« Salivary Melatonin Levels in Pregnant Women with Insomnia: A Prospective Cohort Study with Two Comparison Groups »

By

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A thesis submitted in conformity with the requirements for the degree of « Master of Science »

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Abstract:

Insomnia in pregnancy is associated with depression, preeclampsia, gestational diabetes, and preterm labor. In normal pregnancies, maternal plasma melatonin levels increase significantly as pregnancy proceeds, reaching its peak near term. However, the role of melatonin in insomnia during pregnancy is not known. The objective of this study was to measure nocturnal saliva melatonin levels in pregnant women with and without insomnia. Results did not show a significant difference in melatonin levels between insomniac (treated and untreated) and healthy pregnant women in all trimesters. However, sub-group analysis showed significantly lower melatonin levels in untreated insomniac pregnancies compared to healthy pregnancies and those treated with sleep medications. Results of this study confirmed lower levels of nocturnal melatonin in untreated pregnancies with insomnia. Future research is needed to investigate the safety and efficacy of melatonin supplementation for the treatment of insomnia in pregnancy, replacing psychotropic drugs.
Acknowledgements

With deep gratitude, I want to thank my supervisor Dr. Gideon Koren, a distinguished scientist, clinician and teacher, who is a true leader and inspires leadership. In 2011 I was given the opportunity to work as a Motherisk counselor, and had the pleasure to work with Dr. Koren and his team. I soon learned to appreciate the value of research and its implications in real life. So I decided to pursue graduate studies in research, and Dr. Koren gave me that opportunity, and showed me the excellence in science. His wise advice and comments guided me through all the steps of the research study. I am grateful that I had the chance to work with him both as an employee and as a graduate student.

I want to thank my advisory committee, Dr. Manny Papadimitropoulos and Dr. Bhushan Kapur, whose advice in the past three years was invaluable, and kept me on the right path of research.

I also want to thank you, the Motherisk counselling team and Motherisk’s lab staff for their cooperation throughout the study period.

Last, but not least, I want to give my special thanks to Dr. Shinya Ito. Even with his busy schedule as the head of the Division of Clinical Pharmacology and Toxicology, Dr. Ito spent enough time with me, solved any issues or problems raised during the study period, and during the absence of Dr. Koren. I appreciate all his help and the time he contributed for the work to be done and finished on time.

I would also like to thank to the Department of Pharmaceutical Sciences at the University of Toronto for having this great research program.
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<th>Full Form</th>
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<tr>
<td>5-HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>a-6MTs</td>
<td>6-sulfatoxymelatonin</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADD</td>
<td>attention deficit disorder</td>
</tr>
<tr>
<td>ASPD</td>
<td>advanced sleep phase disorder</td>
</tr>
<tr>
<td>BIQ</td>
<td>brief insomnia questionnaire</td>
</tr>
<tr>
<td>BZD</td>
<td>benzodiazepine</td>
</tr>
<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DLMO</td>
<td>dim light melatonin onset</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual, Fourth Edition</td>
</tr>
<tr>
<td>DSPD</td>
<td>delayed sleep phase disorder</td>
</tr>
<tr>
<td>Ect</td>
<td>ectopic pregnancy</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone releasing hormone</td>
</tr>
<tr>
<td>GHT</td>
<td>intergeniculate tract</td>
</tr>
<tr>
<td>HIOMT</td>
<td>hydroxyindole-o-methyl-transferase</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPT</td>
<td>hypothalamic-pituitary-thyroid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
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<td>hospital for sick children</td>
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<td>International Classification of Diseases-102</td>
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<td>ICF</td>
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<td>ICSD-2</td>
<td>International Classification of Sleep Disorders-24</td>
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<td>IL-1</td>
<td>interlukin-1</td>
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<td>IML</td>
<td>intermediolateral</td>
</tr>
<tr>
<td>IUUGR</td>
<td>intra uterine growth restriction</td>
</tr>
<tr>
<td>LMP</td>
<td>Last Menstrual Period</td>
</tr>
<tr>
<td>MDD</td>
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</tr>
<tr>
<td>MM</td>
<td>major malformation</td>
</tr>
<tr>
<td>MR</td>
<td>motherisk program</td>
</tr>
<tr>
<td>NAS</td>
<td>n-acetyl serotonin</td>
</tr>
<tr>
<td>NAT</td>
<td>n-acetyl transferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NO</td>
<td>nitrous oxide</td>
</tr>
<tr>
<td>NREM</td>
<td>non-rapid eye movement</td>
</tr>
<tr>
<td>NVP</td>
<td>nausea and vomiting of pregnancy</td>
</tr>
<tr>
<td>PGD2</td>
<td>prostaglandin D2</td>
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<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PNAS</td>
<td>poor neonatal adaptation syndrome</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
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<tr>
<td>RDC</td>
<td>research diagnostic criteria</td>
</tr>
<tr>
<td>REB</td>
<td>research ethics board</td>
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<tr>
<td>REM</td>
<td>rapid eye movement</td>
</tr>
<tr>
<td>RHT</td>
<td>retino-hypothalamic tract</td>
</tr>
<tr>
<td>SA</td>
<td>spontaneous abortion</td>
</tr>
<tr>
<td>SCG</td>
<td>superior cervical ganglion</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
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<tr>
<td>SNRI</td>
<td>selective norepinephrine reuptake inhibitor</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>STB</td>
<td>suncytotrophoblast</td>
</tr>
<tr>
<td>T1</td>
<td>first trimester</td>
</tr>
<tr>
<td>T2</td>
<td>second trimester</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>T3</td>
<td>third trimester</td>
</tr>
<tr>
<td>TA</td>
<td>therapeutic abortion</td>
</tr>
<tr>
<td>TCA</td>
<td>tricyclic antidepressant</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>vCTB</td>
<td>villous cytotrophoblast</td>
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</table>
1. **Introduction**

1.1. **Circadian System**

The circadian system is located both in the central nervous system in the suprachiasmatic nucleus (SCN) of the hypothalamus, and peripheral organs such as the heart and liver.¹ The system plays an important role in different physiological arrangements and behaviour. It is the biological clock of the body managing both night and day behaviours. The system functions not only independent of external stimuli such as ambient brightness, but also in a manner dependent on external factors such as food intake.² Any disruption of the system leads to conditions such as sleep disorder, glucose intolerance (diabetes), obesity, and ultimately decreased life expectancy.³

1.2. **Suprachiasmatic Nucleus**

SCN is located in the anterior hypothalamus on top of the optic chiasm, next to the third ventricle with 50,000 neurons.⁴ It is a bilateral structure and is activated by light. In other words, it regulates the circadian rhythm. Although it is now known that circadian rhythm exists in peripheral organs independent of the SCN, their function is lost without input from SCN. For this reason, SCN is called the master circadian clock, or the coordinator of the clocks. A circadian clock has a 24-hour cycle and its most potent stimulus is light that is directly projected from the retina to SCN through the retino-hypothalamic tract (RHT). The indirect pathway of receiving light by the SCN is through the intergeniculate tract (GHT). Malfunctioning of any of the above pathways impairs the phase-shifting and light-dark cycle of the circadian system. For example, the light-dark cycle is impaired by the RHT pathway damage, and phase-shifting is delayed by damage to GHT pathway.¹ Research also shows that the circadian clock can shift a phase independent of light with other stimuli such as exogenous melatonin, temperature, exercise, and food. For example, melatonin supplementation in blind people can regulate the circadian clock.⁵ Core body temperature also affects the peripheral circadian clock but not the SCN.⁶ There are 2 pathways by which the SCN manages the circadian clock complementing each other: endocrine and neural pathways. Circadian clock controls the endocrine system including melatonin, cortisol from the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-thyroid (HPT) axis, and epinephrine.⁷ The neural pathway includes projections from the SCN to the pineal gland (Figure 1).
1.3. Pineal Gland

1.3.1. Description

The pineal gland had several names over-time given its anatomy and function in different animals. It was first identified as a different cerebral organ a few centuries BC, and was initially named “Konareion” for its pine shape, similar to lymph nodes. It was then named “Glandula Pinealis”, pineal. Later Rene Descartes called the gland the “seat of the soul”, coordinator of psychophysiological functions of the body. Bioassay advances resulted in the discovery of pineal “extracts” responsible for lightening frog’s skin, which lead to the isolation of melatonin in 1958. Development of fluorescent techniques allowed the measurement of melatonin and serotonin, their light-dark cycles and their concentrations.

1.3.2. Structure

The location and structure of the pineal gland is species specific and responds to environmental stimuli. The human pineal gland is located at the posterior of the diencephalon. It develops in the second month of gestation as an invagination of the ependymal lining of the diencephalic third ventricle, between the habenular and posterior commissure. The “pineal stalk” consists of a rostral and a caudal lamina surrounded by a pial layer, and is suspended in the CSF- filled pineal recess below the splenium. Average adult dimensions are 5-9mm in length, 1-5mm in width, and 3-5mm in thickness and the average weight of 100-180mg varies in different ages and genders. Its structure is described as two parts. A central core of lobules and peripheral neurons along with pinalocytes are the main cell types of human pineal gland. Pinalocytes are granular shape, and have a nucleus and cytoplasmic processes that end in capillaries, indicative
of an endocrine gland. Neuroglia are distributed unevenly in the periphery. Calcareous deposits are characteristic of the pineal deposits as calcium and magnesium salts on both parenchymal and intracellular tissue. Calcareous deposits are present from birth and increase in concentration by age, starting to decrease in young adulthood. A negative correlation was found between the density of calcification, age and chronic sleepiness. The pineal gland has a rich vascular system receiving blood from posterior choroidal arteries deriving from cerebral arteries. It lacks an endothelial blood-brain barrier, and is sensitive to drugs. The gland is innervated with fibres from sympathetic, parasympathetic, and central nervous systems. The most important pathway is the noradrenergic sympathetic pathway neurons which receive input from the suprachiasmatic nucleus (SCN) of the hypothalamus, which itself receives input from retinal ganglion cells. 

1.3.3. Hormonal Pathway

The secretory characteristic of the pineal gland, or extracts, has been studied extensively. In 1958 melatonin was isolated from the pineal gland. The name “melatonin” comes from the hormone’s effect on frog skin melanophores. So far, melatonin is the most important substance secreted from the pineal gland although other peptides and indoles are known to be produced by the gland. It is produced by pinalocytes in a biochemical cascade. Tryptophan is taken up by the pinalocytes and is hydroxylated and decarboxylated to serotonin. The rate-limiting enzyme N-acetyl-transferase (NAT) converts serotonin to N-acetyl-serotonin (NAS), which in turn becomes methylated to melatonin by the enzyme hydroxyindole-o-methyl-transferase (HIOMT) (Figure 2). Melatonin is a lipophilic molecule. It is released into blood after its biosynthesis. It is 70% bound to albumin, and has a short half-life after intravenous infusion. It is metabolized mainly by the liver, but also by kidneys. So any liver or kidney damage or disease may affect the elimination of the hormone. The main metabolite of melatonin, 6-sulfatoxymelatonin (a-6MTs), is found in urine and counts for 90% of the administered melatonin. Melatonin is found in blood, saliva, urine, and cerebrospinal fluid (CSF) with higher concentration than blood. It is also found in other organs such as reproductive organs and in fluids (semen, amniotic fluid, and breast milk), the gastrointestinal tract, and bone marrow. Pinealectomy eliminates the melatonin in blood, saliva, urine, and CSF, an indication of its main biosynthesis in the pineal gland. Two melatonin receptors MT1 and
MT2 are identified. MT1 is found in the SCN, where the timing of melatonin production is controlled. Melatonin receptors are also found in lymphocytes, prostate epithelial cells, pre-ovulatory follicles, sperm, blood platelets, and placenta.

Figure 2: Biosynthesis and chemical structure of melatonin. Reprinted with permission from Springer.

Pineal melatonin production is regulated by the dark-light cycle. Melatonin levels start to rise in the evening. Maximum concentration is reached by midnight and it starts to decrease before wake up time. This pattern is stable, making melatonin a reliable phase maker of the endogenous clock system. Synchronization with external light-dark is necessary for its constant secretion. Retinal melanopsin-containing ganglion cells transmit light signals through the monosynaptic retino-hypothalamic tract to the SCN. These ganglion cells are sensitive to short wavelength light. Since light suppresses the hormone’s nocturnal production, this is an indication that these cells are responsible in delivering photic signals to pineal gland.

1.3.4. Neural Pathway

The neural pathway regulating the production of melatonin in the pineal gland consists of an input from the GABAergic system from SCN to a subdivision of paraventricular nucleus (PVN), which in turn projects to the intermediolateral (IML) cell column and, by pre-ganglionic adrenergic fibres, to the superior cervical ganglion (SCG). The input then reaches
the pineal gland from SCG by post ganglionic adrenergic fibres and releases norepinephrine (NE). In the pineal gland, beta adrenergic receptors of pinalocytes are stimulated by a subunit of G protein coupled receptors that increase adenylate cyclase. Ca²⁺ and protein kinase C (PKC) are involved in increased beta adrenergic activity and stimulation of cAMP potentiated by Alpha adrenergic receptors. The synergistic activity of alpha and beta adrenergic receptors increases cAMP and production of NAT (Figure 3). The rate of melatonin secretion is negatively correlated with age, and dim light melatonin onset (DLMO) may not reach the threshold concentration, 4 pg/ml, in the elderly. However, concentration is significantly higher in younger people. There are also inter-individual differences in timing, duration, and the amount of nocturnal hormone secretion, but stability within individuals.

