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Sleep Disorder Trends, Epigenetic Markers, And Genetic Variation Of Circadian Genes In Adenomatous Polyp Formation

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Dedication

Without the support of my dissertation committee, family, friends, and mentors, this dissertation would not have been possible. Thank you for providing me with both professional and personal guidance throughout this rigorous process.

Abstract

Introduction: Sleep disturbances and sleep quality have fluctuated over time in the United States (US). Virtually every physiological process follows a circadian pattern, and cellular processes (i.e., cell proliferation, DNA damage response, and apoptosis) are often disrupted by sleep disturbances and other factors, potentially giving rise to adverse health outcomes such as cancer. Additionally, both genetic variation and epigenetic patterns within clock genes, which may affect sleep quality, also may increase one's risk for developing cancer. The role of sleep disturbances in the formation of adenomas, precursor lesions to colorectal cancer (CRC), has been explored previously; however, little is known regarding the influences of genetic variation in the PERIOD3 (PER3) variable number tandem repeat (VNTR) and epigenetic patterns of clock genes on adenoma formation. Furthermore, few studies have attempted to fully characterize sleep disorder trends in a national sample over time, as it has been alleged that sleep quality has declined over time. Examination of sleep disorder trends is of public health significance because individuals affected by sleep disturbances may be at an increased risk for cancer development, particularly CRC. **Materials and Methods**: In order to examine sleep disorder trends over time and to determine the genetic and epigenetic influences of adenoma formation, two studies were conducted. The first study utilizes data from a

national sample of US Veterans across an eleven year span to carry out a serial cross-sectional study to produce annual prevalences of sleep disorders, which were further stratified by additional factors. Cases were defined as patients with at least two outpatient sleep disorder diagnoses at least 30 days apart based on ICD-9 codes and American Academy of Sleep Medicine (AASM) criteria. Additional analyses were carried out to determine factors that were associated with the development of new sleep disorders in the last year of the study using multivariable unconditional logistic regression models to produce odds ratios (OR) and 95% confidence intervals (95% CI).

The second study (Epigenetics and Diet in the Carcinogenesis Process, EDCaP) provided information on epigenetic markers and PER3 VNTR variation. The epigenetic marker study was carried out at local endoscopy center (South Carolina Medical Endoscopy Center, Site 1 [n=107]). For the PER3 VNTR arm of the study, data from Site 1 (n = 93) and an additional endoscopy center were pooled (WJB Dorn Veterans Administration Medical Center [Site 2, n=53]). Cases were defined as individuals with at least one histologically confirmed adenoma, and controls were subjects with a normal colonoscopy, or a normal biopsy not requiring heightened surveillance (e.g., hyperplastic polyp). To determine if cases had differential promoter methylation in selected genes or an increased odds of possessing at least 5-repeat allele in the PER3 VNTR compared to controls, unconditional logistic regression analyses were used to calculate ORs and 95% CIs, while adjusting for confounders. **Results**: In the study using a national sample of US Veterans, apneas and insomnias were the first and

second most common sleep disorders diagnosed among Veteran patients, respectively. Total sleep disorder prevalence rose six-fold across the study period. Additional analyses indicated that several comorbid diseases may influence the development of sleep disorders. In the methylation study, compared to controls, cases were more likely to be hypomethylated in the MINT1, PER1, and PER3 promoters. In the PER3 VNTR study, cases were more likely to possess the 5-repeat PER3 genotype relative to controls. **Discussion**: Given the evidence that diagnosed sleep disorder prevalence has risen over time in the national sample study, a subset of individuals may be increasing their risk for cancer, potentially through genetic or epigenetic mechanisms. Epigenetic and genetic variations in clock genes appear to influence the development of adenomas, which presents an opportunity to develop novel biomarkers in accessible tissues (e.g., blood). It also is important to note that these genetic and epigenetic markers may influence sleep disturbances, which have been linked to cancer formation in previous studies.

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Chapter 1

Introduction and Specific Aims

1.1 Introduction

According to the most recent estimates by the American Cancer Society, there will have been over 93,000 incident colorectal cancers (CRC) patients and more than 39,000 deaths in the United States (US) in 2015, thus making it the third most common cancer in both men and women.[1] While these statistics are alarming, CRC is one of the most preventable cancers. Colorectal adenomatous polyps are considered precursor lesions for CRC.[2] Certain characteristics of adenomas such as size, number, and histology, confer a greater risk within an individual.[3-6] A deeper understanding of the factors related to the formation of these adenomatous polyps and their progression to cancer is needed, but it provides an extremely important opportunity for public health interventions. By intervening in this pathway from adenoma to carcinoma formation, health professionals will have the ability to reduce the number of new CRC cases by as much as 90%.[5,7]

Colorectal adenomas have been estimated to have developed prior to 70-90% of all CRCs.[8] Despite screening programs, the incidence of CRC and deaths due to CRC remains high.[9] Current efforts are directed toward detection and removal of asymptomatic adenomas. However, even after removal, the

recurrence rate for adenomas, an effective surrogate endpoint for CRC, is approximately 40-50%.[10-12] Thus, continued surveillance via follow-up colonoscopies is extremely important in avoiding cancer progression in the colon; however, CRC surveillance compliance is still low (49 – 61% of CRC survivors).[13-17] It also illustrates the importance of finding alternative means of CRC screening.

Because a series of genetic and epigenetic changes occur along the pathway from normal tissue within the gastrointestinal (GI) tract to carcinogenesis, it is important to understand the mechanism behind these changes to help prevent formation of CRC or to better treat current cases of CRC. Also, it has become important to discover novel biomarkers that predict CRC risk, as some heavily-studied genetic markers cannot fully explain CRC formation within certain individuals and populations as a whole especially in readily-accessible tissues derived from the body (e.g., blood). Additionally, several other genes have been implicated in the carcinogenesis process in the GI system;[18] hence, further investigation into other gene families such as the *Period* family (*PER1, PER2,* and *PER3*) is warranted.[19]

The *Period (Per)* gene family plays a role in immunomodulation[20-25] and tumor suppression.[26, 27] Thus, structural variation,[19, 28, 29] deregulated expression within tumor tissues,[30-32] and epigenetic variation [33] within the *Per* genes have shown some promise as novel biomarkers of cancer. For example, Chen et al found dysregulated expression of *PER1*, *PER2*, and *PER3* in breast tumor

tissues compared to matched normal tissues within subjects.[30] Within this same gene family, Zhu et al have found that structural variation in one of these genes in the form of a variable number tandem repeat (*PER3* VNTR), which has been previously been linked to various sleep phenotypes,[34-40] also has been associated with premenopausal breast cancer.[19] Furthermore, this gene family also has been known to influence sleep quality within people, suggesting a mechanistic role in these genes in the pathway of sleep disruption, altered gene variation and function within this family, and cancer formation and mortality in both animals and humans.[41-46] Variation within the *PER3* gene also may explain cancer susceptibility in certain subgroups of people that are more likely to experience sleep disruption.[19, 47] Additionally, little is known on the trends of sleep disorders over time in a national sample. Because prior literature has noted an association between sleep disruption and cancer, it is necessary to characterize an at-risk population. .

1.2 Specific Aims

The specific aims of this proposal are as follows:

Aim 1a: To characterize sleep disorder prevalence among US Veterans over time and determine what factors that may be driving an increased prevalence of sleep disorders in Fiscal Years (FYs) 2000-2010, or new sleep disorder diagnoses in FY2010

> Research Question 1.1: Are sleep disorder diagnoses increasing nationally among U.S. veterans or within specific subgroups?

Research Question 1.2: Are comorbidities such as obesity, diabetes, cardiovascular disease, and post-traumatic stress disorder (PTSD) influencing the increase of sleep disorders in US Veterans?

Research Question 1.3: What factors are increasing one's risk for the development of a new sleep disorder diagnosis?

Aim 2: To determine the association between *Period* (*PER*) gene methylation patterns and other gene methylation markers from a panel of 20 candidate markers and adenoma formation through the use of logistic regression

Research Question 2.1: Do methylation patterns in peripheral blood leukocytes (PBLs) within the *PER1*, *PER2*, and *PER3* genes differ for those with adenomas compared to those without adenomas, after adjustment for confounding factors?

Research Question 2.2: Is there a significant amount of concordance between methylation patterns of the *PER1, PER2,* and *PER3* genes in PBLs, normal tissue, and polyp tissue?

Research Question 2.3: Do methylation levels of *PER1, PER2,* and *PER3* genes in the PBLs, normal tissue, and polyp tissue predict mRNA levels of the *PER1, PER2,* and *PER3* genes in PBLs and tissue, respectively?

Research Question 2.4: Do mRNA levels of *PER1*, *PER2*, and *PER3* in PBLs predict adenoma risk, after adjustment for confounding factors?

Research Question 2.5: Do mRNA levels of *PER1*, *PER2*, and *PER3* in PBLs agree with mRNA levels of *PER1*, *PER2*, and *PER3* found in normal and/or polyp tissue?

Aim 3: To evaluate the influence of the variable tandem repeat (VNTR) in the *PER3* gene on colorectal adenoma risk

Research Question 3.1: Are individuals with the *PER3* 4/5 and 5/5 genotype at an increased risk for adenoma formation compared to individuals with the *PER3* 4/4 genotype?

Research Question 3.2: Does methylation or mRNA expression of the *PER1*, *PER2*, or *PER3* genes differ among those with different *PER3* VNTR genotypes?

Research Question 3.3: Does methylation modify the relationship between genetic variation in the *PER3* VNTR gene and adenoma formation?

Chapter 2

Background

National Sample of US Veterans

2.1 Adverse health outcomes associated with sleep disorders

Previous studies have found that more than 80% of people with clinically significant sleep-disordered breathing are undiagnosed;[48] and prior research has shown that sleep duration and sleep disorders have been associated with adverse health outcomes, which include but are not limited to: earlier mortality (total and cause-specific),[44, 49-51] cardiovascular disease (CVD),[52, 53] type 2 diabetes, [53, 54] obesity, [53, 54] and psychiatric disorders.[55-58] Furthermore, sleep duration or sleep disorders have also been linked with cancer. In a study examining cancer incidence in a large multicenter Spanish cohort, individuals with severe apnea, with the Apnea-hypopnea index (AHI) of 43 or higher as a proxy, had an increased cancer incidence density ratio compared to those with the lowest AHI category; however, this association was only observed in individuals aged 65 or younger. More recently, two meta-analyses revealed inconsistent effects between short sleep duration and other circadian disturbances such as shiftwork and exposure to light at night and breast cancer.[59, 60] Although both meta-analyses did not observe an association in studies that examined short sleep duration, a positive association between

shiftwork and breast cancer risk was detected in the study by He et al.[59] This subgroup tends to have shorter sleep duration compared to permanent day workers.[61] With the co-occurrence of sleep disorders and another chronic disease, quality of life is severely impacted as well.[62, 63] Little research has been conducted on the etiology of sleep disorders in a national sample, and identification of risk factors could improve detection and treatment of sleep disorders.

2. 2 Sleep disorders in the United States

A 2010 study characterized the prevalence of sleep disorders and sleep habits in the United States using the NHANES 2005-2006 sample.[64] Among all physician-diagnosed sleep disorders, sleep apnea was the most prevalent sleep disorder in the civilian, non-institutionalized population (4.2%). In addition to physician-diagnosed sleep disorders, 7% of the sample reported having trouble falling asleep and a similar amount (6.9%) reported waking up in the night and difficulty going back to sleep. Office visits for sleep disorders have significantly increased across a decade,[65] which potentially indicates either a true increase in prevalence, increased awareness, or both. However, common sleep disorders such as sleep apnea and insomnia remain underdiagnosed and therefore go untreated.

Ram et al found several differences in sleep-related metrics and/or symptoms have been observed when stratified by demographic characteristics.[64] Middle-aged adults were more likely to report worse sleep characteristics

compared to younger and older adults. In regard to gender, women were more likely to report worse sleep habits such as trouble falling asleep, feeling unrested during the day, not getting enough sleep and taking sleeping pills. Sleep-related difficulties such as difficulty concentration and remembering were reported at higher rates among women. In regard to race/ethnicity, while EAs had higher rates of sleeping pill use and excessive daytime sleepiness, AAs reported higher rates of sleep-related difficulties, shorter sleep durations, and longer sleep latencies (minutes needed to fall asleep). [65] Despite this study's strengths, several limitations must be noted. First, it relied on data that was self-reported that was not validated through physical examinations or medical chart reviews; second, self-report of these sleep symptoms are subject to recall bias, where subjects with a physician-diagnosed sleep disorder may recall symptoms differently from individuals who do not have a physician-diagnosed sleep disorder; and third, as a result of NHANES being a cross-sectional study design, it is difficult to determine the temporal relationship between sleep disorders/habits and daily activities and trends in sleep habits across time.

Previous studies have also supported some of the findings found in the national sleep disorder prevalence data, particularly in terms of differences by race and ethnicity.[66, 67] Other studies have found that black participants had a doubling of risk for obstructive sleep apnea, reduced sleep satisfaction, and more frequent napping compared to white participants.[66, 68] However, Ram et al's findings could not be replicated in a more recent study examining office visits for sleep disturbances over time.[65] For example, Ford et al reported that men higher

rates of office visits for medical care for sleep difficulties compared to women. Moreover, no racial differences could be detected for any sleep disorder or insomnia, and EAs had higher office visits related to sleep apnea compared to AAs. Lack of consistency may be due to failure to control for factors that may impact prevalence rates, such as physical and mental comorbidities and socioeconomic status;[69, 70] additionally, previous studies have not been adequately powered to examine differences in prevalence rates by various factors. Regardless, increased burden of sleep disorders and disturbances in certain subgroups could partially explain disparities that have been observed in other diseases. Given that sleep disorders in general are undiagnosed, the characterization of sleep disorders and factors contributing to the development of sleep disorders deserve further investigation.

As a result of the drawbacks in the recent prevalence studies and previous studies, it is of high importance to examine populations where data does not rely on self-report of sleep disorder diagnoses across time. One potential source of looking at objective measures of sleep disorder diagnoses is in the Veterans' Health Administration's (VHA) electronic medical record system. Furthermore, this group presents a unique opportunity to see the effects of various comorbidities on sleep disorders. Because this group is particularly susceptible to post-traumatic stress disorder (PTSD), a condition that has been linked to both sleep disorders [71] and early mortality,[72, 73] veterans are at a much higher than usual risk for adverse outcomes. Preliminary de-identified data extracted from Veterans' Integrated Services Network 7 (VISN-7) between 2000 and 2008

suggests that sleep disorder diagnoses may have increased over time among veterans, despite no differences in first time sleep disorder diagnoses and total first time diagnoses from VISN-7 medical centers, community-based outpatient clinics, and primary care clinics. If a relationship between sleep disorders and certain adverse health outcomes (e.g., mortality) exists, this group bears a heavier burden of disease and mortality compared to civilians.

Sleep disruption has increased and sleep quality has decreased in the United States for a variety of reasons and certain subgroups, such as veterans, may be at an increased risk for unfavorable health outcomes. It is critical to assess factors that may be driving the increase in sleep disorder prevalence. Because veterans are a vulnerable population, better behavioral therapies to achieve improved sleep quality could effectively reduce costs associated with subsequent adverse effects of sleep disruption. The public health implications of increased sleep disorder prevalence among veterans are not fully understood. Sleep disorders are associated with increased mortality and cancer, likely through pathways involving immunomodulation and tumor suppression, it stands to reason that sleep disorders may increase cancer risk. In support of this, sleep apnea was associated with increased cancer incidence.[51] A descriptive characterization of the sleep disorder epidemiology is vital for beginning to understand the impact on long term comorbid disease and mortality risks.

2. 3 Colorectal cancer (CRC) in the United States

While the number of incident cases and deaths due to CRC is alarming, these numbers have decreased in the last two decades in the United States due to removal of precancerous lesions during screening, [74, 75] which has increased three to eight-fold during this same time period.[76] Another factor contributing to the decline is the improvement in treatment of CRC, allowing for the survival of over 1 million cases of CRC. Despite the decrease in incident cases of CRC and deaths due to CRC, the lifetime risk for being diagnosed with CRC is 5% for men and women, with men being at a higher risk compared to women (57.2 per 100,000 vs 42.5 per 100,000, respectively). Men are also at a higher risk for death due to CRC compared to women (21.2 per 100,000 vs 14.9 per 100,000, respectively).[77] Differences in rates by gender can be attributed to hormones and exposure to other risk factors that are related to gender.[78-80] In addition to gender differences, burdens in CRC incidence and mortality by race/ethnicity are evident. EAs have a lower incidence rate compared to AAs (44.7 per 100,000 vs 53.1 per 100,000, respectively).[77] Also, AA men are more likely to die from CRC compared to EA men (30.5 per 100,000 vs. 20.9 per 100,000, respectively). Similarly, AA women are more likely to die from CRC compared to EA women.[77] These statistics are reflected at a state level such as in South Carolina.[81] In addition to gender and racial/ethnic differences, there is geographic variation in CRC incidence and mortality that is likely driven by access to screening and treatment centers.[9] Because of this combination of factors, only 62% of eligible

adults, defined as adults 50 to 75 years of age, have been screened for CRC. As a result, there has been an increase in research effort in finding markers of increased risk within readily accessible samples such as blood to improve CRC screening compliance.

2. 4 Risk factors for CRC

Previous studies have indicated several factors that do or may increase the risk for CRC. Lifestyle and environmental factors such as diets high in total fat and red meat, sedentary lifestyle, cigarette smoking, low doses of vitamin D and/or calcium, alcohol consumption, tobacco use, and obesity.[82] Previous studies have also found that personal history of colorectal adenomas increase the risk of developing CRC. Research has shown that patients with adenomas had a 25% increased risk of CRC at that particular site.[83] Removal of adenomas has also been shown to decrease the risk of incident CRC.

Another factor playing a role in the carcinogenesis process is chronic inflammation, where several factors may interfere with the balance between anti-tumor immunity and tumor-promoting inflammation. According to prior studies, up to 20% of cancers are linked to chronic infections,[84] 30% are due to inhalation of pollutants (e.g., tobacco smoke),[85] and 35% to dietary factors.[86] Inflammation also appears to play a role at several stages during the carcinogenesis process. In terms of tumor initiation, a pro-inflammatory environment may increase the likelihood of mutations, genomic instability, and epigenetic modifications, thus extending survival of premalignant cells. These pro-inflammatory conditions

allow premalignant cells to accumulate further mutations and form early tumor nodules. Early tumor nodules continue to thrive in the presence of inflammation, facilitating their growth into advanced tumors, and subsequently, spread of these mutated cells (metastasis).

In addition to these risk factors, several genes have been found to play a significant role in CRC risk, where molecular events involving these genes result in chromosomal instability, gene hypermethylation of cytosine-phosphate-guanine (CpG) islands, and microsatellite instability. In 1990, Fearon and Vogelstein proposed a mechanism explaining how multiple genes play a role in colorectal carcinogenesis.[87] For example, loss of the *Adenomatous Polyposis Coli* (*APC*) gene, which is responsible for tumor suppression, is one of the earliest events in the chromosomal instability colorectal tumor pathway and germline mutations have been shown to be associated with conditions that predispose individuals to colorectal tumors, such as familial adenomatous polyposis (FAP).[87] However, germline mutations that lead to FAP are a minority (5-6%) of CRC cases. A large number of CRC cases (approximately 75%) are sporadic, meaning that there is no apparent family history of CRC and may be due to a common exposure, a genetic background (i.e., sporadic mutation) that would put an individual at a high risk for developing CRC, or a combination of both.[88] More recently, a study looking at 13000 genes found that tumors have an average of 90 mutant genes. Among these genes, 69 genes were relevant to the pathogenesis of CRC, and individual CRCs had an average of nine mutant genes per tumor, revealing a multigenic process in the carcinogenesis in the colon.[18]

Also, this study revealed that there may be alternative explanations as to why certain populations are more susceptible to cancer than others, such as shift workers, who have been found to have gene dysregulation, particularly in the *Period* (*PER*) gene family. Details on molecular changes in the carcinogenesis process are described further in section 2.5 below.

2. 5 Clock genes, circadian disruption, sleep disruption, and cancer

While several genes have been identified as being linked to CRC formation, some genes, known as clock genes, which are involved in circadian rhythm regulation, have not been studied as frequently. In prokaryotic and eukaryotic organisms, most physiological and behavioral (such as sleep and wakefulness, body temperature, blood pressure, immune responses) functions are expressed rhythmically according to light-dark cycles to allow for adaptation to changes in the environment and ensure survival. There are three components in this circadian rhythm: input pathway, central pacemaker, and output pathway. The central pacemaker, which is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, is of particular interest due to its role in synchronization of the rhythm with the environment. At least nine core clock genes have been found to play a role in circadian oscillation: *PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, TIM,* and *CK1ε* ,[33] which are responsible for ~2-10% of the diurnal expression of most tissues in the mammalian genome.[89] Because of the central role that circadian rhythm plays in both homeostatic and non-homeostatic functions,[26, 90] it is essential for the body to maintain this rhythm. However, a variety of environmental (e.g., shiftwork) [91-93] or

genetic factors (genetic polymorphisms or epigenetic modifications) [30, 94] can confuse the master clock, thus disrupting the phase or amplitude of circadian rhythms and the physiological processes they help regulate, such as cell proliferation and apoptosis.[95] Disruption of these processes has been suspected of increasing the risk of cancer in certain populations (e.g., shiftworkers).[96] Effects found in epidemiological studies have been confirmed in studies using mouse model systems. For example, ablation of the SCN in mice resulted in an accelerated tumor growth rate compared to mice with an intact SCN.[97]

Differential methylation of clock genes can lead to differential epigenetic regulation of gene expression, and thus alter individual susceptibility to certain cancers.[30] While consistent evidence between circadian rhythm disruption and cancers of the breast and prostate exists,[42, 98-102] very few studies have examined the role of circadian rhythm disruption due to genetic and/or environmental factors and CRC in human populations; however, these few studies suggest a relationship.[103-105] For example, Schernhammer et al found a 35% increase in risk for CRC for nurses who engaged in long-term rotating shift work three nights per month compared to those who never worked rotating night shifts.[106]

As a consequence of circadian rhythm disruption, disordered sleep may arise. Certain groups such as shiftworkers are more likely to suffer from disordered sleep than those who work day shifts. For example, nurses who work night shifts have shorter sleep duration compared to their sleep duration after a day shift.[107] It is believed that the pathway between this factor and outcome may be mediated by the immune system dysregulation following sleep loss.

Experimental sleep deprivation and epidemiological studies looking at fatigue have found increased circulating levels of inflammatory markers such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP), with significant elevations,[20-25] even after one night of sleep loss.[23] Because previous studies have shown that sleep deprivation affects the inflammatory process and that sleep quality has decreased across time, it may be important to elucidate the relationship between sleep disruption and cancer.

Several mechanisms have been proposed that link sleep disruption to cancer formation. The first may be due to reduced melatonin release as a result of sleep disruption, thus leading to a reduction in natural killer (NK) cells and cytokines responsible for cancer inhibition (IL-2, IL-12, and TNF-α) and development of cancer cells; however, evidence supporting this mechanism is limited. Another mechanism suggests that the carcinogenesis process arises due to issues with melatonin acting upon cells locally by halting the cell proliferation process in cancer cells.[41]

Not only have sleep disorders been implicated in the carcinogenesis process, but factors related to sleep disorders (such as sleep disordered breathing) also played a role on cancer-related mortality,[44] possibly through reduced oxygen delivery to tumor tissues, a factor that predicts cancer progression and poor prognosis.[108, 109] In summary, genes related to circadian rhythm regulation have a role in sleep disruption and inflammatory processes related to tumor suppression/cancer inhibition either through genetic variation or alterations in function due to epigenetic changes. Also, there is reason to believe

that these genes could serve as a marker for both cancer risk and survival due to the physiological role that sleep has on carcinogenesis and cancer-related mortality. While previous studies have made strides in closing the gap, these gaps in research warrant further study.

