide was suggested by the crude mixed effects model (unadjusted $\beta = 65.3$, $p = 0.35$). However, following adjustment for potential confounders, the parameter estimate for melatonin was negative (unadjusted $\beta = -35.8$, $p = 0.49$). None of the mixed effects models reached significance. The results of

the final model did not differ from those observed prior to the removal of an influential individual (data not shown).

**Table 4.9** Spearman's rank correlation between log-transformed melatonin and IGFBP-3 at first day shift of participation

| | **IGFBP-3** |
|---|---|
| | Correlation coefficient (p-value); N |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | 0.06 (0.61); 74* |

* 73 individuals from day shift #1, 1 individual from day shift #2 (who lacked information for all time-dependent characteristics at day shift #1)

**Table 4.10** Associations between log-transformed melatonin and IGFBP-3

| **Predictor** | **Unadjusted** | **Partially Adjusted** | **Final Model*** |
|---|---|---|---|
| | Parameter estimate[a] (p-value) | Parameter estimate[b] (p-value) | Parameter estimate[c] (p-value) |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | 65.3 (0.35) | -2.9 (0.96) | -35.8 (0.49) |

n = 136; 136 observations, 83 individuals; overlap of 53 individuals across day shift collection periods
* n = 134; 134 observations, 82 individuals (one influential individual deleted)
a: unadjusted
b: adjusted for age, BMI, and test batch
c: adjusted for age, BMI, test batch, season, menstrual cycle stage, and recent alcohol consumption

Graphical representation of the final regression for melatonin and IGFBP-3 may be found in Appendix E, Figure E.5.

### 4.3.3 Melatonin and IGF-I/IGFBP-3: correlations, unadjusted and adjusted mixed effects models

No relationship was observed between melatonin and IGF-I/IGFBP-3. Particularly after adjustment for confounders, melatonin did not appear to help determine levels of

IGF-I/IGFBP-3 (Table E.8: β = -0.002, p = 0.57). These observations persisted from those noted prior to removal of three influential individuals (data not shown). Details regarding this analysis may be found in Appendix E (Tables E.7 and E.8, Figure E.6).

### 4.3.4 Associations between melatonin and IGFs: stratified by season

Season was assessed as a potential effect modifier by stratifying the partially adjusted models that tested associations between melatonin and the IGF measures (Table 4.11 and Appendix E, Table E.9). Partially adjusted parameter estimates for melatonin did not appear to differ by season for IGF-I (Table 4.11: summer β = -6.9, p = 0.21 and winter β = 0.7, p = 0.91) or IGF-I/IGFBP-3 (Table E.9: summer β = -0.008, p = 0.27 and winter β = -0.010, p = 0.29). In contrast, the direction of the partially adjusted parameter estimates was observed to differ by season for IGFBP-3 (Table 4.11: summer β = -42.0, p = 0.68 and winter β = 108.2, p = 0.27). This result was considered in the context of the study's average IGFBP-3 concentrations and the project's limited sample size. In light of a median value of 4315.2 ng/ml for IGFBP-3 (at participants' first day shift), parameter estimate values of -42.0 and 108.2 were considered only marginally different, and given the lack of power to formally test for effect modification, the decision was made to keep the data pooled.

**Table 4.11** Partially adjusted associations between log-transformed melatonin and IGF-I and IGFBP-3, by season

| Predictor | IGF-I<br>n = 136<br>Parameter estimate[a]<br>(p-value) | IGFBP-3<br>n = 136<br>Parameter estimate[a]<br>(p-value) |
|---|---|---|
| *Summer* | N = 68 | N = 68 |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -6.9 (0.21) | -42.0 (0.68) |
| *Winter* | N = 68 | N = 68 |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | 0.7 (0.91) | 108.2 (0.27) |

a: adjusted for age, BMI, and test batch

### 4.3.5 Associations between melatonin and IGFs: quartiles of melatonin

Associations between melatonin and the IGF measures were also tested with melatonin quartiles, adjusting for those covariates identified as confounders in the corresponding continuous melatonin model (Table 4.12). No statistically significant association was observed between quartiles of melatonin and IGF-I, IGFBP-3 or IGF-I/IGFBP-3 (see Appendix E, Table E.10 for latter); however, there may have been some suggestion of a possible threshold effect regarding melatonin and IGFBP-3. Average IGFBP-3 values appeared to peak when individuals' melatonin levels (log-transformed) were between 2.5 and 3.4 aMT6s ng/mg creatinine ($\beta = 322.8$, $p = 0.09$), whereas concentrations of melatonin greater than 3.9 aMT6s ng/mg creatinine were observed to plateau or minutely decrease average IGFBP-3 levels ($\beta = -77.9$, $p = 0.67$). These results for IGFBP-3 were somewhat diluted prior to the removal of three highly influential individuals (data not shown).

**Table 4.12** Adjusted associations between log-transformed melatonin and IGF-I and IGFBP-3; melatonin as categorical

| Predictor | IGF-I<br>n = 136*<br>Parameter estimate[a]<br>(p-value) | IGFBP-3<br>n = 130**<br>Parameter estimate[b]<br>(p-value) |
|---|---|---|
| Log-transformed melatonin (aMT6s ng/mg creatinine) | | |
| ≤ 2.5 | Ref | Ref |
| > 2.5 – 3.4 | 1.8 (0.90) | 322.8 (0.09) |
| > 3.4 – 3.9 | 5.2 (0.70) | 205.0 (0.22) |
| > 3.9 | -5.0 (0.73) | -77.9 (0.67) |

*n = 136; 136 observations, 82 individuals
**n = 130; 130 observations, 80 individuals (three influential individuals deleted)
a: adjusted for age, BMI, test batch, season, menstrual cycle stage, current OC use, and number of pregnancies
b: adjusted for age, BMI, test batch, season, menstrual cycle stage, and recent alcohol consumption

## 4.4 Regression diagnostics and sensitivity analysis

Residual analyses and influence diagnostics were completed for the final models to evaluate the assumptions of linear regression. Overall, these assumptions seemed to be met. However, several individuals were identified as highly influential in models pertaining to IGFBP-3 and IGF-I/IGFBP-3. While removal of these individuals generally did not affect parameter estimates or p-values, the fit of the models, for IGF-I/IGFBP-3 in particular, was improved. Thus, the decision was made to report the parameter estimates with those influential subjects deleted.

To evaluate the implications of using imputations to manage missing covariate data, the final predictive models and tests for association with melatonin were completed with imputations removed (see Appendix E, Tables E.11, E.12, and E.13). Overall, the use of imputations did not seem to greatly affect the results of this investigation. One notable difference involved the test for association between melatonin quartiles and

IGFBP-3. After removing imputed values, the borderline positive association between melatonin levels (log-transformed) of 2.5 – 3.4 aMT6s ng/mg creatinine and serum IGFBP-3 concentrations became slightly stronger (Table E.13: $\beta = 413.2$, $p = 0.046$).

## 4.5 Summary

In summary, mixed effects modeling of potential predictors of IGF peptides indicated that age, season, and current OC use were related to circulating IGF-I, while recent alcohol consumption was a determinant of serum IGFBP-3. Similar to IGF-I, values of IGF-I/IGFBP-3, used as a crude estimate of "free" or "bioactive" IGF-I, were predicted by age and OC use. Spearman's rank correlations and mixed effects modeling were used to evaluate melatonin as a determinant of circulating concentrations of IGFs. Results indicated that urinary melatonin was not a predictor of serum IGF peptides, although some suggestion of a possible threshold effect was noted between quartiles of melatonin and circulating levels of IGFBP-3.

# Chapter 5
# Discussion

## 5.1 Introduction

Primarily, this project aimed to cross-sectionally assess the potential relationship between melatonin and insulin-like growth factor (IGF) peptides among premenopausal, rotating shift nurses. Additionally, potential predictors of insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3) were examined. This chapter includes a summary and interpretation of the main observations of the study, as well as a consideration of the strengths, limitations, and contribution of this research.

## 5.2 Summary of main results and interpretation of findings

### 5.2.1 Predictors of insulin-like growth factors

Assessments of possible predictors of IGFs were completed in an attempt to characterize factors that may contribute to the large inter-individual variability commonly seen when quantifying blood levels of these peptides, thus informing the investigation of melatonin as a determinant of IGFs. As expected, age was found to strongly influence levels of both IGF-I and IGF-I/IGFBP-3. Numerous studies have documented inverse relationships between age and IGF-I (92-98) and IGF-I/IGFBP-3 concentrations (92,94,96) in adult populations. Indeed, age is an established determinant of IGF-I, where production of IGF-I is known to increase from birth to adolescence, peaking during this important developmental stage, then decline with age during adulthood (32). Age did not seem to influence circulating concentrations of IGFBP-3 among the participants of this project. Although levels of IGFBP-3 have been generally observed to

decline with age among adults (92,95,96), investigations using subsets of the participants of the Nurses' Health Study (NHS) have noted similarly null findings (94,137). No association was observed between body mass index (BMI) and the IGF measures, contrary to inverse relationships with IGF-I and IGF-I/IGFBP-3 (92,95,102) and positive associations with IGFBP-3 (94) reported in the literature; though similarly null results have also been noted in several other studies (92-96,98). Oral contraceptive (OC) users were observed to have significantly lower levels of IGF-I and IGF-I/IGFBP-3 than non-users, which coincides with results noted elsewhere (96,103,104).

Season also seemed to influence IGF-I concentrations among women of the study population, with serum IGF-I significantly higher in the winter months, as compared to the summer. It is important to consider that season may, in fact, be acting as a surrogate measure for one or more possible lifestyle predictors of IGF-I that were not assessed in this thesis project. For instance, dietary composition and intake are factors that seem to regulate IGF-I, for which some evidence exists regarding seasonality (179-184). Likewise, physical activity may be related to IGF-I levels (88,113) and has also been observed to fluctuate by season and weather (185-187). Limited evidence exists regarding seasonal variation of IGFs in humans; however, the published results are contradictory to those observed in this project. In the small study of 10 normal woman and 15 premenopausal breast cancer survivors conducted by Holdaway and colleagues (54), seasonal differences were noted among the controls, with mean circulating concentrations of IGF-I significantly higher at the summer solstice than in the winter. With a limited sample size of 10 individuals, it is necessary to consider that the direction of the seasonality seen by Holdaway and colleagues could be due to chance. Furthermore, Holdaway and collaborators did not investigate potential confounders

regarding season and IGF-I. As mentioned earlier, season and IGFs may be related to diet and other factors such as physical activity. While Holdaway et al. (54) did control for the acute effects of diet in their study by providing meals at standardized times (clinical setting), assessment of dietary intake over the previous 24 hours or usual consumption (that would better reflect an association with season) were not evaluated by the researchers. Without considering possible lifestyle factors such as diet or physical activity, for which season may be a proxy, discerning if levels of IGFs are truly influenced by season, and in what manner, becomes difficult. Notably, although a positive linear trend was observed in this project when stratifying mean IGF-I levels by quartiles of usual physical activity, a relationship between IGF-I and physical activity was not seen in the unadjusted or partially adjusted mixed effects models.

Recent alcohol consumption was observed to be a determinant of IGFBP-3 levels, where alcoholic beverages ingested in the past 24 hours positively predicted serum concentrations of IGFBP-3, with borderline significance ($p = 0.05$). Physiologically, this finding also suggests that ingestion of alcohol may reduce circulating levels of free IGF-I, as a greater quantity of IGFBP-3 would be available to sequester the peptide. Several investigations, from small controlled feeding studies to larger cross-sectional projects, have assessed the effects of alcohol on circulating levels of IGFBP-3, with positive, negative, and null associations reported (95,96,105,111,112). However, the aforementioned studies focused on the effects of more long-term consumption patterns of alcohol, for which no association was seen in this study population. While the observed relationship between recent, but not usual alcohol ingestion and IGFBP-3 may indicate a transient effect, these results could also reflect the different methods used to collect information for each variable. The use of a study

diary with open-ended questions to capture recent alcohol consumption lowers the likelihood of introducing random error often associated with frequency questionnaires and the assessment of usual consumption patterns. Conversely, the relationship found between recent alcohol ingestion and IGFBP-3 could be an artifact of small sample size and a lack of variability in the data, as a large majority of participants (72.5% of observations) did not consume any alcohol during the two 24 hour periods captured by the study diaries. Finally, as was expected, concentrations of IGF-I and IGFBP-3 were found to be moderately correlated (see Appendix E, Table E.5) with a magnitude similar to reports given elsewhere (96,129,188).

Importantly, these results, beyond indicating sources of variability in IGFs among the study population, add validity to the specimen collection, processing, storage, and quantification methods used in the determination of the serum IGF-I and IGFBP-3 concentrations. In particular, the association between age and IGF-I and the correlation between IGF-I and IGFBP-3 are of importance, as these relationships are well established in the literature.

### 5.2.2 Melatonin and insulin-like growth factors

Spearman's rank correlations, as well as unadjusted and adjusted mixed models were used to assess melatonin as a determinant of circulating levels of IGF peptides. Overall, no association was observed between melatonin and the members of the IGF family investigated. The difficulty of investigating the presence of an association between two variables with high inter-individual variability and the importance of considering and adjusting for possible confounders was highlighted by the analysis of IGFBP-3. While a positive parameter estimate was seen prior to adjustment for confounders ($\beta = 65.3$, $p = 0.35$), a negative beta was observed post-adjustment ($\beta = -35.8$, $p = 0.49$; adjusted for

age, BMI, laboratory test batch, season, menstrual cycle stage, and recent alcohol consumption).

Stratification of the partially adjusted mixed models (adjusted for age, BMI, and laboratory test batch) suggested that season may modify the relationship between melatonin and IGFBP-3 (summer: $\beta = -42.0$, $p = 0.68$ versus winter: $\beta = 108.2$, $p = 0.27$). However, when considered in the context of average IGFBP-3 levels (study median = 4315.2, range = 1476.8 – 7269.1; at first day shift), the parameter estimates' difference in magnitude and direction was not considered to be meaningful. Additional analyses were completed using quartiles of melatonin, with no statistically significant associations observed. Interestingly, there was some indication of a threshold effect between levels of urinary melatonin and serum IGFBP-3. Although not significant, average IGFBP-3 values appeared to peak with the second quartile of melatonin and plateau or minutely decrease with the highest concentrations of melatonin. An explanation for such patterning is unclear and may warrant further investigation in a larger, more appropriately powered study.

Similar to the results of this project, in a study by Kajdaniuk and colleagues, which compared plasma melatonin and IGF-I levels in premenopausal stage II breast cancer patients (n = 24) and healthy volunteers (n = 16), no correlation ($r = 0.24$ $p = 0.36$) was observed between melatonin and IGF-I among the control women (55,56). Intriguingly, a weakly negative correlation of borderline significance was noted among the breast cancer patients ($r = -0.39$ $p = 0.058$) at baseline; an effect which disappeared following treatment with chemotherapy ($r = -0.02$ $p = 0.93$) (55). The results of this study, however, must be interpreted with caution, as the analyses were carried out using extremely small sample sizes. Furthermore, while BMI, medication use, menstrual cycle

stage, and time of day were controlled for by virtue of the study design, the authors failed to assess age or other potentially important confounders, such as diet or alcohol consumption.

One must consider that a relationship between melatonin and IGFs may only be evident in cases of exposure to more extreme levels of melatonin or that different physiological effects may occur at lower versus higher concentrations of melatonin. The latter may be suggested by the patterning observed here with quartiles of melatonin and IGFBP-3 levels. As such, this project, as well as the study by Kajdaniuk et al. (55,56) mentioned above, may not have captured melatonin values with the degree of variation necessary to observe an association. While one may expect a study population consisting of rotating shift nurses to display more altered levels of circadian hormones, such as melatonin, than individuals who maintain a strictly diurnal lifestyle, it is possible that the shift schedule investigated here did not disrupt the daily rhythms in a sufficient manner. Indeed, results from the parent Workplace Safety and Insurance Board (WSIB) study indicated that the rotating shift schedule followed by the participants studied here (two day shifts, two nights, and five days off) did not greatly alter the circadian rhythm of melatonin, when comparing individuals completing a night shift versus those completing a day shift (172,189).