For example, mean melatonin production is estimated to be higher in men than women at night, 60.7 ± 24.6 pg/ml and 29.3 ± 21.8 pg/ml respectively, p<0.01. DLMO also occurs later in women than in men. Inter-individual differences in melatonin production may be due to different metabolism rates.

Day length affects melatonin secretion as well, so there are seasonal variations in the rate of nocturnal melatonin, which affects the physiology, behavior, and reproduction of some species. The use of artificial light or artificial shortening of summer days has been shown to
affect the secretion of the hormone.\textsuperscript{48,49} Evidence shows that the onset of “seasonal affective disorder” is related to shorter days and longer nights in fall and winter.\textsuperscript{50}

Evidence of melatonin as the circadian phase maker comes from studies showing shifts in body temperature and sleep timing following melatonin administration. Therefore, supplementation with melatonin at the right time can adjust circadian rhythm after a phase-shift due to shift work, jetlag, blindness, or sleep disorders.\textsuperscript{51} Evidence shows strong association between melatonin, sleep, and body temperature, energy, activity, and mental ability. The daytime low level of melatonin and high level of cortisol mediate daytime activities. Sleep and the dark-light cycle are also mediated by changes in melatonin and cortisol levels.\textsuperscript{52} The rise of melatonin two hours before bed allows for sleep onset, which in turn decreases the nocturnal body temperature.\textsuperscript{17} Studies show that blocking the beta adrenergic receptors suppresses nocturnal melatonin, and increases body temperature. This can be reversed by administering 5 mg melatonin. This further confirms the role of melatonin in body temperature regulation.\textsuperscript{53} Melatonin levels suppressed either pathologically or artificially by light lead to insomnia (initiation, maintenance, sleep quality).\textsuperscript{54} Figure 4, below shows the immediate effect of light exposure on nocturnal melatonin levels.\textsuperscript{55}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{melatonin_graph.png}
\caption{Effect of light exposure on nocturnal melatonin concentration. Normal pattern of melatonin synthesis in dark environment is shown in grey area. Reprinted with permission from Elsevier.\textsuperscript{55}}
\end{figure}
1.4. Sleep

Sleep is defined as a state characterized by a reduction in voluntary movement, reduced response to stimuli and, ultimately, loss of awareness. Sleep is divided into two parts: non-rapid eye movement (NREM) and rapid eye movement (REM). NREM itself is divided into three stages, which progress to the deeper sleep phase, or REM. Sleep restriction which reduces sleep duration affects the structure of sleep. Regulation of sleep in the brain is a complicated process involving several growth factors synthesized in response to neural activity. These growth factors also affect synaptic efficacy. Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are the two most important factors in the sleep regulation process, released by the production of ATP after neurotransmission. They increase NREM sleep. Inhibition of these two compromises spontaneous sleep and rebound sleep after an episode of sleep deprivation. After sleep deprivation, IL-1 and TNF concentrations rise. However, their over-activation, due to the activation of innate immune response, inhibits sleep. Other factors leading to this upregulation are light and excessive food intake. IL-1 leads to production of corticotropin releasing hormone (CRH) which, in turn, inhibits sleep dependent IL-1 inhibition. CRH also inhibits IL-1 which causes sleep inhibition. Other growth factors involved in sleep regulation are nitrous oxide (NO), growth hormone releasing hormone (GHRH) and nerve growth factor. Nuclear factor kappa B (NFkB), adenosine, and prostaglandin. NFkB is activated by sleep deprivation. Activation of NFkB leads to the activation of adenosine receptor, COX2, and NO synthase. COX2 leads to the synthesis of sleep promoting prostaglandin D2 (PGD2) and sleep inhibiting E2 (PGE2) that inhibits IL-1. Therefore, several positive and negative feedback loops are involved in sleep regulation or dysregulation. The neural system defined above affects the synaptic efficacy and downstream pathways that lead to wakefulness and consolidation of memory, or sleepiness.
1.5. Insomnia

Insomnia, a form of sleep restriction, is an important public health issue.\(^6^0\) A large population-based study calculated the prevalence of insomnia to be 6 - 48% after all diagnostic criteria were brought into consideration.\(^6^1\) It is defined as an inability to initiate sleep, waking up during the night, difficulty falling back to sleep, and non-restorative sleep.\(^6^2\) Delayed sleep phase disorder (DSPD) is a disorder of circadian rhythm and insomnia, in which the patient has difficulty initiating sleep at a normal time, therefore the circadian rhythm is delayed when compared to healthy individuals.\(^4^1\) DSPD patients are able to maintain sleep if they go to bed in their favourite time, but sleep initiation is delayed. This type of insomnia is associated with daytime sleepiness and many physiological dysfunctions. Ultimately long term DSPD insomnia is associated with psychological disorders, substance and alcohol abuse, and other neurobehavioral and physiological disorders. The prevalence of DSPD is 7-16% in the general population.\(^6^3\) To further understand the etiology of DSPD, nocturnal melatonin levels were measured in patients with this disorder and were compared with normal sleepers. Results showed that DSPD patients have significantly delayed dim light melatonin onset (DLMO) by three hours when compared with healthy sleepers (Figure 5). There was no difference in the duration of melatonin secretion but levels did not change significantly from DLMO to acrophase in DSPD patients.\(^6^4\) DLMO is considered an approximate night-time threshold of melatonin synthesis and secretion. DLMO makes an assessment of the circadian rhythm disorder in patients with insomnia easier than a provisional assessment. 10 pg/ml is considered the threshold for plasma DLMO and 3 pg/ml for saliva (Figure 6).\(^6^5\) Melatonin secretion starts at darkness and is suppressed by light, so it is very important to measure hormone levels in dim light to determine its onset time (DLMO) and to assess circadian rhythm in advanced sleep phase disorder (ASPD) or delayed in DSPD\(^4^1\). DLMO can be measured in both blood and saliva. Although DLMO measurement is currently used in research, there is no approved assay or standards in clinical settings.\(^6^6\)
1.6. Physiological Impacts of Insomnia

Chronic medical conditions were reported in 76 - 83% of the population of different ethnic groups due to poor sleep pattern. Adverse psychological and physiological effects of insomnia have a major impact on the quality of life of affected individuals. Recent experimental and epidemiological data have associated insomnia with multiple daytime symptoms and chronic conditions. Among physiological impacts of sleep disorders are metabolic disorders leading to obesity and type 2 diabetes. Insufficient sleep contributes to weight gain via excessive food...
intake associated with increased energy expenditure. Studies show that sleep restriction reduces daytime leptin while increasing daytime ghrelin. These hormonal changes lead to increased hunger and appetite. Increased food intake is beyond the energy needed leading to weight gain. Glucose intolerance increases and its clearance decreases upon sleep loss due to an impaired metabolism system. Studies suggest the potential benefit of recovery sleep.

Hypertension is among other physiological side effects of sleep restriction. It is shown in several studies that insomnia and short sleep duration is associated with hypertension. Duration of sleep is a predictor of the severity of hypertension. When adrenocorticotropic hormone (ACTH) and cortisol levels were compared in insomniac patients and normal sleepers in a lab environment, results were elevated nocturnal levels of those two hormones leading to hyper-activation of hypothalamic-pituitary-adrenal axis. This, in turn, causes several pathological consequences, such as hypertension. Obesity, increased insulin resistance and cardiovascular diseases are associated with elevated levels of ACTH and cortisol. Irwin et al. showed an increase in nocturnal norepinephrine in insomniac patients when compared to depressed and healthy individuals. This may be an indication of increased sympathetic activity, exhibited by increased heart rate, body temperature and metabolic rate. Other studies also showed coronary artery disease mortality after chronic insomnia in middle-aged men and women.

1.7. Psychological Impacts of Insomnia

Psychological effects of insomnia are getting a lot of attention of late. It is now understood that insomnia is a risk factor for the development of depression, anxiety and other comorbid psychiatric conditions. The most common impact of chronic sleep deprivation is daytime sleepiness, or sleep propensity, which is found to be dose-dependent. It is suggested that extracellular adenosine and its subtype receptors in the forebrain are homeostatic regulators of sleep. The nucleotide increases during excessive wakefulness and decreases during the sleep period. The risk of accidents and crashes have been shown to rise in epidemiological studies when sleep is less than 7 hours. Fatigue is another side effect of sleep deprivation, but it is more related to sleep quality rather than its duration. Work place injuries also rise in average due to poor concentration and the inability to maintain focus. Decreased cognitive function and memory impairment have also been documented as neuropsychological side effects of
sleep restriction due to altered hippocampal structure and function as the center of memory and cognitive function.\textsuperscript{82} A pilot study investigated this by performing MRIs on the brain of people with primary insomnia and normal sleepers, and observed a smaller bilateral hippocampal volume in the insomniacs.\textsuperscript{83} In 2012, a larger study replicated the findings of Riemann et. al., and showed that poor sleep quality leads to a smaller hippocampal volume, impaired memory and frontal lobe dysfunction due to chronic insomnia.\textsuperscript{84} A cohort study was conducted to compare the impact of the quality and quantity of sleep on mental status by comparing insomniac patients with shorter sleep duration to insomniac patients with normal sleep duration. Interestingly, a low sleep quality with normal duration was associated with a higher profile of depression, anxiety and low ego strength, while shorter sleep duration was associated with physical morbidities.\textsuperscript{85} A meta-analysis on longitudinal studies of insomnia as a predictor for depression showed that people with insomnia are at two fold increased risk to develop depression when compared to people with no depression.\textsuperscript{86} Although the pathophysiological pathway between insomnia and depression is still unknown, studies started investigating the role of insomnia in the regulation of emotion. It is suggested that insomnia as the product of dysregulation of neuronal pathways of circadian rhythm, affects emotion and its neuropsychological pathway.\textsuperscript{87} A pilot study linked cortisol (wakefulness hormone) and melatonin (sleep hormone) levels and suggested that the phase angle between the peak concentrations of the two hormones can be a biomarker for major depression disorder (MDD). In normal sleepers, cortisol and melatonin work in opposite directions, with melatonin’s onset at night and cortisol’s peak in the morning. Results showed that the phase angle was significantly increased in MDD. It also showed that nocturnal plasma melatonin concentration is significantly lower in MDD (22.67 ± 9.08 pg/ml) compared to healthy (47.82 ± 14.76 pg/ml), $p = 0.015$. This indicates the importance of lower nocturnal melatonin levels in pathophysiology of MDD. Both the phase angle between melatonin onset and cortisol acrophase, and nocturnal melatonin levels can be used as biological markers in MDD diagnosis and treatment (Figure 7).\textsuperscript{88} An association between circadian rhythm and bipolar disorder was also investigated with a structured interview and questionnaire in a multi-center study. Results showed that there is a positive relationship between insomnia, depression and bipolar disorders. The more severe the bipolar symptoms, the more symptoms of insomnia were observed.
Although the direction of causality is not clear, insomnia should also be a target in the treatment of depression and bipolar disorders.\textsuperscript{89}

![Graph of hypothetical phase relationships between cortisol and melatonin markers.](image)

\textit{Figure 7: Relationship between melatonin peak and cortisol acrophase in normal sleepers. Reprinted with permission from Elsevier.}\textsuperscript{88}

### 1.8. Insomnia in Pregnancy

Primary insomnia and/or insomnia related to depression and anxiety affects many pregnant women. Approximately 75\% of pregnant women report multiple awakenings during the night for several reasons.\textsuperscript{90} Research shows that although sleep durations remain within the standard range, the quality of sleep decreases from the beginning of pregnancy, and the risk of a sleep disorder increases with gestational age – with a two fold increase in the third trimester.\textsuperscript{91} 73.5\% of pregnant women experience insomnia in the third trimester.\textsuperscript{92,93}

Although there is a lot of epidemiological data on the impact of insomnia on pregnancy and its outcome, little is known about the pathophysiology involved. Several factors may be involved for the development of sleep disorders during pregnancy. Hormonal changes, such as increased estrogen, progesterone, prolactin, and cortisol may affect sleep patterns.\textsuperscript{94} Increased plasma volume as compared to red cell mass,\textsuperscript{95} leads to a 50-85\% increase in renal blood flow and the urge to go to the bathroom,\textsuperscript{96} all of which lead to sleep interruption. In the first trimester
morning sickness is the main reason for sleep disturbance. Acid reflux may be another reason of pregnancy-induced sleep interruption. In a review of literature by Brzozowska et. al. the role of melatonin in the protection of the esophagus from ulcers or damage by acid reflux was confirmed. Depression symptoms are also known as risk factors for insomnia in pregnancy. There is 2.6 fold increased risk for the development of insomnia in those with depression compared to pregnant women without depression. In line with this, pregnant women with mood and anxiety disorder are at 1.95 fold increased risk for shorter sleep duration, compared to those without a sleep disorder. Perceived stress level in this group is 3.33 times higher, and increased by almost 6 times when BMI is higher than 25 kg/m². In a cohort study, Okun et. al. showed more fragmented sleep in depressed pregnant women at 20 and 30 weeks gestation, but sleep disturbance was the same in both groups at 36 weeks gestation regardless of depression or using selective serotonin re-uptake inhibitors (SSRIs). This indicates that non-depressed pregnant women experience sleep issues towards the end of pregnancy due to physiological and physical changes (such as increased abdominal size and increased physical discomfort), as well as increased progesterone levels, whereas sleep in depressed pregnant women is poor in the beginning and remains the same throughout the pregnancy. The same study also confirmed insomnia as a risk factor for the development of depressive symptoms. Pregnant women who are not depressed at 20 weeks, develop depressive symptoms due to insomnia later in pregnancy.