2. 6 Biological markers for CRC

The adenoma-carcinoma sequence within the colon has been widely studied due to the ability to observe genetic alterations at several stages. Not only do gene variants existing in the form of single nucleotide polymorphisms (SNPs) play a role in conferring risk, but gene expression and the epigenetic modifications that influence gene expression (via methylation, histone modification, phosphorylation) are believed to influence the carcinogenesis process in the GI tract. Epigenetic modifications often involve methylation at the CpG islands within the promoter regions of the genes and methylation of these areas lead to gene silencing (reduced gene expression in the form of lower mRNA levels). Therefore, methylation of genes responsible for tumor suppression, DNA repair, and other physiological processes that control cell division and growth could lead to dysregulation of metabolic processes, thus leading to cancer formation. Epigenetic events are often due to simply inheriting epigenetic markers from parents or from external sources from the environment, such as diet. Because of the reversibility of epigenetic modifications and their influence on the carcinogenesis process, this area is being actively studied as a potential therapeutic modality.

2.6.a Genetic markers in CRC

Genetic markers have been explored to predict risk for cancer. Unique genetic markers are particularly favorable because certain cancers cannot be fully explained by heavily studied genes (*BRCA1*, *BRCA2, APC*), especially among younger individuals with cancer.[110, 111] For example, in 2005, Zhu et al found that women who had at least one 5-repeat allele (~50% are heterozygous for the 5-repeat allele and 10% are homozygous for the 5-repeat allele) in the *PER3* variable number tandem repeat (VNTR) had an increased risk for premenopausal breast cancer.[19] This gene, where individuals may possess a 4/4, 4/5, or 5/5 genotype, may be responsible for variations in circadian rhythm (diurnal preference, delayed sleep phase syndrome, sleep-wake patterns).[34-40] This may be due to the fact that certain individuals with the 4/5 or 5/5 genotype may have a different pattern of secretion of hormones related to sleep-wake cycles (i.e., melatonin). Other studies have revealed apparent tumor suppressor properties of the *Period* genes (*PER1*, *PER2*, and *PER3*), particularly in cancers of the breast, endometrial lining, and prostate.[19, 27, 30, 112-116] Among these genes, *PER3* has been studied extensively and it has been associated with various phenotypes potentially including adverse health outcomes (bipolar disorder, heroin dependence, poorer cognitive performance after sleep deprivation) and recently has been considered a candidate genetic marker of cancer risk.[19, 29] Other genes that play a role in circadian timing, such as *CLOCK*, *BMAL1*, and *NPAS*, have also presented associations with cancer risk, providing evidence of the link between clock gene variation and cancer. The pathophysiological

processes that drive many of these associations remain to be determined. With regard to *PER3*, it is believed that variation in the VNTR affects patterns of cortisol secretion,[117] and disturbances in cortisol secretion have been linked to hyperactivity of pro-inflammatory processes[118] and increases in pro-inflammatory cytokines, such as IL-6,[24] which create an ideal microenvironment for the carcinogenesis process to take place

2.6.b Gene expression in CRC

Due to the series of molecular alterations that lead to CRC, gene expression has also been examined as a way to broaden knowledge on which genes act as either oncogenes or tumor suppressors. Inter-individual variation of gene expression levels has been found by tissue type. For many genes involved or suspected to be involved in CRC formation, tumor tissues in the colon compared to normal, adjacent tissue exhibited differential expression.[30, 119, 120] However, some tumor tissues are difficult to obtain in order to examine gene expression; therefore, it is important to develop other alternative, less invasive methods to detect predictive biomarkers, particularly in blood. Because disease status is believed to cause biochemical changes in blood, it is believed that these changes caused by disease state will alter gene expression levels of certain genes in blood cells.[121, 122]

To complement gene expression levels found in blood, some studies have looked at relationships between gene expression levels in blood, normal tissue, and tumor tissue. For example, in a study by Schena et al, investigators set out to compare expression levels of several genes known to be a part of the

nucleotide excision repair pathway (*ERCC1*, *ERCC2*, *ERCC4*, *XPA*, *XPC*, *XRCC1*, *XRCC3*, *APEX*, *OGG1*, and *MGMT*) in normal and tumor tissues from non-small cell lung cancer (NSCLC) and head and neck squamous cell cancer (HNSCC) patients with blood expression levels within the same person. First, it was found that expression of these genes were generally higher in these target tissues compared to blood. Second, moderately high (Pearson's correlations coefficient (r) of 0.5 to 0.7) positive correlations were found between gene expression levels in blood and tumor tissue, tumor and normal tissue, and blood and normal tissues. This finding highlights the potential usefulness of gene expression levels detected in peripheral blood as a proxy for those levels in tumor tissue. However, there are some drawbacks to using gene expression levels in blood. For example, certain genes that are highly expressed in target tissues may not necessarily be expressed in blood. Also, as gene expression is dependent on cDNA concentration and quality, cDNA quality is dependent on mRNA quality. Because mRNA degrades readily under suboptimal conditions, gene expression levels may not be accurate, thus producing a spurious relationship between gene expression levels and the outcome of interest (e.g., tumor formation). Relationships between gene expression levels in PBLs, normal tissue, and adenoma polyp tissue has yet to be fully characterized for the *Period* gene family.

2.6.c Epigenetic markers in CRC

A 2012 report by the Centers for Disease Control and Prevention showed that both eligible men and women (people over the age of 50 years) still fell short of the *Healthy People 2020* target CRC screening rate of 70.5%. [123] Because of various factors that limit the ability of individuals to be screened for CRC, [124-128] several studies have undertaken the task of identifying epigenetic markers in blood to assess risk of CRC. This method of assessing risk is particularly favorable due to the fact that patient compliance is likely to increase due to the ease of obtaining samples, thereby increasing the chance of detection of disease in earlier stages and improving survival of CRC, especially among groups who have low screening rates (e.g., racial/ethnic minorities, those without health insurance).[123] For example, in a 2007 pilot study by Cui, loss of imprinting (LOI), an epigenetic event in the *IGF2* gene, had a significant relationship with both family history and personal history of CRC, suggesting that LOI of *IGF2* could serve as a predictor of CRC risk.[129] Although LOI in this same gene could not be evaluated in PBLs, another study by Woodson et al confirmed findings by Cui et al by discovering that LOI in *IGF2* was associated with a fivefold increased risk of adenoma formation in women.[130] More recently, a study by Tänzer et al examined the epigenetic markers in *SEPT9* in plasma and its association with colorectal lesions (hyperplastic and adenomatous polyps). Researchers found that prevalences in methylation differed by case status: 9% in controls; 29% of subjects with precancerous lesions presented; and 73% of CRC patients.[131] Additionally, as a result of common differential methylation patterns being found

in tumor tissues (*MLH1*, *p16*, and methylated in tumors [*MINT1*, *MINT2*, and *MINT31*]), a subtype of CRC has been identified: CpG Island Methylation Phenotype (CIMP).[132] This phenotype has been observed in a feature present in variable amounts among adenomas (0-44%),[133, 134] colon cancers (30-40%), and distal and rectal cancers (3-12%).[135-138] Discovery of this particular molecular marker in CRC led to further understanding of alternative carcinogenesis pathways, especially in terms of epigenetics. In regards to the epigenetic profiles of *PER1, PER2,* and *PER3*, and their association with cancer risk, Chen et al found deregulated expression of *PER1*, *PER2*, and *PER3* in breast tumor tissues compared to normal tissues. Furthermore, it was found that promoter methylation influenced expression of these genes.[30] No published studies have looked at epigenetic markers of these genes in circulating blood cells and their association with cancer risk in human populations.

2. 7 Summary and public health significance

Sleep disorders and variation of clock genes have demonstrated associations with various health outcomes that can have a significant impact on disease morbidity and mortality. Thus far, no study has assessed trends in sleep disorders among veterans at a national level (Specific Aim 1), evaluated the performance of PBL methylation levels in *PER1*, *PER2*, and *PER3* on predicting colorectal adenoma formation (Specific Aim 2), or has investigated the impact of *PER3* variation on adenoma risk (Specific Aim 3). Results derived from this project not only would have the ability to identify vulnerable sub-populations among Veterans who are at risk for the development of sleep disorders but also

will add to the scientific body of knowledge on adenoma formation and could provide information on alternative strategies to identify colorectal adenoma cases more effectively. Furthermore, disparities in CRC incidence and mortality observed along racial/ethnic, gender, and geographic lines could partially be explained by sleep disorders and/or differential methylation of certain clock genes.

Chapter 3

The National Veteran Sleep Disorder Study I: Descriptive Epidemiology and Secular Trends, 2000-2010[1]

[1]Alexander M,[1] Ray MA, Hébert JR, Youngstedt SD, Zhang H, Steck SE, Bogan RK, Burch JB. Accepted to *Sleep.* Reprinted here with permission of publisher (Appendix A)

3.1 Abstract

Introduction: Sleep disturbances are alleged to be on the rise in the United States (US), and veterans are particularly vulnerable to factors that elicit or exacerbate sleep disorders. A large proportion of individuals affected by sleep disorders are untreated and susceptible to accidents, injuries, long-term sequelae (e.g., risk of cardiovascular disease, cancer, psychiatric disorders), and earlier mortality. Few studies have examined the scope and magnitude of sleep disorder diagnoses in the US. This serial cross-sectional study characterized secular trends in diagnosed sleep disorders among veterans seeking care in the US over an eleven-year span (Fiscal Years [FY] 2000-2010, N=9,798,034). **Materials and Methods**: Data from the Veterans Administration Informatics and Computing Infrastructure (VINCI) electronic medical record were accessed. Cases were defined as patients with at least two outpatient sleep disorder diagnoses at least 30 days apart using American Academy of Sleep Medicine-specified diagnostic codes. Age-adjusted annual prevalence was summarized by sex, race, combat exposure, body mass index (kg/m^2) and comorbid diagnoses (cardiovascular disease, cancer, mental disorders). **Results**: Sleep apnea (47%) and insomnia (26%) were the most common diagnoses among patients with sleep disorders. There was a six-fold relative increase in total sleep disorder prevalence over the study period. Post-traumatic stress disorder (PTSD) tripled over the same time period, and veterans with PTSD had the highest prevalence of sleep disorders (16%) among the comorbid conditions evaluated.

Discussion: The results indicate a growing need for integration of sleep disorder management in patient care and health care planning among veterans.

3.2 Introduction

Sleep is considered a physiological necessity, and inadequate sleep has been associated with a wide range of adverse physical, mental and behavioral outcomes.[139] Previous studies have linked abnormal sleep duration or a sleep disorder diagnosis with an increased incidence of: obesity, hypertension or metabolic syndrome,[53, 54, 140, 141] type 2 diabetes,[53, 54, 142] cardiovascular disease,[52, 143, 144] stroke,[145, 146] and cancer[59, 60, 147-150] (although results for cancer have been less consistent). Other studies have linked inadequate sleep with an increased risk of psychiatric disorders (e.g., depression, post-traumatic stress disorder [PTSD]), suicides, accidents, injuries, reduced quality of life, and increased mortality.[65, 139, 144, 151-158] The impacts of sleep disruption may be mediated by several pathophysiological processes. These include sympathetic nervous system hyperarousal, the disruption of circadian rhythms, neuroendocrine or immune system dysregulation, inflammation, or metabolic dysfunction.[159-162]

Military personnel are particularly vulnerable to sleep disturbances due to the irregularity of their sleep/wake schedules, austere living conditions (e.g., extremes in temperature, noise, physical exertion), the stress of combat, elevated rates of physical and psychological injury, and issues associated with

post-deployment psychosocial reintegration.[155, 163] Some of these conditions can have residual effects well after the period of service is over. Therefore, veterans can be plagued with multiple comorbidities that adversely influence sleep. Some of these are over-represented in this population (e.g., musculoskeletal pain, traumatic brain injury [TBI], smoking, substance abuse, hypertension, depression, PTSD), while others mirror trends in the general US population (e.g., obesity, aging-related chronic disease).[155, 156, 163-166] Consequently, sleep disruption tends to be more common among veterans than in the general population.[155, 167-170] However, the national scope and characteristics of this problem have not been described previously.

Despite evidence indicating that a lack of recognition or poor management of sleep disorders can have myriad detrimental health consequences, many patients can remain undiagnosed or untreated. Sleep disorders also may be overlooked by physicians;[171, 172] it was estimated that 80-90% of people with clinically significant sleep-disordered breathing may remain undiagnosed.[48] In a representative sample of United States (US) adults, 26% of survey respondents met criteria for high-risk obstructive sleep apnea[173] in contrast to an estimated prevalence of ~4-10%.[64, 174, 175] An increasing trend in office visits for sleep disorders was reported from 1999 to 2010, although it is unclear whether this was due to a true increase in sleep disorder prevalence, increasing awareness among physicians or their patients, or a combination of both.[65] Differences in sleep disorder case definitions, diagnostic criteria, population demographics, or

the presence of co-morbid disease also can contribute to inconsistencies among population-based sleep disorder estimates.

The co-occurrence of a sleep disorder with another co-morbid chronic disease can predict poor quality of life[62, 63] and earlier mortality relative to those without a sleep disorder.[144, 158, 176] This suggests that appropriate sleep disorder management may improve quality of life and longevity. In some studies, psychiatric disorders were predictive of sleep problems,[55, 57] but in other cases sleep disorders predicted increased risks for psychiatric disorders.[177-179] This apparent bi-directionality can complicate etiologic studies, but it also provides an opportunity to implement more effective disease prevention and control strategies through delivery of care that integrates appropriate sleep management.

The information described above suggests that sleep disorder surveillance can play an important role in patient care and long-term health care planning. The characterization of trends in sleep disorder diagnoses among population subgroups may allow clinicians to identify and target high-risk patients who would benefit from management of sleep disorders and their sequelae. Most epidemiologic studies examining sleep disorders have been limited to: highly specific study populations (e.g., sleep clinics), relatively small population sizes, or to a single type of sleep disorder or risk factor of interest. This study evaluated all diagnosed sleep disorders in a national sample of US veterans who utilized health services through the Veterans Health Administration (VHA) between

Fiscal Years (FY) 2000 and 2010. The VHA is the largest integrated health care system utilizing electronic medical records (EMRs) in the US.[180] The approach facilitated the examination of comorbid diseases that appear at higher rates among veterans relative to the general population (e.g., PTSD and other mental health disorders), as well as other individual or clinical factors that may influence the onset or severity of sleep disorders.

3.3 Materials and Methods

Study Population

The study population consisted of all US veterans seeking care in the VHA system between FY2000 and FY2010. Following regulatory approvals, outpatient electronic medical records were accessed from MedSAS Dataset and VA Corporate Data Warehouse files in the VINCI system. Requested data elements from different VA system files were linked via social security number by a system data manager, and then scrambled and replaced with a unique patient ID number. Unique identifiers were inaccessible to the study investigators. Patients excluded were: <18 years old on January 1 of each year, veterans who died in a given fiscal year, spouses of veterans receiving care, and non-veterans.

Case Ascertainment

De-identified outpatient sleep disorder diagnoses were grouped according to American Academy of Sleep Medicine (AASM) International Classification of Sleep Disorder[181] categories based on the following ICD-9 codes: apneas

(organic sleep apnea [327.20-29], unspecified sleep apnea [780.57]); insomnias (organic insomnias [327.00-09], insomnias, unspecified [780.51-780.52], nonorganic insomnias [307.41-307.42]); other sleep disorders (other organic sleep disorder [327.80], specific disorders of sleep of nonorganic origin [307.40-307.49], other sleep disturbances, unspecified [780.50, 780.56, 780.59], other sleep disorders [291.82, 292.85]); hypersomnias (organic hypersomnias [327.10-19], nonorganic hypersomnias [307.43-.44], hypersomnias, unspecified [780.53-780.54], other hypersomnias [347.00-347.01, 347.10-347.11]); parasomnias (organic parasomnias [327.40-327.49], nonorganic parasomnias [307.46-307.47]); sleep disruption movement disorders (organic sleep related movement disorders [327.51-327.59], sleep related movement disorder, unspecified [780.58], Restless Legs Syndrome [333.94]); and circadian rhythm disorders (organic circadian rhythm sleep disorders [327.30-39], circadian rhythm sleep disorder of nonorganic origin [307.45], disruption of 24 hour sleep-wake cycle, unspecified [780.55]). Veterans were assigned a specific sleep disorder diagnosis if their ICD-9 code occurred at least two times within a given fiscal year, and the codes occurred thirty or more days apart.[182] This method was used to reduce the chance of misclassification due to “rule out” diagnoses, which are ICD-9 codes that are entered by providers as the reason for ordering a diagnostic test).

Covariates

Data on covariates (age, race/ethnicity, sex, smoking status, body mass index [BMI], income, comorbid diagnoses, sleep-related medical procedures, service period [World War II, Korean War, Vietnam War, Persian Gulf War, Operation Enduring Freedom/Operation Iraqi Freedom, and other], and combat deployment) were retrieved from the VHA's Vital Status and MedSAS databases. Income was divided into three categories: less than $20,000 a year, $20,000 or more, or unknown.[58] Race and ethnicity were categorized as: Non-Hispanic White, Non-Hispanic Black, Hispanic or Mexican, or "Other" (includes American Indian or Alaska Native, Asian, Native Hawaiian or other Pacific Islander, or more than one race). Missing race may be associated with lower rates of utilization and fewer comorbid diseases,[183] and thus was included as a separate category. Age, in years, was calculated by subtracting the year of birth from the fiscal year of contact with the VHA system and grouped into the following categories: 18-29 years, 30-39 years, 40-49 years, 50-59 years, 60-69 years, 70-79 years, and 80 years or older. The twenty-one Veterans Integrated Service Networks (VISNs) were collapsed into US census regions (VISNs 1-5: Northeast; 10-15 and 23, Midwest; 6-9, 16, 17, South; 18-22, West). Those with missing or infrequent VISN assignment were coded as unknown. BMI was calculated using height and weight after excluding biologically implausible values; height was restricted to 48-84 inches (122-213 cm), and weight was restricted to 75-500 pounds (34-227 kg).[184] If a value for height was missing for a given record, any appearance of height across the study period was used. Multiple entries for biologically

plausible values of weight were averaged within the fiscal year. BMI was categorized into the following groups: underweight (<18.5 kg/m^2), normal (18.5 to <25 kg/m^2), overweight (≥ 25 and < 30 kg/m^2), and obese (≥30 kg/m^2). The underweight BMI category (<1% of study population) was combined with the normal BMI group. Smoking status was assessed as described previously;[185] data extracted from EMRs were compared to self-reported smoking data found in the Veterans Aging Cohort Study (VACS-8) and VACS Virtual Cohort (VACS-VC) study, which yielded kappa (κ) statistics of 0.66 and 0.61, respectively.

ICD-9 codes for comorbid disease were grouped into the following categories: asthma (493), cancer (150-151, 153-155, 157, 162, 172, 174, 183, 185, 188-189, 191, 200, 202, 204-208.9), chronic obstructive pulmonary disease (COPD) (496.0), cardiovascular disease (CVD) (390-459), diabetes (249-250), fibromyalgia (729.1), gastrointestinal (GI) disease (520-579), human immunodeficiency virus (HIV) (042-044), any mental disorder (290-319), renal disease (508-589), and stroke (434.91) using the same criteria as described for sleep disorders. Diagnoses of any mental disorder/mental disability were broken down into the following subgroups for further analyses: depression [296.3], post-traumatic stress disorder [PTSD] [309.81], other mental health disorders with the exception of PTSD and depression [e.g., psychosis [290-299], neurotic disorders, personality disorders, and other nonpsychotic mental disorders [300–316], mental disability [317–319]).

Sleep-related medical procedures were ascertained using the following current procedural technology (CPT) codes: 0203T (sleep study, unattended, simultaneous recording of: heart rate, oxygen saturation, respiratory analysis and sleep type; type II device), 0204T (sleep study, unattended, simultaneous recording of: heart rate, oxygen saturation, respiratory analysis and sleep type; type IV device), 95805 (multiple sleep latency or maintenance of wakefulness testing, recording analysis and interpretation of physiological measurements of sleep during multiple trials to assess sleepiness), 95806 (sleep study, unattended, simultaneous recording of heart rate, oxygen saturation, respiratory airflow and respiratory effort [EEG, thoracoabdominal movement] with Type III device), 95807 (sleep study, simultaneous recording of ventilation, respiratory effort, ECG or heart rate, and oxygen saturation, attended by a technologist), 99508 (home visit for polysomnography and sleep studies), G0398 (home sleep study test [HST] with Type II portable monitor, unattended; minimum of 7 channels: EEG, EOG, EMG, ECG/heart rate, airflow, respiratory effort and oxygen saturation), G0399 (HST with Type III portable monitor, unattended; minimum of 4 channels: 2 respiratory movement/airflow, 1 ECG/heart rate, and 1 oxygen saturation), and G0400 (HST with Type IV portable monitor, unattended; minimum of 3 channels).

Statistical Analysis

Annual sleep disorder prevalence was calculated as the proportion of total sleep disorder diagnoses among eligible patients seeking care in the VHA

system each fiscal year of the study. The numerator was defined as the total number of primary sleep disorder diagnoses (or specific sleep disorder subtype) that occurred during each fiscal year, and the denominator was the total number of Veterans seeking care through the VHA during in the same fiscal year. Annual prevalence was adjusted for possible changes in the age distribution over time via direct age standardization using the 2000 US standard population and the following age groups: 18-29, 30-39, 40-49, 50-59, 60-69, 70-79, and 80 years or older.[186] Absolute change in prevalence across the study period was defined as the age-adjusted prevalence estimate for FY2000 subtracted from the age-adjusted prevalence for a given fiscal year. Two-sample proportion tests were used to compare proportions of select factors in FY2000 and FY2010. Relative changes were described as well. Prevalence and absolute change in prevalence of sleep disorders were stratified by comorbid CVD and cancer diagnoses, as they represent the top causes of mortality in the US, PTSD (a common diagnosis among US Veterans), and other selected factors (sex, race, BMI, combat exposure).

3.4 Results

Data for 9,786,778 veteran patients were included in the analysis (Table 3.1). Changes in the population from FY2000 to FY2010 are found in Table 3.2. The following groups seeking care in the VHA system had the greatest absolute increases over time: white veterans, those aged 60-69 years of age and over 80 years of age, unmarried veterans, those living in the southern region of the US,

those who served in the Persian Gulf, obese veterans, current smokers, and those with at least one mental health disorder. Figure 3.1 summarizes inclusions and exclusions among patients who sought care through the VHA during the study period. The study population was comprised primarily of men (93%) who were married (54%). Among veteran patients diagnosed with at least one sleep disorder (n of unique individuals: 751,502; n of diagnoses: 953,575), apneas made up the majority of the diagnoses (47%, Figure 3.2); followed by insomnias (26%), other sleep disorders (11%), hypersomnias (10%), movement disorders (4%), parasomnias (3%), and circadian rhythm sleep disorders (0.1%). A majority of those diagnosed with a sleep disorder were prescribed at least one sleep medication (91%, data not shown), and the proportion of veterans with any sleep disorder who were prescribed a sleep medication for the entire time they sought VHA care within the study period ranged from 24% to 64% based on the number of years of follow-up (data not shown).

Total sleep disorder diagnoses increased nearly six-fold over the eleven-year study period (Figure 3.3). Sleep apnea and insomnia prevalence exhibited more than a seven-fold relative increase; age-adjusted sleep apnea prevalence increased from 0.4% in FY2000 to 3.0% in FY2010 (a relative increase of 650%); and age-adjusted insomnia prevalence increased from 0.2% in FY2000 to 1.5% in FY2010 (a 650% relative increase). The prevalence of other sleep disorders rose from 0.4% in FY2000 to 0.6% in FY2010 (a relative increase of 50%). Less prevalent sleep disorder subtypes also increased over time (relative increases

were: movement disorders: 1,048%; hypersomnias: 50%; parasomnias: 750%; circadian rhythm disorders: 900%).