Several evaluations of the effect of exogenous melatonin on circulating concentrations of IGF-I have been published, with conflicting results. While afternoon injections of pharmacological melatonin (20 mg) did not influence levels of plasma IGF-I (baseline versus following two months of treatment) among male and female cancer patients (n = 14) (57), treatment with tamoxifen and high doses of oral melatonin (20 mg, evening) produced significant decreases in concentrations of serum IGF-I among

women (n = 14) with metastatic breast cancer ($p < 0.05$ at one month, $p < 0.01$ at two and three months, compared to before treatment) (58). Although the participants for the latter study had not responded or continued to worsen when previously treated with tamoxifen therapy alone, care must be taken when considering these results as a control group was not employed. Consequently, it becomes impossible to delineate the potential effects of the tamoxifen versus the melatonin treatments. More recently, a small trial of elderly female volunteers (n = 14) (59) and a double-blind placebo trial among postmenopausal breast cancer survivors (n = 95) (60) have been completed. While low doses of melatonin (2 mg, nighttime) among the elderly women resulted in marginal, but significant increases in levels of IGF-I (baseline versus following six months of treatment) (59), daily doses of melatonin (3 mg, nighttime) did not affect the plasma concentrations of IGF-I or IGFBP-3 of the postmenopausal breast cancer survivors (Wilcoxon rank-sum $p = 0.98$, placebo versus treatment, at four months) (60). While two of these trials report null results congruous with this project, two do not. With the exception of the study conducted by Schernhammer and colleagues (60), these investigations were completed with extremely small sample sizes and did not account for confounding by variables such as age. Importantly, given that the study populations were cancer patients, survivors, and/or elderly, care must be taken when attempting to generalize the findings of these melatonin trials to healthy, premenopausal women, such as those investigated in this project.

Overall the results of this second objective indicated that endogenous levels of melatonin do not predict circulating concentrations of IGF-I, IGFBP-3, and IGF-I/IGFBP-3, thus coinciding with reports from several other studies. However, one cannot conclude that a relationship between melatonin and the IGF family does not exist. The biologically

relevant time-frame for investigating the potential relationship between melatonin and IGF peptides is unclear and duration of exposure, as well as latency must be considered. With respect to this project, the median number of years of previous shift work was nine (range = zero – 38.0 years). It may be possible that among this study's participants, an inadequate number of individuals had a sufficient cumulative exposure to altered levels of endogenous melatonin to elicit an effect. Regarding latency, overnight levels of melatonin, as measured through urinary 6-sulfatoxymelatonin (aMT6s), were assessed as a predictor of morning IGF-I and IGFBP-3 serum concentrations. Should melatonin influence IGFs in a more acute, short-lived manner or should the effect take longer to manifest, the specimen collection protocol utilized may not have adequately captured IGF peptide levels during the biologically relevant period. Thought must also be given to the possibility that melatonin may interact with the IGF family via mechanisms that may leave levels of endocrine IGFs relatively unchanged. For instance, as suggested by experimental work in rat pancreatic islets (45), melatonin may have the ability to activate the insulin-like growth factor receptor I (IGF-IR) signaling pathway, for which the impact on circulating IGFs and the mechanism of influence in other tissues in humans are unknown.

## 5.3 Strengths, methodological issues, and limitations

### 5.3.1 Strengths

This project had several important strengths. With 85 participants, a majority of whom provided information during both day shift data collection periods, this study is markedly larger than previous studies of melatonin and IGF peptides. Consequently, this project was more powered to detect smaller effects between the hormones than other

work to date. Additionally, by using a study population of rotating shift nurses, an association between melatonin and IGFs, should one truly exist, may have been more easily detected. Shift workers, such as the nurses of this study, are regularly exposed to light at night (LAN) and may have more disrupted circadian rhythms and greater variations in melatonin levels than members of the general public. Importantly, this research is appropriate within the context of shift work, as a relationship between melatonin and IGF peptides could be an intermediary on the pathway between shift work and cancer. Another strength of this study involves the fact that it addressed the obvious gap in the literature by characterizing the relationship between melatonin and IGF-I in a healthy human population. Generally, previous work regarding melatonin and the IGF-axis has been completed among cancer patients or survivors. Research among healthy populations may provide relevant information for upstream aspects of a complex biological pathway. Lastly, this project was able to utilize data from the highly detailed WSIB study questionnaire and participant diaries, which allowed detailed covariate information to be obtained for each participant. This data was used to investigate various potential predictors of IGF-I, IGFBP-3, and IGF-I-IGFBP-3, as well as assess an extensive set of possible confounders during the evaluation of melatonin as a determinant of the IGF measures. A majority of the literature pertaining to melatonin and the IGF-axis among humans have disregarded or only partially controlled for confounding, which could have resulted in the false detection of an association or prevented a true relationship from being identified.

### 5.3.2 Methodological issues and limitations

### 5.3.3 Sample size

As previously mentioned, with 85 participants and two data collection periods, the sample size for this project was quite large, as compared to the existing literature on melatonin and IGFs. Nevertheless, it is important to consider that the project did have a restricted number of subjects, thus increasing the likelihood of type-II error, whereby the power with which a true effect may actually be observed is decreased. *Post hoc* minimum detectable effect (MDE) calculations (see Section 3.7.7) indicated that this project was sufficiently powered to detect unadjusted associations (β) of +/- 12.5, +/- 208.6, and +/- 0.015 between melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3, respectively. Notably, as the MDE calculations do not take into account the dependence of observations in repeated measures data, the computed values therefore overestimate the minimum alternative hypothesis that could be detected. The relationships observed between melatonin and the IGF peptides in this study, unadjusted and adjusted, fell well below the MDEs for which the project was powered, such that it cannot be confidently concluded that these relationships do not exist. Based on investigations conducted among women of the NHS and the ORDET cohort, however, where increased risk of breast cancer was associated with differences of approximately 50 ng/ml (127,129) and 900 ng/ml (129) for IGF-I and IGFBP-3, respectively, beta (β) values as small as those noted in this project may not be clinically meaningful.

### 5.3.4 Volunteer bias, response rates, and loss to follow-up

It is important to recognize that the study population consisted entirely of volunteers. Should those premenopausal women who chose to volunteer differ significantly from the underlying, eligible population of premenopausal shift workers,

selection bias due to selection “in” to the project may have occurred. Consequently, average values of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 may not be representative of all premenopausal rotating shift nurses at Kingston General Hospital (KGH). As the relationships of interest were biologic in nature, however, it does not seem likely that the internal validity of this study was jeopardized by the volunteer sample. It is possible that precision may have been influenced, as it is conceivable that variability in exposures may have been reduced due to self-selection into the study.

Given the different levels of study participation, namely failure to provide any specimens, provision at one data collection, and provision at both, selection bias due to selection “out” of this project may be a concern. Should those women who failed to supply at least one serum sample, or those who only supplied one sample, differ systematically from those who fully participated, the internal validity of the project could have been threatened. Unfortunately, the number of premenopausal rotating shift nurses eligible to participate in the WSIB project and the characteristics of this underlying population are unknown, such that response rates cannot be calculated and an assessment of the representativeness of the volunteer sample to the base population cannot be made. However, characteristics of study participants, as well as outcome measures were assessed, stratified by degree of study participation (See Appendix E, Table E.1 and Table E.2). Generally, individuals that participated in one or both data collection periods had similar baseline characteristics and levels of urinary melatonin. IGF-I levels were comparable between women who provided one versus two blood samples; although, values for IGFBP-3 and IGF-I/IGFBP-3 were slightly increased among women who gave specimens at both data collections. Overall, provision of serum

samples in one or both day shift collection periods did not seem to be related to both exposure and outcome variables and internal validity was likely maintained.

Those who completed the baseline questionnaire, but did not provide serum for either of the day shift periods differed slightly from those who provided at least one blood sample. These four women tended to be younger, had lower BMI values, had never been pregnant or smoked, and had worked fewer years of shift work. Two of the four nurses provided morning urine samples, for which the average level of urinary melatonin was elevated, as compared to those who provided one or both serum samples. Bias may be a concern regarding those individuals who failed to provide a single serum sample. However, as the number of subjects concerned was quite small (4.5% or four of 89 participants), the effect of this bias was expected to be small. Information was not available for those five women who were recruited into the WSIB study, but selected out prior to any data collection.

### 5.3.5 Measurement error and misclassification

### 5.3.6 IGF-I, IGFBP-3, and IGF-I/IGFBP-3

There is a lack of consensus regarding the appropriate method for collection and storage of samples to be quantified for IGFs (161,190). Nonetheless, levels of IGFs seem to be relatively stable when stored over long periods at -80 $^{o}$C and concentrations appear to withstand multiple freeze-thaw cycles (161,190). The samples in this project were collected and stored in 2008 and 2009 and were analyzed in the winter and spring of 2011. Multiple freeze-thaws may have occurred during the process of retrieving samples from storage; however, the exact number of instances and level of thaws per sample is unknown. Additionally, although nurses were instructed to fast for eight hours prior to providing morning serum samples, confirmation of fasting was not obtained.

Error due to multiple freeze-thaws, possible non-fasting, as well as any sources of human error regarding the collection and storage protocol for the blood specimens was likely to be random.

Serum levels of IGF-I and IGFBP-3 were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits, specifically, the R&D Quantikine® ELISAs for total IGF-I and intact IGFBP-3 (See Section 3.5.2). As previously mentioned, there are several complicating factors when attempting to quantify levels of IGF-I and IGFBP-3. One common difficulty of total IGF-I quantification methods is the interference of IGFBPs, such that steps must be taken to remove bound IGFBPs from the IGF-I peptide during the assay procedure (161-163). Regarding IGFBP-3, care must be taken when selecting an assay. While some kits claim to detect intact peptides only, others may quantify total IGFBP-3, which includes both intact IGFBP-3 and (some or all) fragments produced by IGFBP proteases (141,143-145). Indeed, reference ranges for "normal" distributions of IGF-I appear to differ between kits (161,191,192) and studies have indicated that the use of varying assays may partially explain the incongruous results pertaining to IGFBP-3 and cancer (143,145). The potential for kits to systematically over- or underestimate IGF levels would most likely result in non-differential misclassification since it is improbable that the accuracy of the ELISAs would vary systematically with the distribution of the potential predictors, including melatonin, assessed in this project. Several quality control measures were followed and a high degree of intra-plate precision was indicated (see Appendix C, Table C.2). Slightly elevated inter-plate variability was observed in this project (see Appendix C, Table C.3), which was likely due to random human error during the completion of the assay protocol. The high strength of statistical significance continually observed with the laboratory test

batch variables (both in the results of Objective 1, as well as Objective 2) further supports inter-plate variability as a source of error in this project.

Another limitation, levels of “bioactive” IGF-I were crudely estimated via the molar ratio of IGF-I to IGFBP-3. Recently, a kinase receptor activation assay (KIRA) was developed, which measures the ability of sample IGF-I to activate its receptor, IGF-IR, using cell cultures (193). While this assay is believed to be more effective at measuring active IGF-I than assays for total IGF-I or the determination of IGF-I/IGFBP-3 (191,193), use of this protocol was not feasible for this study.

Overall, the large degree of variation in the IGF outcomes of interest was a major limitation of this study and likely explained, in part, by measurement error. Therefore, small associations between the IGF peptides and various determinants would have been difficult to identify due to a reduction in precision and power. In an attempt to identify and control for sources of variation within the data, models of potential predictors of IGF-I, IGFBP-3, and the molar ratio were built. *A priori*, the decision was made to force age, BMI, and laboratory test batch. Predictors of IGF variation were then assessed during the testing of melatonin as a determinant of IGF-I, IGFBP-3, and IGF-I/IGFBP-3. Notably, information on possible determinants of IGFs such as genetics and diet was not available for this project.

### 5.3.7 Melatonin

Overnight urinary concentrations of aMT6s were measured using the Bühlmann ELISA kit, a validated and commonly used technique that produce results highly comparable to those obtained when quantifying via radioimmunoassay, considered the “gold standard” ($r = 0.97$; $p < 0.0001$) (See Section 3.4.2) (151). Similar to the

measurement of the IGF peptides, if deviation of participants from the study instructions regarding specimen collection or human error during the processing and long-term storage of the urinary samples or quantification of the aMT6s in said samples occurred, the measurement error would likely have been random. Notably, several quality control measures were followed and a high degree of precision was indicated (see Appendix C, Table C.1).

### 5.3.8 Other variables

Covariate information captured from the WSIB baseline questionnaire and study diaries may also be subject to measurement error. One must consider that underreporting of socially stigmatized behaviours, such as smoking or increased alcohol consumption, or over-reporting of desirable practices, such as increased levels of physical activity, may have occurred. Additionally, for questions pertaining to past behaviours, recall error may be a concern. Given that participants were unaware of their IGF peptide or melatonin levels, it is doubtful that responses varied systematically by the main exposure or outcomes of interest. Importantly, biasing associations towards the null may have caused true confounders to be observed as unrelated to the exposure and/or outcome of interest (194). Consequently, residual confounding may have occurred, which could have, theoretically, masked, increased, or decreased “true” effects.

Missing data are always problematic. Due to the small sample size of this project, the decision was made to impute median (continuous scale) or mode (categorical scale) values for missing data. The use of median and mode values for imputations may have decreased the natural variation of the variables in question. If this was the case,

relationships would have become more difficult to detect. Overall, errors in measurement due to imputation were expected to be random. To assess the impact of imputation on the final results, a sensitivity analysis was completed, where final models were examined with the imputations removed. Generally, parameter estimates did not differ greatly from those observed with imputed values included.

### 5.3.9 Generalizability

The generalizability of the results of this project is unclear. The representativeness of the study sample to the base population could not be assessed and it is possible that persons self-selecting into the study may have been systematically different from those who did not. Therefore, it becomes difficult to establish a clear, definable population to which some of these results apply. When characteristics of the participants were compared to a group of mostly premenopausal subjects from the Nurses' Health Study II (NHS II) (n = 459), the women of this study were slightly younger (median ages: 37.0 versus 45.0 years for NHS II), a greater percentage of women were nulliparous (44.7% versus 15.7% for NHS II), and fewer women were OC users (22.4% current users at first day shift versus 84.8% current or past use for NHS II) (67). However, the women from both studies had a similar median BMI (25.5 versus 24.0 $kg/m^2$ for NHS II) and a similar percentage had never smoked (62.4% versus 68.6 for NHS II) (67). Also, the use of a study population comprised of working individuals may limit the generalizabilty of this project's findings to the general premenopausal population or even females working shift schedules different than the one observed here. Individuals who are able to work will likely be healthier than those who are not (195) and individuals who better tolerate shift work, and certain levels of rotation, may differ physiologically from those who do not due to morningness or eveningness preferences

(196). Consequently, one should use caution when extending the average melatonin and IGF peptide results to a broader population of premenopausal women. The biological relationships between melatonin and the IGFs, however, are not expected to vary by the factors previously mentioned, and therefore are likely generalizable to a wider population.

## 5.4 Contribution of research and conclusions

Although shift work was recently classified as a “probable carcinogen” (6), and despite the fact that multiple hypotheses have been put forth regarding potential intermediates (10), many questions remain unanswered regarding the nature of the increased risk and the biological mechanisms involved. Importantly, biomarker studies conducted in the context of shift work, and shift work and breast cancer, aid in discerning components of a seemingly complex pathway and eventually inform methods of prevention or intervention.

This project aimed to identify predictors of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 and assess the relationship between melatonin and IGF peptides as intermediate markers in the hypothesized pathway between exposure to LAN and breast cancer (or other cancers). Possible determinants of IGF-I, IGFBP-3, and IGF-I/IGFBP-3 were investigated primarily with the intent of informing the evaluation of melatonin and IGFs. Age, season, and OC use were found to significantly predict serum levels of IGF-I, while a borderline association was observed between recent alcohol consumption and circulating concentrations of IGFBP-3. Age and OC use were determinants of IGF-I/IGFBP-3 levels.

Significantly, this research is the first to assess the possible association between melatonin and IGFs among healthy, premenopausal rotating shift nurses. While

numerous animal studies have reported evidence linking melatonin and IGFs, limited research has been completed among humans and conflicting results have been documented. The observations of this cross-sectional assessment concurred with several of the previously conducted human studies, indicating that melatonin may not be related to IGFs in human physiology. With its 85 participants, a majority of whom contributed to two data collection periods, this study is the largest completed to date, as well as the first to assess an extensive number of potential confounders. Nevertheless, while the findings of this study did not support melatonin and the IGF-axis as intermediates between shift work and breast cancer, the ability to detect small associations may have been limited by a lack of power, sources of non-differential misclassification, and residual or unmeasured confounding.