1.9. Treatment of Insomnia

While insomnia is associated with many adverse lifestyle and health outcomes, pharmacotherapy is a common and promising way of treating the condition. Because sleep disorders such as insomnia are associated with depression and anxiety symptoms, drugs that are effective in the treatment of insomnia may improve depression and anxiety symptoms better than other medications such as antidepressants. Several pharmacological treatments have been approved and used for the treatment of insomnia. Medications approved for insomnia include benzodiazepines (BZDs), hypnotic non-benzodiazepines and melatonin receptors agonists. Off-label use of other medications includes tricyclic antidepressants, trazodone, mirtazapine, antipsychotics, anticonvulsants, and over-the-counter products such as antihistamines and melatonin. Non-pharmacological treatments, including acupuncture, massage, yoga, and
aerobic exercise are also commonly used. Pharmacological treatments differ in their mechanism of action, efficacy and safety, therefore the prescribing clinician should be aware of the type of sleep disturbance individuals have on a case-by-case basis. Another factor in personalizing the medications is the existence of comorbid disorders such as depression and anxiety, mood and bipolar disorders, other psychotic disorders, substance abuse, and chronic pain. Patients with bipolar disorder and depression have the most arrhythmic biological clocks, low melatonin, irregular body temperature, variability in day to day activities, and variability in sleep duration and quality.

1.9.1. Benzodiazepines (BZDs)

BZDs work by their hypnotic and sedating properties. Examples of the class are triazolam, diazepam, lorazepam, temazepam, clonazepam, etc. They exert their effect on the GABAergic system, the primary neurotransmitter inhibitor in the brain. Their mechanism of action is binding to GABA-A receptors, causing conformational change in the chloride ion channel proteins. The change leads to CL- influx, hyperpolarization and reduced neuronal firing. This pathway causes sedation and is sleep-promoting. Sleep onset and maintenance was found to improve with BZD. It also has anti-anxiety and physical pain-alleviating effects in binding to other GABA-A receptors elsewhere in the brain. Therefore, there are side effects to their actions. Although the mechanisms of action of BZDs are the same, they are still different in the rates and pathways of metabolism, half-life and elimination rate. Those with shorter half-life and lower dose have fewer next-day symptoms, while those with longer half-lives (clonazepam) cause daytime sedation and drowsiness, increased incidence of falls and accidents and cognitive impairments. Although the efficacy of BZDs are documented in well-controlled studies, their efficacy with long-term use is still under question. Benzodiazepines are designed for a period of maximum of three months. Withdrawal symptoms upon discontinuing these medications are severe and sometimes prevent the user from ever stopping the medication. The rate of dependence is high, anywhere from 40% to 82% in different patient groups. Insomnia, muscle spasm, gastric upsets, headaches due to scalp muscle stiffness, and jaw clenching at night are amongst the milder withdrawal symptoms. Seizure, psychosis, confusion, paranoia, and visual hallucinations are among the more severe withdrawal symptoms requiring pharmacotherapy.
1.9.2. Non-benzodiazepine hypnotics (Z-drugs)

While BZDs are the conventional therapy for insomnia, Z-drugs are now the drugs of choice. Their mechanism of action is quite similar to BZD, although they are chemically and structurally different from BZD. One main advantage of this class is its faster elimination rate and their relative affinity for alpha binding sites of GABA-A subunit receptors, which in turn reduces some of the side effects of the BZDs. Zolpidem binds to the alpha-1 subset of receptors, and therefore has anticonvulsant, amnestic and motor impairment as side effects. Eszopiclone binds to alpha-2 and alpha-3 subsets and acts as an anti-anxiety and muscle relaxant agent. As mentioned, they have shorter elimination half-lives than BZD, so they aid in decreasing sleep latency and initiating sleep. Less daytime sedation is expected with these agents. Evidence for the efficacy and safety of z-drugs is reassuring in several studies with anti-depressants in patients with comorbid psychiatric conditions, but their long-term efficacy has yet to be determined. However, they are not side effect-free. The most common side effects of these, and similar to BZDs, include dependence, higher risk of accidents and falls, cognitive disturbances, sedation, dizziness, and psychomotor performance.

Hypnotics are designed for the short-term treatment of insomnia or situational insomnia (such as acute episodes of stress, jet lag, shift work, or medical illness). Despite these guidelines, many individuals continue therapy with hypnotics for a long period of time – often years – either for continuing sleep problems or prophylactic use. Yet they complain about significant sleep issues. This is called hypnotic-dependant insomnia. Symptoms include the return of sleep difficulties, rebound insomnia, and exacerbation of the original insomnia. There are several psychological and behavioural effects of the long-term use of hypnotics. Tolerance will develop with continuous use and increasing dose seems to be the solution. However, despite reaching the maximum therapeutic dose, sometimes the patient still suffers from insomnia. Therefore, loss efficacy leads the patient to stop taking the medication resulting in withdrawal symptoms following the cessation of the medication. Rebound insomnia is one of the symptoms of withdrawal, where the patient feels that she needs to get back to taking the medication or she cannot sleep. Sleep is conditioned in chronic hypnotic use by alleviating sleeplessness, so hypnotic use is negatively reinforced, and this chain reaction could continue for years. Reverse sleep state misperception is another reason for continuous therapy, where un-medicated
individuals underestimate their sleep time and overestimate their waking time, while it is the other way in medicated individuals. Very similar effects have been seen with benzodiazepine dependent individuals.

1.9.3. Antidepressants

1.9.3.1. Tricyclic antidepressants (TCAs)

TCAs include doxepin and trimipramine in lower doses than they are used for depression. They act by inhibiting norepinephrine, histamine and acetylcholine, all of which are involved in wakefulness as described before. Studies on these agents are limited, and mostly come from those with comorbid depression. Their side effects are mostly weight gain and sedation along with the anticholinergic side effects described above. They are not potentially abusive, but are fatal in overdose, so prescribing them to patients with suicidal thoughts should be done with caution.

1.9.3.2. Trazodone

Trazodone is the most common drug used off-label in low dose for the treatment of insomnia. Although there is a lack of data in the effectiveness of the drug, it has been prescribed commonly. It acts by inhibiting serotonin 5HT2 receptors, norepinephrine alpha-1 receptors and histamine-1 receptors. Genetic polymorphism plays an important role in the therapeutic effects of this medication, where some patients may experience daytime sedation with it. Its side effects are mostly anticholinergic causing dry mouth, constipation, urinary retention and blurred vision. It doesn’t have the potential to be an abuse drug so it can safely be used. Since it is an antidepressant it can be used for the treatment of mood disorders or depression in higher doses, but in the preliminary research it is recommended mostly with bupropion or fluoxetine in the treatment of both depression and insomnia.

1.9.3.3. Mirtazapine

Mirtazapine works by inhibiting serotonin 5HT2 and 5HT3 receptors, alpha-1 and alpha-2 adrenergic receptors, and histamine-1 receptors. Efficacy studies on this drug are limited to those where it has been used with fluoxetine for the treatment of depression, and a pilot study
in patients with depression.\textsuperscript{113,114} Mirtazapine is associated with weight gain, sedation, constipation, and dry mouth. Also it has a low potential for abuse, too.\textsuperscript{100}

1.9.3.4. Ramelteon (approved in the USA, but not in Canada)
Ramelteon is a prescription melatonin receptor agonist that binds to MT1 and induces sleep by inhibiting signals from the SCN. Many studies evaluate the effectiveness and safety of ramelteon, mostly in elderly population. It is also studied in children with neurodevelopmental disorders. Headache and somnolence has been reported with its use, but it has no potential for abuse.\textsuperscript{100}

1.9.3.5. Agomelatine (approved in Europe)
Agomelatine is a melatonin receptor agonist with a high affinity for MT1 receptors. It causes inhibition of neural activity in the SCN. Agomelatine is also considered an antidepressant because of its activity and inhibition of serotonin 5HT2 receptors. The efficacy of agomelatine has been assessed in many studies including both healthy volunteers and people with insomnia, and was found to improve the structure of sleep by increasing REM and the quality of sleep. An increase in the quality of NREM sleep was suggested as one of the mechanisms of action of agomelatine.\textsuperscript{115}

1.9.4. Antipsychotics
Low dose antipsychotics have been in use for insomnia. The most commonly used are quetiapine and olanzapine. They work through inhibition of dopamine receptors, histamine, serotonin 5HT2, acetylcholine, and norepinephrine. The long absorption half-life of olanzapine makes it a good choice for sleep maintenance, while quetiapine can be used for both onset and maintenance due to the short absorption half-life and long elimination half-life.\textsuperscript{100} Efficacy studies on these two medications are limited to studies in primary insomnia patients and healthy volunteers. Quetiapine has a relatively lower side effect profile than olanzapine. It may cause sedation, dizziness, and the anticholinergic effects described elsewhere in this paper. Olanzapine has been shown to cause metabolic disorder and insulin resistance, tardive dyskinesia, akathisia, and increased appetite among other side effects. They both have low potential for abuse, so they may be used safely.\textsuperscript{100}
1.9.5. Anticonvulsants

Examples of anticonvulsants are pregabalin and gabapentin. They bind to alpha-2-delta subunit of N-type voltage-gated calcium channels that inhibit the activity of norepinephrine and glutamate. Sleep-enhancing properties of these medications have been tested in several studies and different patient populations, as well as in healthy volunteers. These include patients with primary insomnia, neurologic pain, restless leg syndrome, and seizures. Gabapentin has fewer side effects than pregabalin that include ataxia and diplopia. Pregabalin, though, can cause increased appetite, dry mouth, peripheral edema, and cognitive impairment. It is also associated with abuse, and should be used with caution.100

1.9.6. Over-the-Counter Products

1.9.6.1. Antihistamines

The most common over-the-counter (OTC) products used for insomnia are diphenhydramine, dimenhydrinate, and doxylamine. They act as histamine-one receptor blockers and promote sleep by blocking wake-promoting histamine. Muscarinic anticholinergic properties also induce sleep, and side effects include dry-mouth, blurred vision, urinary retention, and delirium. The efficacy of doxylamine for insomnia has not yet been established. The efficacy of diphenhydramine has been established for sleep maintenance but not sleep onset.103 Their potential for abuse is almost none, and they can be used for both insomnia and comorbid allergies. Consecutive use of diphenhydramine is reported to lose its effect on sleep, but more research is still needed.100

1.9.6.2. Melatonin

As described earlier, melatonin is an endogenous hormone produced in the pineal gland and is mainly involved in the sleep-wake cycle by acting on MT1 and MT2 receptors. Melatonin has a very short half-life and is therefore used mostly for sleep onset. No tolerance or dependence has been seen with it and there is no potential for abuse. There are no regulations or guidelines for melatonin dosage or time of administration. Doses can be as low as 1 mg up to 10 mg in Canada. Time of administration has not been agreed upon among clinicians and researchers, and it can be any time from three hours before bedtime to bedtime itself. As a result, studies so far could not establish a unique efficacy profile for the exogenous hormone’s use in the
treatment of insomnia. There are still a few studies that showed some efficacy and a high safety profile in some patient populations. Currently, melatonin is available with different doses over-the-counter. Prolonged-release melatonin 2mg was studied in two different treatment periods of 6 months and 12 months. At the end of the 12 months, and after discontinuing therapy, urine samples were collected for melatonin metabolite (6-sulfatoxymelatonin). Sleep quality was significantly better when compared to pre-treatment sleep. Sleep quality was still higher than baseline when treatment stopped after 6 or 12 months. There was no tolerance reported. No dependence has been observed and the endogenous production of the hormone after the treatment was stopped was no different than baseline. A meta-analysis of 19 studies showed that melatonin significantly reduces sleep latency and increases total sleep time. It also showed that long term melatonin therapy does not decrease effects. Although the benefit of taking melatonin compared with a placebo is not as significant as other pharmacological treatments, its low side effect profile makes it a potential candidate for the treatment of insomnia.