When the pattern of CPT codes for sleep procedures was inspected over time, there was nearly a three-fold increase in the proportion of veteran patients receiving a sleep-related procedure during the study period (0.2% in FY2000, 0.15% in FY2010). These proportions were considerably lower than diagnosed sleep disorders; 1/6th to 1/13th of the observed prevalence of sleep disorder diagnoses (Figure 3.4). CPT code 95807 (sleep study, simultaneous recording of ventilation, respiratory effort, ECG or heart rate, and oxygen saturation, attended by a technologist for obstructive sleep apnea) was the most common code in FY2000 (0.09%) and exhibited a small increase in prevalence by the end of the study period (0.11%). CPT code 95806 (sleep study, unattended, simultaneous recording of, heart rate, oxygen saturation, respiratory airflow, and respiratory effort) was the second most common procedure for sleep in FY2000 (0.05%), but became the most common sleep procedure by FY2010 (0.30%), which corresponds to a five-fold relative increase during the study period.

Veterans diagnosed with CVD had a larger absolute increase in sleep apnea prevalence over time compared to veterans without CVD (5.8% and 1.6%, respectively, Figure 3.5). Veterans with a CVD diagnosis also experienced an absolute increase of insomnia prevalence (2.6%) more than double the absolute increase among veterans without CVD (1.1%). Less common sleep disorder subtypes also exhibited greater increases in sleep disorder prevalence among those with CVD compared to those without CVD.

Figure 3.6 presents changes in sleep disorder prevalence stratified by cancer diagnosis. From FY2000 to FY2010, absolute increases sleep apnea and insomnia prevalence were 3.7% and 2.0%, respectively, among veterans with cancer. These absolute rate increases were greater than those recorded among veterans who were not diagnosed with cancer (2.5% and 1.3%, respectively). The remaining sleep disorders, with the exception of other sleep disorders, exhibited larger increases in prevalence among those diagnosed with cancer compared to those who were not.

Absolute changes in sleep apnea and insomnia over the study period were the largest among veterans with PTSD (5.7% and 4.3%, respectively, Figure 3.7). Smaller absolute increases in prevalence were observed for the other sleep disorder subtypes, ranging from 0.02% (circadian rhythm disorders) to 1.4% (other sleep disorders). Increases in sleep disorder subtypes over time also were observed when the data were stratified by any mental health disorder diagnosis (Figure 3.8), any depression diagnosis (Figure 3.9), or by mental health disorders other than PTSD or depression (Figure 3.10), the largest absolute increases were observed among those with sleep apnea.

Since combat can be related to development of PTSD and mental health disorders, sleep disorder types were stratified by combat exposure as well (Figure 3.11). In FY2010, the prevalence of sleep apnea and insomnia was 7.6 times and 6.3 times greater among combat veterans than noncombat veterans, respectively. A similar pattern was observed for other sleep disorders; by 2010,

there was a greater than seven-fold relative difference in total sleep disorder prevalence among veterans or without combat experience.

Sleep disorder prevalence also was examined across BMI categories (Figures 3.12a-3.12g). As expected, obese veterans (BMI ≥30 kg/m^2) experienced the largest increase (5.6%) in sleep apnea prevalence over time (1.4% in FY2000, 7.0% in FY2010, Figure 3.12a). For insomnia, veterans who were underweight or had normal BMI were the fastest growing group, with a 1.8% absolute increase in insomnia prevalence over time (0.3% in FY2000, 2.1% in FY2010, Figure 3.12b).

Figure 3.13 presents sleep disorder prevalence stratified by gender. Male veterans consistently had a higher prevalence of sleep apnea across the study period. Female veterans either had the same or higher insomnia prevalence until FY2008; thereafter, their rates were exceeded by those of male veterans. Figures 3.14a-3.14g present trends in sleep disorder prevalence by race and ethnicity. The prevalence of sleep apnea increased by 3.1% among Hispanic or Mexican-American veterans over the study period (0.5% in FY2000 to 3.6% in FY2010, Figure 3.14a), the largest increase among all race/ethnicity groups that were evaluated. This group also had the highest prevalence of sleep apnea among those studied. Veterans of unknown race/ethnicity had the largest absolute increase (1.5%) in insomnia prevalence over time (0.3% in FY2000 to 1.8 % in FY2010, Figure 3.14b). The northeast and southern US regions dominated increases observed in sleep disorder prevalence (Figures 3.15a-3.15g). Specifically, the northeast region experienced the greatest absolute

increases in sleep apnea, circadian rhythm disorders, parasomnias, and other sleep disorders; whereas the southern region experienced increases in insomnias, hypersomnias, and movement disorders.

3.5 Discussion

Results from this study indicate a notable rise in sleep disorder diagnoses, especially for sleep apnea and insomnia, among US veterans who sought care through the VHA from FY2000 through FY2010. The largest increases were noted among veterans grouped according to certain demographic or clinical characteristics, particularly those with PTSD, other mental health disorders, or combat experience. The increasing trends observed in this study are consistent with recent results from the National Ambulatory Medical Care Survey,[65] where a 442% relative increase in office visits for sleep apnea was reported within a similar time period (1999-2010).[65] Those investigators acknowledged uncertainty as to whether this represented a true increase in prevalence or if it was due to heightened awareness, or a combination of factors. In 2004, veterans requiring the use of breathing assistance equipment, such as continuous positive airway pressure (CPAP) devices, were granted a service-connected disability benefit for sleep apnea,[187] which likely led to more veterans discussing sleep complaints with their provider. However, increases in sleep disorders in the present study were still observed prior to 2004; for example, apnea prevalence nearly doubled between FY2000 and FY2003. Thus, the changes in sleep disorder prevalence may not have been attributable solely to this administrative change. In addition, rates for other sleep disorders not covered by the new disability rating also

increased during the same period. Potential administrative bias was evaluated in this study by examining trends in the utilization of clinical procedures (CPT codes) for a sleep disorder diagnosis. The increases in prevalence observed for sleep apnea or other sleep disorders did not match the magnitude or frequency of diagnostic procedures for sleep that were recorded during the same period (Figure 3.4). Some veterans may have sought medical care outside of the VHA system.[188] A previous investigation found that ~28% of veterans reported dual-use primary care (VA and non-VA).[189] It is unknown whether such patients may have preferentially sought care for sleep disorders at non-VHA facilities, but the possibility cannot be excluded.

Other study uncertainties included a lack of information on the relative proportions or influx of certified sleep physicians entering the VHA system during the study period, or the extent to which updates in diagnostic criteria may have influenced national trends in sleep disorder diagnoses among VHA providers. The Accreditation Council on Graduate Medical Education (ACGME) initiated a national sleep medicine training and certification program in 2004-2005,[190] which coincides with an increase in movement disorder diagnoses that were virtually absent within the VHA medical record in prior years. This suggests that providers may not have been trained to recognize these sleep disorders, and that veterans may have been under-diagnosed for these conditions prior to the initiation of this program. The trajectory of sleep disorder diagnoses over the study period suggests that upward trends may continue beyond FY2010.

Only a few studies have examined the prevalence of diagnosed sleep disorders in a national sample, and several of those studies focused on veterans.[58, 183, 191] Prior investigations examined prevalence over a shorter time frame (i.e., across one or two fiscal years) and/or evaluated a single type of sleep disorder.[58, 183, 191] In the current study, sleep apnea prevalence ranged from 0.4% to 3.0%, and insomnia prevalence ranged from 0.4% to 1.5%. A large cohort study examining the relationship between sleep apnea and psychiatric comorbidities reported a sleep apnea prevalence of 2.9% in a two-year period (FY1998-FY2001),[191] which exceeds the current study's prevalence of 0.4% in FY2001. Another investigation of sleep apnea among veterans ≥65 years old utilized criteria similar to the present study (apnea cases were defined as individuals with two outpatient diagnoses at least seven days apart, or one inpatient diagnosis) and reported a sleep apnea prevalence of 4.4%.[183]

In the current study, age-adjusted sleep disorder prevalence did not differ notably from crude rates (data not shown). When the current study population was restricted to veterans ≥65 years old in FY2005, a sleep apnea prevalence of 3.6% was obtained, which is reasonably consistent with the previous studies.[183, 191] A recent study of insomnia prevalence reported that 3.4% of veterans in FY2010 had insomnia,[58] which is more than double the prevalence found in the current study in the same year (1.5%), but is consistent with the prevalence of self-reported, physician-diagnosed insomnia that was recently described in a nationally representative sample of the non-institutionalized US population (1.2%).[64] Inconsistencies in sleep disorder prevalence reported among different

studies may be due to differences in case definitions (e.g., some studies used a single occurrence of sleep disorder, which could overestimate case frequencies), or a tendency for physicians to consider sleep impairment as a comorbid symptom rather than a diagnosable disorder. Differences in population demographics, comorbidities, or the time period studied also may contribute to differences among published studies.

An increase in the prevalence of comorbid diseases may have contributed to the increasing trend of sleep disorder diagnoses in the current study. Veterans with cardiovascular disease, cancer, or mental health disorders (particularly PTSD) experienced higher rates of sleep disorder diagnoses, a phenomenon that has been observed in other populations.[192-194] Veterans tend to have more mental disorders compared to the general population,[49, 144, 158, 176, 195-197] and the co-occurrence of sleep disorders among those with PTSD or other psychiatric disorders has been described previously.[55, 57, 177-179, 198] The current study used two occurrences of a PTSD diagnosis to identify veterans with PTSD, which has a positive predictive value (PPV) of 82%, whereas a single diagnosis only had a PPV of 75%.[199] A 12-month prevalence study of DSM-IV disorders indicated that 3.5% of Americans had PTSD,[200] which is similar to what was observed in the present study during the same time period (FY2001-2003, 3.3-3.5%). PTSD prevalence in this study also was consistent with the previously reported range among veterans (2-17%).[201] PTSD rates among veterans increased three-fold over the eleven-year period, an absolute increase of almost 7%. It was the greatest change among the mental health disorders evaluated, resulting in a

PTSD prevalence of 10% by FY2010. The US' multiple-front war effort that occurred during this period likely contributed to this striking trend.[202]

Sleep disturbances are a significant component of PTSD; some studies report that sleep disturbances can predict the development of PTSD or depression.[156] However, there is no clear consensus on the cause-effect relationship between sleep disorders and psychiatric outcomes, and these relationships may be bi-directional. For example, one study found that insomnia preceded a current anxiety disorder in less than 20% of cases, that anxiety appeared prior to insomnia in 44% of cases, and that these disorders co-occurred in approximately 40% of the cases.[179] In a community-based sample of adolescents with comorbid disorders, nearly 75% of subjects had anxiety prior to their insomnia diagnosis, but prior insomnia was not associated with onset of anxiety disorders in that study. Another investigation found that generalized anxiety disorder and/or depression predicted increases in sleep problems over time.[177] This suggests that mental health disorders including PTSD may drive increases in sleep disorder prevalence among veterans over time, although the cross-sectional nature of the current study precludes an ability to infer causality. Some studies indicate that sleep problems can persist even after amelioration of PTSD or TBI symptoms, and that sleep improvement can facilitate better responses to PTSD therapy.[156] Others reported that successful treatment of sleep apnea led to clinically meaningful improvements in PTSD symptoms.[203] This highlights the importance of integrated strategies to ameliorate the long-term combined impacts of mental and sleep-related disorders among VHA patients.

An aging population has been frequently cited as a factor that could be driving an increase in sleep disorder prevalence, particularly sleep apnea. The use of age-standardization in the present study eliminated potential biases that may have been introduced due to a shift in the age distribution among veteran patients during the study period. Additionally, examination of changes within specific age groups over time did not provide strong evidence for aging to explain the observed trends. One unexpected finding was that sleep apnea prevalence did not display a monotonic increase with age (data not shown), which has been previously cited as a strong predictor of this disorder.[204] Instead, the prevalence of sleep apnea was highest among veterans 40-69 years old (data not shown). Also, veterans in both the 40-49 and 50-59 age groups decreased by almost 7%, while those aged 60-69 years increased by 6%. A previous study using either clinical or laboratory-based criteria found that sleep apnea prevalence peaked among middle-aged men.[205] Discrepancies with other reports may be due to differences in the criteria used to diagnose sleep apnea, the types of sleep apnea included in the analyses (i.e., obstructive, central, and mixed), not accounting for apnea therapy or other comorbid conditions, or a combination of these factors. Other reasons to explain this finding also have been offered.[58] First, older veterans may be less likely to seek medical care for sleep disruption symptoms. Moreover, as older veterans are more likely to be retired and the consequences of sleep disruption may be less likely to interfere with daily activities. Although veteran patients ≥80 years old exhibited the largest increase among age groups over time (Table 3.2), they consistently had among the lowest rates of sleep

disorder diagnoses in this population. Another explanation may be related to provider practices.[58] As more veterans return from recent conflicts, more PTSD or other comorbid diagnoses arise, which may make it more likely that providers diagnose these veterans with sleep disorders relative to older veterans.[58]

The obesity epidemic has been cited as another factor driving elevated sleep disorder prevalence, and veterans have experienced increases in obesity over time similar to the general population.[165, 184] The increased prevalence of obesity could account, in part, for increases in sleep apnea prevalence observed in this study over time. Previous investigations have reported an increased incidence of insomnia among obese individuals.[206] However, the prevalence of insomnia, the second-most common sleep disorder, rose fastest among veteran patients in the normal BMI category, which suggests that other factors, such as comorbid disease, may have had a predominant role in the trends observed in the present study. Obesity and overweight are risk factors for many major chronic diseases including CVD and cancer.

The strengths of this study included its large sample size, use of validated methods for characterizing sleep outcomes and important covariates, and a medical care system that ensured equal access to care. Furthermore, prevalence estimates were age-adjusted to account for changes in age distribution, and CPT codes for sleep procedures were examined to evaluate potential reporting bias over time. This study consisted of serial cross-sectional data, and was therefore subject to limitations typically associated with this type of study design, including a lack of appropriate temporality between sleep disorder diagnoses and their

predictors or modifiers. Furthermore, this investigation used only data from veterans who sought care through the VHA system, resulting in an over-representation of older men with multiple comorbidities; thus potentially limiting the generalizability of the results to other populations or to veterans who did not utilize VHA services.[207] Sleep disorder prevalence may have been overestimated due to the distribution of age or comorbid disease in this population. However, prevalence values were age-adjusted and stricter criteria for case definitions (two diagnostic codes thirty or more days apart) were used to reduce potential misclassification. This likely resulted in more conservative estimates of sleep disorder prevalence relative to some other studies.

In summary, results from this study suggest that prevalence of sleep disorder diagnoses increased among US veterans between FY2000 and FY2010, which may have important implications for the health and longevity of this population. Sleep disturbances are linked with increased mortality; for example recent meta-analyses reported 10-12% increases in mortality risk among those with short sleep duration.[152, 153] Also, clinically significant, untreated sleep apnea has been associated with increases in cardiovascular disease and all-cause mortality.[208] Since many sleep disorders are undiagnosed and therefore untreated, clinicians should take special care to recognize high-risk patients. Early identification and adequate treatment of individuals in the expanding population of sleep disorder patients may lead to lower health care utilization and improved quality of life. The cross-sectional nature of the current study design precludes resolution of the complex interrelationships between obesity, comorbid

disease, and sleep disorder diagnoses. Thus, further examination of these issues using other study designs is warranted.

Table 3.1. Characteristics of veterans who sought care through the Veterans Health Administration (VHA) system: FY2000 to FY2010)

Characteristic	Categories	Total population (N =9,786,778)[1]
		N (%)
Sex	Female	664,880 (6.8)
	Male	9,121,898 (93.2)
Race	White	2,730,235 (27.9)
	Black or African American	575,770 (5.9)
	Hispanic	241,914 (2.5)
	Other	162,966 (1.7)
	Unknown	6,075,893 (62.1)
Marital Status	Married	5,272,298 (53.9)
	Not Married	3,755,025 (38.4)
	Unknown	759,455 (7.8)
Comorbid diseases[2]	Asthma	263,306 (2.7)
	Cancer	768,351 (7.9)
	CVD	5,088,157 (52.0)
	COPD	875,006 (8.9)
	Diabetes	1,873,235 (19.1)
	Fibromyalgia	114,406 (1.2)
	GI Disease	3,077,094 (31.4)
	HIV	33,755 (0.3)
	Hypertension	4,258,724 (43.5)
	Mental disorder	3,469,637 (35.5)
	Depression	420,284 (4.3)
	PTSD	757,673 (7.7)
	Other mental disorder	3,224,565 (33.0)
	Renal Disease	430,010 (4.4)
	Stroke	11,324 (0.1)

Abbreviations: COPD, Chronic Pulmonary Obstructive Disease; CVD, Cardiovascular Disease; HIV, Human Immunodeficiency Virus; PTSD, Post-Traumatic Stress Disorder. [1]Number of veterans entering the system with age and gender information; [2]Disease categories are not mutually exclusive.

Table 3.2. Distribution and absolute percent change in population characteristics in veterans seeking care through the Veterans Health Administration, FY2000 - FY2010

Characteristic	Categories	FY2000 (n=3,467,444) (%, SE)	FY2010 (n=5,634,519) (%, SE)	Percentage point change from FY2000 to FY2010 % (95% CI)
Sex	Female	12.10 (0.02)	13.84 (0.01)	1.74 (-1.77, 5.25)
	Male	87.90 (0.02)	86.16 (0.01)	-1.74 (-5.25, 1.77)
Race/Ethnicity	White	20.60 (0.02)	33.22 (0.02)	12.62 (8.63, 16.62)
	Black/African American	7.82 (0.01)	9.88 (0.01)	2.06 (-1.16, 5.28)
	Hispanic	2.43 (0.01)	4.60 (0.01)	2.17 (-0.39, 4.73)
	Other	1.57 (0.01)	2.75 (0.01)	1.18 (-1.10, 3.46)
	Unknown	67.58 (0.03)	49.55 (0.02)	-18.03 (-22.25, -13.82)
Age Category (years)*	18-29	2.97 (0.01)	4.82 (0.01)	1.85 (-0.79, 4.48)
	30-39	6.71 (0.01)	5.69 (0.01)	-1.02 (-4.00, 1.97)
	40-49	15.65 (0.02)	9.11 (0.01)	-6.54 (-10.02, -3.05)
	50-59	23.17 (0.02)	16.28 (0.02)	-6.89 (-10.72, -3.06)
	60-69	23.38 (0.02)	29.76 (0.02)	6.38 (2.37, 10.40)
	70-79	23.56 (0.02)	17.68 (0.02)	-5.89 (-9.75, -2.02)
	80 years or older	4.56 (0.01)	16.66 (0.02)	12.10 (8.88, 15.31)
Marital Status	Married	48.59 (0.03)	47.67 (0.02)	-0.92 (-0.99, -0.85)
	Not Married	41.58 (0.03)	45.19 (0.02)	3.64 (3.57, 3.71)
	Unknown	9.52 (0.03)	6.72 (0.02)	-2.72 (-2.72, -2.72)
Annual Income	Less than 20K	67.90 (0.03)	59.58 (0.02)	-8.31 (-12.51, -4.12)
	More than 20K	25.82 (0.02)	32.98 (0.02)	7.16 (3.08, 11.24)
	Unknown	6.29 (0.01)	7.44 (0.01)	1.15 (-1.89, 4.20)

VA Region	Northeast	6.77 (0.01)	7.18 (0.01)	0.41 (-2.65, 3.47)
	South	19.31 (0.02)	23.87 (0.02)	4.56 (0.68, 8.44)
	Midwest	7.83 (0.01)	9.06 (0.01)	1.22 (-1.97, 4.42)
	West	9.93 (0.02)	11.56 (0.01)	1.63 (-1.73, 5.00)
	Unknown	56.16 (0.03)	48.33 (0.02)	-7.83 (-12.11, -3.55)
Service Period	World War II	11.26 (0.02)	3.00 (0.01)	-8.26 (-11.30, -5.21)
	Korean War	6.55 (0.01)	4.48 (0.01)	-2.07 (-4.98, 0.83)
	Vietnam War	26.76 (0.02)	18.42 (0.02)	-8.34 (-12.27, -4.42)
	Persian Gulf War	28.88 (0.02)	50.83 (0.02)	21.95 (17.77, 26.13)
	OEF/OIF	0.23 (0.00)	1.83 (0.01)	1.60 (-0.17, 3.37)
	Other	26.32 (0.02)	21.44 (0.02)	-4.88 (-8.84, -0.91)
Combat Exposure	No	83.72 (0.02)	83.75 (0.02)	0.03 (-3.66, 3.72)
	Yes	16.28 (0.02)	16.25 (0.02)	-0.03 (-3.72, 3.66)
BMI (kg/m^2)	Normal	14.76 (0.02)	15.73 (0.02)	0.97 (-2.67, 4.60)
	Overweight	20.81 (0.02)	27.43 (0.02)	6.63 (2.68, 10.58)
	Obese	18.24 (0.02)	30.80 (0.02)	12.56 (8.63, 16.49)
	Unknown	46.19 (0.03)	26.04 (0.02)	-20.15 (-24.32, -15.98)
Smoker Status	Never	2.36 (0.01)	17.45 (0.02)	15.09 (12.05, 18.14)
	Former	8.07 (0.01)	17.73 (0.02)	9.66 (6.22, 13.09)
	Current	2.09 (0.01)	20.48 (0.02)	18.39 (15.31, 21.47)
	Unknown	87.49 (0.02)	44.35 (0.02)	-43.14 (-47.00, -39.28)
Comorbid diseases	Asthma	1.25 (0.01)	1.66 (0.01)	0.41 (-1.68, 2.50)
	Cancer	1.81 (0.01)	1.83 (0.01)	0.02 (-2.20, 2.24)
	CVD	22.07 (0.02)	23.47 (0.02)	1.40 (1.34, 1.46)
	COPD	2.56 (0.01)	2.15 (0.01)	-0.41 (-2.78, 1.96)
	Diabetes	7.90 (0.01)	9.66 (0.01)	1.76 (-1.46, 4.98)
	Fibromyalgia	0.38 (0.00)	0.74 (0.00)	0.36 (-1.27, 1.99)

	GI Disease	12.69 (0.02)	14.57 (0.01)	1.88 (-1.67, 5.43)
	HIV	0.58 (0.00)	0.45 (0.00)	-0.13 (-1.76, 1.50)
	Hypertension	16.02 (0.02)	19.48 (0.02)	3.46 (-0.28, 7.20)
	Mental disorder	19.81 (0.02)	30.67 (0.02)	10.86 (6.90, 14.82)
	Depression	2.16 (0.01)	3.41 (0.01)	1.25 (-1.19, 3.69)
	PTSD	3.43 (0.01)	10.07 (0.01)	6.64 (3.70, 9.58)
	Other	17.81 (0.02)	23.04 (0.02)	5.23 (1.39, 9.07)
	Renal Disease	0.61 (0.004)	1.33 (0.005)	0.72 (0.71, 0.73)
	Stroke	0.02 (0.00)	0.01 (0.00)	-0.01 (-0.68, 0.66)

*Not age-standardized. Abbreviations: FY, Fiscal Year; SE, Standard Error; CI, Confidence Interval; OEF/OIF, Operation Enduring Freedom/Operation Iraqi Freedom; BMI, Body Mass Index; CVD, Cardiovascular Disease, COPD, Chronic Obstructive Pulmonary Disease; GI, Gastrointestinal; HIV, Human Immunodeficiency Virus; PTSD, Post-Traumatic Stress Disorder