### 5.5 Future directions

Before definitive conclusions can be made regarding melatonin and the IGF family, there are several areas that future studies should address. Firstly, there is a need for studies with larger samples sizes, and consequently greater power, given that studies conducted to date have been relatively small. Adjustment for potential confounders, either by way of study design or during the analysis stage, must also be more commonly considered. Indeed, the importance of considering confounders when assessing melatonin as a determinant of IGFs was highlighted by the analysis for melatonin and IGFBP-3, where the direction of the parameter estimate changed upon adjustment.

Greater thought must be given to realistic biological windows, particularly latency and durations of exposure, during study design, as associations may be missed simply due to the timing of specimen collection or length of study follow-up. One must also recognize that while this study did not find a cross-sectional relationship between

melatonin and IGFs, this does not necessarily indicate that changes in levels of melatonin over time will not be associated with changes in concentrations of IGF peptides. Research focusing on such a relationship may provide additional insight. Moreover, associations among individuals completing other shift work schedules should be assessed; particularly those that may theoretically result in more extreme variations of melatonin levels, as greater disruption of circadian rhythms may be required in order to see an effect. Given the suggestion of a possible threshold effect between melatonin and IGFBP-3, further investigation into the possibility of non-linear relationships should be also considered.

While this project did assess the relationship between melatonin and two of the more commonly studied IGF peptides with respect to cancer, the possibility of associations between melatonin and other members of the IGF family may be of interest as well. Additionally, although inclusion of the molar ratio of IGF-I to IGFBP-3 did not appear to contribute any additional knowledge to the investigation, it is important to remember that this measure serves only as a crude surrogate for “bioactive” IGF-I. Where economically and logistically feasible, it may be prudent to more accurately assess “bioactive” IGF-I using the recently developed KIRA, as greater insight might be gained regarding the effect of melatonin on IGF-I activity. Finally, further exploration as to whether season acts as an independent predictor of IGF-I levels, or simply as a surrogate for other lifestyle factors, may be warranted.

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# Appendix A
# Biological Mechanisms

As previously mentioned, the biological framework on which this study is based involves the hypothesis that a reduction in levels of melatonin will result in elevated circulating concentrations of insulin-like growth factor-I (IGF-I), thus increasing the risk of breast cancer. Several mechanisms exist, which could possibly link melatonin and the insulin-like growth factor (IGF) peptide family. Based on experimental evidence, a short summary of a number of possibilities has been provided.

The most intuitive mechanism involves the GH-IGF axis, as growth hormone (GH) is an important governing factor in the production of IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) (31,32,90). Several of the animal studies previously mentioned found evidence to support GH as the possible mediator between light at night (LAN) and/or melatonin-induced changes in IGF-I levels, as trends seen for IGF-I were likewise observed for concentrations of GH (42,48,52). The potential involvement of gonadal hormones has also been suggested by the sexual dimorphism observed in Syrian hamsters (46-49), while a third possibility concerns mediation via thyroid hormones. Although thyroid involvement was not indicated in male hamsters, females receiving melatonin; thiourea, as a means of inducing hypothyroidism; or melatonin+thiourea injections, in order of impact, had significantly reduced serum IGF-I levels, compared to controls ($p < 0.01$ for all three) (46,47). Coupled with the finding that the female hamsters receiving melatonin injections also had statistically significant decreases in circulating thyroxine, a hormone produced by the thyroid gland, these results suggest a pathway that involves the thyroid (47).

In addition, preliminary evidence for a melatonin-IGF pathway via sirtuin 1 (Sirt1), a histone deacetylase, has been documented by Jung-Hynes et al. (147) using transgenic adenocarcinoma of the mouse prostate (TRAMP) mice. Observations of decreased circulating levels of IGF-I/IGFBP-3, as well as lowered IGF-I and elevated IGFBP-3 mRNA levels in the prostate coincided with melatonin-moderated suppression of Sirt1 mRNA in the prostrate (147). Melatonin has been observed to interact directly with the IGF family in isolated adult and juvenile rat pancreatic islets, with Picinato and colleagues demonstrating that the melatonin G-protein-coupled receptor, melatonin receptor 1 (MT1), was able to activate insulin-like growth factor receptor I (IGF-IR) via tyrosine phosphorylation, consequently initiating its signaling pathway (45). Finally, melatonin may influence IGFs indirectly through its proposed role in glucose homeostasis, as experimental research has shown melatonin to aid in regulating insulin release (146); a hormone, which in turn, can alter the levels of IGFs (87). However, studies explicitly exploring this pathway could not be found. In conclusion, a concrete pathway has yet to be proven and given the complexity and incomplete knowledge of the elements involved, it is possible that more than one intermediary pathway may be responsible.

# Appendix B

# WSIB Study Material

## Recruitment poster

### How does ambient light and physical activity affect cancer risk factors in nurses?

*The International Agency for Research on Cancer has recently classified long-term night shift work as a probable carcinogen. Disruption of circadian rhythms due to light exposure at night has been suggested to explain this effect through its impact on the hormone melatonin.*

### Researchers at the Queen's Cancer Research Institute are looking for female nurses:

- who have worked DDNN rotational shifts for the last year
- not currently pregnant
- not pregnant or lactating within the past 6 month
- not taking melatonin supplements

**Participants will be offered a $200 honourarium**

If you are interested in learning more about this project, please contact:
**Deborah Emerton Tel.: 613-548-2389 or ext. 2389**
**E-mail: emertond@kgh.kari.net**

Queen's UNIVERSITY

WSIB ONTARIO CSPAAT

## Consent form

**STUDY OF LIGHT AT NIGHT, PHYSICAL ACTIVITY AND MELATONIN IN NURSES**
***Consent form***

**Study No:**_____

By signing below, I am indicating that I have read and understand the information for the study on physical activity, light and melatonin levels. I have been assured that records relating to my study participation will be kept confidential and that no information will be released or printed that would disclose my personal identity without my permission. I understand that my participation is voluntary, will not affect my work status, and I am free to withdraw from the study at any time without any penalty.
If you are a First Nations person or an indigenous person who has contact with spiritual elders, you may want to talk with them before you proceed with being part of this study. Elders may have reservations about genetic procedures.

❑ I agree to participate and do the following:

- Provide questionnaire responses
- Wear a light data-logger
- Provide sixteen saliva samples
- Provide diary responses
- Wear a pedometer
- Provide eight urine samples

❑ Pre-menopausal subjects only: I agree to provide one 30mL and one 15 mL blood sample.

❑ I agree to provide access to my mammograms to be assessed for breast density

If yes: Date and location of most recent mammogram: _____

❑ I agree that this information can be used for research purposes in future studies

❑ I would like a copy of study results sent to me at study completion

❑ I do NOT consent to participate

| _____ | _____ | _____ |
|---|---|---|
| Printed Name of Participant | Signature of Participant | Date |
| _____ | _____ | _____ |
| Printed Name of Witness | Signature of Witness | Date |

**Contact Name:** If you requested study information to be sent to you (see above), please give us the name, address, and phone number of a relative or close friend who will know your whereabouts so we can get your new address to send you the results of this study if you move.

**Name:**_____ **Phone #:**_____

**Address:**_____

**Please complete and sign two copies of this consent form and <u>return</u> one copy to us during your first scheduled meeting with the study team. <u>Retain</u> the duplicate copy of the consent form and the study guide for your information.**

**Principal Investigator**
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If you have any questions concerning your rights as a study participant, please contact:
Dr. Albert Clark, Chair of Ethics Board at Queen's University
Telephone: (613) 533-6081 Fax: (613) 533-6806 E-mail: clarkaf@queensu.ca

**Questionnaire**

Study ID | | | | | |

Queen's UNIVERSITY

# STUDY OF LIGHT AT NIGHT, PHYSICAL ACTIVITY AND MELATONIN IN NURSES

## QUESTIONNAIRE

This questionnaire is part of our research study to understand the relationship between a woman's environment, behavioural patterns and melatonin production. The specific objectives are to investigate the association between melatonin levels produced by the body and exposure to certain environmental factors, including light exposure and physical activity.

The following questions should be completed on _____, the first day of study participation.

The answers that you share with us will be kept strictly confidential and identified by a study ID number, known only by selected members of our research team. Your honesty is important for the success of this research, and a partial answer is better than no answer at all.

*We appreciate your cooperation.*

*Thank you!*

**Study ID** | | | | | |

**Please answer each question as completely as possible. If you are unsure of an exact answer, give your best estimate.**

Today's Date: _____
*Month / day / year*

1) What is your date of birth? _____
*Month / day / year*

2) What was your weight at age 30? _____ (kg) **or** _____ (lbs)

3) At age 14 what was your height? _____ (feet and inches) **or** _____ (cm)

4) Relative to your peers at age 14 (grade 8 or 9), were you:
❑ Taller ❑ Shorter ❑ Average ❑ Don't remember

5) How would you best describe you and your grandparents' race, ethnicity or colour? Please specify as many as applicable:

| Race, ethnicity or colour | Yourself | Maternal Grandmother | Maternal Grandfather | Paternal Grandmother | Paternal Grandfather |
|---|---|---|---|---|---|
| **White** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Black** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Native/Aboriginal peoples of North America** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Filipino** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Chinese** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Japanese** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Korean** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **South Asian** *(e.g. East Indian, Pakistani, Punjabi, Sri Lankan)* | ❑ | ❑ | ❑ | ❑ | ❑ |
| **South East Asian** *(e.g. Cambodian, Indonesian, Laotian, Vietnamese)* | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Arab/West Asian** *(e.g. Armenian, Egyptian, Iranian, Lebanese, Moroccan)* | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Latin America** | ❑ | ❑ | ❑ | ❑ | ❑ |
| **Other** *(Specify)* | | | | | |

**Study ID** | | | | | |

6) What is the highest degree or diploma you have obtained?
   - ❑ High school
   - ❑ Postsecondary (certificate/diploma)
   - ❑ University undergraduate degree
   - ❑ Graduate degree (Master's, PhD)
   - ❑ Other _____

**HEALTH BACKGROUND**

7) How old were you when you had your first menstrual period?

_____ years of age ❑ Have never menstruated (*go to question #11)*

8) Are you still menstruating?

❑ No → How many years has it been since your last menstrual period? _____ years

❑ Yes (*go to question #8*)

9) How did your menstrual periods stop?

❑ Naturally (through onset of menopause) ❑ As a result of radiation or chemotherapy

❑ As a result of a hysterectomy ❑ Other – please specify: _____

8) Have you ever been pregnant?

❑ No (*go to question #10)* ❑ Yes → Please indicate the total number of times you have been pregnant (please include any live births, miscarriages or abortions).

_____

9) Do you have any biological children?

❑ No (*go to question #10*) ❑ Yes → Please indicate how old you were when you gave birth to your first child._____

10) Have you ever had a mammogram (i.e. a breast x-ray?)

❑ Yes ❑ No (*go to question #13*)

↓

How old were you the first time you went for a mammogram?_____ Years

What was the reason? _____

How many times have you had a mammogram since the first time? _____

When was the last time? (ie. 6 months ago? 5 or more years ago?) _____

11) Have you ever been told that you have high breast (mammographic) density?

❑ Yes ❑ No (*go to question #13*)

**HEALTH BACKGROUND (Cont'd)**

12) These questions are about breast lumps or cysts that you may have had.

| | |
|---|---|
| Have you ever had a lump or cyst in your breast? | No ❑ (*go to question #14*)<br>Yes ❑ |
| How old were you when the first lump/cyst appeared? | Age *(years)* _____ |
| Did you have any of the lumps/cysts examined by a doctor? | No ❑<br>Yes ❑ |
| Did you have a biopsy or fine needle aspiration for any of the lumps/cysts? | No ❑<br>Yes ❑<br>If yes, how many? _____ |
| Did a doctor diagnose any of the lumps/cysts as *atypical hyperplasia?* | No ❑<br>Yes ❑ |
| Did a doctor diagnose any of the lumps/cysts as *carcinoma in situ?* | No ❑<br>Yes ❑ |
| Did a doctor diagnose any of the lumps/cysts as breast cancer? | No ❑<br>Yes ❑ |

13) These questions are about your sleep patterns.

| | |
|---|---|
| Do you experience problems sleeping? | No ❑<br>Yes ❑<br>If yes, how often/when? _____ |
| Have you ever been diagnosed with a sleep disorder? | No ❑<br>Yes ❑<br>If yes: Name: _____<br>Date of diagnosis: _____ |

Study ID | | | | | | |

**FAMILY**

14) Have any of the following relatives been diagnosed with cancer? *(A full sibling is one who has both the same mother and father as you)*

| Relative | Have they ever been diagnosed with cancer? | Type(s) of cancer |
|---|---|---|
| **Mother** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Father** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Full Sister 1** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Full Sister 2** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Full Brother 1** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Full Brother 2** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Son 1** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Son 2** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Daughter 1** | ❑ Yes ❑ No<br>❑ Don't know | |
| **Daughter 2** | ❑ Yes ❑ No<br>❑ Don't know | |

*If more space is required please use an additional page.*

**Study ID** | | | | | |

**LIFESTYLE HABITS**

15) Have you ever smoked more than 100 cigarettes in your lifetime?

❑ Yes ❑ No (*go to question #18*)

16) How old were you when you STARTED smoking? _____ years of age

17) Are you currently smoking?

❑ Yes ❑ No – If no, at what age did you quit? _____ years of age

18) How many years in total have you smoked cigarettes? (excluding the years that you quit)

_____ years

19) For the entire time you smoked, on average, how many cigarettes a day did you usually smoke?

_____ cigarettes/day OR _____ cigarettes/week

20) This question asks about your family's smoking habits when you were 19 or younger.

| **FAMILY'S SMOKING HABITS** | **FATHER / GUARDIAN** | **MOTHER / GUARDIAN** | **OTHER MEMBER** |
|---|---|---|---|
| Did your parent(s) or other household member(s) ever smoke in your presence when you were 19 or younger? (*go to question #22 if "no" for all*) | ❑ Yes<br>❑ No | ❑ Yes<br>❑ No | ❑ Yes<br>❑ No |
| For the entire time that s/he smoked, on average, how many cigarettes a day did s/he usually smoke? **(cigarettes/day)** | _____ cigarettes/day | _____ cigarettes/day | _____ cigarettes/day |
| What age were you when first exposed to your father's &/or mother's tobacco smoke? **(years)** | _____ years old | _____ years old | _____ years old |
| What age were you when no longer exposed to your father's &/or mother's tobacco smoke? **(years)** | _____ years old | _____ years old | _____ years old |

Study ID | | | | | |

**LIFESTYLE HABITS (Cont'd)**

21) During this time period (19 or younger), on average, how many hours per week were you exposed to someone else's tobacco smoke?

| | Hours per week exposed to "second-hand" tobacco smoke | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **<1** | **1-2** | **3-4** | **5-6** | **7-9** | **>9** |
| **Age: 19 years and younger** | | | | | | | |

22) In the past (during different decades of your life), on average, how many hours per week, outside of the workplace, were you exposed to someone else's tobacco smoke?

| | Hours per week exposed to "second-hand" tobacco smoke | | | | | | |
|---|---|---|---|---|---|---|---|
| **Age (decades)** | **0** | **<1** | **1-2** | **3-4** | **5-6** | **7-9** | **>9** |
| **20-29 years** | | | | | | | |
| **30-39 years** | | | | | | | |
| **40-49 years** | | | | | | | |
| **50-59 years** | | | | | | | |
| **2 years ago (if >60)** | | | | | | | |

23) This question asks about your alcohol consumption habits.

| | | **Beer** | **Wine** | **Spirits** |
|---|---|---|---|---|
| Have you ever drank the following more than twice a year? *(if "no" to all 3, i.e. beer, wine **and** spirits, go to question #41)* | Yes<br>No | ❑<br>❑ | ❑<br>❑ | ❑<br>❑ |
| On average, how many drinks per week did you consume during different decades of your life? ***One drink=1 glass of wine or 1 bottle of beer or 1 oz of spirits*** | | | | |
| **Teens (15-19)?** | # Drinks/week | ___per wk | ___per wk | ___per wk |
| **20-29 years** | # Drinks/week | ___per wk | ___per wk | ___per wk |
| **30-39 years** | # Drinks/week | ___per wk | ___per wk | ___per wk |
| **40-49 years** | # Drinks/week | ___per wk | ___per wk | ___per wk |
| **50-59 years** | # Drinks/week | ___per wk | ___per wk | ___per wk |
| **2 years ago (if over 60 years of age)** | # Drinks/week | ___per wk | ___per wk | ___per wk |

Study ID | | | | | |

**EMPLOYMENT HISTORY (Cont'd)**

24) Please tell us about EACH job or occupation you had for **at least** 6 months, including volunteer and military service, but not including schooling or homemaker. Include only seasonal or part-time work that is equivalent to 6 months or more. Begin with your most recent job and continue back to your first job. Include any absences from the work force and jobs you have done outside of Canada.

| Job No. | Time Period | | Type of Industry, Business or Service | Company Name and Location | Job Title | Intensity* (Please check one for each job) |
|---|---|---|---|---|---|---|
| | Start (Month-Year) | End (Month-Year) | | | | |
| *Eg.* | *Nov-1993* | *Feb-2003* | *Hairdressing* | *Suki's Hair Salon, Vancouver, BC* | *Colour Specialist* | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |
| **1** | | | | | | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |
| **2** | | | | | | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |
| **3** | | | | | | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |
| **4** | | | | | | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |
| **5** | | | | | | ❑ Sedentary ❑ Moderate ❑ Light ❑ Heavy |

Intensity of job or occupation defined as:
**Sedentary** = work that involves sitting only, with minimal walking,
**Light** = work that involves minimal physical effort such as standing and slow walking with no increase in heart rate and no perspiration,
**Moderate** = work that is not exhausting, that increases the heart rate slightly and may cause some light perspiration, such as those that require carrying light loads (5-10 lbs) or that have continuous walking,
**Heavy** = work that is vigorous, increases the heart rate substantially and causes heavy sweating such as those that involve lifting, carrying heavy loads (>10 lbs), brisk walking, or climbing.