1.10. Effects of Treatments on Melatonin Levels

A clinical study comparing duloxetine and fluoxetine with a placebo found increased concentration of the urinary metabolite of melatonin (a-MT6s) after 8 weeks of antidepressant therapy. Authors of this paper concluded that the increased melatonin concentration was due to actual pharmacological actions of antidepressants on melatonin synthesis (Figure 8). This has been shown by previous studies with TCAs by 5-HT uptake inhibition. Increased melatonin synthesis by SSRIs, duloxetine and fluoxetine in this study were shown to be the result of modulating neurotransmission. Fluoxetine increases 5-HT and norepinephrine (NE). NE stimulates the noradrenergic pathway and interacts with 5-HT, increasing the activity of N-acetylserotonin (NAS) the enzyme necessary for melatonin biosynthesis.
1.10.1. Zopiclone

Zopiclone has no effect on melatonin concentration when compared to zolplon and a placebo, and it has a non-benzodiazepine hypnotic in rabbits. Similar results have been found in healthy volunteers following acute and subchronic administration of zopiclone. Therefore, the hypnotic effects of zopiclone are due to changes on EEG (electroencephalogram) parameters of sleep. Rebound insomnia was observed in this patient population after subchronic discontinuation (Figure 9).
1.10.2. Benzodiazepines

It is suggested that sleep-promoting effects of BZD are through their effect on peripheral GABAergic systems. As part of the neural pathway to melatonin release, GABA plays an important role. GABA receptors in the dorsal hypothalamus are responsible in modulating melatonin synthesis. The activation of GABA and its release from SCN terminals results in transmission of light to the pineal gland, hence a reduction in melatonin synthesis. Benzodiazepines modulate the GABAergic system by upregulation of their receptors, increase their inhibitory action, and suppress nocturnal melatonin synthesis.

1.10.3. Bupropion

Bupropion as an antidepressant increases melatonin synthesis indirectly by reducing IL-6. IL-6 is a pro-inflammatory cytokine, which is increased in people with depression. Cortisol, TNF, and other cytokines also rise with depression. Bupropion reduces IL-6 and indirectly enhances melatonin production in counteraction behaviour to inflammation.
1.10.4. Mirtazapine

Mirtazapine decreases cortisol and dehydroepiandrosterone, which is produced in the adrenal gland and results in hyperactivity of the HPA axis and insomnia. Decreased levels of these hormones promotes melatonin synthesis.\textsuperscript{123}

1.10.5. Trazodone

An increased melatonin level and improved sleep with trazodone has been documented in clinical studies, as well.\textsuperscript{124,125}

1.10.6. Quetiapine

Quetiapine is indirectly involved in increased melatonin concentration. A randomized double-blind placebo-controlled study in healthy volunteers showed a significant decrease in nocturnal cortisol levels, but a non-significant change in melatonin levels directly after treatment with low dose quetiapine.\textsuperscript{126} However, lower cortisol levels are associated with higher melatonin levels.\textsuperscript{127}

1.11. Melatonin Levels in Pregnancy

In normal pregnancies, an increase in nocturnal maternal melatonin levels after 24 weeks to term have been documented (Figure 10).\textsuperscript{128} Serum melatonin levels were measured in all trimesters of pregnancy, with first trimester 29.7±9.9, second trimester 39.1±11.2, and third trimester 76.5±38.3 (Mean ± SD), units presented in pmol/l.\textsuperscript{129} Moreover, when plasma melatonin concentration was compared between the maternal vein and umbilical vein, no significant differences were observed. A significant positive correlation was observed in melatonin levels in the umbilical vein and maternal vein (r=0.979). Similar levels were seen in the umbilical artery and vein (Figure 11). Two hours after oral administration of 3 mg of melatonin at term, similar levels were observed in maternal and umbilical veins. This finding suggested a free exchange of melatonin between maternal and fetal circulation due to the permeability of the feto-placental barrier. The concentration of melatonin declined after two hours in similar rates in both maternal and umbilical veins. Maternal melatonin levels declined at a similar rate after their decline upon oral administration.\textsuperscript{130} Several suggestions have been made as to why melatonin levels were similar in maternal and fetal veins. Previous investigators reported that the pineal gland of the neonate secretes melatonin but not in a
circadian way, indicating that fetal melatonin is mainly a supply from the maternal pineal gland and permeability of the fetal-placental barrier. Later, in 2001, Nakamura and colleagues investigated changes in melatonin levels during pregnancy in normal pregnancies and those complicated by preeclampsia and intrauterine growth restriction (IUGR). They also investigated the source of melatonin during pregnancy. Higher melatonin concentration was found in night time compared to daytime in normal singleton pregnancies. The daytime melatonin level rose after 32 weeks, while it increased after 28 weeks gestation in twin pregnancies. Night time melatonin levels started a significant rise after 24 weeks gestation. Melatonin levels were found to be significantly lower in IUGR and preeclampsia pregnancies with no significant rise even in late pregnancy (Figure 12). The pathophysiological reason for lower melatonin levels in pre-eclamptic pregnancies was explained by Lanoix et. al. They found reduced activity of melatonin synthesizing enzymes, AANAT and HIOMT, in the placenta. MT1 and MT2 receptors were also expressed lower in these placentas. This indicates down-regulation of melatonin receptors by lower melatonin levels in placenta. Melatonin and its receptors protect the placenta against myocardial ischemia. Melatonin concentration was not different in daytime and night time deliveries. Findings of this study suggested a role for fetal-placental unit in melatonin concentration because levels are lower in pre-eclamptic and IUGR pregnancies. In 2005, Iwasaki and coworkers showed the presence of mRNA transcripts of melatonin-synthesizing enzymes and both melatonin receptors in the first-trimester placenta. They also showed significant involvement of melatonin in the regulation of hCG-B in the first trimester trophoblast cells, which is very important in maintaining pregnancy. hCG-B is used as a marker in pregnancy diagnosis. Later in 2006, Lanoix et. al. for the first time showed the expression of nuclear and membrane MT1 and MT2 G-protein coupled receptors (GPCR) in human term placenta and choriocarcinoma cell lines. The results of this study confirmed that human trophoblast are targets for melatonin acting in autocrine/paracrine way and its role in fetal development. Previously, MT1 and MT2 were discovered and located in the brain and retinas, in other tissues such as ovarian follicles, prostate, kidney, and the immune system. In 2008, the expression of melatonin receptors in human term placenta, and synthesis of melatonin in placenta and properties of the melatonin receptors were investigated. Using RT-PCR and western blot analysis it was found that arylalkilamine N-acetyl transferase (AANAT) and hydroxyindole o-methyltransferase
(HIOMT) melatonin synthesizing enzymes are present in human villous cytotrophoblast (vCTB), syncytiotrophoblasts (STB) and placenta. STB is responsible for gas and nutrition exchanges between the maternal and fetal circulations, hCG and hPL are both responsible in maintaining pregnancy. The activity of AANAT is three and five fold higher in vCTB and STB, respectively. The activity of HIOMT is also two and 2.5 fold higher in vCTB and STB, suggesting that local synthesis in these two sites are more than in the placenta. Melatonin synthesizing enzymes and its receptors are also found in endothelial cells surrounding fetal capillaries. Their activities in the placenta are similar to those in the pineal gland at night time. Considering the weight of a human term placenta (470g) and a human pineal gland (0.14g), N-acetylserotonin (NAS) and melatonin production are 10,000 and 225 fold more in the placenta, respectively. This finding suggest a very important paracrine, autocrine and intracrine role for melatonin involved in placental development and function, and it significantly contributes to increased maternal melatonin levels during pregnancy. However, whether placental melatonin production follows a circadian rhythm system is not yet known.

Figure 10: Changes of serum melatonin levels in pregnancy at night (solid) and day (dotted line). Significant rise after 24 weeks and peak at 36 weeks gestation is shown. Reprinted with permission from John Wiley and Sons
1.12. Impacts of Insomnia in Pregnancy

There are physiological and psychological consequences following insomnia in pregnancy. Glucose intolerance and gestational diabetes mellitus (GDM) are amongst the physiological impacts of insomnia in pregnancy. The prevalence of GDM in the US is approximately 7%, affecting 200,000 women every year. Risk increases by obesity and/or type 2 diabetes (T2D). Earlier it was mentioned that insomnia is a risk factor for type 2 diabetes. As a result, insomnia and T2D may increase the risk of a pregnancy to develop GDM. In a cohort study following up
pregnant women from early pregnancy with their sleep duration, it was found that women with shorter sleep duration are at 1.86 fold increased risk for development of gestational diabetes. GDM adversely affects both maternal and fetal health and pregnancy outcomes. Mothers with GDM are at increased risk for cardiovascular disease and metabolic dysfunction. GDM put the pregnancy at risk for pre-eclampsia, preterm labor and caesarean section due to the accumulation of excessive amniotic fluid, and infection. In addition to maternal risks associated with GDM, children of diabetic mothers are at increased risk for macrosomia, related birth injuries, respiratory problems, neonatal hypoglycemia, and jaundice.

Hypertension is one of the other physiological impacts of insomnia in individuals. In a pregnancy prospective cohort study, mean diastolic, systolic and mean arterial pressures were measured. It was found that systolic blood pressure is highest in those who sleep less than six hours at night in both first trimester and third trimester, a 1.62 and 4.41 fold increase respectively. Also, diastolic blood pressure blood pressure was highest in short time sleepers in both first and third trimesters, 1.85 and 3.52 fold increase respectively. Mean arterial pressure was significantly higher in both short and long sleep durations. In the previous chapter mechanisms responsible for hypertension associated with insomnia have been explained. Changes in body’s hemodynamics such as temperature, heart rate and hyper-activation of the HPA axis are all factors in increased risk for hypertension. Circadian system dysregulation and insomnia is now known to be a risk factor in depression and mood disorders in pregnancy. In line with this, studies investigated plasma melatonin levels in pregnant women with and without depression. Levels were measured at baseline, synthesis onset, and offset times. It was found that daytime melatonin levels are significantly higher in depressed pregnant women, while night time levels were significantly lower when compared to healthy pregnancies. Melatonin synthesis onset time and offset time were also significantly earlier in depressed pregnant women. Second finding of this study was that the number of previous episodes of depression was negatively correlated with melatonin offset time \( r=-0.66 \). Therefore, previous episodes of depression may be a risk factors in earlier onset and offset times of the hormone production and its low levels in pregnancy. Depression disorders in pregnancy are explained by loss of sensitivity of melatonin receptor to estradiol and progesterone levels (Figure 13). Therefore, rise in gonadal hormone in healthy pregnancy results in an increase in melatonin levels but not in depressed pregnancies.
1.13. Impact of Insomnia on Pregnancy Outcome

While insomnia has severe physiological and psychological impact on maternal health, it can impact the outcome of pregnancy too. Micheli and colleagues investigated the question by following up women throughout pregnancy and post-partum. They excluded pregnancies complicated by preeclampsia, gestational diabetes, and obesity. They found that insomnia in the third trimester can significantly affect the outcome of pregnancy. Sleeping less than five hours at night is associated with 1.7-fold increase for preterm birth. Relative risk for medically indicated preterm birth was 2.4. Medically indicated preterm birth was defined as emergency C-sections or vaginally induced birth. Spontaneous preterm birth was 1.6 fold increased when sleep was disturbed in the third trimester. The mechanisms for preterm birth due to insomnia were suggested by Okun et. al. proposing that short sleep duration (<6 hours per night) is associated with a rise in IL-6. IL-6 is a pro-inflammatory cytokine, and the role of cytokines in initiating labor has been studied. Pro-inflammatory cytokines can stimulate the synthesis of prostaglandin, which plays a major role in uterine contraction during labor. Arntzen and colleagues measured levels of TNF, IL-1, IL-6, IL-8, and soluble TNF in the amniotic fluid of...
three groups of pregnant women. The first group delivered preterm by c-section, the second group delivered prematurely, and the third group had term vaginal births. IL-1, IL-6, and IL-8 were found higher in preterm deliveries.\textsuperscript{150} The same result was achieved by Hillier et. al. in women who delivered before 24 weeks and women who delivered after 34 weeks.\textsuperscript{151}

1.13.1. Postpartum Depression

Post-partum depression is defined as the onset of depression approximately 4 weeks after delivery, lasting for as long as 6 months. Contributing factors to post-partum depression are defined as increased demand in infant care, changes in family and marital relationships and the impact on work and social activities, previous histories of depression, and decreases in progesterone and estrogen after delivery.\textsuperscript{152} 10-15\% of women experience postpartum depression. Symptoms are almost the same as depression but may differ in a few, such as anxiety about caring for the baby or an inability to fall asleep when baby is sleeping. The role of sleep deprivation or insomnia in depression has been studied and explained earlier in this paper. It is documented that people with insomnia experience depression 31.1\% more than those without insomnia.\textsuperscript{152} Goyal et. al. followed up pregnant women in their last month of pregnancy and 3 months post-partum, and found an association between sleep quality and quantity in the last month of pregnancy with post-partum depressive symptoms.\textsuperscript{153}

1.14. Treatment of Insomnia in Pregnancy

1.14.1. Non-Pharmacological Treatments

1.14.1.1. Acupuncture

In non-pregnant individuals acupuncture was shown to be an effective treatment.\textsuperscript{154} Guerreiro da Silva et. al. investigated the safety and efficacy of acupuncture in the treatment of insomnia in pregnancy and found that insomnia was significantly lowered in the study group, compared with the control group. No adverse effects were found in the 17 pregnant women on whom the acupuncture was performed. People with a history of insomnia or taking sleep medications were excluded. First trimester pregnancies were excluded too. Further research is needed to investigate the safety of acupuncture in the first trimester.\textsuperscript{155}
1.14.1.2. Yoga

Yoga has been looked at in the treatment of insomnia in pregnancy, and shown to be effective. A pilot study investigated the effectiveness of mindful yoga in treating insomnia in pregnancy. Performing yoga in the second and third trimester showed sleep improvement as gestation increases with a 7-week program. It further showed that yoga may be more effective if it starts in the second trimester. Pregnant women in the first trimester were excluded. Pregnant women with a history of mental illness or insomnia or currently on medication were also excluded.