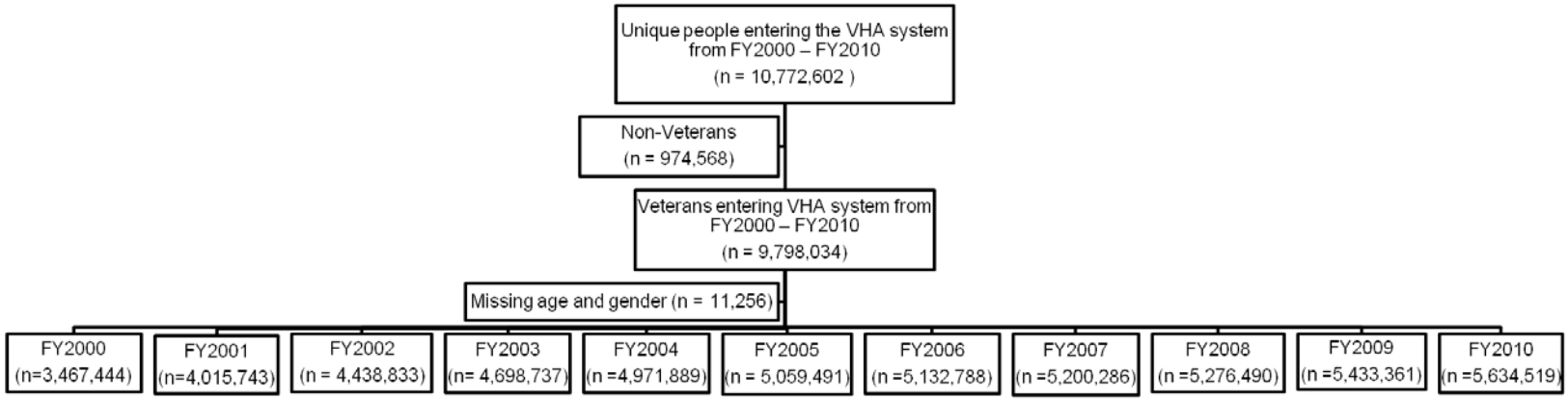

Figure 3.1. Flowchart of veterans who entered the Veterans Health Administration (VHA) system from FY2000 to FY2010

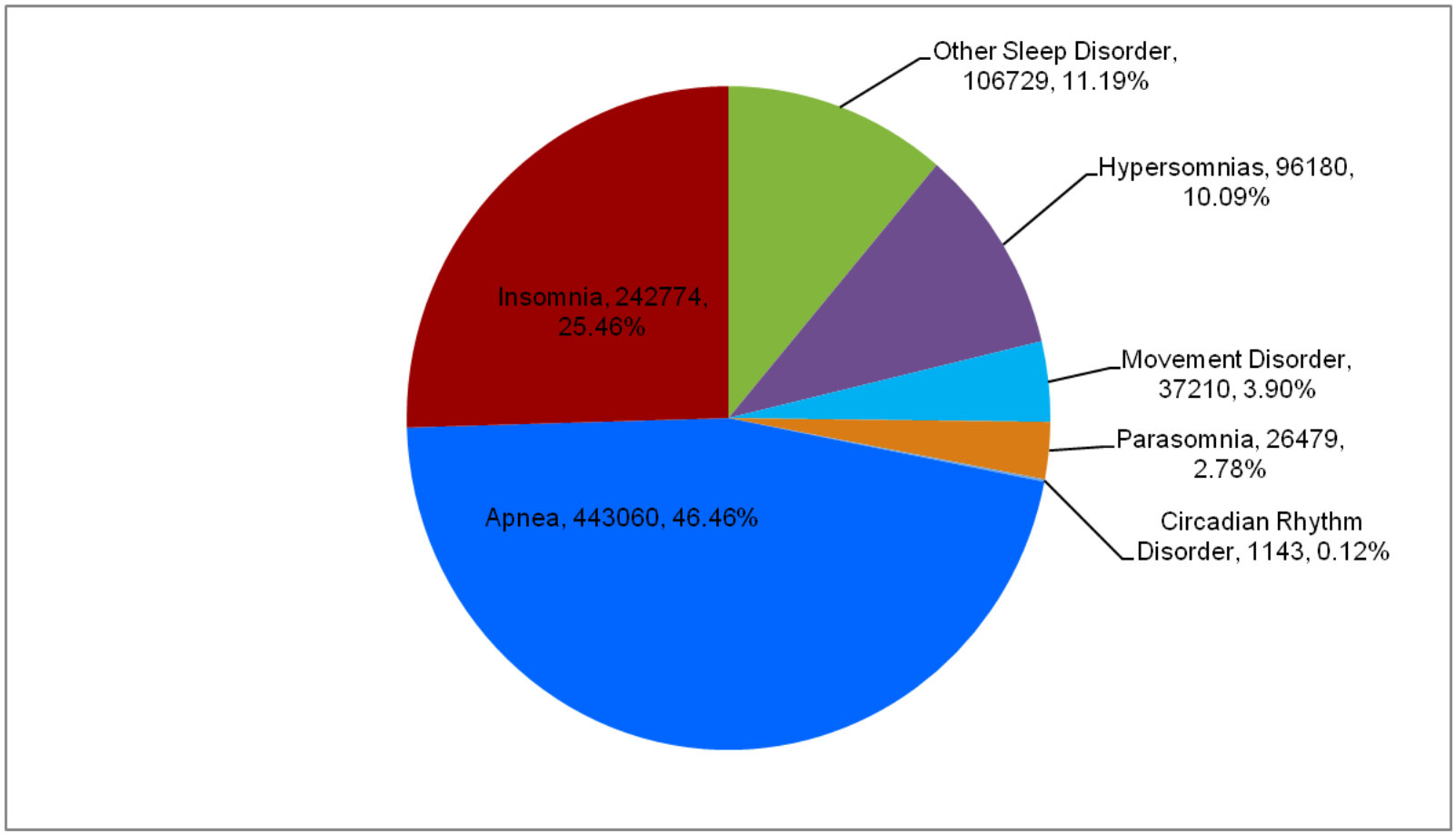

Figure 3.2. Proportions of sleep disorder subtypes diagnoses among all sleep disorder diagnoses (n of unique individuals=751,502; n of diagnoses = 953,575), FY2000 – FY2010

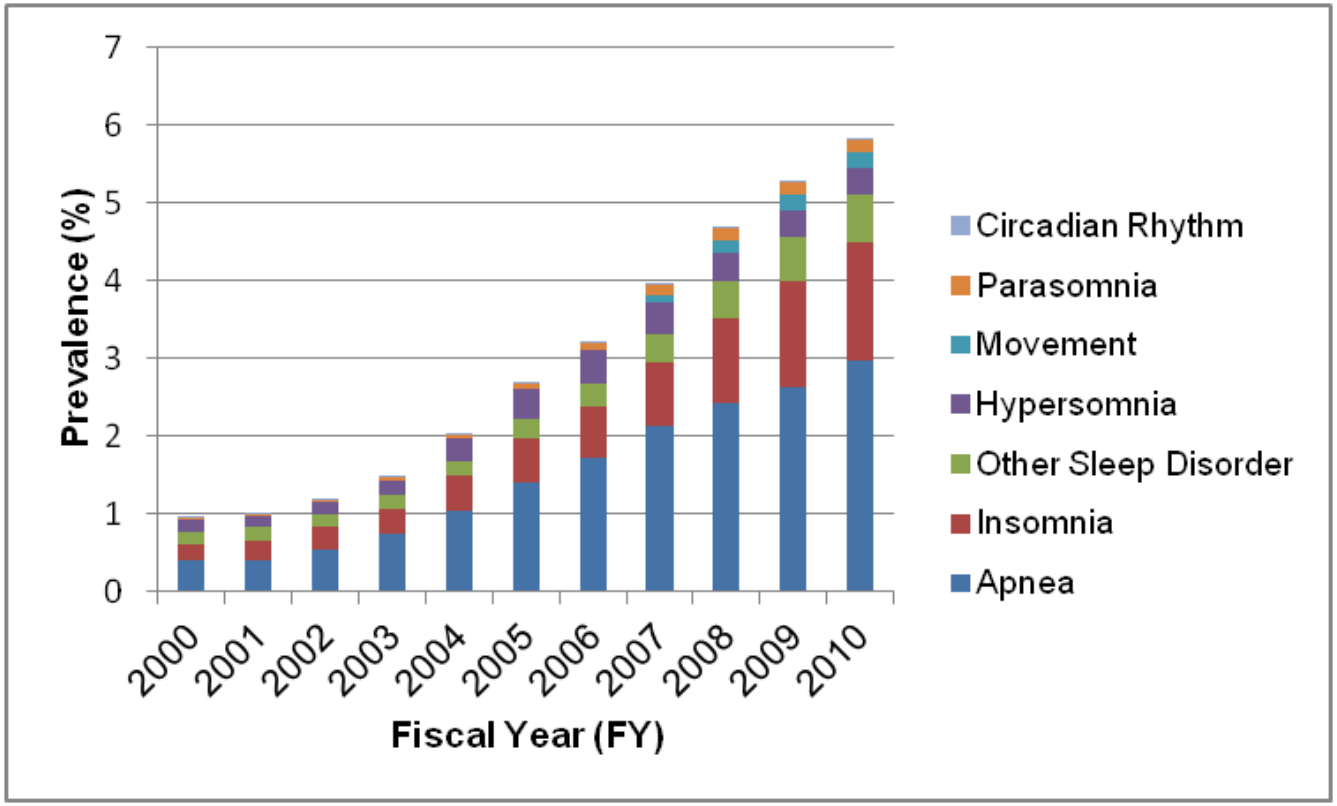

Figure 3.3. Age-adjusted prevalence of sleep disorders among United States Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

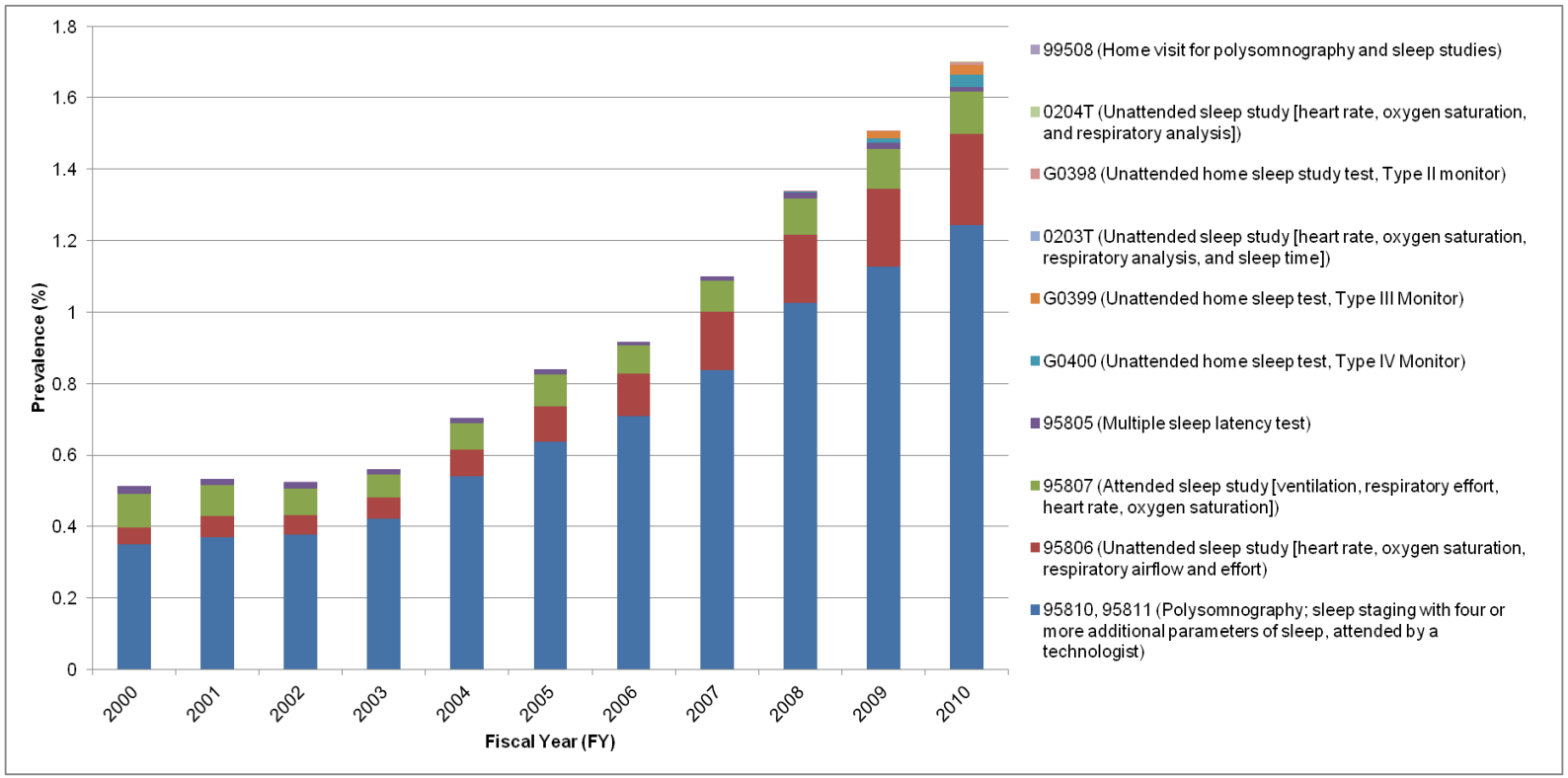

Figure 3.4. Age-adjusted prevalence of CURRENT PROCEDURAL TERMINOLOGY (CPT) CODES for sleep procedures among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

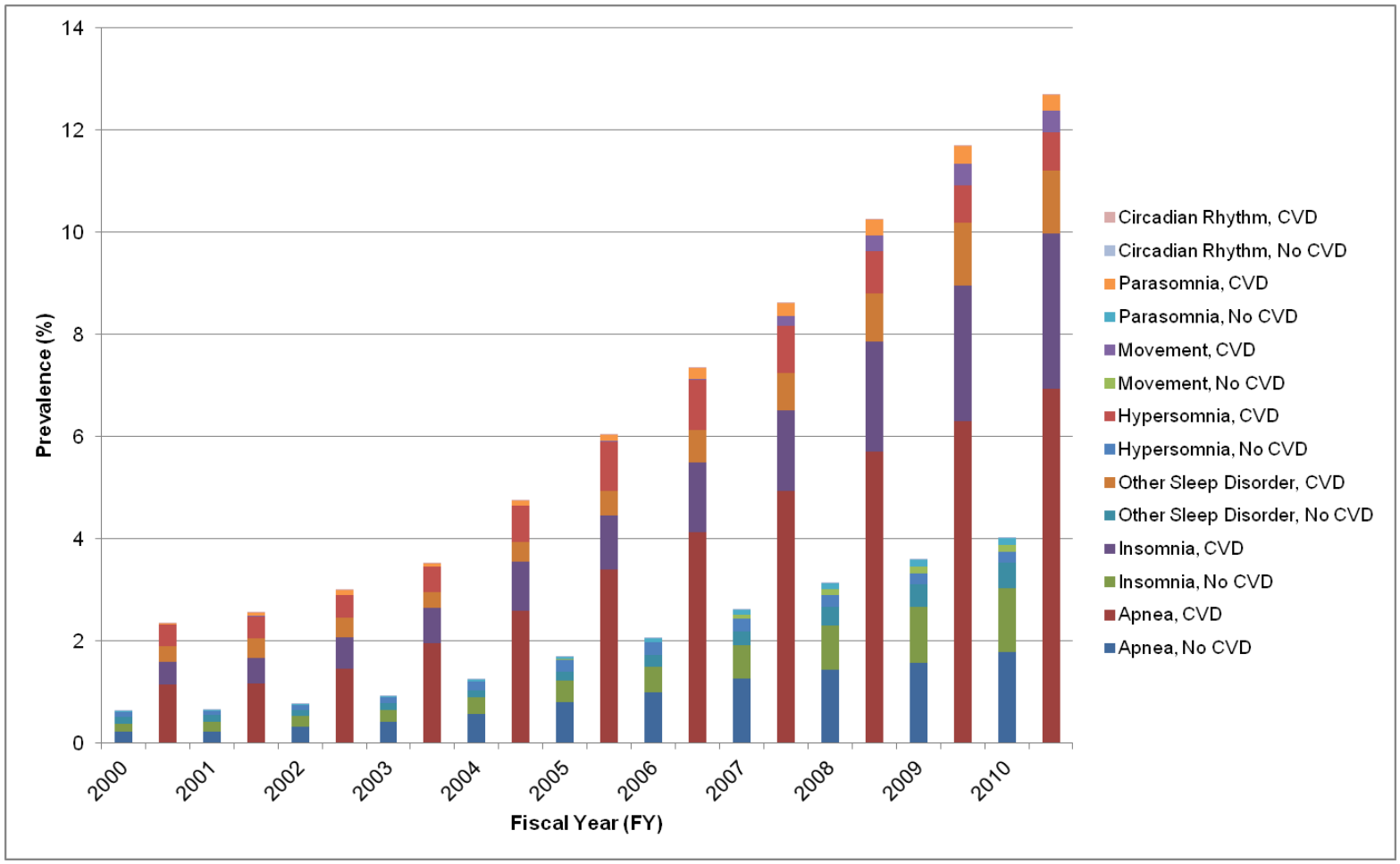

Figure 3.5. Age-adjusted prevalence of sleep disorders by CARDIOVASCULAR DISEASE status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

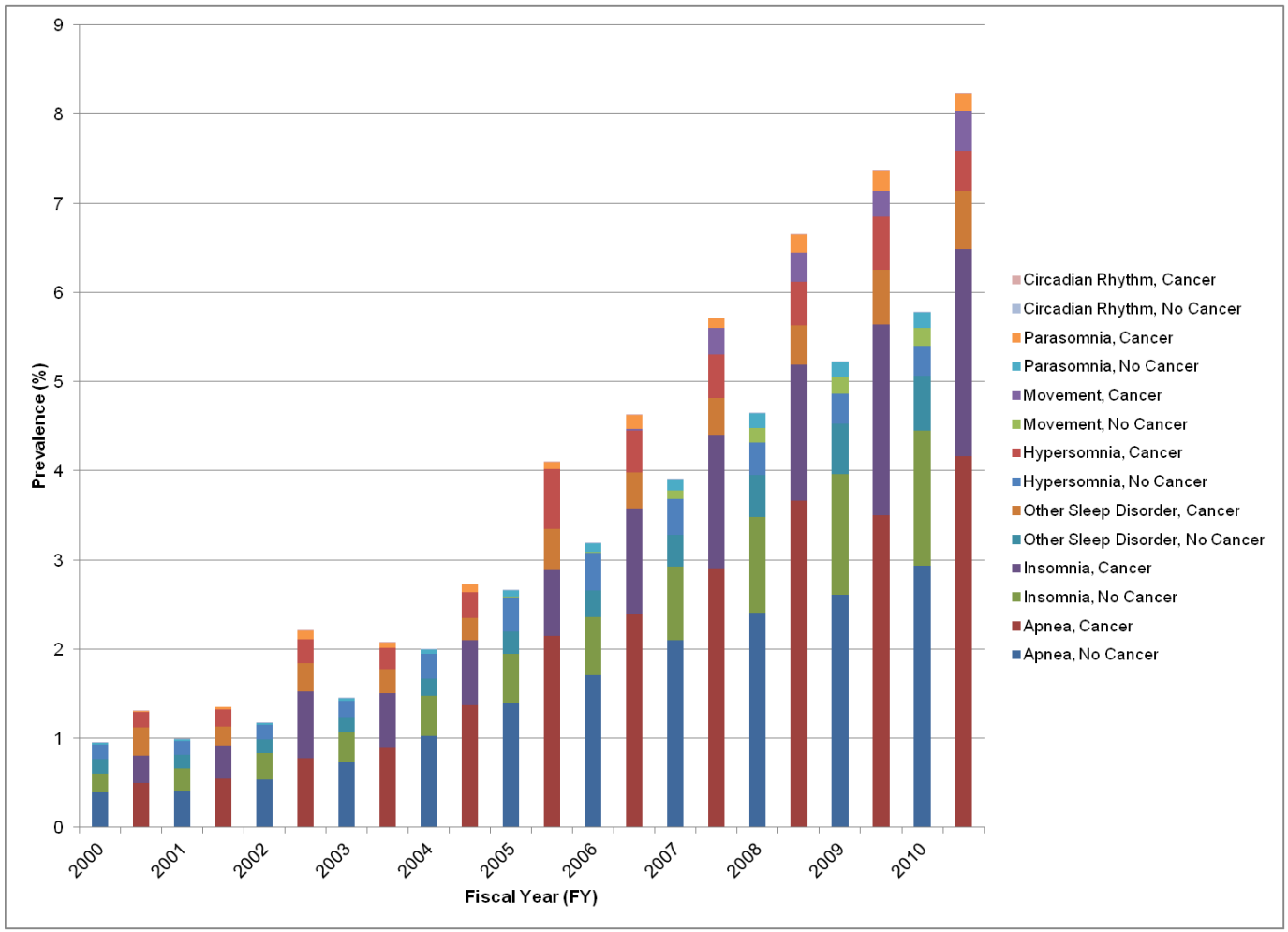

Figure 3.6. Age-adjusted prevalence rate of sleep disorders by CANCER status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

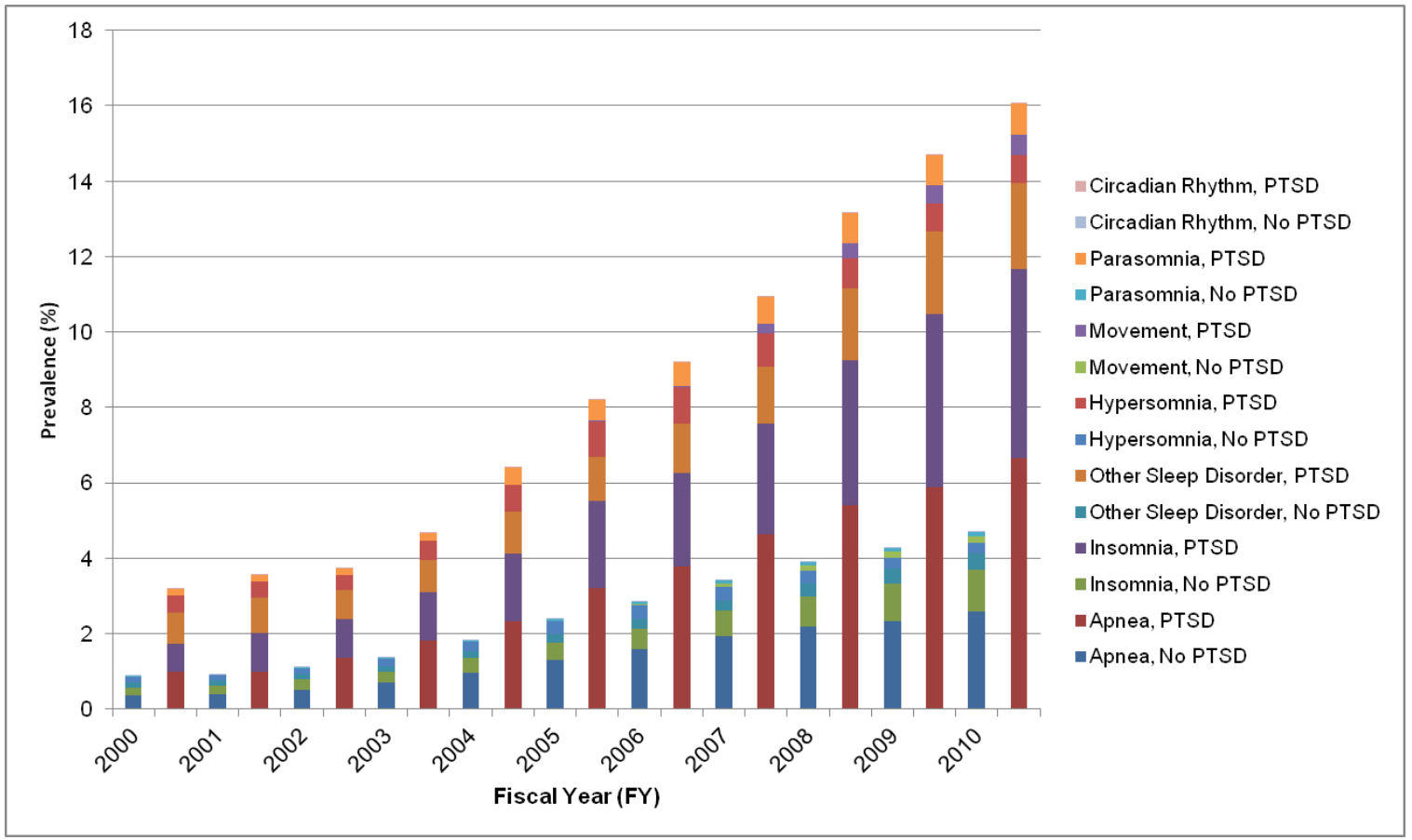

Figure 3.7. Age-adjusted prevalence of sleep disorders by POST-TRAUMATIC STRESS DISORDER (PTSD) status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