Study ID | | | | | |

**EMPLOYMENT HISTORY (Cont'd)**

25) Please tell us about the corresponding shift work for each job you listed.

| Job No. | Average number of hours worked per week | Percentage of time worked at each shift | | Usual hours worked at each shift | | | |
|---|---|---|---|---|---|---|---|
| | | Day Shifts | Night Shifts | Day Shifts | | Evening Shifts | |
| | | | | Start | End | Start | End |
| Eg. | 35 | 80% | 20% | 10:00 am | 5:30 pm | 5:30 pm | 9:00 pm |
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |
| 5 | | | | | | | |

*Note: If more than 5 jobs, please use an additional page.*

***Thank you very much for completing this questionnaire***

.

**One-day diary**

Queen's UNIVERSITY

Study ID | | | | | |

# STUDY OF LIGHT AT NIGHT, PHYSICAL ACTIVITY AND MELATONIN IN NURSES

## ONE-DAY DIARY

This one-day diary is part of our research study to understand the relationship between a woman's environment, behavioural patterns and melatonin production. This one-day diary should be completed over a twenty-four hour period, beginning _____ and ending _____. **All questions included in this one-day diary pertain to activities completed and conditions experienced within this twenty-four hour period only.**

The answers that you share with us will be kept strictly confidential and identified by a study ID number, known only by selected members of our research team. Please note that although there are questions in this one-day diary that bear some similarity to those found in the questionnaire completed on the first day of study participation, it is imperative that you answer all questions. Your honesty is important for the success of this research, and a partial answer is better than no answer at all.

*We appreciate your cooperation.*

*Thank you!*

**Study ID** | | | | | |

## SAMPLE TIMES AND PEDOMETER VALUES

Please record the exact time and date of collection for all urine and saliva samples. Please also record the value on your pedometer at the time of each saliva sample collection.

Saliva Samples:

Time and date of saliva sample #1 collection: _____

Time and date of saliva sample #2 collection: _____

Time and date of saliva sample #3 collection: _____

Time and date of saliva sample #4 collection: _____

Urine Samples:

Time and date of urine sample #1 collection: _____

Time and date of urine sample #2 collection: _____

Pedometer Values:

Value at time of saliva sample #1 collection: _____

Value at time of saliva sample #2 collection: _____

Value at time of saliva sample #3 collection: _____

Value at time of saliva sample #4 collection: _____

**Study ID** | | | | | |

**Today's Date**: _____ / _____ / _____ Shift worked during specified 24-hr period (day/night): _____
Day Month Year Shift worked yesterday (day/night/off): _____

## HEALTH BACKGROUND

1) Are you still menstruating?

❑ Yes → what was the date of the first day of your last menstrual period? _____
*month/day/year*

❑ No

2) Have you taken aspirin, ibuprofen, other nonsteroidal anti-inflammatory (NSAIDs) pain medication or Tylenol/other acetaminophen pain medication in the 24-hour period specified on the front cover of this one-day diary??

❑ No (*go to question #3*) ❑ Yes → Please provide details. If you do not remember the brand name, fill in the type, dose and number of tablets taken.

| ***Brand Name*** | ***Dose (milligrams)*** | ***Number of Tablets*** |
|---|---|---|
| *Example: Tylenol* | *200* | *1* |
| | | |
| | | |

3) Have you used sedatives or muscle relaxants in the 24-hour period specified on the front cover of this one-day diary??

❑ No (*go to question #4*) ❑ Yes → Please provide details. If you do not remember the brand name, fill in the type, dose and number of tablets taken.

| ***Brand Name*** | ***Dose (milligrams)*** | ***Number of Tablets*** |
|---|---|---|
| *Example: Methocarbamol (Robaxin)* | *200* | *1* |
| | | |
| | | |

**Study ID** | | | | | |

**HEALTH BACKGROUND (cont'd)**

4) During the 24-hour period specified on the on the front cover of this one-day diary, did you suffer from a migraine?

❑ No (*go to question #5*) ❑ Yes → Please provide details of the medication taken (including brand name, dose and number of tablets)

| ***Brand Name*** | ***Dose (milligrams)*** | ***Number of Tablets*** |
|---|---|---|
| | | |
| | | |

5) Have you taken prescribed birth control medication in the 24-hour period specified on the front cover of this diary? *(eg. Norplant, Norinyl, Demulen, Depo-Provera, Tri-Cyclen, Alesse, etc.)*

❑ No (*go to question #6*) ❑ Yes → Please provide details of the medication taken (including brand name, dose and number of tablets)

| ***Brand Name*** | ***Dose (milligrams)*** | ***Number of Tablets*** |
|---|---|---|
| | | |
| | | |

**LIFESTYLE HABITS - Smoking**

6) Did you smoke during the 24-hour period specified on the cover of this one-day diary?

❑ No (*go to question #7*) ❑ Yes → Please estimate the number of cigarettes you smoked today. _____

7) On average, how many hours, during the 24-hour period specified on the cover of this one-day diary, were you exposed to *someone else's tobacco smoke*?

| **The number of hours exposed to "second-hand" tobacco smoke** | | | | | | |
|---|---|---|---|---|---|---|
| **0** | **<1** | **1-2** | **3-4** | **5-6** | **7-9** | **>9** |
| ❑ | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |

**Study ID** | | | | | |

**LIFESTYLE HABITS – Alcohol and caffeinated products consumption**

8) Did you drink any of the following in the 24-hour period specified on the front cover of this one-day diary?

| | *Beer* | *Wine* | *Spirits* | *Coffee* | *Tea* | *Other caffeinated beverages* |
|---|---|---|---|---|---|---|
| Yes | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| No | ❑ | ❑ | ❑ | ❑ | ❑ | ❑ |
| On average, how many drinks did you consume? ***One drink=1 glass of wine or 1 bottle of beer or 1 oz of spirits. For caffeinated beverages, specify size (e.g. small/single shot) and type (e.g. espresso).*** *(If "no" to all, i.e. beer, wine, spirits, coffee, tea, other caffeinated beverages, go to question #9)* | | | | | | |
| Number of Drinks | _____ | _____ | _____ | _____ *Specify average size and type* _____ | _____ *Specify average size and type* _____ | _____ *Specify average size and type* _____ |

**LIGHTING CONDITIONS**

9) Please answer the following questions:

| | |
|---|---|
| What time did you wake-up at the start of the 24-hour period specified on the front cover of this one-day diary? | |
| What time did you go to sleep? | |
| What time did you wake-up at the end of the 24-hour period specified on the front cover of this one-day diary? | |
| If sleep was interrupted, were lights turned on? *(Please choose N/A if sleep was not interrupted.)* | ❑ NO ❑ YES ❑ N/A |
| If the lights were on, were they on for more than 1 hour? *(Please choose N/A if sleep was not interrupted.)* | ❑ NO ❑ YES ❑ N/A |

10) When you sleep at night do you usually wear a sleep mask?
❑ Yes ❑ No

11) When you sleep during the day do you usually wear a sleep mask?
❑ Yes ❑ No

**Study ID** | | | | | |

**PHYSICAL ACTIVITY (Past 24 hours)**

12) In the table below, please record which physical activities that you have participated in during the **24-hour period** specified on the front cover of this one-day diary. It is important to indicate the duration you performed each of these activities in the morning-afternoon (7:00 AM - 3:00 PM), afternoon-evening (3:00 PM – 11:00 AM), or evening-morning (11:00 PM – 7:00 AM). It is also important to indicate whether the intensity of the physical activity was light, moderate, and heavy. These activities include both *sports/exercise* and active forms of *transportation* (e.g., walking to work).

The 3 intensity categories can be defined as follows:

**Light**: Require minimal physical effort such as slow walking

**Moderate:** Activities that are not exhausting, but that increase heart rate and breathing rate slightly and may cause some light sweating

**Heavy:** Activities that substantially increase heart rate and breathing and cause heavy sweating.

| **Type of Activity** | **Duration Performed (in minutes) at Different Times of the Day** | | | **Typical Intensity of Activity (Please check only one for each activity)** | | |
|---|---|---|---|---|---|---|
| | Morning-Afternoon (07:00-15:00) | Afternoon-Evening (15:00-23:00) | Evening-Morning (23:00-07:00) | Low | Moderate | High |
| Walking (at least 10 minutes) | | | | ❑ | ❑ | ❑ |
| Bicycling (stationary or outdoor) | | | | ❑ | ❑ | ❑ |
| Elliptical Trainer | | | | ❑ | ❑ | ❑ |
| Jogging or running (outdoors or treadmill) | | | | ❑ | ❑ | ❑ |
| Aerobics class | | | | ❑ | ❑ | ❑ |
| Yoga | | | | ❑ | ❑ | ❑ |
| Strength training (including lifting weights) | | | | ❑ | ❑ | ❑ |
| Rowing | | | | ❑ | ❑ | ❑ |
| Tennis | | | | ❑ | ❑ | ❑ |
| Squash/racquetball | | | | ❑ | ❑ | ❑ |
| Calisthenics (sit-ups, push-ups, etc.) | | | | ❑ | ❑ | ❑ |

**Study ID** | | | | | |

**PHYSICAL ACTIVITY (Cont'd)**

12) (cont'd)

| Type of Activity | Duration Performed (in minutes) at Different Times of the Day | | | Typical Intensity of Activity (Please check only one for each activity) | | |
|---|---|---|---|---|---|---|
| | Morning-Afternoon (07:00-15:00) | Afternoon-Evening (15:00-23:00) | Evening-Morning (23:00-07:00) | Low | Moderate | High |
| Hiking | | | | ❑ | ❑ | ❑ |
| Swimming | | | | ❑ | ❑ | ❑ |
| Golfing | | | | ❑ | ❑ | ❑ |
| Dancing | | | | ❑ | ❑ | ❑ |
| **OTHER (please list below)** | | | | ❑ | ❑ | ❑ |
| | | | | ❑ | ❑ | ❑ |
| | | | | ❑ | ❑ | ❑ |
| | | | | ❑ | ❑ | ❑ |

*In the above box be sure to indicate up to 3 additional activities that were not listed but which you performed in the specified 24 hour period. For these activities also list the duration and intensity of the physical activity session.*

13) The following are questions about your household activity levels.

a) In the **24 hour period** specified on the cover of this one-day diary, about how many hours did you watch television (including videos and DVDs)? *Please mark one box for each of the 3 times of day listed*

| Morning – Afternoon (07:00 – 15:00 hours) | Afternoon – Evening (15:00 – 23:00 hours) | Evening – Morning (23:00 – 07:00 hours) |
|---|---|---|
| ❑ None at all | ❑ None at all | ❑ None at all |
| ❑ About half an hour | ❑ About half an hour | ❑ About half an hour |
| ❑ About 1 hour | ❑ About 1 hour | ❑ About 1 hour |
| ❑ About 2 hours | ❑ About 2 hours | ❑ About 2 hours |
| ❑ 3 or more hours | ❑ 3 or more hours | ❑ 3 or more hours |

**PHYSICAL ACTIVITY (Cont'd)**

13) (cont'd)

b) In the **24 hour period** specified on the cover of this one-day diary, about how many hours did you use the computer (including Internet, email, chatting, etc.)? *Please mark one box for each of the 3 times of day listed*

| Morning – Afternoon (07:00 – 15:00 hours) | Afternoon – Evening (15:00 – 23:00 hours) | Evening – Morning (23:00 – 07:00 hours) |
|---|---|---|
| ❑ None at all | ❑ None at all | ❑ None at all |
| ❑ About half an hour | ❑ About half an hour | ❑ About half an hour |
| ❑ About 1 hour | ❑ About 1 hour | ❑ About 1 hour |
| ❑ About 2 hours | ❑ About 2 hours | ❑ About 2 hours |
| ❑ 3 or more hours | ❑ 3 or more hours | ❑ 3 or more hours |

c) In the **24 hour period** specified on the cover of this one-day diary, about how many hours did you sit quietly around the home doing things such as reading, knitting, playing board games, etc?. This does not include time spent watching television or on the computer. *Please mark one box for each of the 3 times of day listed*

| Morning – Afternoon (07:00 – 15:00 hours) | Afternoon – Evening (15:00 – 23:00 hours) | Evening – Morning (23:00 – 07:00 hours) |
|---|---|---|
| ❑ None at all | ❑ None at all | ❑ None at all |
| ❑ About half an hour | ❑ About half an hour | ❑ About half an hour |
| ❑ About 1 hour | ❑ About 1 hour | ❑ About 1 hour |
| ❑ About 2 hours | ❑ About 2 hours | ❑ About 2 hours |
| ❑ 3 or more hours | ❑ 3 or more hours | ❑ 3 or more hours |

d) In the **24 hour period** specified on the cover of this one-day diary, about how many hours did you perform light chores around the home such as cooking and cleaning? *Please mark one box for each of the 3 times of day listed*

| Morning – Afternoon (07:00 – 15:00 hours) | Afternoon – Evening (15:00 – 23:00 hours) | Evening – Morning (23:00 – 07:00 hours) |
|---|---|---|
| ❑ None at all | ❑ None at all | ❑ None at all |
| ❑ About half an hour | ❑ About half an hour | ❑ About half an hour |
| ❑ About 1 hour | ❑ About 1 hour | ❑ About 1 hour |
| ❑ About 2 hours | ❑ About 2 hours | ❑ About 2 hours |
| ❑ 3 or more hours | ❑ 3 or more hours | ❑ 3 or more hours |

**Study ID** | | | | | |

**PHYSICAL ACTIVITY (Cont'd)**

14) The following questions are about activities you performed while at work in the **24 hour period** specified on the front of this diary.

a) About how much time did you spend on your feet while at work?

- ❑ None at all
- ❑ About half an hour
- ❑ About 1 hour
- ❑ About 2 hours
- ❑ About 3 hours
- ❑ About 4 hours
- ❑ About 5 hours
- ❑ About 6 hours
- ❑ 7 or more hours

b) About how much time do you spend doing heavier activities such as lifting or bathing patients?

- ❑ None at all
- ❑ About half an hour
- ❑ About 1 hour
- ❑ About 2 hours
- ❑ About 3 hours
- ❑ About 4 hours
- ❑ About 5 hours
- ❑ About 6 hours
- ❑ 7 or more hours

**Study ID** | | | | | |

**PHYSICAL ACTIVITY (Past Month)**

15) Please indicate which of the following physical activities you have performed **in the last 30 days** (1 month). For each activity you performed, indicate the number of times you have participated in the past 30 days, the average length/duration of participation for a given session, and whether the typical intensity was light, moderate, and heavy. These activities include both *sports/exercise* and active forms of *transportation* (e.g., walking to work).