1.14.1.3. Aerobic Exercise

Studies have been conducted on the effect of exercise on the overall pregnancy well-being, and found lower perceived pregnancy symptoms, but insomnia has not been looked at as a primary outcome of the study. A longitudinal study compared exercise and non-exercise pregnant women in pregnancy and found that overall symptoms of anxiety and insomnia were significantly lower in the exercise group. Pregnant women were recruited in the second trimester and continued exercise into the third trimester. Also, women with a high risk pregnancy, unable to perform exercise and Over-weight women, BMI>30, were excluded.

1.14.1.4. Massage Therapy

Massage therapy has been shown to be effective in reducing stress, improving mood and sleep when pregnant women were followed up in a 16-week program starting in the second trimester and through to the end. Urine samples were collected on the first and last day of the program for levels of norepinephrine (NE), cortisol, serotonin, and dopamine. While higher levels of dopamine and serotonin was observed at the end of study, (NE) and cortisol levels were found lower, associated with the lower level of anxiety, depression and mood problems. Better neonatal outcomes were also seen. Massage therapy has also been studied with yoga to treat depression in pregnancy. Pregnant women in the second trimester and diagnosed with depression started a 12-week massage and/or yoga program until 32 weeks. Results showed a decrease in depression and anxiety. No difference was seen in neonatal outcomes such as gestational age at birth and birth weight between the two treatment groups of yoga and massage therapy.
1.14.2. Pharmacological Treatments of Insomnia in Pregnancy

Limitations with drug safety studies in pregnancy are that in many cases concomitant drugs are used for other conditions as well. These may include pregnancy induced conditions or pre-existing physical and mental conditions. Pregnancy complications may result from an underlying maternal condition and a drug study may not be able to rule it out. When discussing the risk for major malformations, it should be compared with the general population’s risk, which is suggested to be 2 - 3%. A major malformation is defined as one that affects survival (anencephaly) and has a major impact on the quality of life of the survived child (mental retardation), or requires a major operation (cleft palate/lip, cardiac defects).\textsuperscript{161}

1.14.2.1. Benzodiazepines and BZD receptor agonists

There is a critical review\textsuperscript{162} on the exposure to BZDs during pregnancy especially first trimester included 12 studies. Results are as follows:

1.14.2.2. Alprazolam

There is no increased risk for major malformations (MM) observed in a small sample size. A high rate of spontaneous abortion (SA) was seen.

1.14.2.3. Clonazepam

There is no increased risk for MM upon exposure in the first trimester.

1.14.2.4. Chlorodiazepoxide

There is no increased risk observed for MM, but a higher rate of SA and lower birth weight was reported.

1.14.2.5. Diazepam

Different MMs were reported in two case control studies. A recall bias was also reported in these studies. Two other studies investigating impacts of this medication didn’t find any increased risk for birth defects when studied in the first trimester. However, a higher rate of SA was shown.\textsuperscript{162}
Overall, benzodiazepines have not been shown to be a strong teratogenic when used in the first trimester. They are shown to be associated with lower birth weight and preterm birth.\textsuperscript{163, 164} The small increased risk seen for oral cleft palate/lip in a meta-analysis, was not confirmed by later studies.\textsuperscript{165}

**1.14.3. Antidepressants**

Antidepressants include: selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), bupropion, mirtazapine, and tricyclic antidepressants (TCAs) among others. A meta-analysis specifically on SSRIs found paroxetine an increased risk for cardiac malformations and recommended their avoidance in the first trimester. Escitalopram, citalopram, and sertraline were not found teratogens in the first trimester.\textsuperscript{166} A meta-analysis investigating the relationship between antidepressant exposure and neonatal outcome found significantly lower birth weight and preterm birth (<36 weeks) in those exposed sometime during pregnancy.\textsuperscript{167} Another meta-analysis was also conducted on the neonatal risks after exposure to antidepressants prenatally. Results show that exposure to these drugs is significantly associated with poor neonatal adaption syndrome (PNAS). Some of the symptoms may include respiratory depression, jitteriness and tremors.\textsuperscript{168}

**1.14.4. Antipsychotics**

As mentioned before, low dose atypical antipsychotics are now commonly prescribed as sleep aids and the most common ones are quetiapine and olanzapine. A population-based cohort study used prescription filled data to investigate the maternal and neonatal outcome after exposure to these drugs in the first and second trimesters of pregnancy. Results from the matched-cohort showed no significant difference in the rate of hypertension and gestational diabetes in users and non-users of these drugs. In the neonatal outcomes results, birth weight and preterm weight were not significantly different between the groups. However, results from the unmatched-cohort and a comparison with general population showed significant increased risk for hypertension and gestational diabetes. Delivery complications included operative vaginal delivery, premature birth and c-section rates, which were all higher in the exposed group. Also, lower birth weight, preterm birth and poor neonatal adaptation syndrome were observed in infants exposed, compared with the general population.\textsuperscript{169}
1.14.5. Antihistamines

Mitchell et. al. showed in a retrospective study that antihistamines as a class are not associated with any increased risk for birth defects and malformations. However, there is controversy over the safety of antihistamines, in particular doxylamine in the first trimester. Doxylamine is used as a treatment for both nausea and vomiting of pregnancy and insomnia for its histamine-1 blockade properties. A meta-analysis showed that antihistamine use in the first trimester of pregnancy is not associated with any increased risk for major malformations with an odds ratio of 0.76. Later in 2014, Persaud et. al. re-analysed the previous meta-analysis and found a higher odds ratio of 1.04 for taking antihistamines in the first trimester. Based on this meta-analysis more studies are needed to reach a conclusive safety profile. Contrary to this, an older meta-analysis on Bendectin, the drug of choice for the treatment of morning sickness in the first trimester (containing doxylamine and vitamin B6), did not conclude teratogenicity associated with Bendectin. In previous studies the rate of birth defects were not significantly different between exposed and non-exposed infants.
2. Rationale

Sleep is important for the physiological and psychological wellness, quality of life, and overall well-being. Primary insomnia or insomnia secondary to depression and anxiety are major health issues amongst pregnant women. Elevated blood pressure, gestational diabetes, preterm birth, preeclampsia, prolonged labor, increased cesarean section rates, prenatal and post-partum depression are amongst adverse pregnancy outcomes associated with sleep disorders in pregnancy. Poor sleep quality and quantity in the last month of pregnancy are associated with post-partum depression.

Although there is a lot of epidemiological data on the impact of insomnia on pregnancy and its outcome, little is known about the pathophysiology involved. Understanding the pathophysiology and etiology of sleep helps address the issue with better treatment regimen. Physiological and psychological impacts of untreated insomnia on maternal and fetal health and on pregnancy outcome lead to more in-depth investigation of the disorder and treatment options. Since melatonin is a naturally occurring hormone in our body and is responsible for the sleep-wake cycle and circadian rhythm, it is very important to find out if pregnant women with insomnia have lower levels of the hormone. To our knowledge no study has examined the effects of melatonin levels on the pathophysiology of insomnia.

In normal pregnancies, an increase in nocturnal maternal melatonin levels after 24 weeks to term has been documented. Maternal and umbilical cord melatonin levels are highly and positively correlated when measured at term, suggesting that the hormone freely crosses the human placenta. Local synthesis of the hormone by the human trophoblast have been identified as indication of autocrine, paracrine, intracrine and endocrine function of the hormone. However, there is still a question of whether placental melatonin levels play a role in the sleep patterns of pregnant women.

The rationale for doing this study was to find out if salivary melatonin levels in pregnant women with insomnia was lower than in healthy pregnancies. An understanding of the melatonin levels in insomniac pregnancies aids in choosing a better treatment such as melatonin as a natural hormone therapy as opposed to psychotropic medications and their potential risk for the health of pregnancy and the unborn child. If lower melatonin levels are found it leads us
to further research the safety and efficacy of melatonin supplementation to treat insomnia in pregnancy (without the side effects of pharmacotherapy on both mother and fetus/baby, which are explained in detail earlier in this paper). Melatonin is a natural hormone, and it was explained above that it has a high efficacy profile in treating insomnia with minimal side effects in non-pregnant individuals. To our knowledge there is no safety and efficacy study on the hormone in pregnancy.

The rationale behind choosing saliva measurement was that it is non-invasive, and patients can collect the samples in the convenience of their home before bed.

Melatonin was measured three times in pregnancy – at the end of all trimesters to get a better understanding of the pattern of increase, in each patient and between patients.

2.1. **Hypothesis**

The hypothesis of this study was to expect a lower melatonin levels in pregnancies with insomnia when compared to healthy pregnancies.

2.2. **Objective**

The objective of this study was to measure and determine salivary melatonin levels in pregnant women with insomnia, treated or untreated, and to compare their levels with healthy pregnant women in all trimesters of pregnancy.
3. Methods

3.1. Measuring Melatonin in Saliva

3.1.1. Saliva

Healthy individuals produce 500-1500 ml of saliva per day, or 0.5 ml/minute salivary output, and composition may be different in terms of viscosity, ions and protein concentrations. The flow of the saliva depends on autonomic stimulation. Parasympathetic stimulation results in a large flow, with low levels of inorganic and non-protein organic compounds, such as cholesterol, uric acid, glucose, bilirubin, and creatinine. Sympathetic stimulation results in a small flow high in inorganic compounds such as K⁺ and proteins. Compounds are released into saliva by different means, such as passive diffusion for lipophilic compounds, active transport and ultrafiltration through gap junctions.¹⁷⁶

3.1.2. Saliva Melatonin and its Interactions

Melatonin transfers into saliva by passive diffusion.¹⁷⁶ Composition of the saliva is affected by food intake, increasing the release of total proteins. Caffeine should be avoided 12-24 hours before saliva sampling because it stimulates the activity of CYP 1A2.¹⁷⁷ Banana with low melatonin content is shown to increase urinary melatonin metabolite after consumption. This can be explained by the rate of absorption or metabolism of melatonin due to CYP1A2 polymorphism. Chocolate increases melatonin concentration by its flavonoid content.¹⁷⁸ Some drugs such as NSAIDS (ibuprofen, naproxen, diclofenac, ketorolac, etc.) may also reduce melatonin concentrations by inhibiting prostaglandin and COX-2.¹⁷⁹ Therefore, in the current study, patients were instructed to avoid the above mentioned to prevent any interaction with saliva melatonin levels.

3.1.3. Measuring Melatonin in Saliva

Salivary measurement of melatonin is now a practical and reliable tool for diagnosis of the condition and research purposes. Saliva melatonin as a circadian phase maker is validated and compared to plasma levels. There is a significant positive correlation between saliva and plasma melatonin onset \( r=0.64 \), and saliva and plasma acrophase concentrations, \( r=0.83 \) with saliva levels being one-third of plasma’s (Figure 14).¹⁸⁰ Saliva sampling should be done in dim
light and taken every 30-60 minutes for at least one hour to determine its onset. Reliability of saliva melatonin measurement to determine dim light melatonin onset (DLMO) was assessed. Results showed that saliva measurement of melatonin levels is valid, and it is a reliable tool for the measurement of hormones. This was further investigated by comparing in-home methods to laboratory measurements. Patients with insomnia were instructed to collect saliva samples at home and the following evening saliva samples were collected in the lab under lab conditions. It was found that at-home saliva melatonin levels are positively correlated with in-lab measurements $r=0.85$. However, a delay of approximately forty minutes was seen with the in-home method. This study confirmed the previous work that in-home saliva melatonin measurement is practical and valid to determine DLMO and to assess circadian rhythm (Figure 15).

*Figure 14: Relationship between plasma and saliva melatonin levels $r=0.64$. Reprinted with permission from SAGE publications.*
3.2. Study Design

This was an observational prospective cohort study.

3.3. Study Groups

- **Exposed group:**

  Pregnant women with insomnia treated with sleep medications

- **Comparison group 1 - Disease Matched Controls:**

  Pregnant women with untreated insomnia, matched by maternal age (±2 years) and gestational age (±2 gestational weeks).

- **Comparison group 2 - Healthy Pregnant Women:**

  Healthy pregnant women, matched by maternal age (±2 years) and gestational age (±2 gestational weeks).
3.4. Participants

3.4.1. Inclusion Criteria

- First trimester pregnant women with insomnia (treated and untreated)
- First trimester healthy pregnant women without insomnia
- Signed consent for participation
- Able to provide saliva samples as instructed.