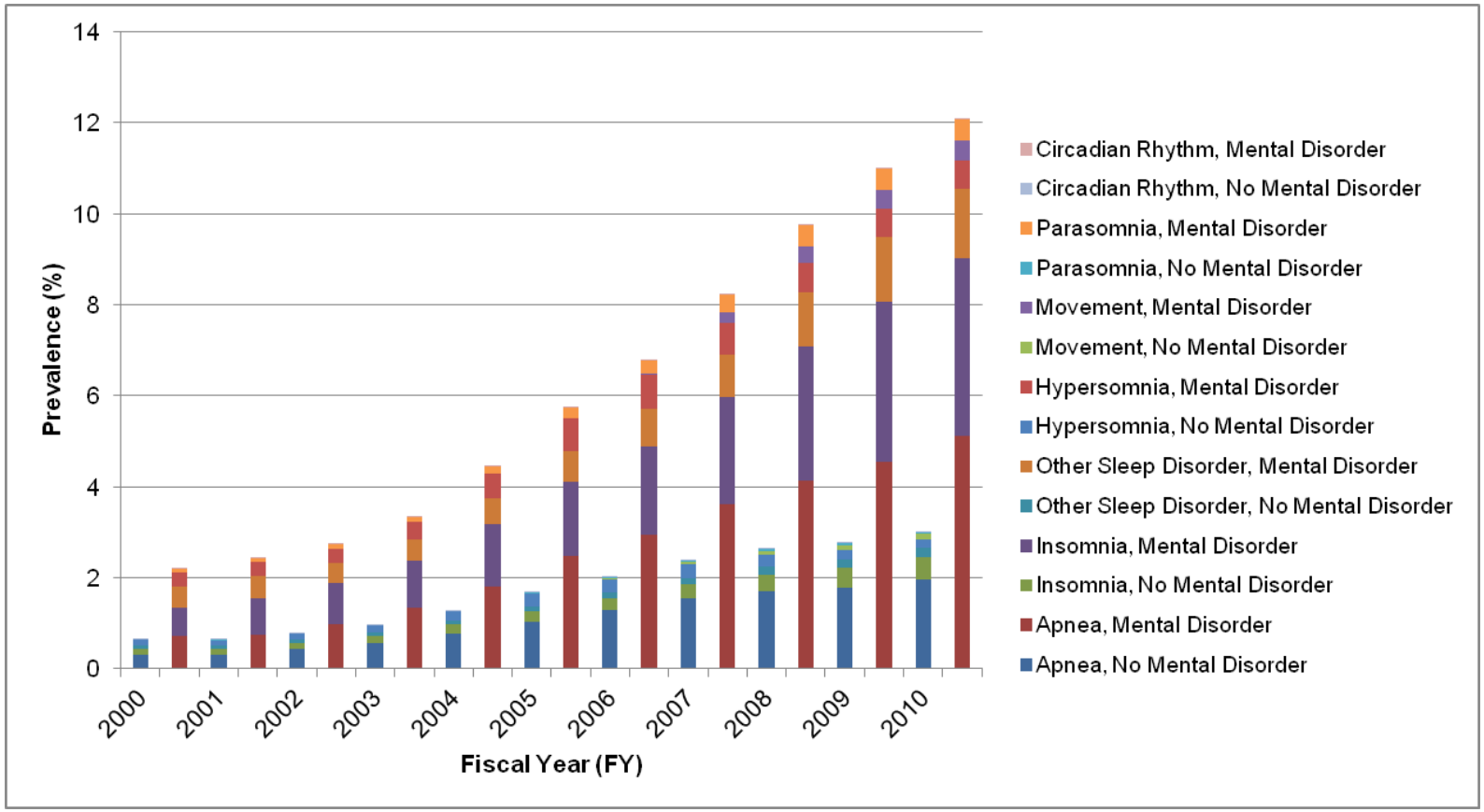

Figure 3.8. Age-adjusted prevalence of sleep disorders by MENTAL DISORDER status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

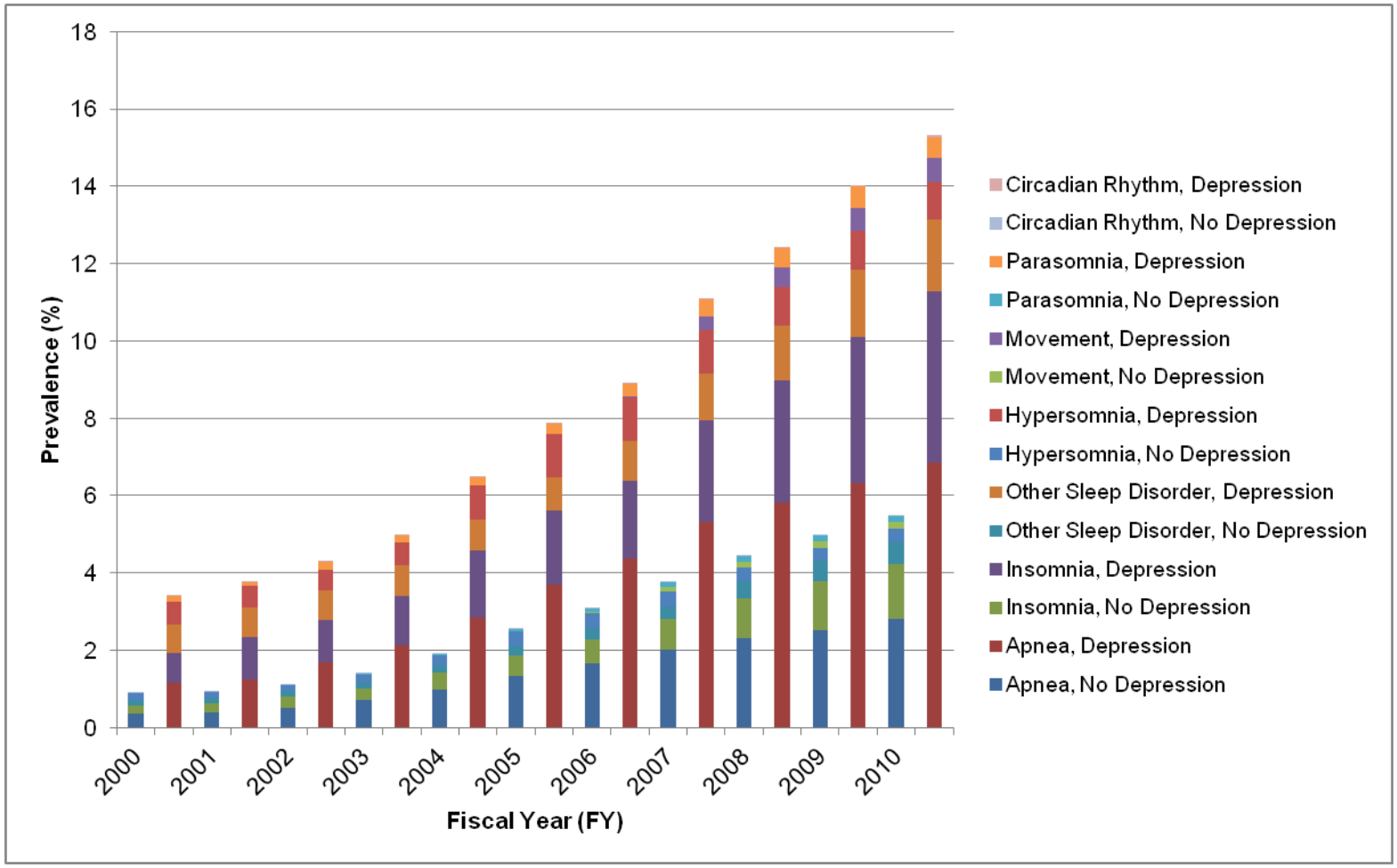

Figure 3.9. Age-adjusted prevalence of sleep disorders by DEPRESSION status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

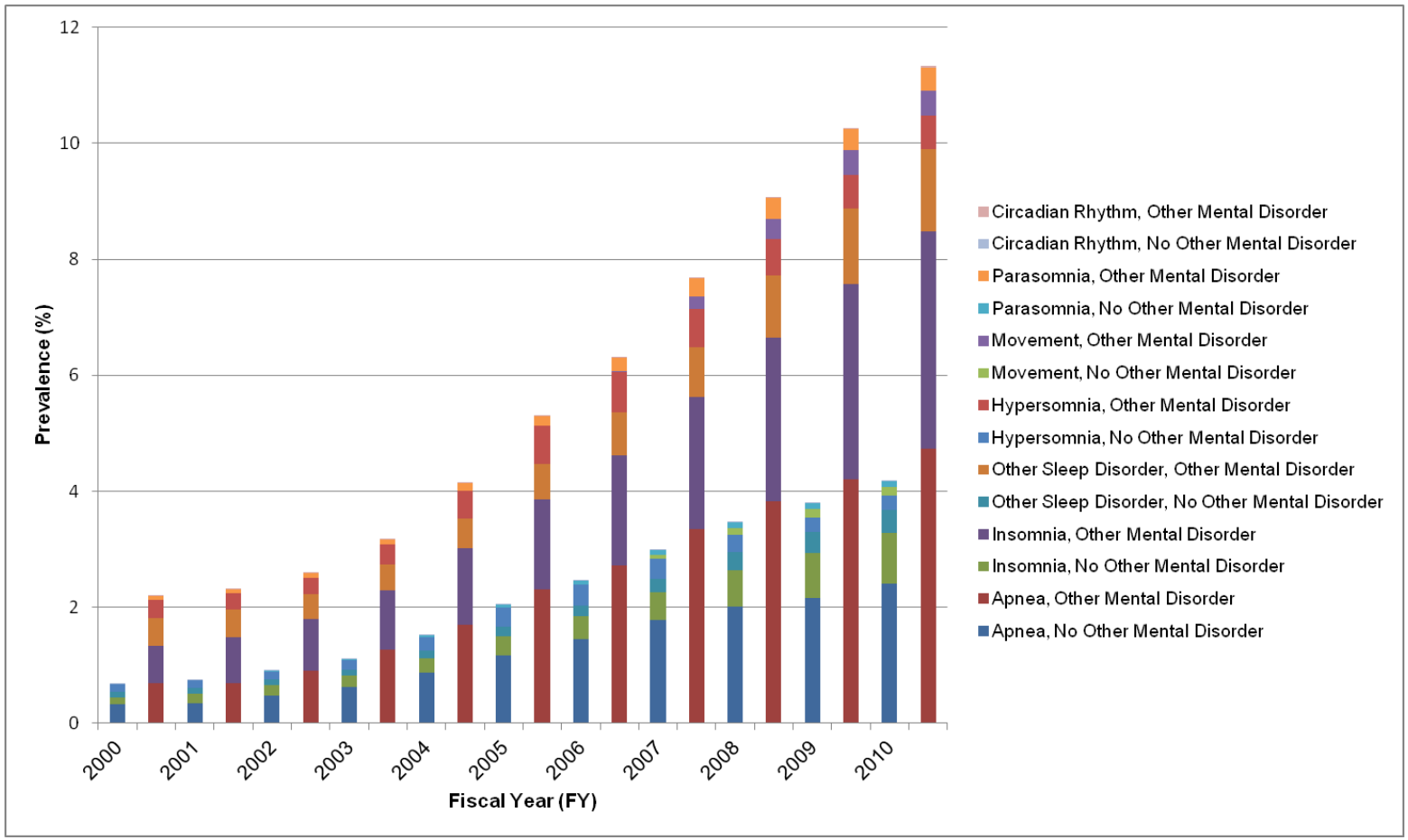

Figure 3.10. Age-adjusted prevalence of sleep disorders by OTHER MENTAL HEALTH DISORDER status among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

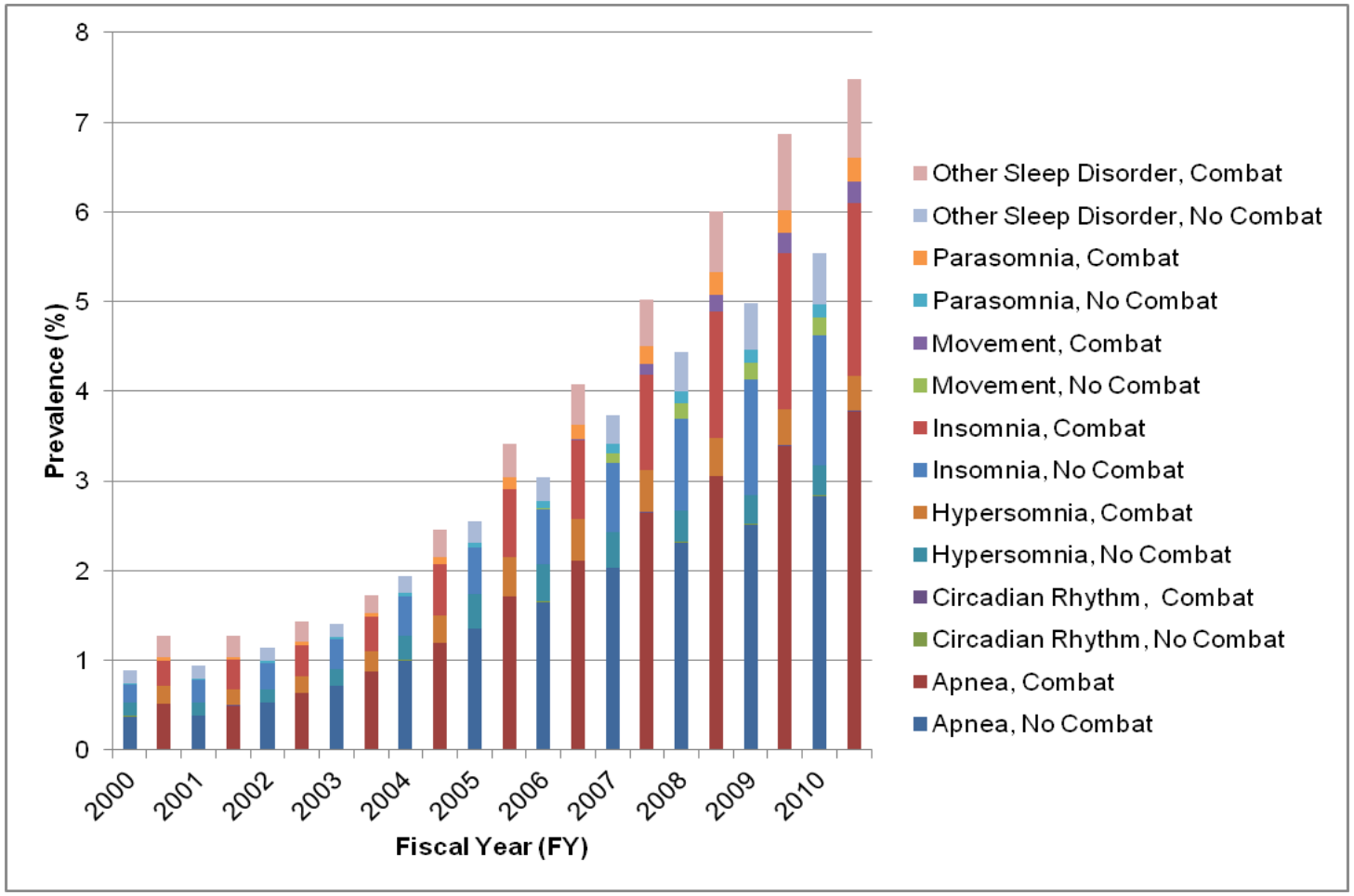

Figure 3.11. Age-adjusted prevalence of sleep disorders by COMBAT EXPOSURE among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

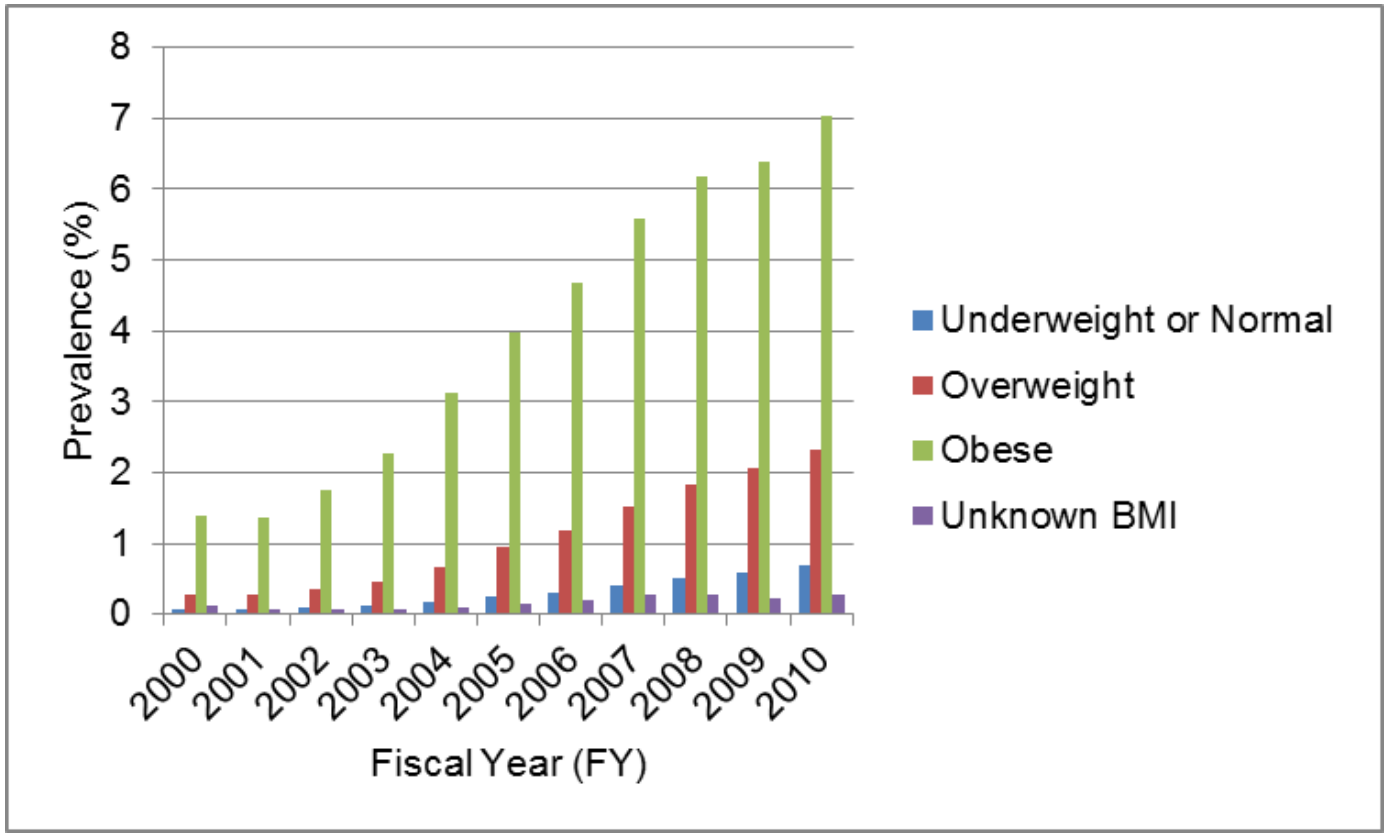

Figure 3.12a. Age-adjusted prevalence of APNEAS by BODY MASS INDEX (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

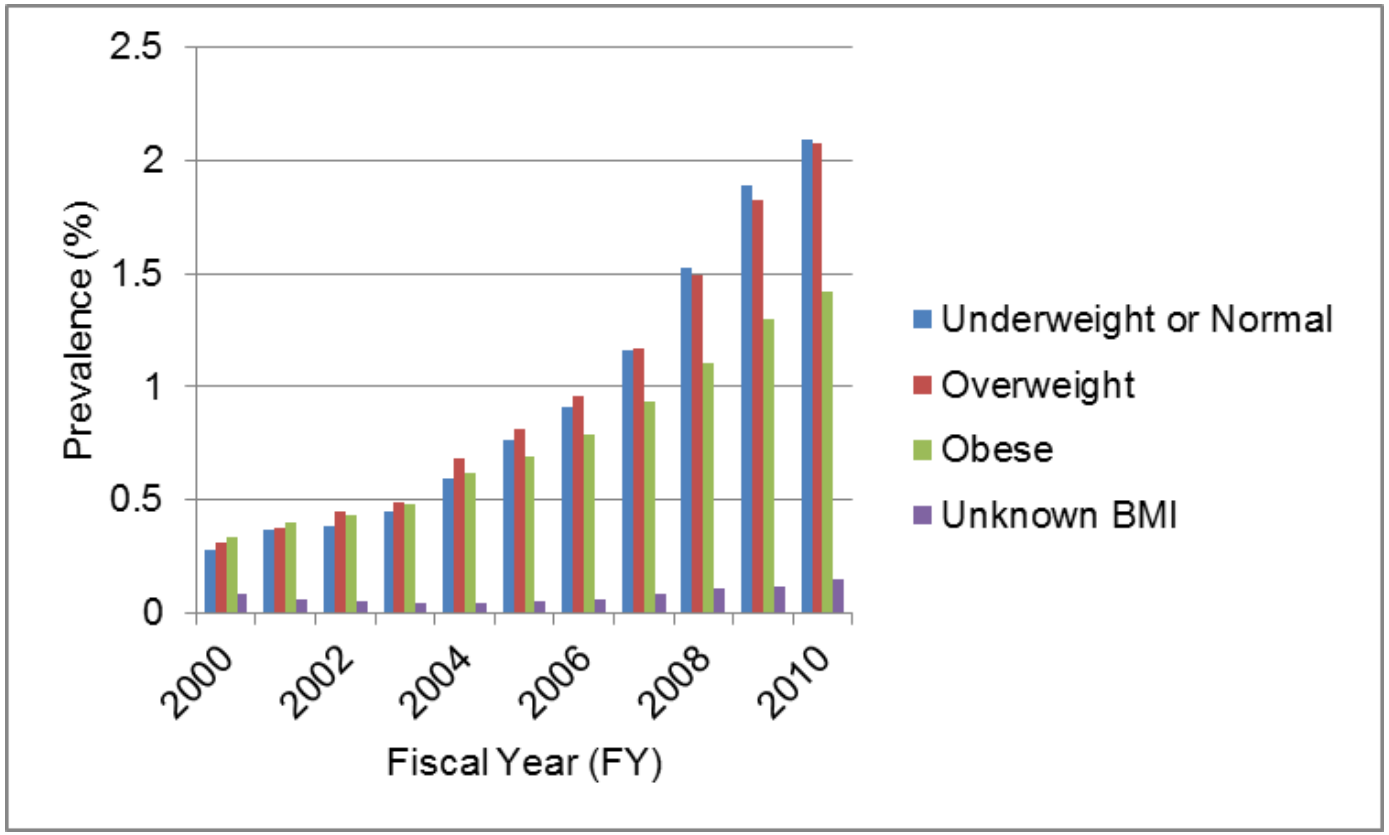

Figure 3.12b. Age-adjusted prevalence of INSOMNIAS by BODY MASS INDEX (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