The 3 intensity categories can be defined as follows:

**Light**: Require minimal physical effort such as slow walking

**Moderate:** Activities that are not exhausting, but that increase heart rate and breathing rate slightly and may cause some light sweating

**Heavy:** Activities that substantially increase heart rate and breathing and cause heavy sweating.

| **Type of Activity** | **Number of times performed in past 30 days** | **Average duration of physical activity session** | **Typical Intensity of Activity (Please check only one for each activity)** | | |
|---|---|---|---|---|---|
| | | | **Low** | **Moderate** | **High** |
| Walking (at least 10 minutes) | | | ❑ | ❑ | ❑ |
| Bicycling (stationary or outdoor) | | | ❑ | ❑ | ❑ |
| Elliptical Trainer | | | ❑ | ❑ | ❑ |
| Jogging or running (outdoors or treadmill) | | | ❑ | ❑ | ❑ |
| Aerobics class | | | ❑ | ❑ | ❑ |
| Yoga | | | ❑ | ❑ | ❑ |
| Strength training (including lifting weights) | | | ❑ | ❑ | ❑ |
| Rowing | | | ❑ | ❑ | ❑ |
| Tennis | | | ❑ | ❑ | ❑ |
| Squash/racquetball | | | ❑ | ❑ | ❑ |
| Calisthenics (sit-ups, push-ups, etc.) | | | ❑ | ❑ | ❑ |
| Hiking | | | ❑ | ❑ | ❑ |
| Swimming | | | ❑ | ❑ | ❑ |
| Golfing | | | ❑ | ❑ | ❑ |

Study ID | | | | | |

**PHYSICAL ACTIVITY (Cont'd)**

15) (cont'd)

| Type of Activity | Number of times performed in past 30 days | Average duration of physical activity session | Typical Intensity of Activity (Please check one for each activity) | | |
|---|---|---|---|---|---|
| | | | Low | Moderate | High |
| Dancing | | | ❑ | ❑ | ❑ |
| Hiking | | | ❑ | ❑ | ❑ |
| **OTHER (please list below)** | | | ❑ | ❑ | ❑ |
| | | | ❑ | ❑ | ❑ |
| | | | ❑ | ❑ | ❑ |
| | | | ❑ | ❑ | ❑ |

*In the above box be sure to indicate up to 3 additional activities that were not listed but which you performed in the past month. For these activities also list the number of times you have participated, the average duration, and the typical intensity.*

16) Was the amount of physical activity you performed in the **past month** typical for you? Please check the correct response option.

- ❑ Yes
- ❑ No, I usually perform more physical activity
- ❑ No, I usually perform less physical activity

17) The following questions are about your household activity levels.

a) About how many hours a day in the **past 30 days** did you usually watch television (including videos and DVDs) in your free time? *(Please mark one box for days that you work and one box for days that you have off)*

Days that you work
- ❑ None at all
- ❑ About half an hour a day
- ❑ About 1 hour a day
- ❑ About 2 hours a day
- ❑ About 3 hours a day
- ❑ About 4 hours a day
- ❑ About 5 hours a day
- ❑ About 6 hours a day
- ❑ About 7 hours or more a day

Days that you have off
- ❑ None at all
- ❑ About half an hour a day
- ❑ About 1 hour a day
- ❑ About 2 hours a day
- ❑ About 3 hours a day
- ❑ About 4 hours a day
- ❑ About 5 hours a day
- ❑ About 6 hours a day
- ❑ About 7 hours or more a day

Study ID | | | | | |

**PHYSICAL ACTIVITY (Cont'd)**

17) (cont'd)

b) About how many hours a day in the past 30 days did you use the computer (including Internet, email, chatting, etc.)? (*Please mark one box for days that you work and one box for days that you do not work)*

| Days that you work | Days that you have off |
|---|---|
| ❑ None at all | ❑ None at all |
| ❑ About half an hour a day | ❑ About half an hour a day |
| ❑ About 1 hour a day | ❑ About 1 hour a day |
| ❑ About 2 hours a day | ❑ About 2 hours a day |
| ❑ About 3 hours a day | ❑ About 3 hours a day |
| ❑ About 4 hours a day | ❑ About 4 hours a day |
| ❑ About 5 hours a day | ❑ About 5 hours a day |
| ❑ About 6 hours a day | ❑ About 6 hours a day |
| ❑ About 7 hours or more a day | ❑ About 7 hours or more a day |

c) About how many hours a day in the **past 30 days** do you sit quietly around the home doing things such as reading, knitting, playing board games, etc. This does not include time spent watching television or on the computer? *(Please mark one box for days that you work and one box for days that you do not work)*

| Days that you work | Days that you have off |
|---|---|
| ❑ None at all | ❑ None at all |
| ❑ About half an hour a day | ❑ About half an hour a day |
| ❑ About 1 hour a day | ❑ About 1 hour a day |
| ❑ About 2 hours a day | ❑ About 2 hours a day |
| ❑ About 3 hours a day | ❑ About 3 hours a day |
| ❑ About 4 hours a day | ❑ About 4 hours a day |
| ❑ About 5 hours a day | ❑ About 5 hours a day |
| ❑ About 6 hours a day | ❑ About 6 hours a day |
| ❑ About 7 hours or more a day | ❑ About 7 hours or more a day |

d) About how many hours a day in the **past 30 days** do you perform light chores around the home such as cooking and cleaning? *(Please mark one box for days that you work and one box for days that you do not work)*

| Days that you work | Days that you have off |
|---|---|
| ❑ None at all | ❑ None at all |
| ❑ About half an hour a day | ❑ About half an hour a day |
| ❑ About 1 hour a day | ❑ About 1 hour a day |
| ❑ About 2 hours a day | ❑ About 2 hours a day |
| ❑ About 3 hours a day | ❑ About 3 hours a day |
| ❑ About 4 hours a day | ❑ About 4 hours a day |
| ❑ About 5 hours a day | ❑ About 5 hours a day |
| ❑ About 6 hours a day | ❑ About 6 hours a day |
| ❑ About 7 hours or more a day | ❑ About 7 hours or more a day |

Study ID | | | | | |

**PHYSICAL ACTIVITY (Cont'd)**

17) (cont'd)

e) About how many hours a day in the **past 30 days** do you perform moderate chores around the home such as cooking and cleaning? *(Please mark one box for days that you work and one box for days that you do not work)*

| Days that you work | Days that you have off |
|---|---|
| ❑ None at all | ❑ None at all |
| ❑ About half an hour a day | ❑ About half an hour a day |
| ❑ About 1 hour a day | ❑ About 1 hour a day |
| ❑ About 2 hours a day | ❑ About 2 hours a day |
| ❑ About 3 hours a day | ❑ About 3 hours a day |
| ❑ About 4 hours a day | ❑ About 4 hours a day |
| ❑ About 5 hours a day | ❑ About 5 hours a day |
| ❑ About 6 hours a day | ❑ About 6 hours a day |
| ❑ About 7 hours or more a day | ❑ About 7 hours or more a day |

f) About how many hours a day in the **past 30 days** do you perform heavy chores around the home such as gardening, shoveling snow, etc.? *(Please mark one box for days that you work and one box for days that you do not work)*

| Days that you work | Days that you have off |
|---|---|
| ❑ None at all | ❑ None at all |
| ❑ About half an hour a day | ❑ About half an hour a day |
| ❑ About 1 hour a day | ❑ About 1 hour a day |
| ❑ About 2 hours a day | ❑ About 2 hours a day |
| ❑ About 3 hours a day | ❑ About 3 hours a day |
| ❑ About 4 hours a day | ❑ About 4 hours a day |
| ❑ About 5 hours a day | ❑ About 5 hours a day |
| ❑ About 6 hours a day | ❑ About 6 hours a day |
| ❑ About 7 hours or more a day | ❑ About 7 hours or more a day |

**PHYSICAL ACTIVITY (Cont'd)**

18) The following questions are about activities you performed while at work in the **past 30 days**.

a) About how much time do you spend on your feet while at work?

- ❑ None at all
- ❑ About half an hour a day
- ❑ About 1 hour a day
- ❑ About 2 hours a day
- ❑ About 3 hours a day
- ❑ About 4 hours a day
- ❑ About 5 hours a day
- ❑ About 6 hours a day
- ❑ About 7 hours or more a day

b) About how much time do you spend doing heavier activities such as lifting or bathing patients?

- ❑ None at all
- ❑ About half an hour a day
- ❑ About 1 hour a day
- ❑ About 2 hours a day
- ❑ About 3 hours a day
- ❑ About 4 hours a day
- ❑ About 5 hours a day
- ❑ About 6 hours a day
- ❑ About 7 hours or more a day

**Study ID** | | | | | |

**CHANGES MADE TO LIGHTING CONDITIONS**

19) By participating in this study, have you changed the lighting conditions in your home and/or bedroom?

❑ No

❑ Yes → Please provide details in the space provided specifying what type of changes you have made.

20) By participating in this study, will you change the lighting conditions in your home and/or bedroom?

❑ No

❑ Yes → Please provide details in the space provided specifying what type of changes you will make.

Because we want to be able to use all the information you have provided, please take a moment to review each page, making sure that you did not skip any pages.

If you have any additional comments, please provide them in the space provided below.

***Thank you again for the information you have provided!***
***Your input is very valuable to us.***

# Appendix C
# Laboratory Procedures

## Introduction

This appendix contains summaries of the laboratory protocols followed during the quantification of urinary melatonin and creatinine, as well as serum insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3).

## Melatonin

### 6-sulfatoxymelatonin assessment

As previously mentioned, concentrations of urinary 6-sulfatoxymelatonin (aMT6s) were measured using the Bühlmann aMT6s enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, Salem, NH), a commonly used competitive immunoassay, which operates through the capture antibody approach (151). The protocol followed for each assay test plate is outlined below.

After washing the wells of the microtiter plate with a reconstituted wash buffer, six standard solutions of known concentration (A-F); two control solutions (high and low), also of known concentration; and the urinary samples, diluted with an incubation buffer solution, were pipeted, in duplicate, into the wells of the test plate (197). A solution of aMT6s conjugated to biotin was then added to each well, followed by an antiserum reagent; aMT6s present in the standards, controls, and samples competed with the biotinylated aMT6s to bind to the highly specific rabbit anti-aMT6s antibodies that comprise the antiserum solution (197). Subsequently, the biotinylated aMT6s – anti-aMT6s antibody complexes were able to bind to antibodies precoated on the microtiter

plate (197). Following a three-hour incubation at 2 – 8 $^{o}$C, the wells of the test plate were washed with a buffer solution, then treated with an enzyme label capable of binding to biotin (a streptavidin – horseradish peroxidase (HRP) conjugate) and incubated for 30 minutes at 2 – 8 $^{o}$C (197). Unbound enzyme label was rinsed from the microtiter plate using the wash buffer solution (197). Next, tetramethylbenzidin (TMB) substrate, which in the presence of HRP generates a coloured product, was added to each well and the plate was incubated at room temperature, protected from direct light, for 15 minutes (197). An acidic stop solution was then added to the wells of the test plate, to halt the colour-producing reaction, and within 30 minutes, the absorbance of each well measured via a spectrophotometer set to 450 nm (197). The level of absorbance of the coloured product present in each well was proportionate to the concentration of biotinylated aMT6s bound to the microtiter plate and thus, was inversely proportionate to the amount of aMT6s initially present in the diluted samples, standards, and controls (197). Using the absorbance readings for the standards of known concentrations, a standard curve was generated and the levels of aMT6s in the participants' urine samples were calculated (197).

**Creatinine assessment**

Values of aMT6s were standardized using urinary creatinine concentrations as a means of controlling inter-individual variability due to varying urine output volumes, a practice commonly employed (21,26,27,66,152-154). Urinary creatinine concentrations were measured via the Parameter$^{TM}$ Creatinine Assay (R&D Systems, Minneapolis, MN), which uses the Jaffé reaction in its method of quantification (155); the procedure followed is summarized below.

After preparing a set of standard solutions through a serial dilution of the stock standard solution supplied, the urinary samples, diluted with distilled water, as well as the standard solutions of known concentration (A-H) were pipeted, in duplicate, into the wells of the microtiter plate (155). Alkaline picrate solution, prepared from the sodium hydroxide and picric acid solutions provided, was then added to each well (155). Following a 30-minute incubation at room temperature, the absorbance of each well was measured via a spectrophotometer set to read at 490 nm (155). The optical density of each well was proportionate to the concentration of creatinine present (155). Using the standards of known concentration, a standard curve was generated and the creatinine concentration of each urine sample determined (155). Finally, the aMT6s results were adjusted using the urinary creatinine concentrations, giving units of ng aMT6s / mg creatinine for the surrogate melatonin variable.

**Quality control**

All urine samples quantified for aMT6s and creatinine were tested in duplicate and the mean concentration of the two wells used. Coefficients of variation (CVs) between the sample duplicates were calculated and the median values for both aMT6s and creatinine, as well as details regarding manufacturer-provided precision and accuracy estimates, may be found in Table C.1. If absorbance values for either sample duplicate fell outside the range of the standard curve, the sample was appropriately diluted and reanalyzed. Test plates where the standard curve generated an $R^2$ value less than 0.95 were also re-assayed. Finally, manufacturer-provided controls of known concentration were assayed on each microtiter plate as a means of assessing the accuracy of the plate's results.

**Table C.1** Precision and accuracy of the Bühlmann 6-Sulfatoxymelatonin ELISA and Parameter™ Creatinine Assay kits

| Manufacturer intra-assay CV (%) | | Manufacturer inter-assay CV (%) | | Manufacturer minimum detectable concentration | | Median experimental CV (%) | |
|---|---|---|---|---|---|---|---|
| | | | | (ng/ml) | (mg/dl) | | |
| **aMT6s** | **Creatinine** | **aMT6s** | **Creatinine** | **aMT6s** | **Creatinine** | **aMT6s** | **Creatinine** |
| 7.1 | 3.5 | 11.9 | 5.5 | 0.14 | 0.01 - 0.07 | 9.1 | 10.0 |

(155,197)

## Insulin-like growth factors

### Insulin-like growth factor-I assessment

Serum levels of IGF-I were quantified using the R&D Systems Quantikine® assay (R&D Systems, Minneapolis MN), a non-competitive "sandwich" ELISA (160), one of several commercially available assays commonly used. The protocol followed for each Quantikine® IGF-I assay test plate is outlined below.

As per the pretreatment protocol, serum samples were first treated with an acidic solution used to dissociate any bound insulin-like growth factor binding proteins (IGFBPs), followed by a neutralizing reagent containing an IGFBP-blocking agent (160,161). After adding assay diluent to each well of the microtiter plate, eight standard solutions of known concentration (A-H), prepared from a reconstituted stock solution; control solutions, also of known concentration; and the pretreated serum samples were pipeted, in duplicate, into the wells of the test plate (160). IGF-I present in the standards, controls, and samples were able to bind to an IGF-I-specific monoclonal antibody pre-coated on the test plate (160). Following a two-hour incubation at 2 – 8 $^{o}$C, the wells of the test plate were washed with a buffer solution to remove unbound factors, then treated with a cold reagent consisting of an IGF-I-specific polyclonal antibody conjugated

to HRP (160). During the subsequent one-hour incubation at 2 – 8 $^{o}$C, the polyclonal antibody-enzyme complexes were able to bind to the IGF-I peptides secured to the microtiter plate by the monoclonal antibodies (160). Unbound IGF-I conjugate was rinsed from the microplate using the wash buffer solution (160). Next, a prepared substrate solution, which produces a coloured product when exposed to HRP, was pipeted into each well and the microtiter plate was incubated at room temperature, protected from direct light, for 30 minutes (160). The wells of the test plate were then treated with an acidic stop reagent, preventing the colour-producing reaction from continuing, and within 30 minutes, the absorbance of each well measured using a spectrophotometer set to 450 nm (160). Plates were placed on an orbital shaker for several minutes prior to measuring colour intensity as a means of facilitating the thorough mixing of the stop and substrate solutions. Using the absorbance readings for the eight standards of known concentration, a standard curve was produced and the concentrations of IGF-I in the participants' serum samples were calculated (160). Specifically, the optical density of the coloured product present in each well was proportionate to the amount of IGF-I bound to the test plate (160). Absorbance readings were taken twice, approximately 10 minutes apart, and the concentration values averaged in an attempt to achieve greater accuracy, given that the complete mixing of the stop solution, characterized by a change in well colour from blue to yellow, often required several minutes.