3.4.2. Exclusion Criteria

- Pregnant women exposed to known teratogenic drugs.
- Pregnant women currently taking melatonin.
- Women who are not proficient in the English language.
- Women not able to provide clean saliva samples. Saliva samples with contamination will exclude the participant from the study.
- Shift workers
- Drug abuse

3.5. Sample Size

Salivary melatonin levels have not been measured in pregnancy. Therefore, due to lack of delta a convenient sample size of 31 pregnant women per group was estimated. Alpha was set at 0.05 and a power of 0.8.

3.6. Recruitment

Eligible pregnant women were identified and recruited through The Motherisk Program (MR) help line, at the Hospital for Sick Children (HSC). MR is a Teratogen Information Service affiliated with the Organization of Teratology Information Specialists (OTIS). It is designed to help pregnant women, and health care providers with evidence-based information on benefits or risks associated with different exposures in pregnancy. These include drugs, chemicals, radiations, infectious diseases, alcohol, and drugs of abuse. Information is provided either over the phone, or an in-person session with one of MR’s clinicians at the MR clinic located at HSC by appointment only. The help line operates between 8am and 8pm providing service to all
Canadians nation-wide. Women call the helpline through phone numbers 1-877-439-2744 and 416-813-6780 for all exposures except cigarette, alcohol and drugs of abuse for which another phone number is available. There is also a nausea and vomiting of pregnancy (NVP) helpline with a different phone number at the center, which provides recommendations on managing NVP symptoms with safe non-pharmacological and pharmacological approaches. All incoming calls to MR are documented in a confidential MR-exclusive database and are property of HSC. At the time of call MR counselors fill an intake form and documents patients’ information confidentially. This information includes patients’ personal information (such as first and last name, phone number, date of birth), patients’ medical history (such as all acute health issues, chronic medical condition, and medications) and patients’ obstetric information (including total number of pregnancies, live children, spontaneous abortions, terminations, ectopic pregnancies, and fetal death in their life time). Current pregnancy information is noted with details on last mensuration period (LMP), expected delivery date, number of ultrasounds and reasons, weight and height, current medications and their dose along with start and stop times if discontinued. Data on the safety of medications in pregnancy is obtained from an exclusive MR resource containing all studies and researches done on the medications.

At the end of a counselling call, the counselor mentioned the ongoing study of measuring melatonin in saliva in pregnancy, provided a brief explanation about the study and, if agreed, got the caller’s verbal consent to be called back by the researcher. The caller’s information was then referred to the researcher for future contact. The researcher called the pregnant woman to verify the information given in the initial call and on the intake form, and provided her with detailed information about the study and its procedure. The potential participant received information on why this study was being conducted and the rationale behind it, her role in the study and any direct or indirect risk or benefit to her was explained in detail. Participation was voluntary and she could say “no” at any stage during the conversation or later during the study. If the candidate agreed to participate, verbal consent was obtained, the initial phone interview was done, and a pregnancy questionnaire was filled out. A written informed consent form (Appendix 2) was sent to participants to sign and return.
3.7. Initial study Interview

At the first outgoing call to the potential participant, and after the verbal consent was obtained, the researcher completed a more detailed pregnancy questionnaire similar to the initial intake form (Appendix 3,4). A series of questions about sleep pattern were also asked using the Brief Insomnia Questionnaire (BIQ). The questionnaire included the following questions:

1. How many nights out of seven do you have difficulty falling sleep?

2. How long does it take you to fall sleep on those nights?

3. In the past seven nights, how many of them did you have problems staying asleep throughout the night?

4. How many times per night do you usually wake up on the nights you have trouble sleeping?

5. How long does it take to go back to sleep once you wake up at night?

6. How many mornings out of seven do you wake up before your alarm clock goes off?

7. How much earlier than you want to do you wake up on those days?

8. In the past seven days how many mornings did you wake up still feeling tired or un-rested?

9. Do you experience any of the following symptoms during the day attributed to your night sleep: fatigue, malaise, attention, concentration or memory impairment; social/vocational dysfunction or poor school performance; mood disturbance/irritability, daytime sleepiness, motivation, energy /initiative reduction; proneness for errors/accidents at work or while driving; tension headaches, and/or GI symptoms in response to sleep loss?

10. Concerns or worries about sleep?

3.7.1. Brief Insomnia Questionnaire (BIQ)

Approximately one third of the adult population in first world countries experience sleep issues over initiation, maintenance and poor quality. Many of these people meet the criteria for insomnia diagnosis with diverse diagnostic tools. These diagnostic tools are quite different in
terms of severity and specificity of the criteria, although all of them share the same questions about sleep pattern, including initiation, maintenance, restorative sleep, and daytime symptoms due to sleep issue. The brief insomnia BIQ was developed in response to the differences in other insomnia measurements, and incorporates all the criteria for diagnosis of insomnia such as the *Diagnostic and Statistical Manual, Fourth Edition (DSM-IV-TR)*, *International Classification of Diseases-102 (ICD-10)*, *research diagnostic criteria3 (RDC)*, and *International Classification of Sleep Disorders-24 (ICSD-2)*. DSM-IV Inclusion Criteria A includes: predominant complaint of difficulty falling sleep, or maintaining sleep, or non-restorative sleep for at least one month. DSM-IV Inclusion Criteria B includes: sleep problem causing daytime impairments. ICD-10 inclusion criteria A includes: difficulty falling sleep or poor sleep quality. ICD-10 inclusion criteria B includes: having the problem at least 3 times per week for at least one month. In the BIQ, symptoms are required to happen more than 3 times a week for 30 or more minutes, and at least for one month. Daytime distress or impairment is required by all the above measures but differ in specificity and severity. BIQ operationalizes and standardizes all these criteria in one single questionnaire. However, no attempt was made for differential diagnosis of insomnia and comorbid conditions associated with insomnia in this questionnaire, because it is difficult to distinguish between primary insomnia and insomnia due to other medical conditions.

### 3.7.2. Written Informed Consent Form (ICF)

The written informed consent form was designed and written according to the Research Ethics Board (REB) of the Hospital for Sick Children’s policy on “research consent form for clinical trial” and was approved by the board. There were different sections in the ICF. The language was easy to read and understand, and included detailed information a participant needed to know before signing the form. Sections included: the title of the research project; the name, phone number and affiliation of all the principal investigators, co-investigators, the study coordinator and other research team members. The next section explained the objective of the research study. The third section was a description of the research in which the step-by-step procedures of the study and role of the subject was explained. In this section recruitment methods, groups of study, the subject’s role and necessary instructions for saliva sampling was given. The next section described potential harms and benefits. In the current study there was
no potential harm to the patient, except the inconvenience of collecting a saliva sample one hour before bed. There were no direct potential benefits to the participants by participating in this study however, the study would benefit many pregnant women in the same situation in future. Participation was voluntarily and withdrawal from study at any time was allowed, without any impact on the service they got at HSC or the Motherisk Program. In the section of “confidentiality”, subjects were informed that their privacy was respected. No personal information would be shared or published anywhere without their permission, unless the law required that information. Examples of such disclosed information included abuse, contagious disease, suicidal thoughts, or a court order. Therefore, by signing the form they agreed that their information might be shared or disclosed if needed.

Two copies of the written informed consent form were mailed or e-mailed to the participant. Both copies were signed by both the study coordinator (the person who explained the study) and the subject. The subject kept one copy for their records and e-mail/mail the other copy back to the coordinator. Upon receiving the ICF, a saliva collection kit, instruction sheet, and a sample collection log sheet were mailed to participant.

3.8. Study Methodology

Saliva collection was done three times during pregnancy; at 12-14 weeks, 24-26 weeks, and 34-36 weeks. I was interested to know the pattern of melatonin rise from the beginning of pregnancy in all three groups described above. Plasma melatonin levels were measured in all trimesters. Participants collected saliva samples 3 times every 30-60 minutes, in dim light, starting at least 1 hour prior to bedtime. Therefore, in total there were 9 saliva samples from each participant. No chocolate or bananas, alcohol, caffeine, or drinks with artificial colorants should have been taken on the day of sampling. No aspirin or medicines containing ibuprofen, would be taken on the day of sampling. The participant needed to remain in dim light during the sampling hours with a night light or a low wattage lamp. They should not have sat closer than 6 feet to a TV; and if using a computer/laptop/tablet, they should have adjusted the contrast to low. To avoid contamination with food, participants needed to finish their main meal at least 30 minutes before sampling time and to brush their teeth without toothpaste then rinse with water 10 minutes before sampling. For saliva sampling a cotton swab was used, which was chewed or held in the mouth for 1-2 minutes, and then placed in a vial when soaked
enough. Participants documented each sample time on a log provided to them along with the
duration of staying in dim light. Participants were instructed to refrigerate or freeze their saliva
samples within 30 minutes of sampling and mail them back in a cold box within 1-3 days
(Appendix 5,6). All storage and shipment accessories were provided to participants. The saliva
samples were tested for melatonin levels using the enzyme-linked immunosorbent assay
(ELISA) method in the Motherisk lab.

3.9. Saliva Sample Storage

Saliva storage is of great importance. The method of storage can affect the concentration of the
compound. For saliva melatonin it was instructed to refrigerate the saliva vial and, if not sent or
picked up within 7 days, to freeze the sample.

3.10. Laboratory Process

3.10.1. Preparing Saliva Samples in Laboratory

Saliva was recovered by centrifuge for 5 minutes at 3,000 rpm. 200 µl of saliva was then
transferred to polypropylene tubes where the pre-treatment solution (sodium hydroxide) was
added and vortexed for 5 seconds. After a10 minute wait, the neutralizing solution
(hydrochloric acid) was added and vortexed for 5 seconds. Pre-treated samples were
centrifuged for 5 minutes at 10,000 rpm.

3.10.2. ELISA

In the current study the BÜHLMANN Direct Saliva Melatonin ELISA (EK-DSM) Salem, NH,
USA was used. It uses a competitive binding antibody technique. The microtiter plate is coated
with an anti-melatonin antibody. Pretreated samples and controls were incubated for 16-20
hours in 2-8 degrees Celsius. Biotin conjugate was added to wells and was incubated for the
next three hours in 2-8 degrees Celsius. Saliva melatonin competed with biotinylated melatonin
for the binding sites of this highly specific antibody. After washing, for the next 60-minute
incubation on a plate rotator and in room temperature, the enzyme label called streptavidin, and
conjugated to horseradish peroxidase (HRP) was added. This binds to the melatonin-biotin
antibody complexes captured on the coated wells. The unbound fraction of the enzyme label
was washed, and tetramethylbenzidine (TMB) substrate was added to the wells. In the last
incubation step of 30 minutes in room temperature and on a plate rotator, a chromophore of blue color was formed. The amount of the chromophores inversely correlated to the melatonin concentration in the sample. In the last step, stop solution was added to the wells that turns the color from blue to yellow. At this stage the plate was run and measured at 450 nm.

3.11. Statistical Analysis

All statistical analysis was done with student SPSS version 23.

All data is presented in mean ± SEM. In order to compare melatonin concentrations between pregnant women with insomnia either treated or untreated (combined in one group) with healthy pregnant women a t-test was used. The null-hypothesis was that there was no difference in melatonin levels in insomniac pregnant women and healthy pregnancies.

For sub-group analysis, one-way ANOVA was used. Subgroups included pregnant women with treated insomnia, pregnant women with untreated insomnia and healthy pregnant women.

In the general linear model, repeated measure analysis was used to compare the pattern of increase of melatonin levels in all two groups of untreated insomniacs, treated insomniacs and healthy subjects.

Alpha was set at p<0.05 in all statistical analysis.
4. Results

In the first group, Exposed, a total of 24 people were recruited. 13 were lost to follow up. They did not respond to emails or phone calls after the ICF was signed. Two had miscarriages and automatically were excluded from the study. One patient provided a very small amount of saliva sample that was not measurable. One patient’s saliva melatonin measure was an outlier, and was deleted from the analysis. In the first trimester seven samples were used in (T1) melatonin measurement. In the second trimester (T2). This is while one patient withdrew after T1, but one patient was recruited in the second trimester due to her severe insomnia symptoms. In the third trimester (T3) measurement, there were five samples because two patients withdrew after T2.

In the second group, Disease-match, 19 people were recruited. Six were lost to follow up, and 13 samples were received for the T1 measurement. For the T2 measurement there were 12 samples, as one patient withdrew after T1. For the T3 measurement there were 9 samples because one patient withdrew. Two other patients had sampling dates much later than the expected end date of study.

In the third group, Healthy, a total of 28 people were recruited. 7 were lost to follow up, two had miscarriages and one provided small amount of saliva that was not measurable. A total of 18 samples were used for the T1 measurement. For the T2 there were 11 samples. There were 4 withdrawals, 2 miscarriages, and one was lost to follow up in T2. In T3, there were 12 samples, because one patient that was lost to follow up in T2 came back and continued the study (Figure 16).
Mean (SD) age was not significantly different among the groups, 35.43±3.7, 35.54±4.4, 33.73±3.7, respectively. Ethnic background, marital status, education, occupational status, number of pregnancies (gravidity), number of live children (parity), spontaneous abortions (SA), therapeutic abortion (TA), ectopic pregnancies (Ect), pre-pregnancy BMI, third trimester BMI, and total weight gain are shown in table 5 in appendix 1.