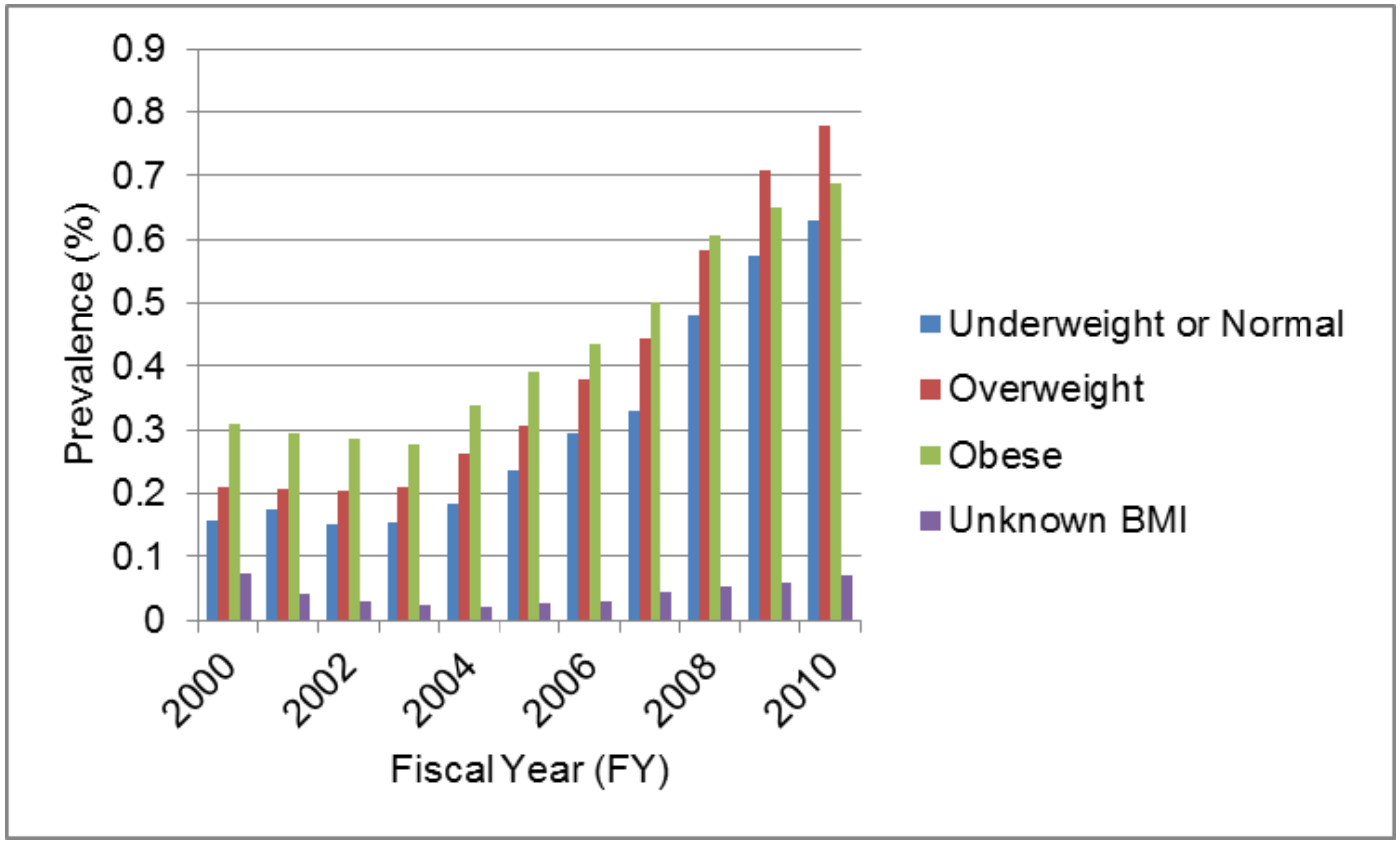

Figure 3.12c. Age-adjusted prevalence of OTHER SLEEP DISORDERS by Body Mass Index (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

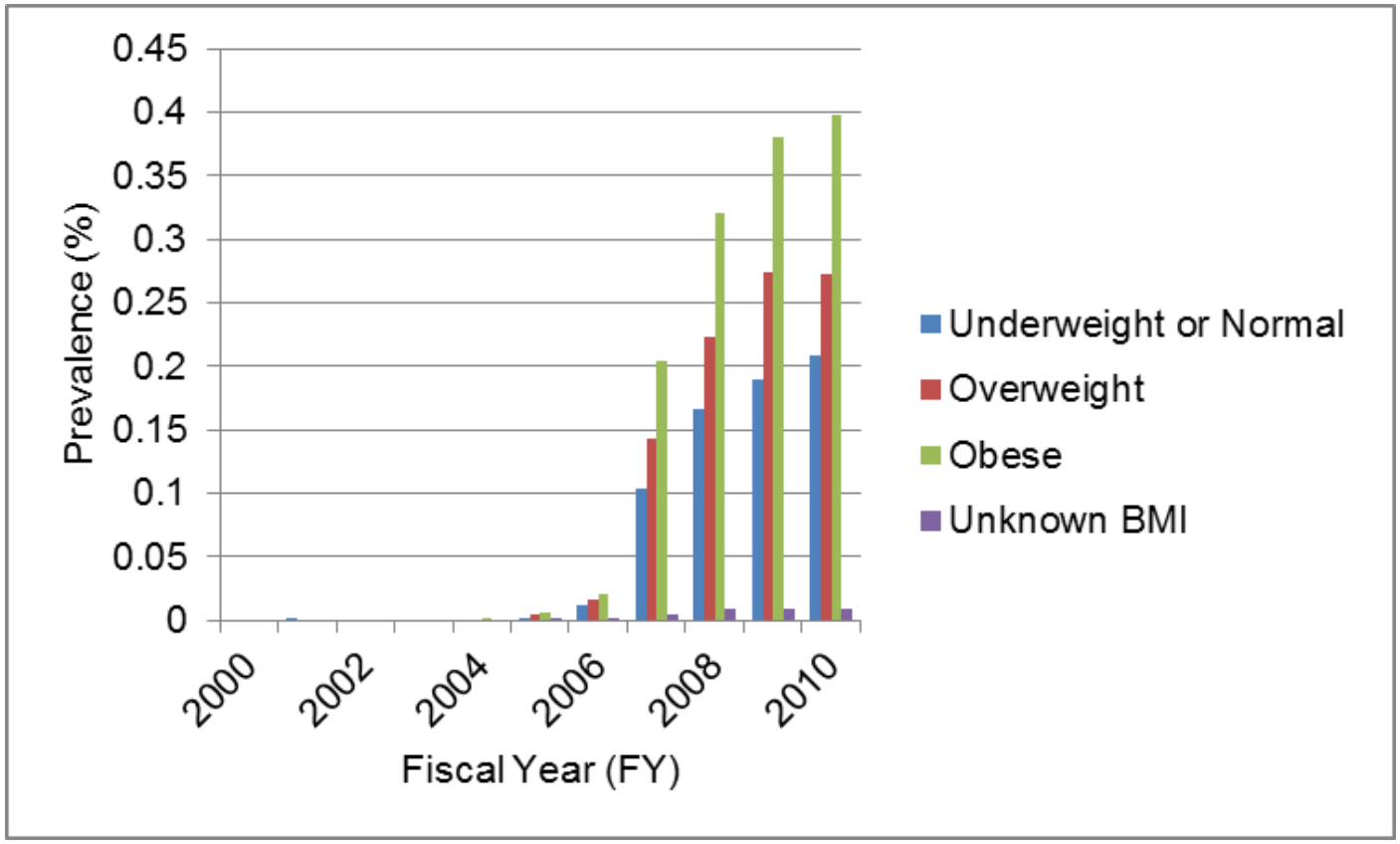

Figure 3.12d. Age-adjusted prevalence of HYPERSOMNIAS by Body Mass Index (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

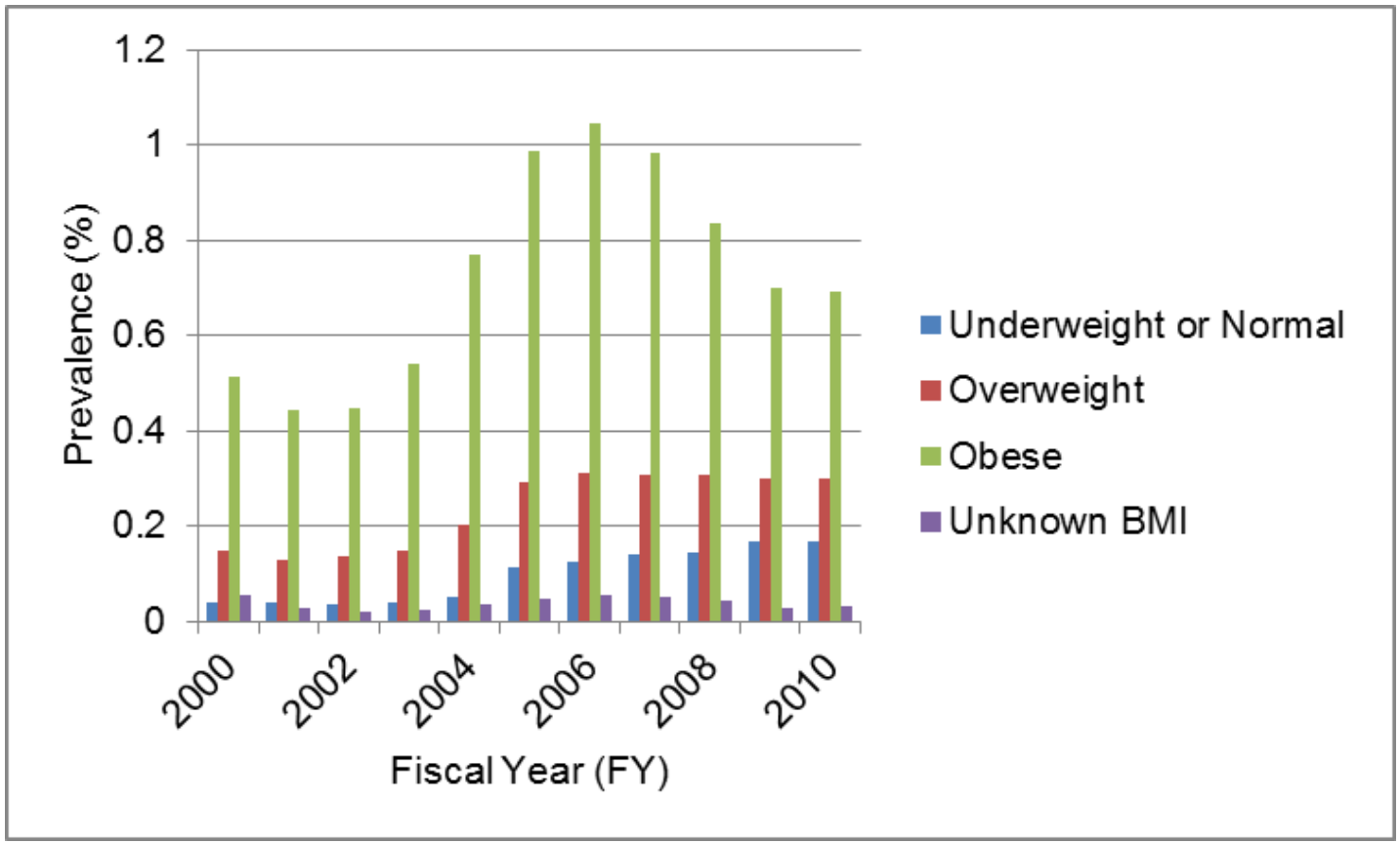

Figure 3.12e. Age-adjusted prevalence of MOVEMENT DISORDERS by Body Mass Index (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

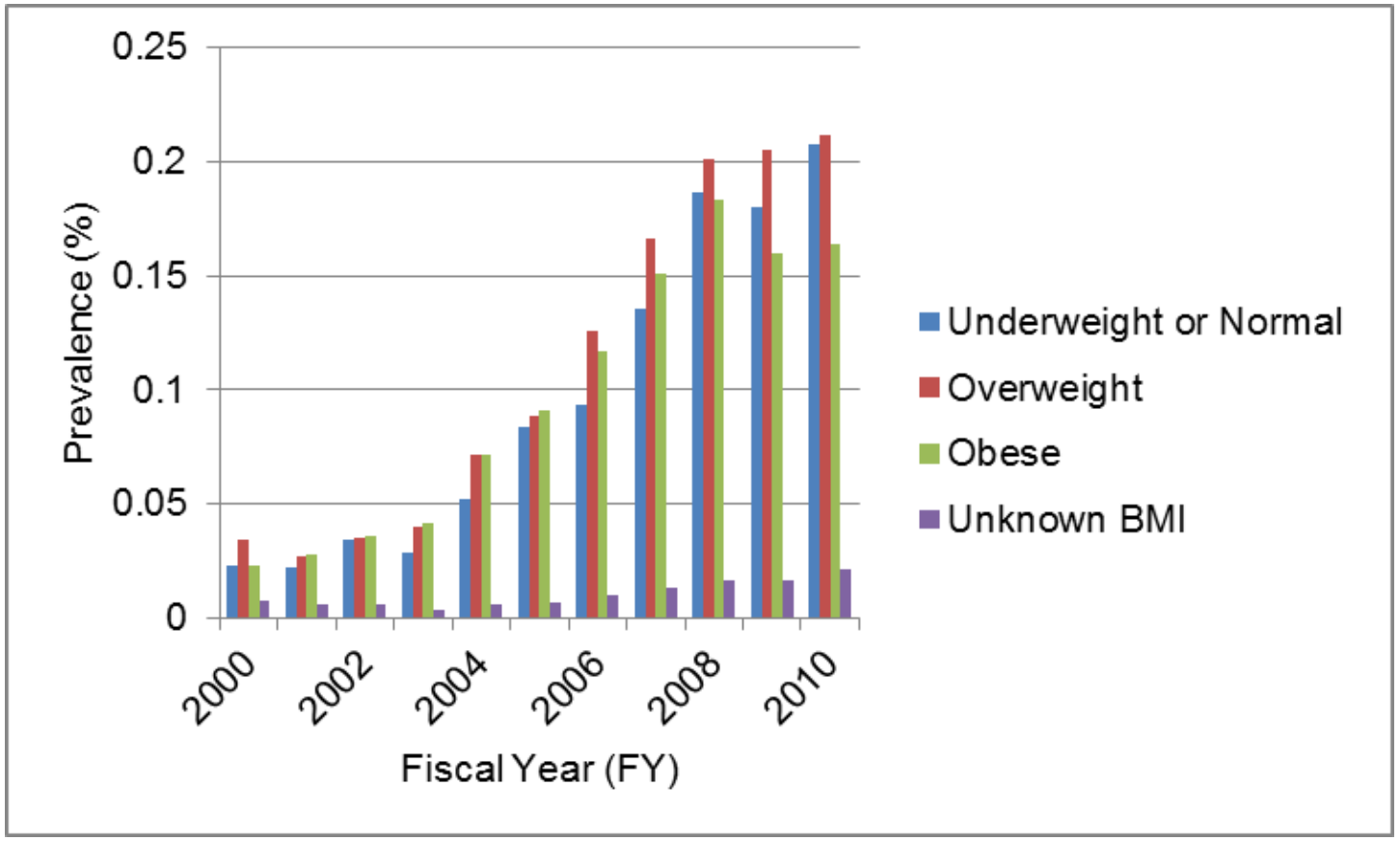

Figure 3.12f. Age-adjusted prevalence of PARASOMNIAS by Body Mass Index (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

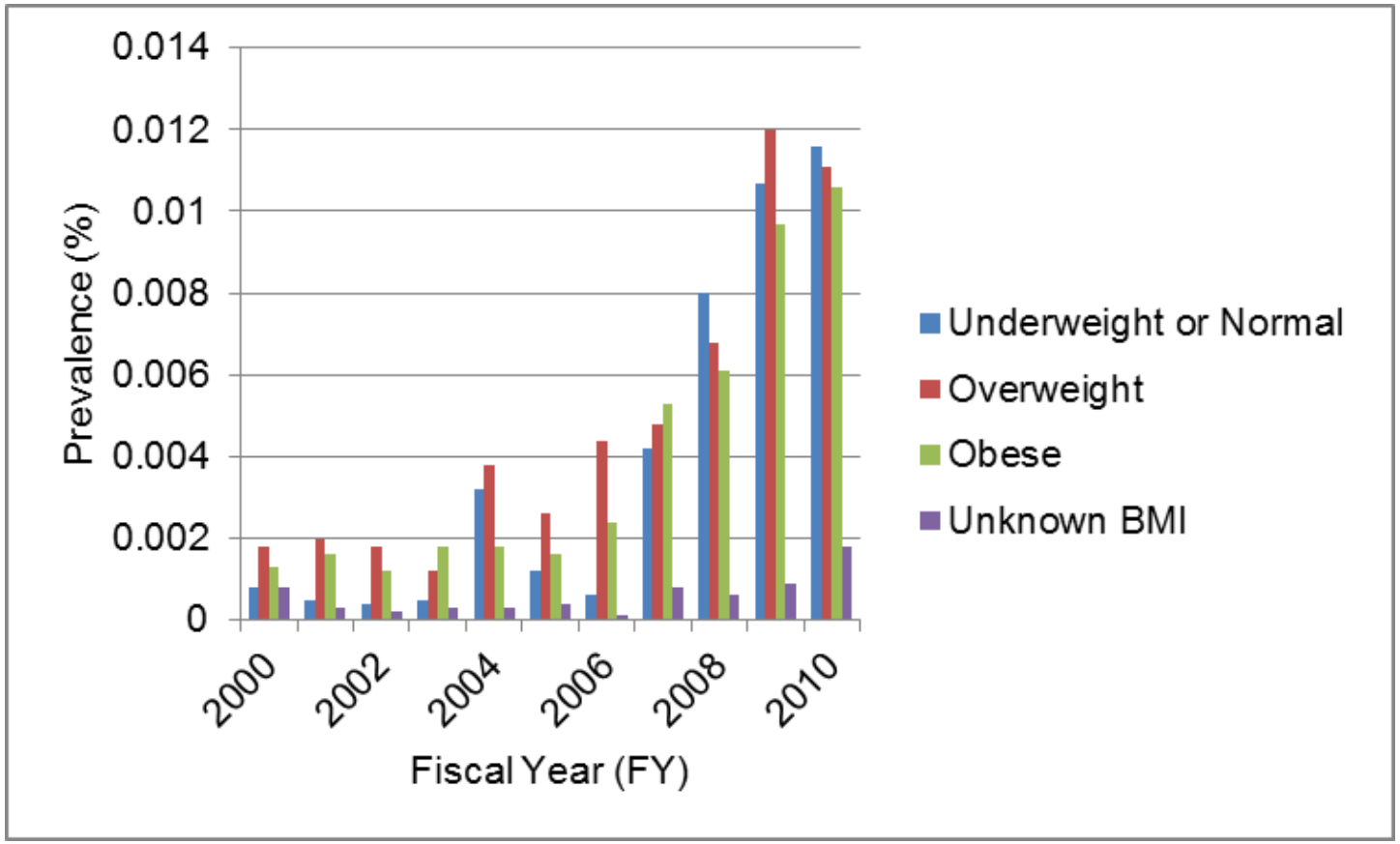

Figure 3.12g. Age-adjusted prevalence of CIRCADIAN RHYTHM DISORDERS by Body Mass Index (BMI) groups among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

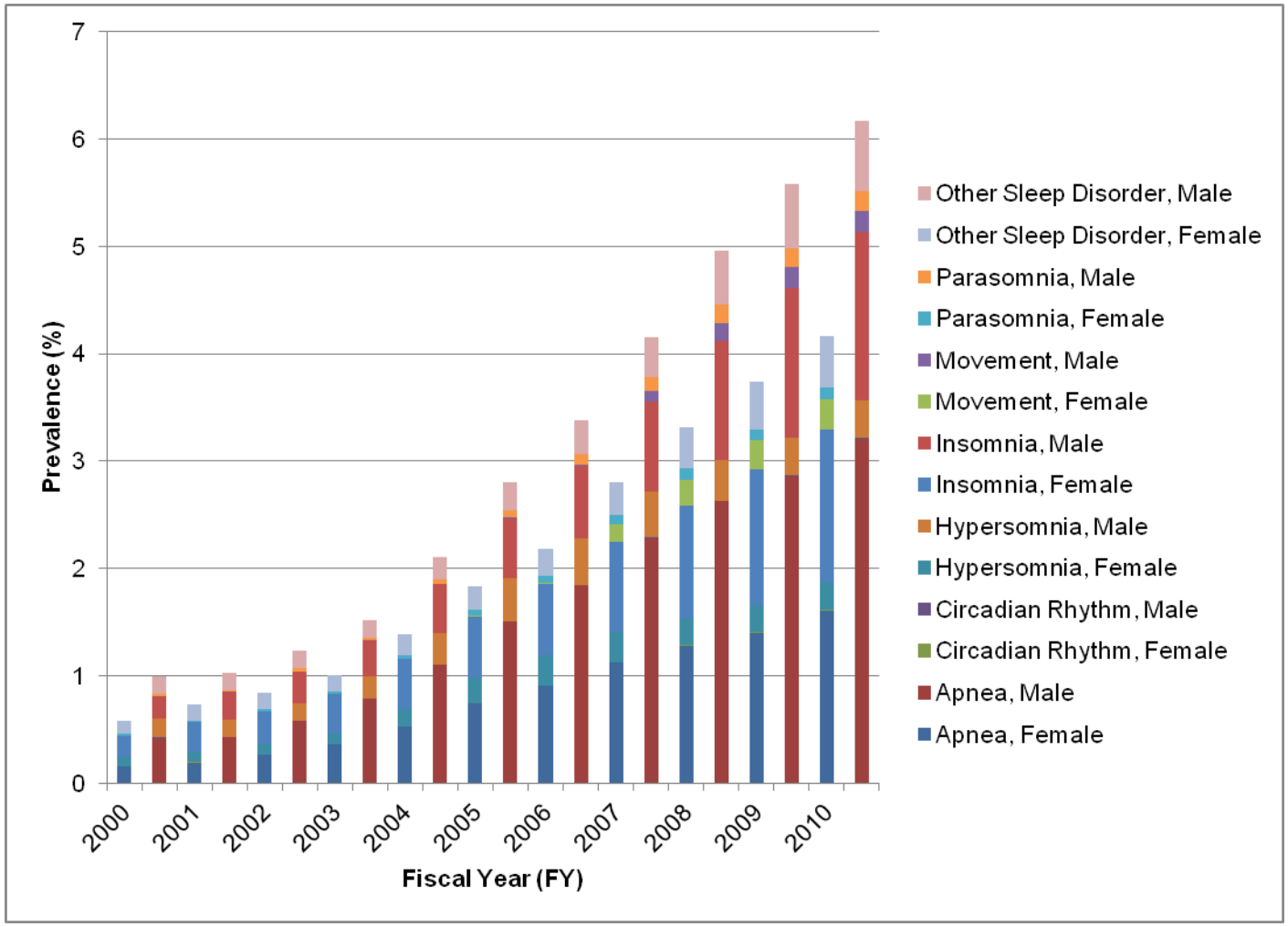

Figure 3.13. Age-adjusted prevalence of sleep disorders by gender among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010