**Insulin-like growth factor binding protein-3 assessment**

Serum concentrations of IGFBP-3 were determined via the R&D Systems Quantikine® IGFBP-3 ELISA kit (R&D Systems, Minneapolis MN), a non-competitive "sandwich" ELISA (164), similar to that used to quantify levels of IGF-I. The protocol followed for each Quantikine® IGFBP-3 microplate is summarized below.

After adding assay diluent to the wells of the test plate, eight standard solutions of known concentration (A-H), prepared from a reconstituted stock solution; control solutions, also of known concentration; and the serum samples, diluted with calibrator diluent, were pipeted, in duplicate, into the wells of the microplate (164). The test plate was then incubated for two hours at 2 – 8 $^{o}$C (164). IGFBP-3 peptides present in the standards, controls, and samples were able to bind to an IGFBP-3-specific monoclonal antibody pre-coated on the test plate (164). Next, the microtiter plate was washed with a buffer solution to remove unbound factors, then treated with a cold reagent containing an IGFBP-3-specific polyclonal antibody conjugated to HRP (164). The microplate was incubated for two hours at 2 – 8 $^{o}$C, during which time the polyclonal antibody-enzyme complexes were able to bind to the IGFBP-3 peptides immobilized to the test plate (164). Unbound IGFBP-3 conjugate was rinsed from the microplate using the wash buffer solution and a prepared substrate solution, which generates a coloured product in the presence of HRP, was added to each well (164). Following a 30-minute incubation at room temperature, with the test plate protected from direct light, an acidic stop reagent was pipeted into each well, preventing the colour-producing reaction from continuing (164). To promote the mixing of the stop and substrate solutions, the test plate was placed on an orbital shaker for several minutes. Within 30 minutes of adding the stop solution, the colour intensity of each well was determined using a spectrophotometer set to 450 nm, the absorbance of the wells being proportionate to the amount of IGFBP-3 (164). The optical density of the eight standards of known concentration was used to create a standard curve and the levels of IGBP-3 in the participants' serum samples were calculated (164). As with the IGF-I assays, absorbance readings were taken twice,

approximately 10 minutes apart, and the mean of the two concentrations used in an attempt to acquire the most accurate results.

**Quality control**

All serum samples analyzed for IGF-I and IGFBP-3 were assayed in duplicate and the mean absorbance of the two wells was used to determine each sample's concentration for that reading. As previously mentioned, absorbance readings were taken twice, approximately 10 minutes apart, and the concentrations, as well as the CVs between sample duplicates, averaged for each serum sample. Manufacturer-provided precision and accuracy estimates for both IGF-I and IGFBP-3 may be found in Table C.2.

Samples were diluted accordingly and reanalyzed if the spectrophotometer reading for either sample duplicate fell outside the range of the standard curve. In addition, for test plates where the standard curve generated an $R^2$ value less than 0.95, the most influential value was deleted and the curve regenerated. If the $R^2$ value remained less than 0.95, the microtiter plate was to be re-assayed; however, none of the plates met this final requirement for reanalysis. Manufacturer-provided controls of known concentration were also assayed on each test plate as a means of evaluating the accuracy of the results. Finally, two small, random samples, one for IGF-I and one for IGFBP-3, were selected to be re-assayed as an assessment of reproducibility. Median values of the experimentally determined intra- and inter-assay CVs for both IGF-I and IGFBP-3 may be found in Table C.3.

**Table C.2** Manufacturer-determined precision and accuracy of the Quantikine® IGF-I and IGFBP-3 ELISA kits

| Manufacturer intra-assay CV (%) | | Manufacturer inter-assay CV (%) | | Manufacturer minimum detectable concentration (ng/ml) | |
|---|---|---|---|---|---|
| **IGF-I** | **IGFBP-3** | **IGF-I** | **IGFBP-3** | **IGF-I** | **IGFBP-3** |
| 4.3 | 5.0 | 8.3 | 8.0 | 0.007 – 0.056 | 0.02 – 0.14 |

(160,164)

**Table C.3** Experimentally-determined precision of the Quantikine® IGF-I and IGFBP-3 ELISA kits

| Median experimental intra-assay CV (%) | | Median experimental inter-assay CV (%) | |
|---|---|---|---|
| **IGF-I** | **IGFBP-3** | **IGF-I*** | **IGFBP-3‡** |
| 4.3 | 4.3 | 17.6 | 15.8 |

* n = 34
‡ n = 21

# Appendix D

# Ethics

**QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD**

January 20, 2011

This Ethics Application was subject to:

☐ Full Board Review
Meeting Date:
☒ Expedited Review

Ms. Kirstin E. Boehme
Department of Community Health and Epidemiology
Division of Cancer Care and Epidemiology
Queen's Cancer Research Institute
10 Stuart Street
Queen's University

Dear Ms. Boehme,

**Study Title:** **Melatonin, insulin-like growth factor-1, and insulin-like growth factor binding protein-3 in premenopausal rotating shift nurses**
**Co-Investigators:** **Dr. Harriet Richardson**

I am writing to acknowledge receipt of your recent ethics submission. We have examined the protocol for your project (as stated above) and consider it to be ethically acceptable. This approval is valid for one year from the date of the Chair's signature below. This approval will be reported to the Research Ethics Board. Please attend carefully to the following list of ethics requirements you must fulfill over the course of your study:

- **Reporting of Amendments**: If there are any changes to your study (e.g. consent, protocol, study procedures, etc.), you must submit an amendment to the Research Ethics Board for approval. (see http://www.queensu.ca/vpr/reb.htm).

- **Reporting of Serious Adverse Events**: Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

- **Reporting of Complaints**: Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board.

- **Annual Renewal**: Prior to the expiration of your approval (which is one year from the date of the Chair's signature below), you will be reminded to submit your renewal form along with any new changes or amendments you wish to make to your study. If there have been no major changes to your protocol, your approval may be renewed for another year.

Yours sincerely,

Chair, Research Ethics Board

Jan 20, 2011
Date

ORIGINAL TO INVESTIGATOR - COPY TO DEPARTMENT HEAD- COPY TO HOSPITAL – BINDER COPY - FILE COPY

**Study Code: EPID-337-11**

- **Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete**

# Appendix E
# Additional Results

## Introduction

Additional analyses were completed, which were deemed supplemental to the main results presented in Chapter 4. Contained within the appendix are baseline characteristics and insulin-like growth factor (IGF) peptide values by level of subject participation, as well as by data collection period, graphical representations of the distributions of urinary melatonin and the IGF outcome measures, and mean IGF levels, stratified by covariates. Results from the Spearman's rank correlation between insulin-like growth factor-I (IGF-I) and insulin-like growth factor binding protein-3 (IGFBP-3), as well as a graph illustrating the relationship between melatonin and IGFBP-3 are presented. Lastly, investigations pertaining to IGF-I/IGFBP-3 and results from the sensitivity analysis may also be found here.

## Descriptive statistics by level of participation

**Table E.1** Baseline characteristics of study population, by level of participation

| Characteristic | Blood available for both day shifts n = 64 | Blood available for one day shift only n = 21 | Blood not available for either day shift n = 4 |
|---|---|---|---|
| Age (years) median (range); N | 38.5 (23.0 – 54.0); 64 | 32.0 (23.0 – 48.0); 21 | 24.5 (23.0 – 48.0); 4 |
| BMI (kg/m$^2$) (N, %) | | | |
| ≤ 22.7 | 18 (28.1) | 4 (19.1) | 2 (50.0) |
| > 22.7 – 25.5 | 16 (25.0) | 4 (19.1) | 1 (25.0) |
| > 25.5 – 29.4 | 16 (25.0) | 5 (23.8) | 1 (25.0) |
| > 29.4 | 13 (20.3) | 8 (38.1) | 0 (0) |
| *Missing* | 1 (1.6) | 0 (0) | 0 (0) |
| Ethnicity (N, %) | | | |
| White | 61 (95.3) | 20 (95.2) | 2 (50.0) |
| Non-white | 2 (3.1) | 0 (0) | 2 (50.0) |
| *Missing* | 1 (1.6) | 1 (4.8) | 0 (0) |
| Number of pregnancies (N, %) | | | |
| None | 27 (42.2) | 11 (52.4) | 4 (100.0) |
| 1 | 5 (7.8) | 2 (9.5) | 0 (0) |
| 2 | 17 (26.6) | 3 (14.3) | 0 (0) |
| ≥ 3 | 11 (17.2) | 4 (19.1) | 0 (0) |
| *Missing* | 4 (6.3) | 1 (4.8) | 0 (0) |
| Smoking status (N, %) | | | |
| Never | 40 (62.5) | 13 (61.9) | 4 (100.0) |
| Past | 14 (21.9) | 6 (28.6) | 0 (0) |
| Current | 10 (15.6) | 2 (9.5) | 0 (0) |
| Alcohol consumption (avg. # drinks/week)* median (range); N | 2.0 (0 – 12.5); 64 | 2.0 (0 – 8.0); 21 | 1.5 (0 – 6.0); 4 |
| History of night shift work (years) median (range); N | 10.0 (0 – 38.0); 63 | 6.1 (0 – 25.0); 21 | 1.8 (0.8 – 20.0); 4 |

* average value for current decade of life

**Table E.2** Biological samples, by level of participation

| Characteristic | Blood available from both day shifts | Blood available from one day shift only | Blood not available for either day shift |
|---|---|---|---|
| *Biological samples* | | | |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | | | |
| geometric mean (back-transformed range); N | 22.5 (0.8 – 1195.6); 119* | 31.3 (0.9 – 553.1); 19** | 66.6 (53.1 – 102.5); 4*** |
| IGF-I (ng/ml) | | | |
| median (range); N | 155.5 (65.3 – 344.1); 126‡ | 165.1 (56.0 – 337.1); 21‡‡ | -- |
| IGFBP-3 (ng/ml) | | | |
| median (range); N | 4062.8 (1476.8 – 7269.1); 124† | 3872.3 (2436.9 – 6148.4); 23†† | -- |
| IGF-I/IGFBP-3 | | | |
| median (range); N | 0.141 (0.052 – 0.670); 122Ψ | 0.119 (0.049 – 0.331); 23ΨΨ | -- |

* 119 observations; 60 individuals from day shift #1, 59 individuals from day shift #2
‡ 126 observations; 63 individuals (missing two observations, same individual)
† 124 observations; 62 individuals (missing two observations, different individuals)
Ψ 122 observations; 61 individuals (missing four observations, two from same individual and two from different individuals)
** 19 observations; 19 individuals (15 from day shift #1, 4 from day shift #2)
‡‡ 21 observations; 21 individuals (15 from day shift #1, 6 from day shift #2)
†† 23 observations; 23 individuals (15 from day shift #1, 8 from day shift #2)
ψψ 23 observations; 23 individuals (15 from day shift #1, 8 from day shift #2)
*** 4 observations; 2 individuals (missing four observations)

**Table E.3** Time-dependent characteristics of study population, by data collection period

| Characteristic | Day shift collection #1<br>n = 79* | Day shift collection #2<br>n = 70* |
|---|---|---|
| *Study diary measures* | | |
| Sleep duration (hours) | | |
| median (range); N | 7.0 (3.5 – 13); 77 | 7.1 (3.8 – 9.5); 67 |
| Menstrual cycle stage (N, %) | | |
| Follicular | 30 (38.0) | 16 (22.9) |
| Mid-cycle | 14 (17.7) | 9 (12.9) |
| Luteal | 23 (29.1) | 39 (55.7) |
| *Missing* | 12 (15.2) | 6 (8.6) |
| Current oral contraceptive use (N, %)‡ | | |
| No | 54 (68.4) | 53 (75.7) |
| Yes | 18 (22.8) | 12 (17.1) |
| *Missing* | 7(8.9) | 5 (7.1) |
| Recent alcohol consumption‡ (# drinks/day) | | |
| median (range); N | 0 (0 – 3); 73 | 0 (0 – 3); 66 |
| Recent caffeine consumption‡ (# drinks/day) | | |
| median (range); N | 2.0 (0 – 14); 68 | 3.0 (0 – 9); 62 |
| Total physical activity† (avg. METmin/day) | | |
| median (range); N | 1449.3 (657.1 – 3314.8); 73 | 1403.6 (421.7 – 2574.1); 66 |

* overlap of 64 individuals across day shift collection periods
‡ last 24 hours
† last 30 days

## Distributions of melatonin, IGF-I, IGFBP-3, and IGF-I/IGFBP-3

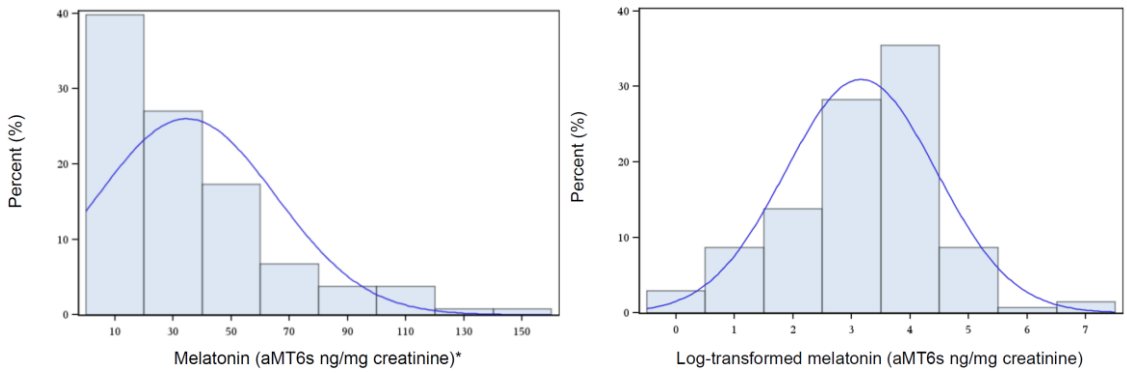


**Figure E.1** Distribution of melatonin: untransformed versus log-transformed

(* untransformed values greater than the 75$^{th}$ percentile plus three times the interquartile range removed for graphing purposes only)

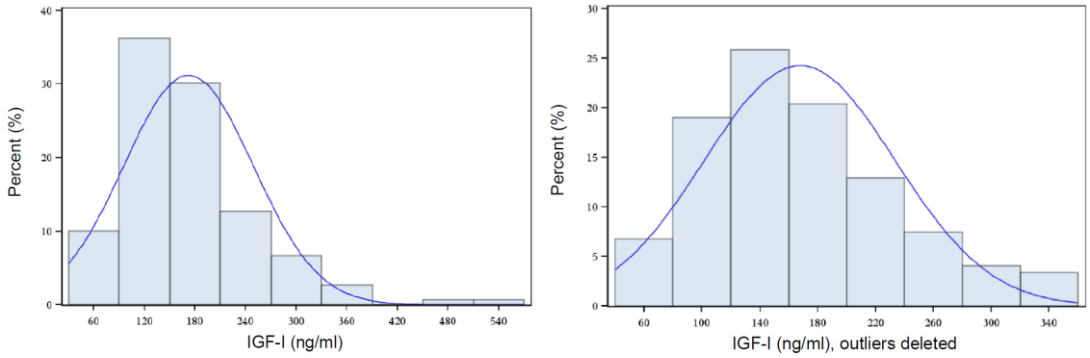


**Figure E.2** Distribution of IGF-I: with outliers versus outliers removed

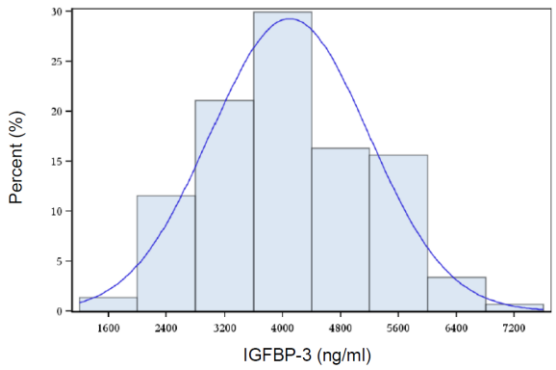


**Figure E.3** Distribution of IGFBP-3

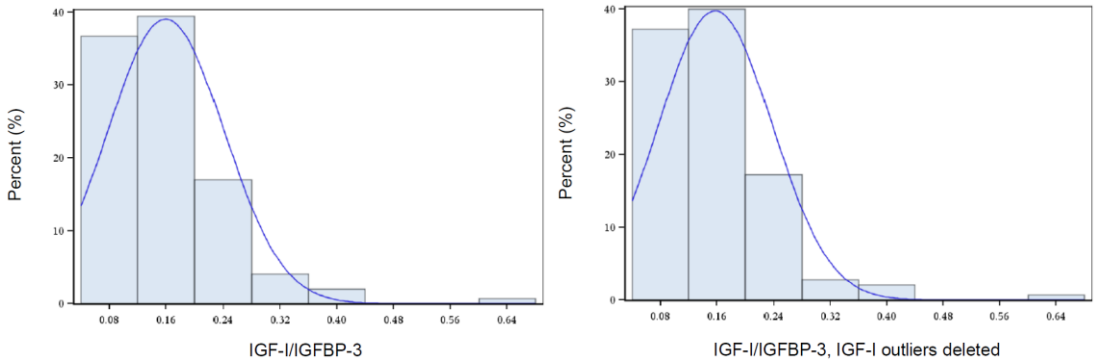


**Figure E.4** Distribution of IGF-I/IGFBP-3: with IGF-I outliers versus IGF-I outliers removed

## Mean concentrations of IGF-I, IGFBP-3, and IGF-I/IGFBP-3, stratified by covariates

Mean serum concentrations of IGF-I, IGFBP-3, and IGF-I/IGFBP-3, stratified by various potential predictors, were also studied in hopes of gaining a more clear understanding of the distribution of IGF in the study population (Table E.4). IGF-I and IGF-I/IGFBP-3 concentrations were found to decrease significantly with increasing age (p-trend = 0.0004 and p-trend= 0.03, respectively). In addition, average concentrations of IGF-I were also observed to decline with a greater history of night shift work (p-trend = 0.002) and increase with greater levels of regular physical activity (p-trend = 0.02). Mean serum IGFBP-3 levels appeared to significantly decrease as the participants' menstrual cycles progressed (p-trend = 0.003) and significantly increase with greater recent consumption of alcoholic beverages (p-trend = 0.02).