4.1. Disease Characteristics of the Insomnia Group

Five patients in the Exposed group suffered from insomnia only. One patient had insomnia, depression, anxiety and attention deficit disorder (ADD). One patient had insomnia, is bipolar, and had mania. One patient had insomnia and anxiety, and one patient suffered from insomnia and depression.

In the Disease-match group, eight patients suffered from insomnia only. One patient had insomnia, depression, and anxiety. One patient had insomnia and anxiety, two patients had insomnia and depression.

All nine patients in the Exposed (treated) group took medications for either sleep issues or comorbid symptoms such as depression and anxiety. Five patients took one or combinations of zopiclone, quetiapine, lorazepam, dimenhydrinate, and progesterone as a sleep aid. One patient
took a combination of bupropion, amitriptyline, lisdexamphetamine, and clonazepam for insomnia, depression/anxiety, and ADD. Trazodone and aripiprazole were taken by another patient for bipolar disorder, mania and insomnia. Zopiclone, duloxetine and trazodone were used in combination for insomnia and comorbid anxiety. A combination of bupropion and quetiapine was taken for insomnia and comorbid depression (Table 1).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Exposed Group N=9</th>
<th>Disease-Match Group N=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insomnia Only</td>
<td>5(55%)</td>
<td>8(67%)</td>
</tr>
<tr>
<td>Insomnia with other psychiatric comorbidities</td>
<td>4(44%)</td>
<td>4(33%)</td>
</tr>
</tbody>
</table>

*Table 1: Disease composition of exposed and disease-match group.*

T-tests compared insomniac (exposed and disease-match) pregnant women (N=20) with healthy pregnant women. It was found that mean melatonin levels in all three trimesters were not significantly different between the two groups, p=0.4, p=0.9, p=0.1 for T1, T2, and T3 respectively (Table2) (Figure 17).

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Mean melatonin pg/ml</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Insom. H</td>
<td>20</td>
<td>9.4</td>
<td>6.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Insom. H</td>
<td>18</td>
<td>11.0</td>
<td>6.3</td>
<td>1.5</td>
</tr>
<tr>
<td>T2</td>
<td>Insom. H</td>
<td>19</td>
<td>11.9</td>
<td>12.6</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Insom. H</td>
<td>11</td>
<td>11.9</td>
<td>11.7</td>
<td>3.5</td>
</tr>
<tr>
<td>T3</td>
<td>Insom. H</td>
<td>14</td>
<td>11.3</td>
<td>6.5</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Insom. H</td>
<td>12</td>
<td>17.4</td>
<td>12.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Table 2: Descriptive statistics and t-test results. No significant difference found between the groups in all trimesters. T1: first trimester, T2: second trimester, T3: third trimester, Insom: insomnia, H: healthy*
Sub-group analysis by one-way ANOVA (Table 3) showed that Disease-match (untreated) insomniac pregnant women have significantly lower melatonin levels (p=0.04) in the third trimester (mean (pg/ml)=8.2±1.33 SEM), when compared to the treated (mean (pg/ml) = 16.8±3.1 SEM) and Healthy pregnant women (mean (pg/ml)=17.4 ±3.5 SEM). No significant difference was found in melatonin levels among the groups in the first trimester with the following means: Exposed: 8.9±1.6 pg/ml, Disease-match: 9.7±2.0 pg/ml, Healthy: 11.0±1.5 pg/ml. Pairwise comparison showed significant lower melatonin levels in Disease-match compared to healthy group (p=0.03) in the third trimester. Pairwise comparison also found Disease-match group’s melatonin levels significantly lower (7.4±2.1 pg/ml) compared to the Exposed group (19.5±6.25 pg/ml) in the second trimester, p=0.03.
Trimester | Groups | N | Mean Melatonin pg/ml | Std. Deviation | Std. Error | P-value |
--- | --- | --- | --- | --- | --- | --- |
T1 | EXP | 7 | 8.9 | 4.3 | 1.6 | 0.7 |
| DM | 13 | 9.7 | 7.1 | 1.9 | |
| H | 18 | 11.0 | 6.3 | 1.5 | |
T2 | EXP | 7 | 19.5 | 16.5 | 6.2 | 0.2 |
| DM | 12 | 7.4 | 7.4 | 2.1 | 0.2 |
| H | 11 | 11.9 | 11.7 | 3.5 | |
T3 | EXP | 5 | 16.8 | 6.9 | 3.1 | 0.04 |
| DM | 9 | 8.2 | 4.0 | 1.3 | 0.04 |
| H | 12 | 17.4 | 12.0 | 3.4 | |

Table 3: One-way ANOVA analysis of the three groups in all trimesters. Significant difference observed only in third trimester. EXP: exposed group, DM: Disease-Match, H: Healthy, T1: first trimester, T2: second trimester, T3: third trimester.

Repeated measure analysis was performed for all three groups separately, investigating the pattern of change in melatonin levels in all trimesters as secondary observation. No significant change was found in all groups when all trimesters were compared, p=0.1, p=0.3, p=0.1 for the Exposed, Disease-match, and Healthy groups respectively (Table 4) (Figure 18).

Table 4: Repeated measure analysis did not show a significant rise of melatonin levels in all trimesters. Exp: exposed, DM: disease-match, H: healthy. ns: non-significant
Figure 18: Dot plot of repeated measure analysis of melatonin levels in all trimesters. EXP: exposed, DM: disease-match, H: healthy. T1: first trimester, T2: second trimester, T3: third trimester.
5. Discussion

Both groups with insomnia were associated with comorbid mental disorders. Melatonin levels in treated women with psychotropic drugs were similar to healthy controls. A non-significant difference in melatonin levels in insomniac pregnancies (Exposed and Disease-match combined group) compared with Healthy subjects is indicative of higher melatonin level in the exposed group due to the effects of sleep medications. All nine patients in the Exposed (treated) group took medications for either only sleep issues or comorbid symptoms such as depression and anxiety. Five patients took one or combinations of zopiclone, quetiapine, lorazepam, dimenhydrinate, and progesterone as a sleep aid. One patient took a combination of bupropion, amitriptyline, lisdexamfetamine, and clonazepam for insomnia, depression/anxiety and ADD. Trazodone and aripiprazole were taken by another patient for bipolar, mania, and insomnia. Zopiclone, duloxetine and trazodone were used in combination for insomnia and comorbid anxiety. A combination of bupropion and quetiapine was taken for insomnia and comorbid depression.

Amitriptyline decreases sleep latency while treating depression symptoms. It also increases melatonin synthesis by increasing NAS (N-acetyl transferase) in the pineal gland. SSRIs cause increased sleep latency, REM suppression and frequent waking up during the night. As a result, pharmacotherapy addressing the treatment of insomnia should be considered with SSRIs. This is in conflict with a clinical study where duloxetine was shown to increase 6-sulphatoxymelatonin (aMT6s) levels after treatment. Duloxetine was used in one patient for anxiety and trazodone and zopiclone were used concomitantly to improve sleep. Zopiclone has no effect on melatonin concentration when compared to zaleplon placebo and a non-benzodiazepine hypnotic in rats. Similar results have been found in healthy volunteers following acute and subchronic administration of zopiclone. A sleep-inducing effect may be due to its hypnotic properties. Increased melatonin levels and improved sleep with trazodone has been documented in clinical studies. Bupropion is associated with increased REM (rapid eye movement) sleep, however, a meta-analysis found bupropion was one of the antidepressants that caused insomnia the most often (Figure 19).
Low dose quetiapine is shown to have a non-significant effect on melatonin level but significantly reduces nocturnal cortisol levels. A lower cortisol level is associated with a higher melatonin level.

Melatonin levels in women with untreated insomnia have been low throughout pregnancy. This indicates the potential role of melatonin in sleep regulation. Moreover, it further shows the effect of psychotropic medications on sleep and improvement of symptoms which was observed in treated group. Significant lower levels of melatonin observed in the untreated (disease-match) group in the third trimester of pregnancy, compared to the Exposed and Healthy groups, is an indication of the negative role insomnia and sleep disorders can play in modulating melatonin levels.

The observed lower melatonin levels in the healthy group than what was shown in previous research and expected may be due to a few reasons. It is documented that melatonin crosses freely between maternal circulation and the placenta. The level increases when gestation increases after 24 weeks and it reaches its peak at 36 weeks gestation. The maternal plasma levels is shown to reach above 100 pg/ml in late pregnancy. The validation of saliva melatonin levels vs. plasma levels shows a significant positive correlation between the two methods, with saliva being 30% of that of plasma, and the onset is shown to be 40 minutes later than plasma. Taking these into consideration, expected saliva melatonin levels in the Healthy
group in the third trimester in the current study is above 30 pg/ml. Therefore, the observed lower levels in the Healthy group may be because five subjects had <10 pg/ml saliva levels throughout pregnancy. One was a smoker with 10 cigarettes a day. Lower levels may be explained by the negative impact of smoking on melatonin. One patient reported having vivid dreams and broken sleep in the third trimester of pregnancy. Lastly, with its small sample size, we would suspect the result found may not be representative of what we would expect in the population, i.e. a chance finding.

The non-significant finding in the repeated measure analysis was due to small sample size, since samples from all trimesters were not available from all study participants, the Exposed group N=4, Disease-match group N=9 and Healthy group N=11 (Table 4).

There were limitations in this study. First and most important was a lack of power due to small sample size. Unmeasured patient compliance was another challenge in this study. Whether patients followed all the instructions they were given from staying in dim light to refrain from food and drugs listed in the instruction sheet was not measured. It was also difficult recruiting pregnant women with insomnia who may also suffer from other comorbid psychiatric conditions. Furthermore, retaining these patients for the duration of pregnancy was another challenge in this study. Therefore, we stopped the study without reaching our optimal calculated sample size.

This study opened the door to future research on the important role of melatonin in the regulation of sleep in pregnancy. More research is needed to confirm the validity of salivary method of melatonin measurement during pregnancy. Assessment of efficacy and reproductive safety of melatonin supplementation in the treatment of insomnia during pregnancy is an important subject of future investigations.
6. Conclusion

Presently, pregnant women suffering from insomnia are treated with pharmacotherapy, most commonly with sedative hypnotics, benzodiazepines, antihistamines, and antipsychotics. Side effects vary from daytime sleepiness to dependence. Many studies looked at the benefits and risks associated with sleep medications in pregnancy. Fetal risks associated with some of these medications have been confirmed in the studies. Side effects include low risk oral cleft palate, withdrawal symptoms after birth, preterm birth, and low birth weight. Meanwhile, studies examined the efficacy of the exogenous melatonin in treating sleep disorders. A recent meta-analysis on the efficacy of melatonin for the treatment of insomnia showed a significant decrease in sleep latency, increased sleep time, and a significant positive effect on sleep quality. Results from an open-labeled long-term study showed the efficacy and safety of prolonged-release melatonin (2mg) without any withdrawal symptoms. Results also showed that long term melatonin treatment is not associated with suppression of endogenous secretion of the hormone as levels measured in urine two weeks after discontinuation. No dependence, relapse of symptoms, or withdrawal symptoms have been reported after discontinuation of treatment. Pilot studies at this time are investigating the role of antenatal melatonin administration as an antioxidant in late pregnancies to prevent or treat IUGR and preeclampsia. The prospect of treating pregnant women with insomnia with the natural sleep hormone melatonin has received very little attention.

This study confirmed the lower saliva levels of melatonin in pregnant women with untreated insomnia. Therefore, melatonin supplementation may be considered an intervention for insomnia in pregnancy. Melatonin is inexpensive, is available over-the counter and it has a low side effect profile. This study confirmed a need for future research to introduce melatonin as a natural hormone therapy for insomnia. The results of this study may have a significant positive effect on the quality of life and health of many pregnant women worldwide currently on sleep medication who have no choice but to continue pharmacotherapy to maintain a stable mental and physical health during pregnancy.
7. References

References


8. Appendices

8.1. Appendix 1: Characteristics of Study Population

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8.2. Appendix 2: ICF

Research Consent Form

Title of Research Project:

Salivary Melatonin Levels in Pregnant Women with Insomnia: A Prospective Cohort Study with Two Comparison Groups

Investigator(s):

- Principal Investigator: Dr. Shinya Ito Telephone: 416-813-5776
- Research Study Coordinator: Kamelia Mirdamadi Telephone: 416-813-7283
  E-mail: kamelia.mirdamadi@sickkids.ca
  Affiliation: Hospital for Sick Children, University of Toronto
  Research Site Telephone: 1-877-439-2744

Please read this document carefully before you sign it!

- Your participation is voluntary.
- You will be asked to sign this consent form.
- You may refuse to take part or can withdraw from this study at any time without penalty or loss of benefits to which you are otherwise entitled.
- The purpose of this form is to give you information about the research study and, if signed, to show your decision to take part in the study. This form describes the purpose, procedures, benefits, risks, discomforts and precautions of the research study.
- The following information will describe the study and your role in it.
- Please read this Subject Information and Consent Form and ask as many questions as needed. This Consent Form may contain words you do not understand. If you do not understand some terms and words, please ask the study team to explain any part that is unclear.
- A copy of this form will be given to you to review at your leisure; please feel free to ask for advice from others before signing.