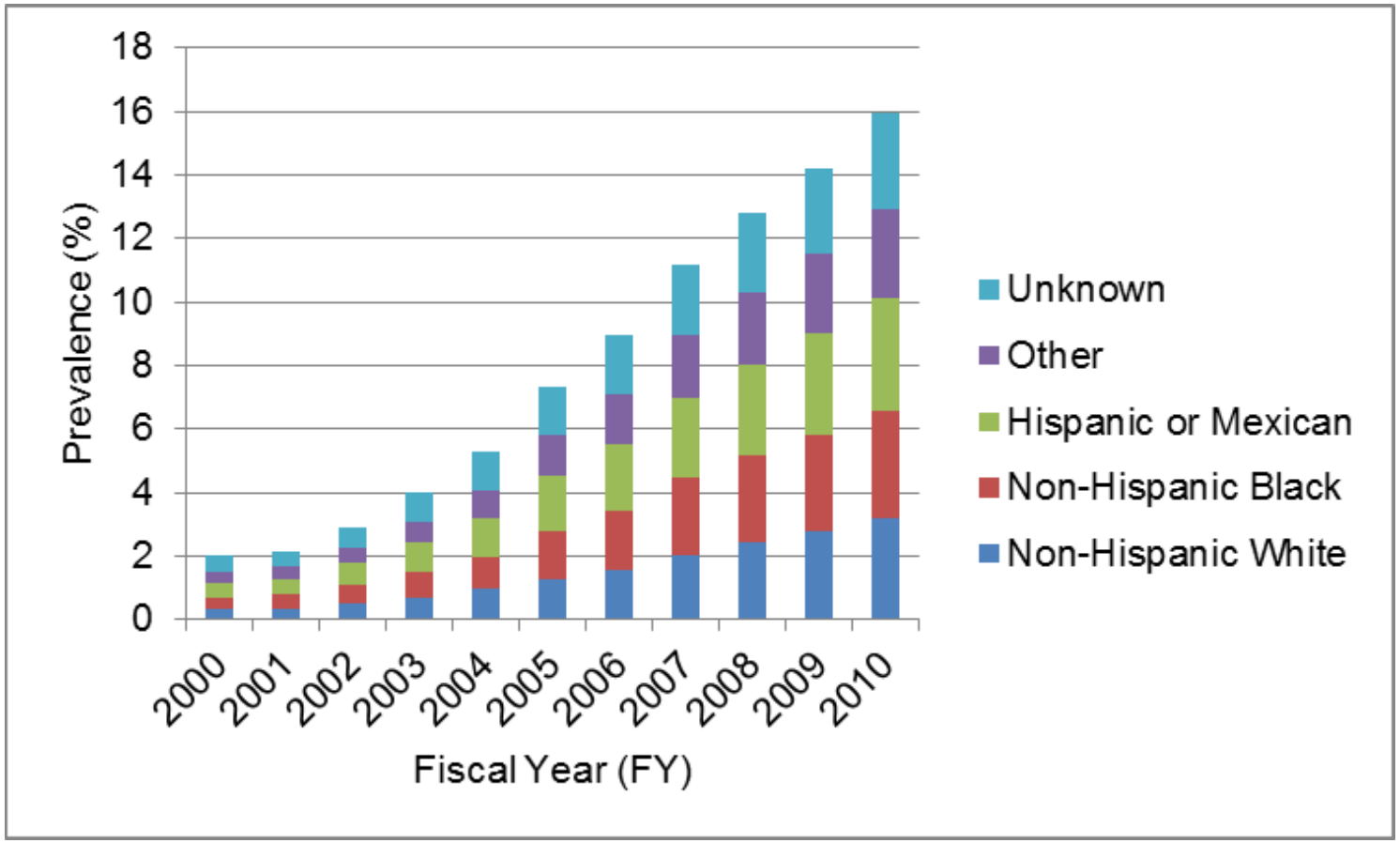

Figure 3.14a. Age-adjusted prevalence of APNEAS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

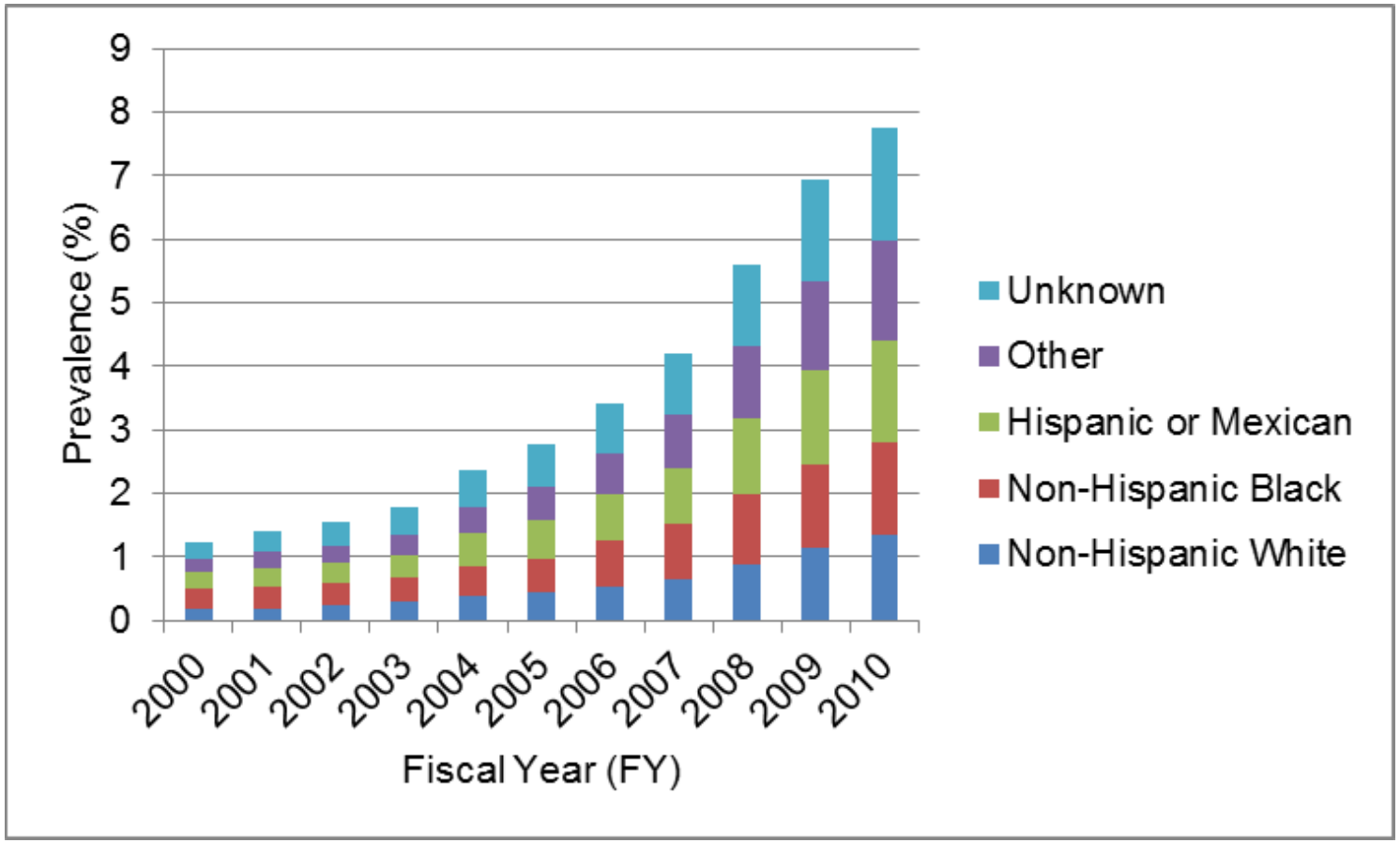

Figure 3.14b. Age-adjusted prevalence of INSOMNIAS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

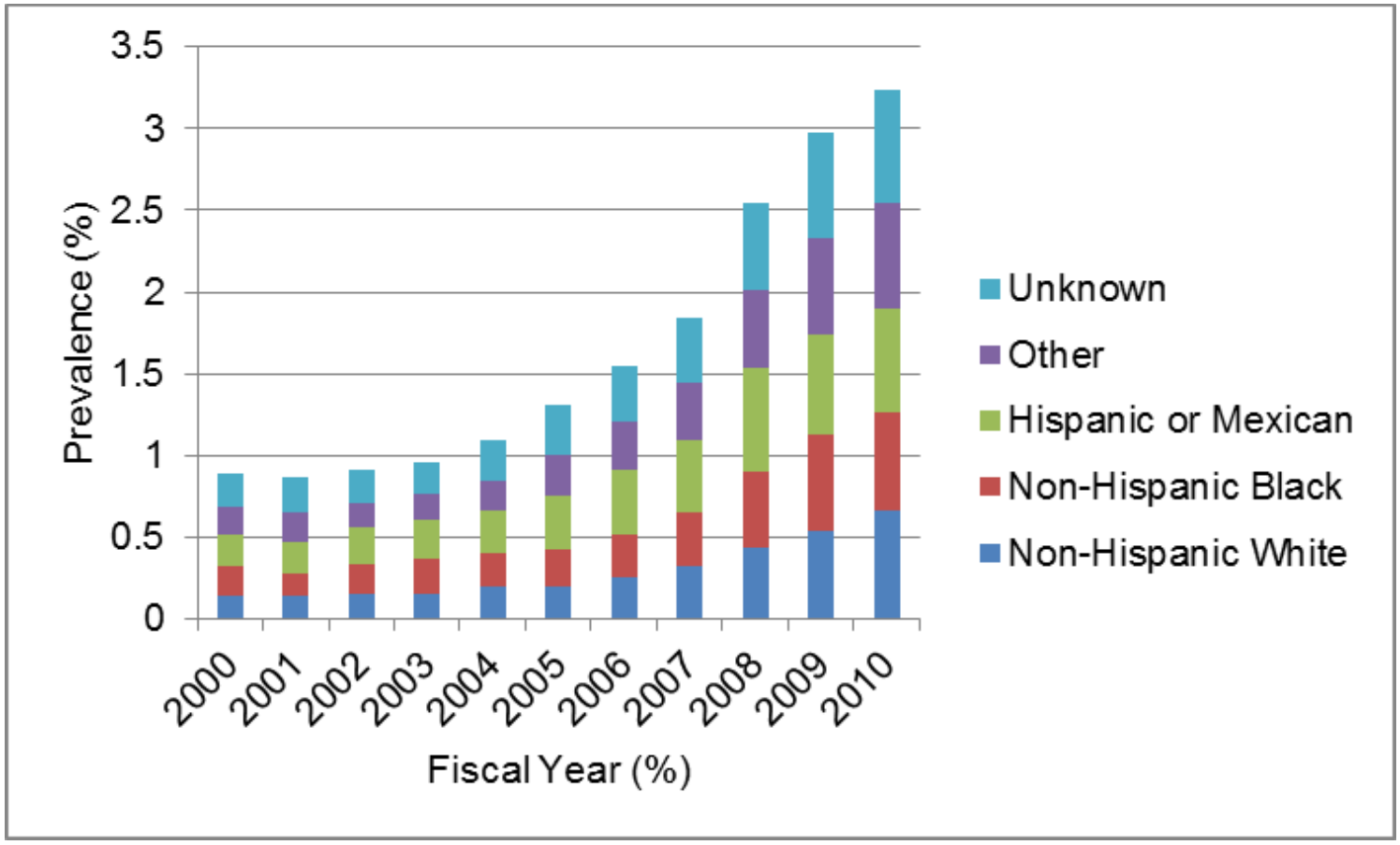

Figure 3.14c. Age-adjusted prevalence of OTHER SLEEP DISORDERS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

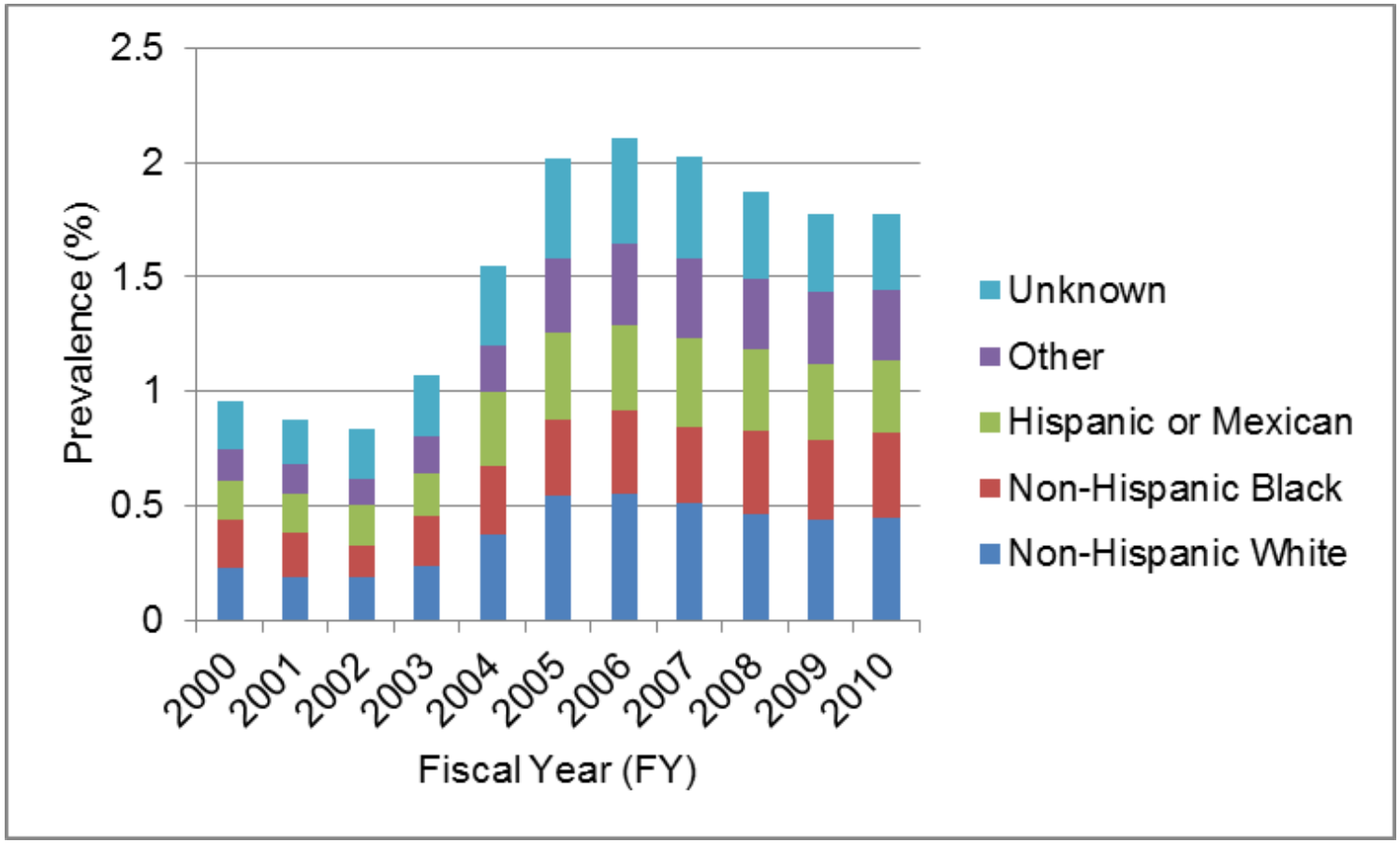

Figure 3.14d. Age-adjusted prevalence of HYPERSOMNIAS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

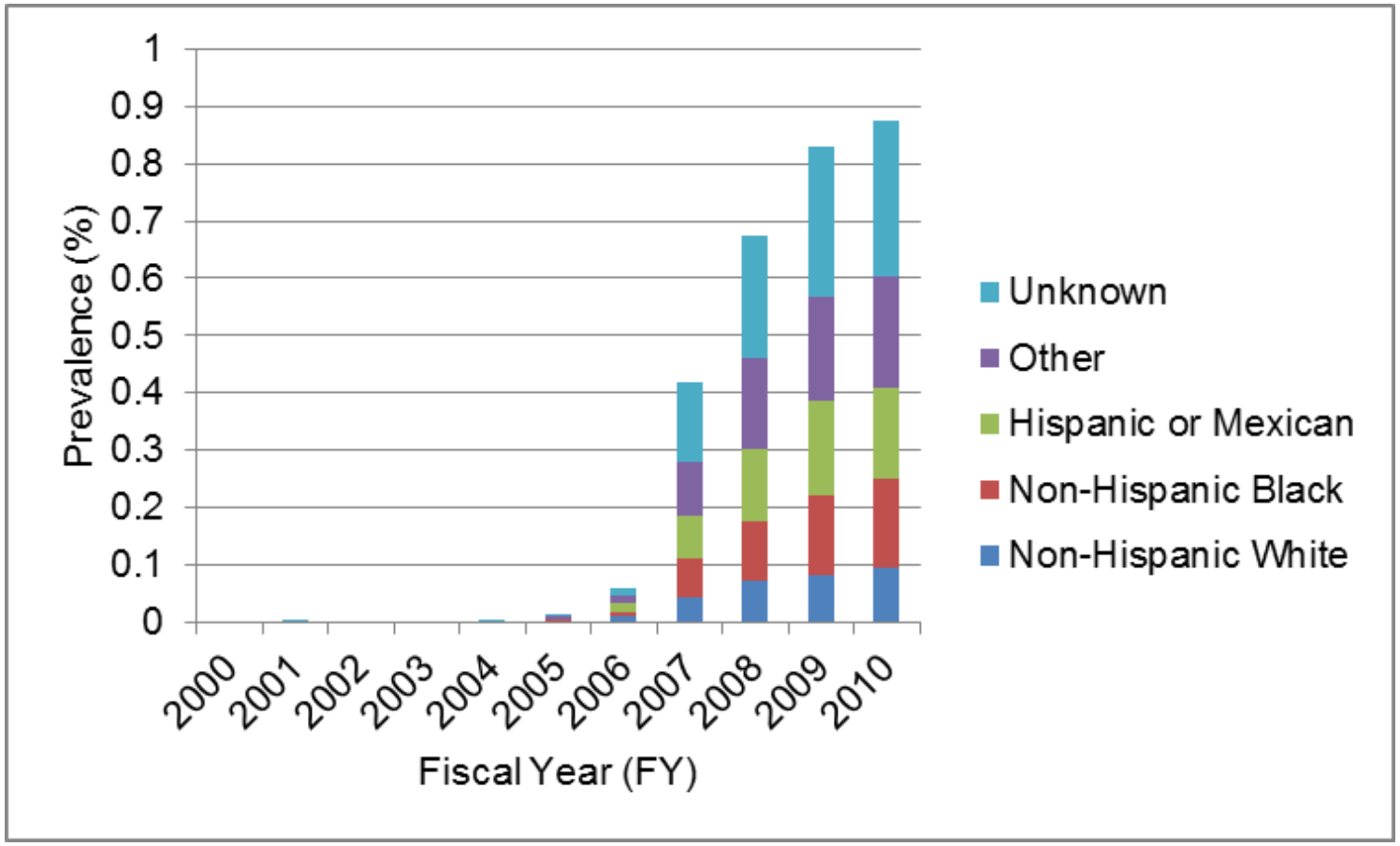

Figure 3.14e. Age-adjusted prevalence of MOVEMENT DISORDERS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

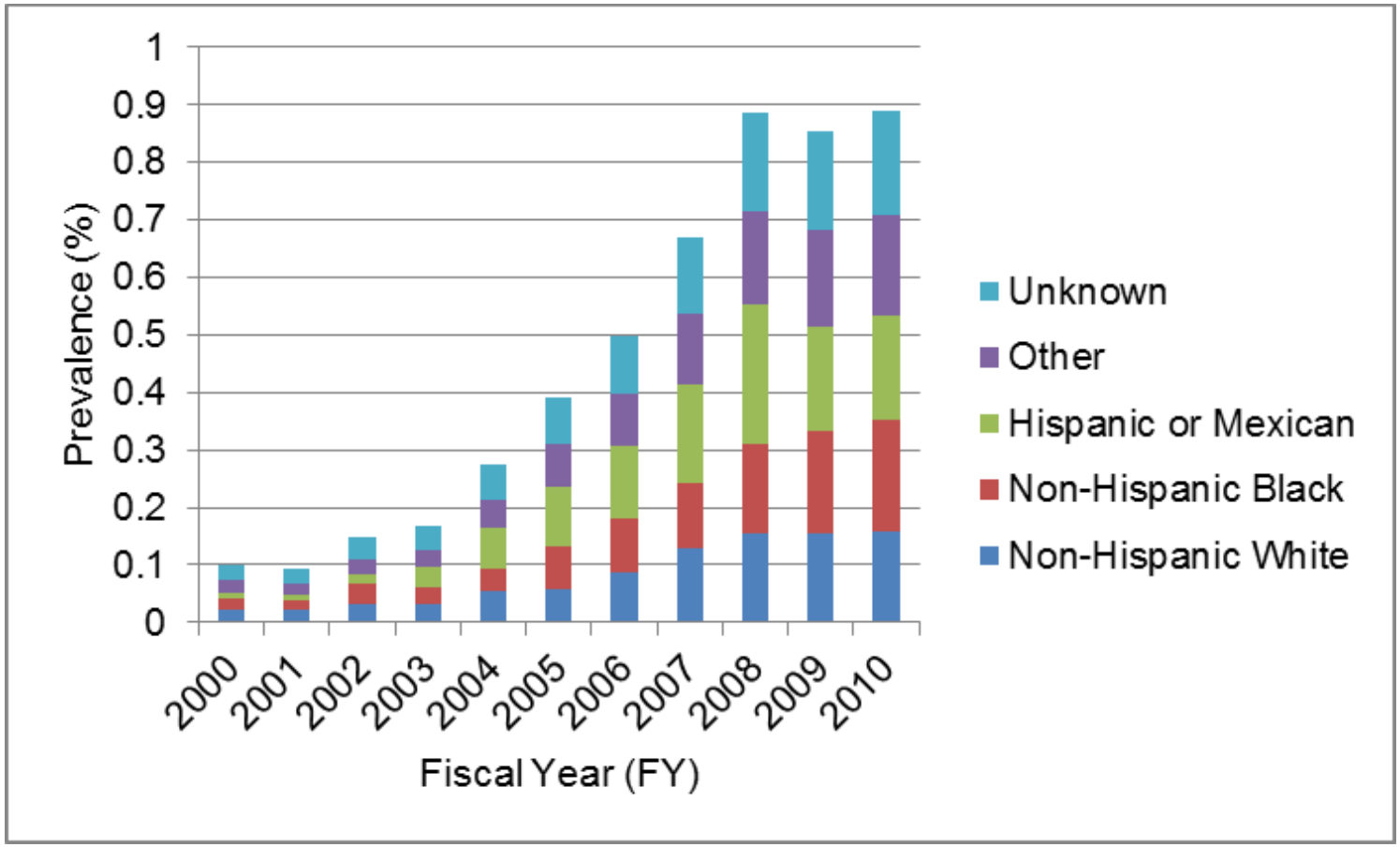

Figure 3.14f. Age-adjusted prevalence of PARASOMNIAS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

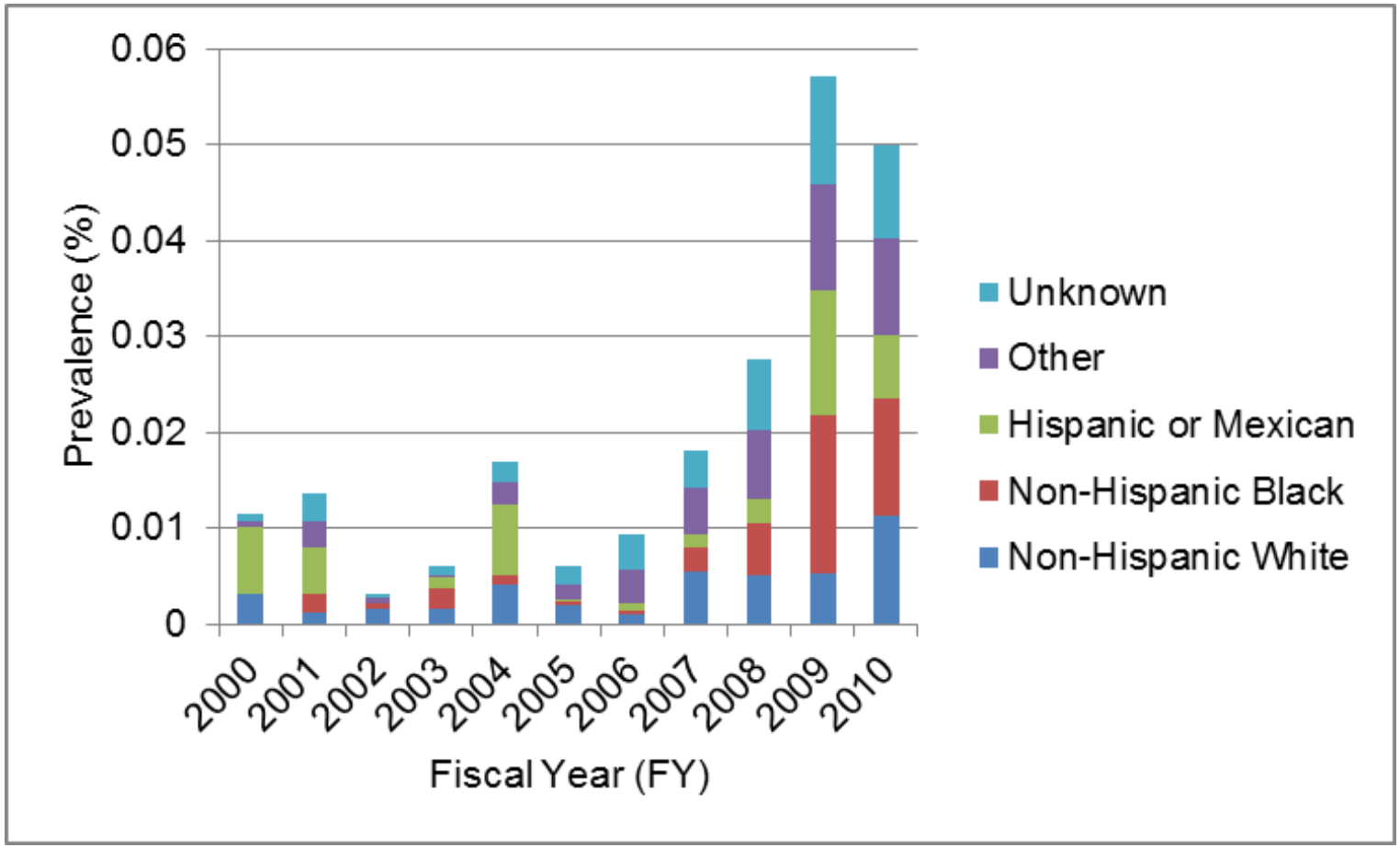

Figure 3.14g. Age-adjusted prevalence of CIRCADIAN RHYTHM DISORDERS by race/ethnicity among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