Marked differences were also noted between summer and winter concentrations of all three IGF measures, with mean winter levels registering higher than those observed in the summer. Similarly, differences in serum IGFBP-3 and IGF-I/IGFBP-3 were seen between oral contraceptive (OC) users and non-users; although, while users were noted to have greater IGFBP-3 concentrations than non-users, the opposite effect

was seen for IGF-I/IGFBP-3, as users appeared to have less “free” IGF-I than non-users.

Mean concentrations of the IGF measures, stratified by the different covariates, were also considered for each of the two data collections (results not shown). Generally, patterns identical to those noted in the pooled data were observed for at least the first day shift collection, if not both. A reduced sample size for the second data collection was suspected as the probable explanation for any inconsistencies.

**Table E.4** Mean IGF-I, IGFBP-3, and IGF-I/IGFBP-3 concentrations stratified by covariates

| Characteristic | IGF-I (n = 147)* ‡ Mean ± SD; N (ng/ml) | IGFBP-3 (n = 147)** ‡ Mean ± SD; N (ng/ml) | IGF-I/IGFBP-3 (n = 145)*** ‡ Mean ± SD; N |
|---|---|---|---|
| Age (years)† | | | |
| ≤ 30 | 203.3 ± 63.7; 37 | 4362.1 ± 1076.1; 38 | 0.175 ± 0.055; 36 |
| > 30 – 37 | 168.1 ± 71.2; 37 | 3975.7 ± 1059.4; 37 | 0.170 ± 0.115; 37 |
| > 37 – 43 | 151.4 ± 60.4; 39 | 3905.7 ± 1154.7; 39 | 0.148 ± 0.068; 39 |
| > 43 | 146.6 ± 52.9; 34 | 4143.4 ± 1045.0; 33 | 0.135 ± 0.064; 33 |
| *p-trend* | *0.0004* | *0.46* | *0.03* |
| BMI (kg/m$^2$)† | | | |
| ≤ 22.7 | 173.7 ± 62.3; 38 | 4102.6 ± 1184.4; 39 | 0.164 ± 0.063; 37 |
| > 22.7 – 25.5 | 175.7 ± 55.9; 36 | 4247.2 ± 1345.4; 36 | 0.170 ± 0.109; 36 |
| > 25.5 – 29.4 | 152.0 ± 63.0; 39 | 3869.6 ± 827.6; 39 | 0.149 ± 0.074; 39 |
| > 29.4 | 170.0 ± 80.6; 34 | 4184.9 ± 932.0; 33 | 0.146 ± 0.068; 33 |
| *p-trend* | *0.57* | *0.91* | *0.35* |
| History of night shift work (years)† | | | |
| ≤ 5 | 184.2 ± 69.4; 37 | 4197.2 ± 1019.0; 37 | 0.160 ± 0.045; 35 |
| > 5 – 10 | 181.5 ± 66.7; 53 | 4249.5 ± 1099.8; 53 | 0.169 ± 0.101; 53 |
| > 10 – 15 | 153.8 ± 53.5; 15 | 3680.2 ± 887.2; 15 | 0.162 ± 0.074; 15 |
| > 15 – 20 | 134.6 ± 54.4; 14 | 3910.5 ± 1128.8; 14 | 0.130 ± 0.050; 14 |
| > 20 | 143.2 ± 58.9; 28 | 3980.2 ± 1226.8; 28 | 0.143 ± 0.085; 28 |
| *p-trend* | *0.002* | *0.30* | *0.19* |
| Season | | | |
| Summer | 145.1 ± 55.3; 73 | 3809.0 ± 1061.6; 73 | 0.148 ± 0.071; 72 |
| Winter | 189.8 ± 68.0; 74 | 4376.5 ± 1050.8; 74 | 0.167 ± 0.088; 73 |
| Sleep duration (hour) | | | |
| ≤ 6.5 | 167.7 ± 67.2; 45 | 4195.3 ± 1061.9; 45 | 0.150 ± 0.057; 44 |
| > 6.5 – 7.0 | 159.2 ± 63.4; 29 | 4040.7 ± 969.5; 28 | 0.144 ± 0.058; 28 |
| > 7.0 – 7.5 | 185.4 ± 73.8; 37 | 4012.6 ± 1232.1; 38 | 0.188 ± 0.119; 37 |
| > 7.5 | 155.8 ± 54.9; 36 | 4097.5 ± 1090.4; 36 | 0.146 ± 0.064; 36 |
| *p-trend* | *0.91* | *0.97* | *0.55* |

Con't

| | | | |
|---|---|---|---|
| Menstrual cycle stage | | | |
| Follicular | 168.4 ± 69.9; 57 | 4276.4 ± 1138.9; 57 | 0.150 ± 0.089; 56 |
| Mid-cycle | 154.6 ± 57.2; 23 | 4121.7 ± 929.4; 22 | 0.138 ± 0.048; 22 |
| Luteal | 171.3 ± 65.3; 67 | 3933.6 ± 1086.8; 68 | 0.170 ± 0.080; 67 |
| *p-trend* | *0.83* | *0.003* | *0.18* |
| Current oral contraceptive use‡‡ | | | |
| No | 166.7 ± 65.8; 118 | 3995.2 ± 1086.3; 117 | 0.161 ± 0.086; 116 |
| Yes | 171.1 ± 66.6; 29 | 4482.7 ± 1034.2; 30 | 0.142 ± 0.050; 29 |
| Number of pregnancies† | | | |
| None | 177.2 ± 67.9; 63 | 4201.0 ± 997.7; 64 | 0.155 ± 0.055; 62 |
| 1 | 164.8 ± 63.7; 14 | 3620.4 ± 1213.3; 14 | 0.204 ± 0.165; 14 |
| 2 | 152.8 ± 62.5; 44 | 4036.1 ± 1192.8; 43 | 0.145 ± 0.068; 43 |
| ≥ 3 | 170.8 ± 66.3; 26 | 4185.0 ± 1053.3; 26 | 0.158 ± 0.080; 26 |
| *p-trend* | *0.34* | *0.79* | *0.87* |
| Smoking status† | | | |
| Never | 169.8 ± 67.9; 91 | 4196.5 ± 1160.9; 92 | 0.157 ± 0.088; 90 |
| Past | 162.6 ± 63.8; 34 | 3896.7 ± 1036.9; 34 | 0.157 ± 0.070; 34 |
| Current | 166.4 ± 62.4; 22 | 3969.1 ± 801.3; 21 | 0.158 ± 0.060; 21 |
| *p-trend* | *0.74* | *0.29* | *0.99* |
| Chronic alcohol consumption (avg. # drinks/week)† Ψ | | | |
| None | 159.7 ± 56.3; 46 | 4176.6 ± 1242.5; 45 | 0.155 ± 0.103; 45 |
| > 0 – 2 | 162.8 ± 70.5; 37 | 4115.2 ± 1127.1; 37 | 0.147 ± 0.063; 37 |
| > 2 – 4 | 184.0 ± 77.2; 38 | 4108.6 ± 852.8; 37 | 0.163 ± 0.070; 37 |
| > 4 | 164.3 ± 54.9; 26 | 3917.1 ± 1097.4; 28 | 0.168 ± 0.073; 26 |
| *p-trend* | *0.53* | *0.37* | *0.62* |
| Recent alcohol consumption (# drinks/day)‡‡ | | | |
| None | 169.2 ± 66.2; 117 | 3992.3 ± 1083.9; 116 | 0.163 ± 0.082; 115 |
| > 0 – < 2 | 156.5 ± 60.7; 15 | 4303.2 ± 973.1; 16 | 0.144 ± 0.084; 15 |
| ≥ 2 | 166.2 ± 70.2; 15 | 4664.1 ± 1117.5; 15 | 0.131 ± 0.055; 15 |
| *p-trend* | *0.74* | *0.02* | *0.14* |

Con't

| | | | |
|---|---|---|---|
| Recent caffeine consumption (# drinks/day)‡‡ | | | |
| ≤ 2 | 174.0 ± 66.5; 69 | 4152.0 ± 1202.9; 70 | 0.163 ± 0.090; 68 |
| > 2 – 2.5 | 112.8 ± 44.2; 5 | 4368.6 ± 908.6; 5 | 0.092 ± 0.023; 5 |
| > 2.5 – 4 | 163.8 ± 62.8; 50 | 3922.7 ± 1011.8; 50 | 0.158 ± 0.071; 50 |
| > 4 | 168.4 ± 71.0; 23 | 4240.8 ± 918.4; 22 | 0.152 ± 0.074; 22 |
| *p-trend* | *0.61* | *0.57* | *0.94* |
| Total physical activity (avg. METmin/day)†† | | | |
| ≤ 1178.7 | 150.9 ± 61.9; 36 | 4113.1 ± 1213.3; 38 | 0.145 ± 0.073; 36 |
| > 1178.7 – 1435.7 | 167.2 ± 66.1; 37 | 3733.6 ± 930.8; 37 | 0.176 ± 0.109; 37 |
| > 1435.7 – 1712.3 | 168.1 ± 59.8; 37 | 4356.5 ± 1109.2; 35 | 0.145 ± 0.058; 35 |
| > 1712.3 | 183.7 ± 73.0; 37 | 4189.0 ± 1034.4; 37 | 0.163 ± 0.069; 37 |
| *p-trend* | *0.02* | *0.20* | *0.35* |

* 147 observations, 84 individuals
** 147 observations, 85 individuals
*** 145 observations, 85 individuals
‡ overlap of 63 individuals across day shift collection periods for IGF-I, 62 for IGFBP-3, and 61 for IGF-I/IGFBP-3
† measured at baseline only
Ψ average value for current decade of life
‡‡ last 24 hours
†† last 30 days

## Correlation between IGF-I and IGFBP-3

**Table E.5** Spearman's rank correlation between IGF-I and IGFBP-3 at first day shift of participation

| | **IGFBP-3** (ng/ml) |
|---|---|
| | Correlation coefficient (p-value); N |
| **IGF-I** (ng/ml) | 0.4 (0.003); 77* |

* 77 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)

## IGFBP-3 analysis

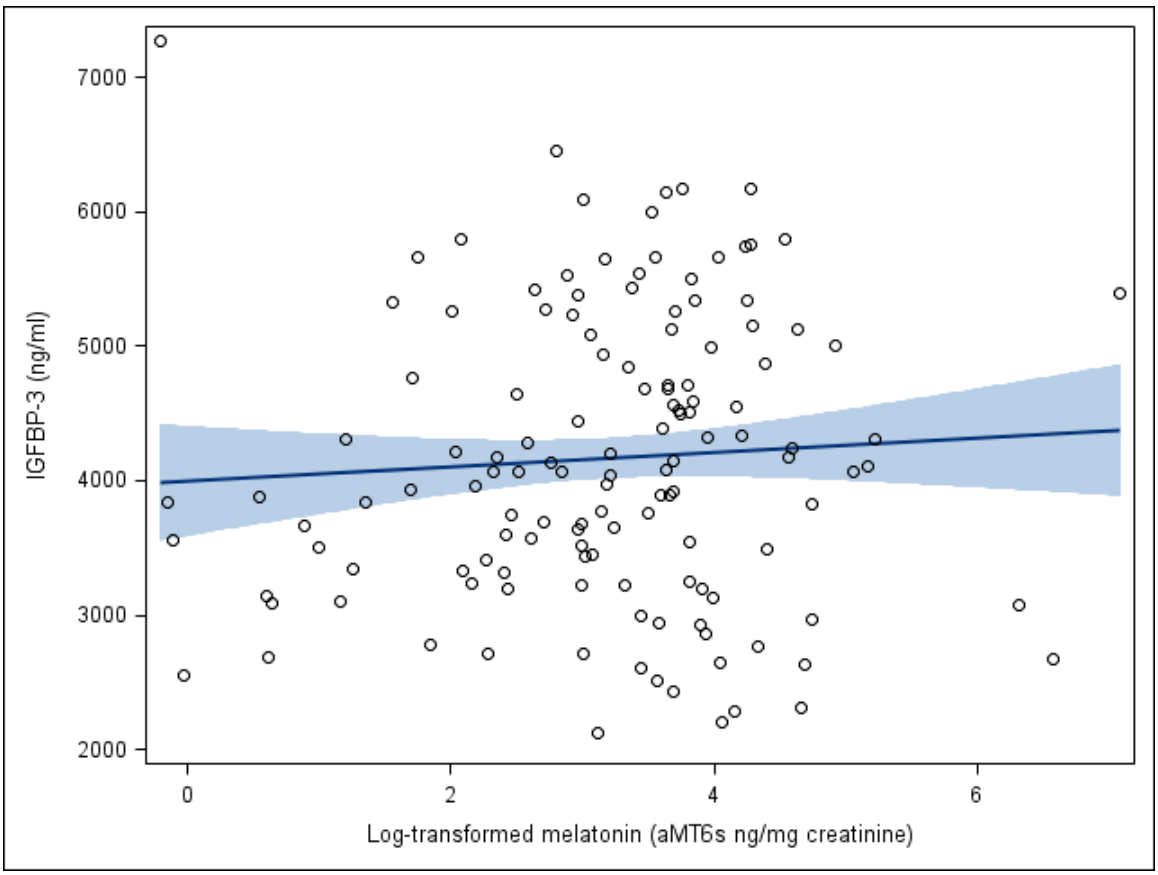


**Figure E.5** Graph of log-transformed melatonin (aMT6s ng/mg creatinine) vs. IGFBP-3 (ng/ml)

## IGF-I/IGFBP-3 analysis

### Objective 1: Predictive models

**Table E.6** Predictive models for IGF-I/IGFBP-3

| Predictor | Unadjusted<br>Parameter estimate[a]<br>(p-value) | Partially Adjusted<br>Parameter estimate[b]<br>(p-value) | Final Model**<br>Parameter estimate[c]<br>(p-value) |
|---|---|---|---|
| *Forced covariates*<br>('Partially Adjusted' = mutual adjustment for age, BMI, and test batch predictors) | | | |
| Age (years)* | -0.002 (0.02) | -0.002 (0.11) | -0.003 (0.0004) |
| BMI (kg/m$^2$)* | | | |
| ≤ 22.7 | Ref | Ref | Ref |
| > 22.7 – 25.5 | 0.003 (0.89) | 0.010 (0.64) | -0.0008 (0.95) |
| > 25.5 – 29.4 | -0.017 (0.44) | -0.00005 (1.0) | -0.004 (0.79) |
| > 29.4 | -0.015 (0.50) | -0.006 (0.79) | -0.006 (0.68) |
| IGF-I test batch | | | |
| 1 | Ref | Ref | Ref |
| 2 | -0.032 (0.03) | -0.031 (0.16) | -0.036 (0.03) |
| 3 | 0.049 (0.003) | 0.059 (0.0003) | 0.045 (0.0003) |
| 4 | 0.045 (0.008) | 0.031 (0.07) | 0.025 (0.049) |
| 5 | 0.004 (0.92) | -0.0005 (0.99) | -0.010 (0.72) |
| IGFBP-3 test batch | | | |
| 1 | Ref | Ref | Ref |
| 2 | -0.029 (0.05) | -0.040 (0.009) | -0.036 (0.004) |
| 3 | -0.019 (0.26) | -0.033 (0.05) | -0.029 (0.04) |
| 4 | -0.046 (0.008) | -0.007 (0.74) | -0.0001 (0.99) |
| 5 | -0.102(<0.0001) | -0.042 (0.14) | -0.027 (0.20) |
| *Potential covariates* | | | |
| History of night shift work (years)* | -0.0009 (0.35) | 0.0008 (0.46) | -- |