Purpose of the Research:

The purpose of this prospective study is to determine saliva melatonin levels in pregnant women with insomnia.
Description of the Research:

There are three groups in this study:

**Group one:** pregnant women with insomnia who take sleep medications  
**Group two:** pregnant women with insomnia who don’t take any medications  
**Group three:** Pregnant women with no medical conditions

When you initially called the Motherisk Helpline, we filled an intake form given advice on products and exposures. Our counselor described the study and asked if you were interested in getting more information on the study to participate. You were called back by the study coordinator and agreed to take part in this study.

In this study you will be asked to provide 3 saliva samples three times during your pregnancy; 12-14 weeks, 24-26 weeks, 34-36 weeks, in total 9 samples. Sample collection should be done one hour before bed time three times every 30-60 minutes. During the sampling time you should be in a low light area with a night light or a low wattage lamp. You should not sit closer than 6 feet to TV, and adjust your compute light and contrast to low. In order to prevent contamination with food you should finish your main food or snack at least 30 minutes before sampling time, and to brush your teeth without toothpaste and thoroughly rinse your mouth with water 10 minutes before saliva collection. You can collect the saliva at any position you are i.e. seated, lying, or standing. You will then freeze the samples in the freezer within 30 minutes after sampling and mail it back to us on dry ice within 3 days. All you need for sample shipping will be provided to you.

Saliva tubes will be provided after we receive this consent form signed. You need to sign the informed consent form and return it either by mail, e-mail or fax. You will also return the samples separately for each stage of pregnancy.

**Potential Harms:**

We know of no harm that taking part in this study could cause you.

**Potential Discomforts or Inconvenience:**

Although saliva collection is easy to do, you may feel some discomfort or inconvenience during collection.

**Potential Benefits:**

There is no known direct benefit to you individually by taking part in this study. However, it is a big step in finding a new approach to treat insomnia in pregnancy with least side effects for many mother and baby pairs in near future.

This study may take long to complete, but, if you are interested you can provide us with an email address to provide you with the results of the study or the published paper.
**Confidentiality:**

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law requires us to do this. For example, the law requires us to give information about you if a child has been abused, if you have an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.

Personal information including ethnicity, living arrangement, education, and working status will be collected upon verbal consent for analysis of study population characteristics. Study population will be identified with a given study number. Address, phone number, and e-mail will be collected for mailing and courier services. Demographic information will be kept locked and secure, separate from study files, in a double locked space for confidentiality.

The data produced from this study will be stored in a secure, locked location. Only members of the research team and clinical research monitors will have access to the data. This could include external research team members. By signing this consent form, you agree to let these people look at your research records. We will keep your original signed consent and give you a copy for your files. Following completion of the research study, the data will be kept as long as required and then destroyed as required by Sick Kids policy. Published study results will not reveal your identity.

**Reimbursement:**

No reimbursement will be provided for participation in this study.

**Participation:**

It is your choice to take part in this study. You can stop at any time. The care you may seek from Sick Kids (including the Motherisk Program) in the future will not be affected in any way by whether you take part in this study.

New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information, and we will ask you again if you want to be in this study.

Your signing this consent form doesn’t interfere with your legal rights in any way. The staff of the study, any people who gave money for the study, or the hospital are still responsible, legally and professionally for what they do.

**Sponsorship:**

The study is sponsored by The Motherisk Program and the Hospital for Sick Children.

**Conflict of Interest:**

Dr. Shinya Ito and the other research team members, have no conflict of interest to declare.
Consent:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.

2) You have explained the possible harms and benefits (if any) of this study.

3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect the care I may seek at Sick Kids (including the Motherisk Program) in future.

4) I am free now, and in the future, to ask questions about the study.

5) I have been told that my medical records will be kept private except as described to me.

6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.

7) I have read and understood pages 1 to 4 of this consent form. I agree, or consent, to take part in this study.

____________________________________  ______________
Printed Name of subject & Age            Subject’s signature & date

________________________________________
Printed name of person who explained consent    Signature & date

If you have any questions about this study, please call Kamelia Mirdamadi at 416-813-7283

If you have questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manager at 416-813-5718.
8.3. Appendix 3: Pregnancy Questionnaire

Study: Salivary Melatonin Levels in Pregnant Women with Insomnia: A Prospective Cohort Study with Two Comparison Groups

Name of investigator completing the questionnaire and obtaining consent _______________________

Verbal permission to participate in the study: Yes ☐ (If No, ‘then write ‘refused to participate melatonin study’ at the top of the MR Intake Form)

GENERAL

At the time of this follow-up: G___ P___ SA___ TA___ Ectopic___ Molar___ Other___

If miscarriage, fetal death or therapeutic abortion: at how many weeks_____ months_____ Any defects detected (describe) ______________________

How detected?  By ☐ ultrasound  ☐ amniocentesis

PREGNANCY

Have you used fertility drugs in this pregnancy?

☐ No  ☐ Yes _________________________________

LMP:_________ CYCLE:______ EDC:__________

PPW:_______lb/kg WT:_________lb/kg HGT:______ cm/ft

BMI:___

GA:_______wks   HT:________________

Last ultrasound scan: ☐ No  ☐ Yes __________wks

Reason:_____________________________________

Result:_____________________________________

PREVIOUS PREGNANCY HISTORY

Please complete table.  Eff’y = Efficacy; 1 = no effect, 10 = best.

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<th>SEV</th>
<th>MED</th>
<th>EFF’Y(1-0)</th>
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</table>
MATERNAL HEALTH HISTORY

For Non-Disease matched controls: Have you ever been diagnosed with insomnia? No ☐ Yes ☐

For exposed and Disease matched controls or if a control answers yes to the above question:
When were you diagnosed with insomnia: ________________________________?
Details: ________________________________________________________________

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<td>UGR/Growth Problems</td>
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EXPOSURES DURING PREGNANCY

Please record all medications used during pregnancy (prescription or over-the-counter)

Do you use any herbal medications?
Do you use any vitamins (prenatal or other supplements)?
Do you use anything for allergies, anxiety, cold, constipation, depression, diarrhea, headache, heartburn, weight loss?

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Tests During Pregnancy

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at _____ weeks, Reason/results __________________________
2. Amniocentesis  No ☐    Yes ☐
at _____ weeks, Reason/results __________________________
3. Glucose Tolerance Test  No ☐    Yes ☐
at _____ weeks, Reason/results __________________________
4. Ultrasounds:
at _____ weeks, Reason/results __________________________
at _____ weeks, Reason/results __________________________
at _____ weeks, Reason/results __________________________
at _____ weeks, Reason/results __________________________
5. Other: ________ ______  No ☐    Yes ☐
at _____ weeks, Reason/results __________________________

Sleep Pattern (BIQ)

“On average how long does it take you to fall asleep?

In the past 7 nights how many nights did you have problems STAYING asleep throughout the night?

How many times per night do you usually wake up on the nights you have trouble sleeping?

How long does it take to go back to sleep once you wake up at night?

How many mornings out of 7 do you wake up before your alarm clock goes off?
How much earlier than you want do you wake up on those days?

In the past seven days how many mornings you wake up still feeling tired or un-rested?"

Do you experience any of the following symptoms during the day attributed to your night sleep?

1. Fatigue, Malaise

2. Attention, concentration, or memory impairment

3. Social/Vocational dysfunction or poor school performance

4. Mood disturbance/Irritability

5. Daytime sleepiness

6. Motivation, Energy /Initiative reduction

7. Proneness for errors/accidents at work or while driving

8. Tension headaches, and/or GI symptoms in response to sleep loss

9. Concerns or worries about sleep “(17)

Was this the pattern lasted for a while?

On a scale of 0-10, how would you rate your Well Being?

0 (Worst possible) ____________________________10 (The best you felt before pregnancy)

Can you tell me what causes you to feel that way?

_____________________________________________________________________________________

_____________________________________________________________________________________

Following completion of questionnaire:

“If you have any questions/concerns about what we’ve talked about, please feel free to call my supervisor
Dr. Gideon Koren at 416-813-5778. Finally, if you need additional information about your exposures
during pregnancy and breastfeeding you can contact our Motherisk helpline at 416-813-6780 or 1-877-
436-2744.

The finding from this study will be published in a medical journal and can be sent to you if you are
interested in receiving a copy. Are you interested in getting the results?”

Yes □ No □

If yes, by which method mail □ email□ (write the address on the Master file)

“Thank you very much for taking the time to participate in our research”
8.4. Appendix 4: Demographic form

Melatonin Study

THE MOTHERISK PROGRAM - HOSPITAL FOR SICK CHILDREN, UNIVERSITY OF TORONTO, PHARMACEUTICAL SCIENCES

<table>
<thead>
<tr>
<th>Contact date:</th>
<th>Study Group:</th>
<th>Study ID No.:</th>
</tr>
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MATERNAL DATA

<table>
<thead>
<tr>
<th>Name:</th>
<th>☐ GP ☐ OB ☐ midwife/nurse practitioner</th>
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<tbody>
<tr>
<td>Participant D.O.B:</td>
<td>Name:</td>
</tr>
<tr>
<td>phone: (H)</td>
<td>(W)</td>
</tr>
<tr>
<td>E-mail address:</td>
<td></td>
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<tr>
<td>Address:</td>
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MOTHERISK PROGRAM - HOSPITAL FOR SICK CHILDREN, UNIVERSITY OF TORONTO, PHARMACEUTICAL SCIENCES

DOCTOR’S INFORMATION

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<tbody>
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<tr>
<td>-----------</td>
</tr>
<tr>
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</tr>
<tr>
<td>☐ Black</td>
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<tr>
<td>☐ Oriental</td>
</tr>
<tr>
<td>☐ Other</td>
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</tbody>
</table>

JOB: ________________________
8.5. Appendix 5: Saliva Collection Instruction Sheet

Please read the instructions below before saliva collection:

1. 3 samples every 30 minutes one hour before bed time in one single night.
2. **Bananas and chocolate** should not be eaten during the day of sample collection. **Caffeine, alcohol, or drinks containing artificial colorants** should not be drunk on the sampling day as they may cause **dry mouth**.
3. On the day of the collection, if possible, **no aspirin** and medicines that contain **ibuprofen** should be taken.
4. Please remain in **dim light during the sampling hours** with a night light or a low wattage lamp.
5. Please do not sit closer than 6 feet to TV; and if using a computer/laptop/tablet, adjust the contrast to low.
6. To avoid contamination with food, please finish your main **meal at least 30 minutes before sampling** time,
7. Please **brush your teeth without toothpaste, and rinse your mouth with water ten minutes before sampling**.
8. Open the top of the tube (the swab is in the top) and remove the top from the tube.
9. Put the swab between your teeth and cheek and move it around with your tongue for 3-5 minutes, until the swab is thoroughly soaked with saliva.
10. Put the swab straight from your mouth into the smaller tube, without touching it with your fingers.
11. Using the top, push the swab into the smaller tube and put on the top
12. Please document **each sample time** and the duration of staying in dim light.
13. Please **refrigerate** your saliva samples within 30 minutes of sampling.
14. Please let me know when you are planning the sampling, and the courier company will pick up the samples from you, if applicable.

If you have any questions please contact me (Kamelia Mirdamadi) at: kamelia.mirdamadi@sickkids.ca , 1-877-439-2744, cell#647-838-4014
8.6. Appendix 6: Saliva Collection Log Sheet

Study: Salivary Melatonin Levels in Pregnant Women with Insomnia: A Prospective Cohort Study with Two Comparison Groups

<table>
<thead>
<tr>
<th>First name:</th>
<th>Last name:</th>
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</thead>
<tbody>
<tr>
<td>D.O.B.</td>
<td>Study ID#:</td>
</tr>
</tbody>
</table>

**T1 Saliva Collection:**
Gestational age: ------ Weeks
Duration of staying in dim light: ------:------- hr(s):min(s)
Bedtime: --------:-------- hr(s):min(s)
Medication(s) taken during the past 24 hrs, if any:
1. ------------------------ Dose: ------------------------
2. ------------------------ Dose: ------------------------
3. ------------------------ Dose: ------------------------

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time:</td>
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<td></td>
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</tbody>
</table>

**T2 Saliva Collection:**
Gestational age: ------ Weeks
Duration of staying in dim light: ------:------- hr(s):min(s)
Bedtime: --------:-------- hr(s):min(s)
Medication(s) taken during the past 24 hrs, if any:
1. ------------------------ Dose: ------------------------
2. ------------------------ Dose: ------------------------
3. ------------------------ Dose: ------------------------

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time:</td>
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</tbody>
</table>

**T3 Saliva Collection:**
Gestational age: ------ Weeks
Duration of staying in dim light: ------:------- hr(s):min(s)
Bedtime: --------:-------- hr(s):min(s)
Medication(s) taken during the past 24 hrs, if any:
1. ------------------------ Dose: ------------------------
2. ------------------------ Dose: ------------------------
3. ------------------------ Dose: ------------------------

<table>
<thead>
<tr>
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<th>Sample 3</th>
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<tbody>
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