Con't

| | | | |
|---|---|---|---|
| Season | | | |
| Summer | Ref | Ref | |
| Winter | 0.020 (0.04) | 0.002 (0.91) | -- |
| Sleep duration (hour) | 0.0004 (0.94) | 0.002 (0.73) | -- |
| Menstrual cycle stage | | | |
| Follicular | Ref | Ref | |
| Mid-cycle | -0.021 (0.24) | -0.011 (0.43) | -- |
| Luteal | 0.016 (0.18) | -0.006 (0.59) | -- |
| Current oral contraceptive use† | | | |
| No | Ref | Ref | Ref |
| Yes | -0.024 (0.17) | -0.046 (0.008) | -0.037 (0.003) |
| Number of pregnancies* | | | |
| None | Ref | Ref | |
| 1 | 0.051 (0.07) | 0.072 (0.01) | -- |
| 2 | -0.008 (0.65) | 0.025 (0.23) | -- |
| ≥ 3 | 0.004 (0.84) | 0.043 (0.08) | -- |
| Smoking status* | | | |
| Never | Ref | Ref | |
| Past | 0.001 (0.95) | 0.009 (0.62) | -- |
| Current | -0.0003 (0.99) | -0.007 (0.76) | -- |
| Chronic alcohol consumption (avg. # drinks/week)* ‡ | 0.002 (0.48) | -0.0004 (0.87) | -- |
| Recent alcohol consumption (# drinks/day)† | -0.010 (0.25) | -0.008 (0.30) | -- |
| Recent caffeine consumption (# drinks/day)† | -0.002 (0.60) | -0.0006 (0.84) | -- |
| Total physical activity (avg. METmin/day)Ψ | $4.23 \times 10^{-6}$ (0.78) | $2.78 \times 10^{-6}$ (0.84) | -- |

n = 145; 145 observations, 84 individuals; overlap of 61 individuals across day shift collection periods
* measured at baseline only
** n = 139; 139 observations, 81 individuals (three influential individuals deleted)
‡ average value for current decade of life
† last 24 hours
Ψ last 30 days
a: unadjusted
b: adjusted for age, BMI, and test batch
c: variables mutually adjusted; all variables remaining after backwards selection, inclusion cutoff of $p \leq 0.10$

**Objective 2: Associations between melatonin and the IGF family**

*Spearman's rank correlation*

**Table E.7** Spearman's rank correlations between log-transformed melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3 at first day shift of participation

| | **IGF-I** | **IGFBP-3** | **IGF-I/IGFBP-3** |
|---|---|---|---|
| | Correlation coefficient (p-value); N | Correlation coefficient (p-value); N | Correlation coefficient (p-value); N |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -0.1 (0.32); 75* | 0.06 (0.61); 74** | -0.1 (0.32); 73‡ |

* 74 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)
** 73 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)
‡ 72 individuals from day shift collection #1, 1 individual from day shift collection #2 (participated in day shift collection #2 only)

*Unadjusted and adjusted mixed effects modeling*

**Table E.8** Associations between log-transformed melatonin and IGF-I/IGFBP-3

| **Predictor** | **Unadjusted** | **Partially Adjusted** | **Final Model*** |
|---|---|---|---|
| | Parameter estimate[a] (p-value) | Parameter estimate[b] (p-value) | Parameter estimate[c] (p-value) |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -0.007 (0.16) | -0.005 (0.28) | -0.002 (0.57) |

n = 134; 134 observations, 82 individuals; overlap of 52 individuals across day shift collection periods
* n = 128; 128 observations, 79 individuals (three influential individuals deleted)
a: unadjusted
b: adjusted for age, BMI, and test batch
c: adjusted for age, BMI, test batch, current OC use, and number of pregnancies

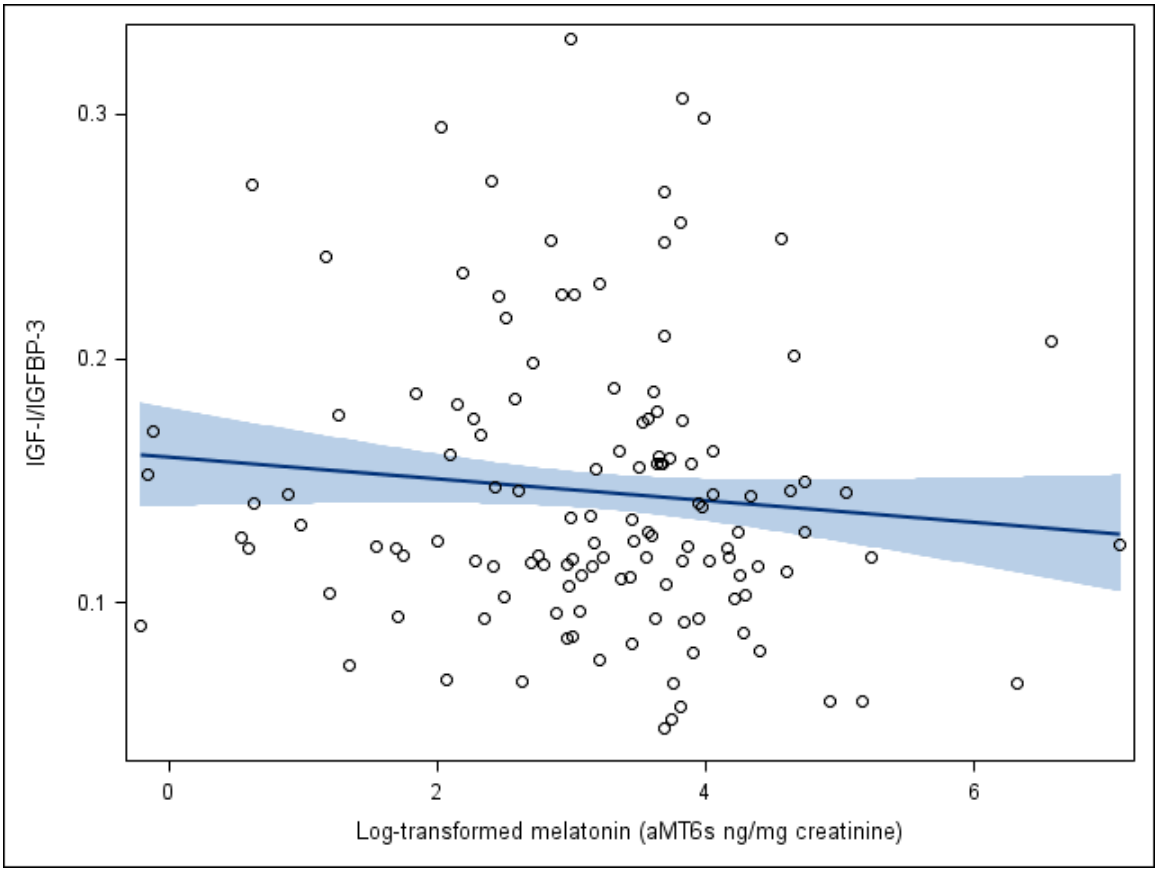


**Figure E.6** Graph of log-transformed melatonin (aMT6s ng/mg creatinine) vs. IGF-I/IGFBP-3

*Associations between melatonin and IGFs: stratified by season*

**Table E.9** Partially adjusted associations between log-transformed melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3, by season

| Predictor | **IGF-I** **n = 136** Parameter estimate[a] (p-value) | **IGFBP-3** **n = 136** Parameter estimate[a] (p-value) | **IGF-I/IGFBP-3** **n = 134** Parameter estimate[a] (p-value) |
|---|---|---|---|
| *Summer* | N = 68 | N = 68 | N = 67 |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -6.9 (0.21) | -42.0 (0.68) | -0.008 (0.27) |
| *Winter* | N = 68 | N = 68 | N = 67 |
| Log-transformed melatonin (aMT6s ng/mg creatinine) | 0.7 (0.91) | 108.2 (0.27) | -0.010 (0.29) |

a: adjusted for age, BMI, test batch

*Associations between melatonin and IGFs: quartiles of melatonin*

**Table E.10** Adjusted associations between log-transformed melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3; melatonin as categorical

| Predictor | IGF-I<br>**n = 136***<br>Parameter estimate[a]<br>(p-value) | IGFBP-3<br>**n = 130****<br>Parameter estimate[b]<br>(p-value) | IGF-I/IGFBP-3<br>**n = 128‡**<br>Parameter estimate[c]<br>(p-value) |
|---|---|---|---|
| Log-transformed melatonin (aMT6s ng/mg creatinine) | | | |
| ≤ 2.5 | Ref | Ref | Ref |
| > 2.5 – 3.4 | 1.8 (0.90) | 322.8 (0.09) | -0.010 (0.41) |
| > 3.4 – 3.9 | 5.2 (0.70) | 205.0 (0.22) | -0.006 (0.60) |
| > 3.9 | -5.0 (0.73) | -77.9 (0.67) | -0.010 (0.47) |

* n = 136; 136 observations, 82 individuals
** n = 130; 130 observations, 80 individuals (three influential individuals deleted)
‡ n = 128; 128 observations, 79 individuals (three influential individuals deleted)
a: adjusted for age, BMI, test batch, season, menstrual cycle stage, current OC use, and number of pregnancies
b: adjusted for age, BMI, test batch, season, menstrual cycle stage, and recent alcohol consumption
c: adjusted for age, BMI, test batch, current OC use, and number of pregnancies

## Sensitivity analysis

**Table E.11** Final predictive models for IGF-I, IGFBP-3, and IGF-I/IGFBP-3, imputations removed

| Predictor | IGF-I<br>n = 133*<br>Parameter estimate[a]<br>(p-value) | IGFBP-3<br>n = 134**<br>Parameter estimate[a]<br>(p-value) | IGF-I/IGFBP-3<br>n = 126‡<br>Parameter estimate[a]<br>(p-value) |
|---|---|---|---|
| *Forced covariates* | | | |
| Age (years)† | -3.5 (<0.0001) | -19.6 (0.13) | -0.003 (0.0004) |
| BMI (kg/m$^2$)† | | | |
| ≤ 23.5 | Ref | Ref | Ref |
| > 23.5 – 25.8 | 8.6 (0.56) | 212.8 (0.46) | -0.0008 (0.95) |
| > 25.8 – 29.2 | -9.0 (0.55) | -262.4 (0.35) | -0.002 (0.89) |
| > 29.2 | -13.5 (0.38) | 31.5 (0.91) | -0.013 (0.38) |
| IGF-I test batch | | | |
| 1 | Ref | | Ref |
| 2 | -8.7 (0.59) | | -0.041 (0.02) |
| 3 | 54.2 (<0.0001) | -- | 0.044 (0.0004) |
| 4 | 31.8 (0.04) | -- | 0.020 (0.11) |
| 5 | 34.9 (0.26) | -- | -0.015 (0.58) |
| IGFBP-3 test batch | | | |
| 1 | | Ref | Ref |
| 2 | -- | 1309.1 (<0.0001) | -0.033 (0.009) |
| 3 | -- | 838.8 (0.0002) | -0.026 (0.07) |
| 4 | -- | 442.4 (0.03) | 0.005 (0.77) |
| 5 | -- | 1434.2 (<0.0001) | -0.022 (0.30) |
| *Other covariates* | | | |
| Season | | | |
| Summer | Ref | | |
| Winter | 24.1 (0.06) | -- | -- |

Con't

| | | | |
|---|---|---|---|
| Menstrual cycle stage | | | |
| Follicular | | | |
| Mid-cycle | -- | -- | -- |
| Luteal | -- | -- | -- |
| Current oral contraceptive use[Ψ] | | | |
| No | Ref | | Ref |
| Yes | -43.1 (0.003) | -- | -0.045 (0.001) |
| Number of pregnancies[†] | | | |
| None | | | |
| 1 | -- | -- | -- |
| 2 | -- | -- | -- |
| ≥ 3 | -- | -- | -- |
| Recent alcohol consumption (# drinks/day)[Ψ] | -- | 210.4 (0.04) | -- |

* n = 133; 133 observations, 79 individuals (14 observations missing)
** n = 134; 134 observations, 79 individuals (11 observations missing)
‡ n = 126; 126 observations, 76 individuals (13 observations missing)
† measured at baseline only
Ψ Last 24 hours
a: variables mutually adjusted

**Table E.12** Final adjusted associations between log-transformed melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3, imputations removed

| **Predictor** | **IGF-I** **n = 109*** Parameter estimate[a] (p-value) | **IGFBP-3** **n = 117**** Parameter estimate[b] (p-value) | **IGF-I/IGFBP-3** **n = 112[‡]** Parameter estimate[c] (p-value) |
|---|---|---|---|
| Log-transformed melatonin (aMT6s ng/mg creatinine) | -5.3 (0.17) | -14.9 (0.77) | -0.004 (0.28) |

* n = 109; 109 observations, 67 individuals (27 observations missing)
** n = 117; 117 observations, 73 individuals (17 observations missing)
‡ n = 112; 112 observations, 69 individuals (16 observations missing)
a: adjusted for age, BMI, test batch, season, menstrual cycle stage, current OC use, and number of pregnancies
b: adjusted for age, BMI, test batch, season, menstrual cycle stage, and recent alcohol consumption
c: adjusted for age, BMI, test batch, current OC use, and number of pregnancies

**Table E.13** Final adjusted associations between log-transformed melatonin and IGF-I, IGFBP-3, and IGF-I/IGFBP-3; melatonin as categorical, imputations removed

| Predictor | IGF-I<br>n = 109*<br>Parameter estimate[a]<br>(p-value) | IGFBP-3<br>n = 114**<br>Parameter estimate [b]<br>(p-value) | IGF-I/IGFBP-3<br>n = 112‡<br>Parameter estimate[c]<br>(p-value) |
|---|---|---|---|
| Log-transformed melatonin (aMT6s ng/mg creatinine) | | | |
| ≤ 2.5 | Ref | Ref | Ref |
| > 2.5 – 3.4 | -11.8 (0.41) | 413.2 (0.046) | -0.015 (0.23) |
| > 3.4 – 3.9 | 6.8 (0.62) | 198.8 (0.28) | -0.011 (0.36) |
| > 3.9 | -23.0 (0.12) | -33.0 (0.86) | -0.015 (0.26) |

* n = 109; 109 observations, 67 individuals (27 observations missing)
** n = 114; 114 observations, 71 individuals (16 observations missing)
‡ n = 112; 112 observations, 69 individuals (16 observations missing)
a: adjusted for age, BMI, test batch, season, menstrual cycle stage, current OC use, and number of pregnancies
b: adjusted for age, BMI, test batch, season, menstrual cycle stage, and recent alcohol consumption
c: adjusted for age, BMI, test batch, current OC use, and number of pregnancies