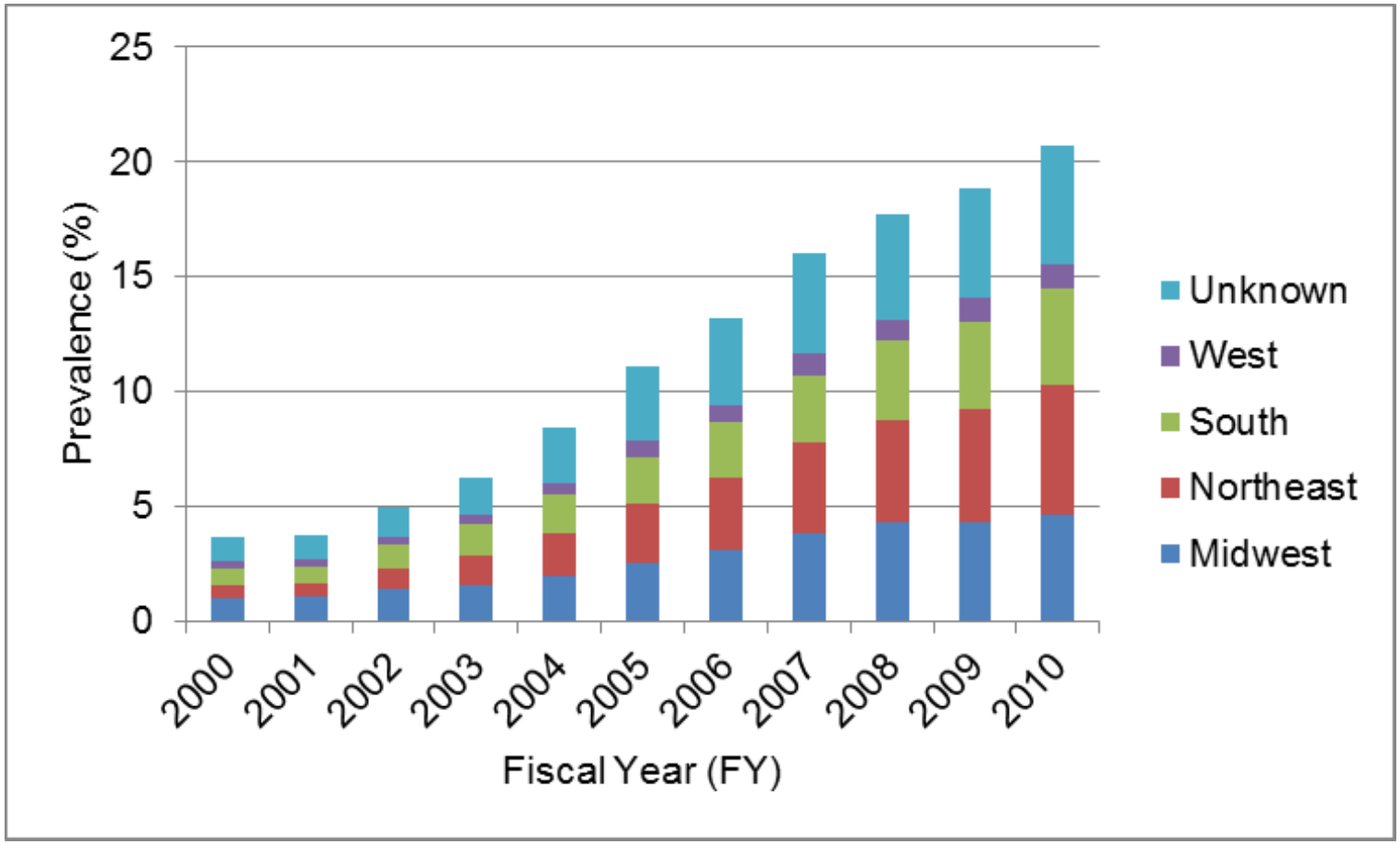

Figure 3.15a. Age-adjusted prevalence of APNEAS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

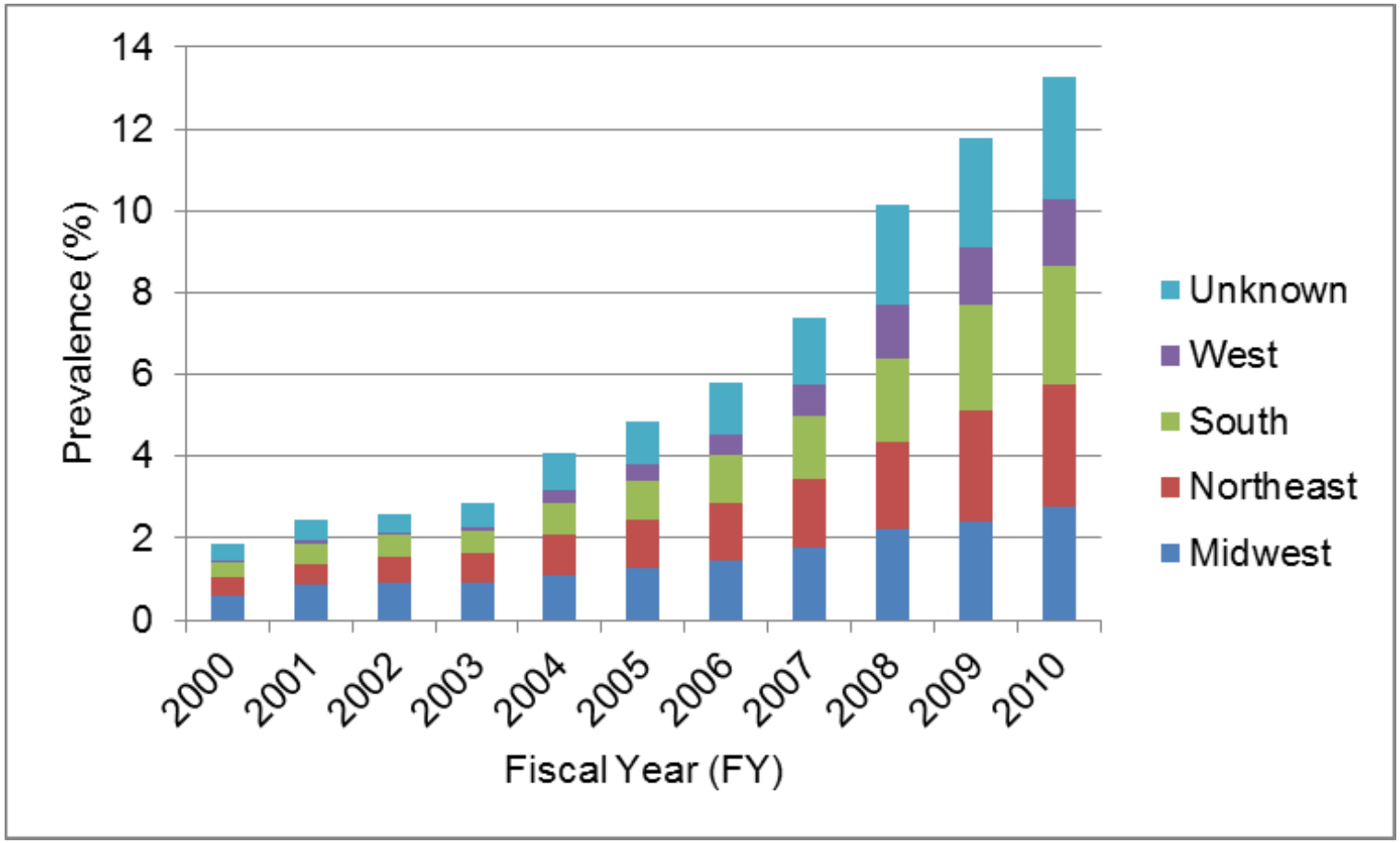

Figure 3.15b. Age-adjusted prevalence of INSOMNIAS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

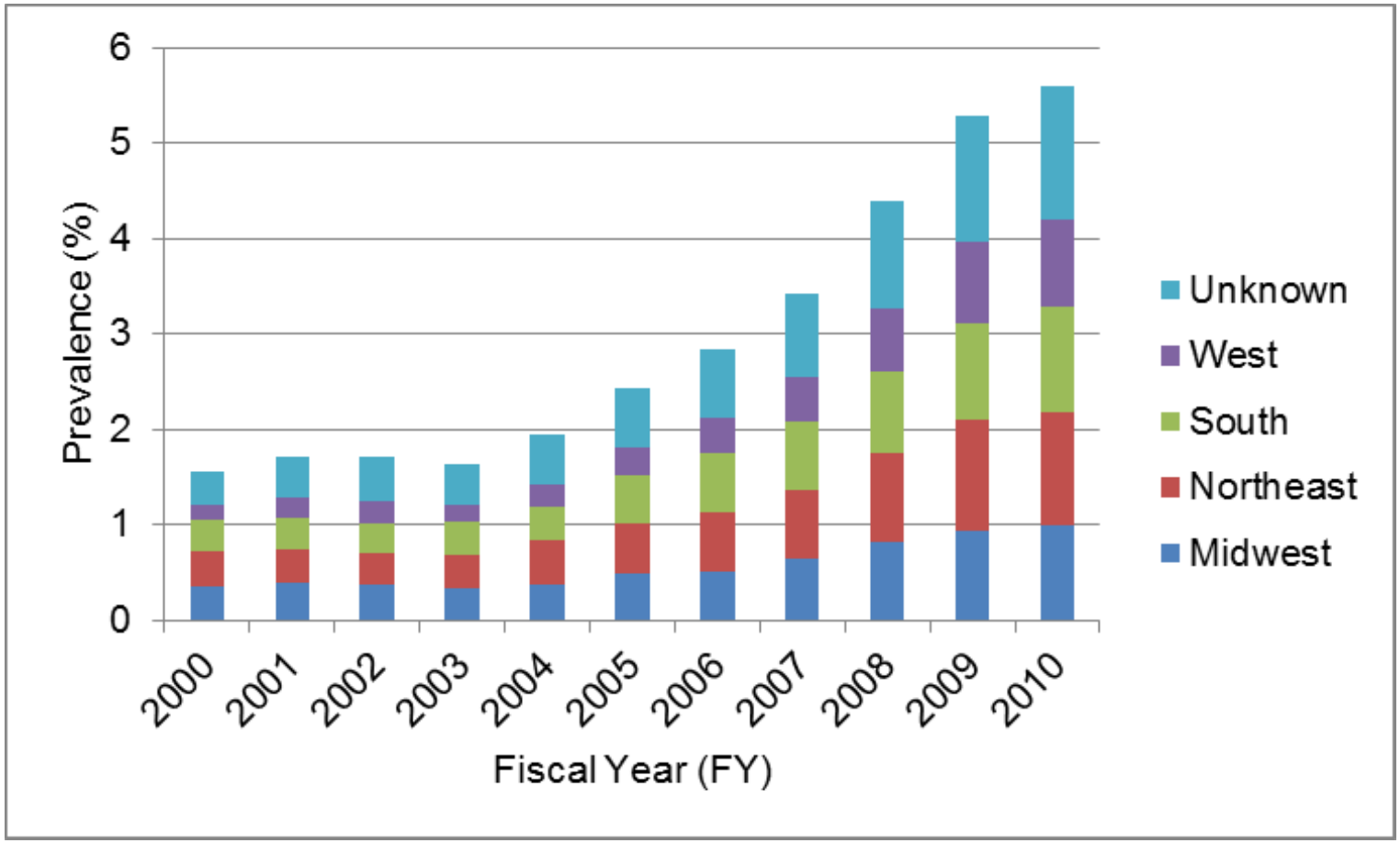

Figure 3.15c. Age-adjusted prevalence of OTHER SLEEP DISORDERS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

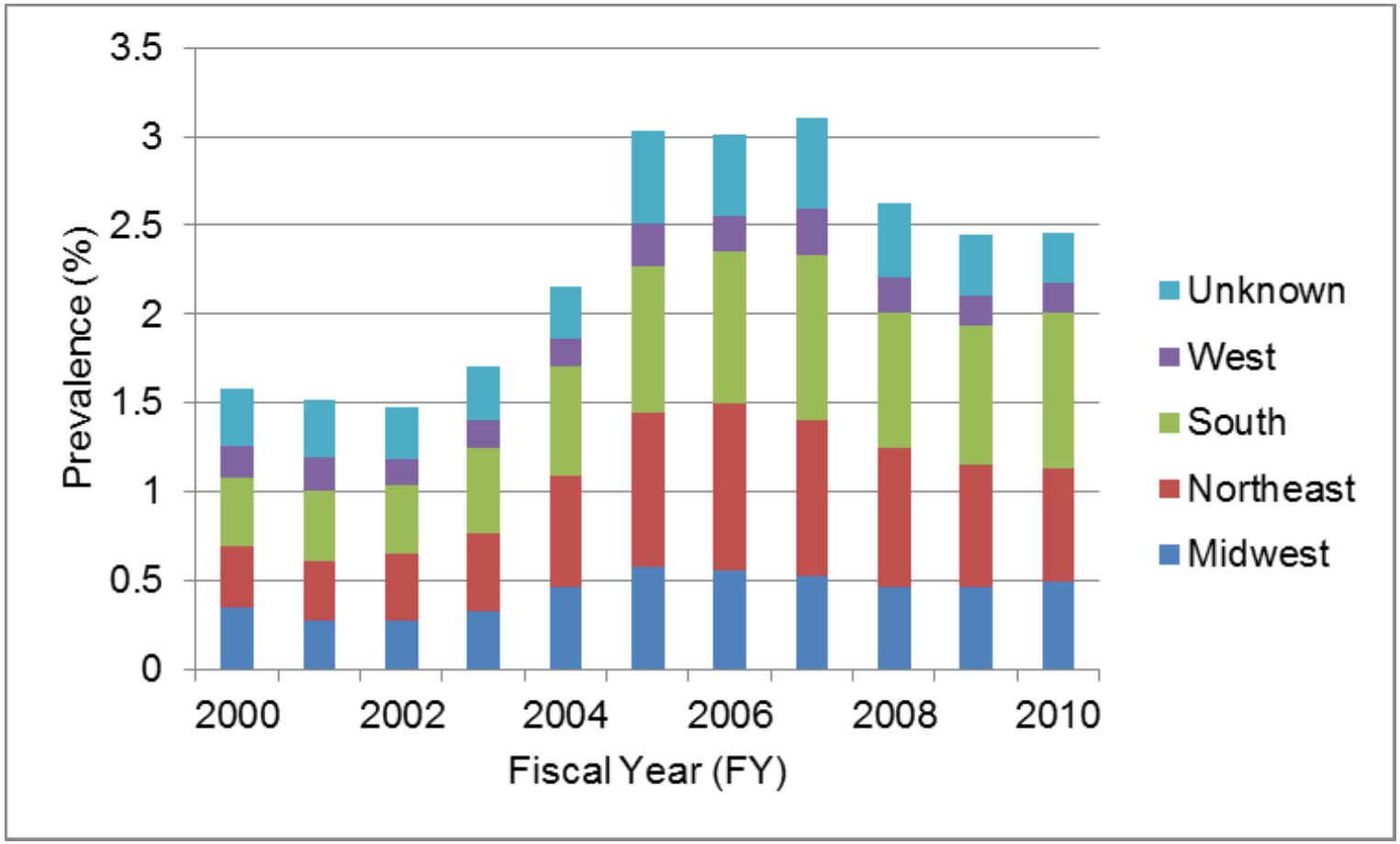

Figure 3.15d. Age-adjusted prevalence of HYPERSOMNIAS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

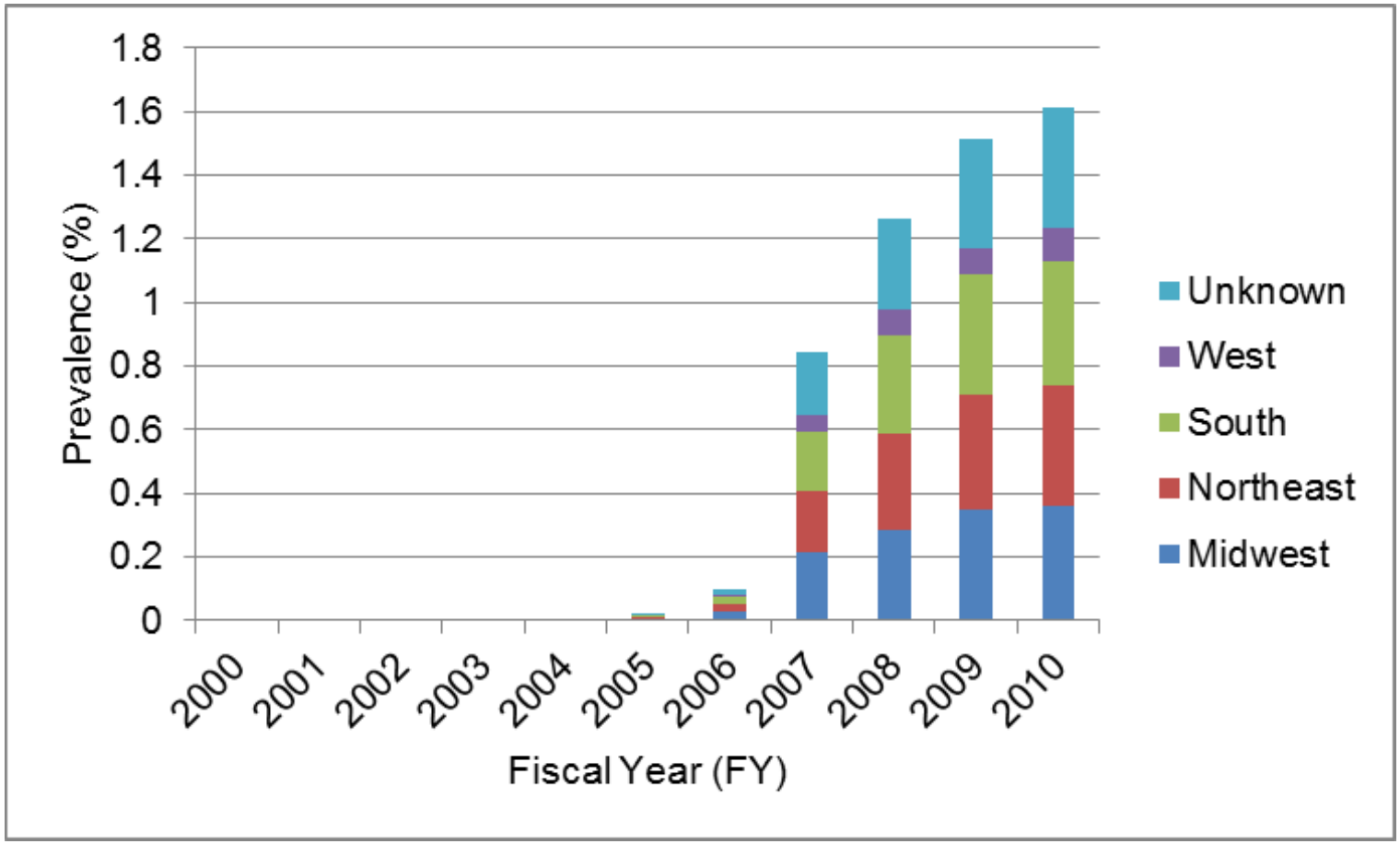

Figure 3.15e. Age-adjusted prevalence of MOVEMENT DISORDERS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

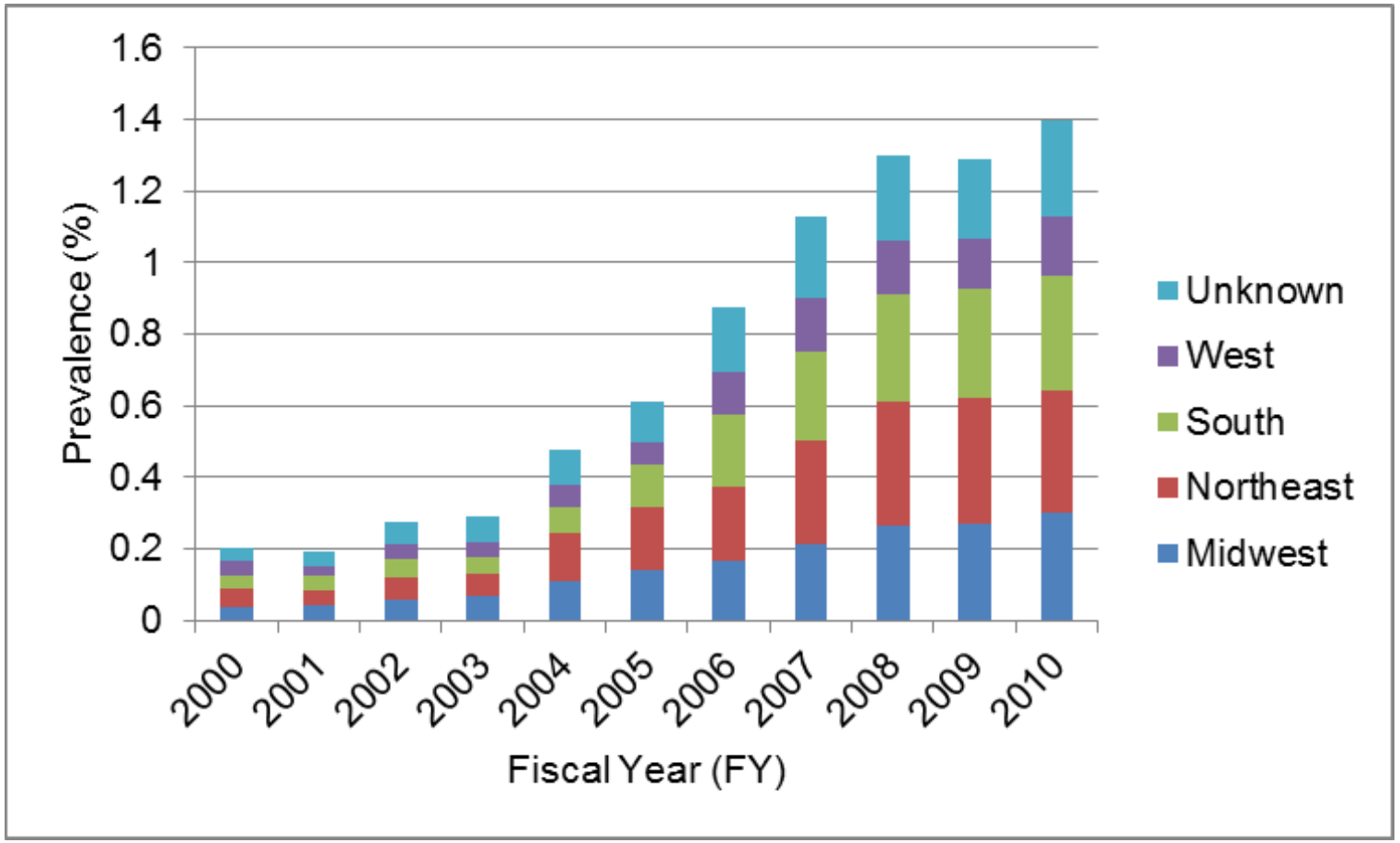

Figure 3.15f. Age-adjusted prevalence of PARASOMNIAS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

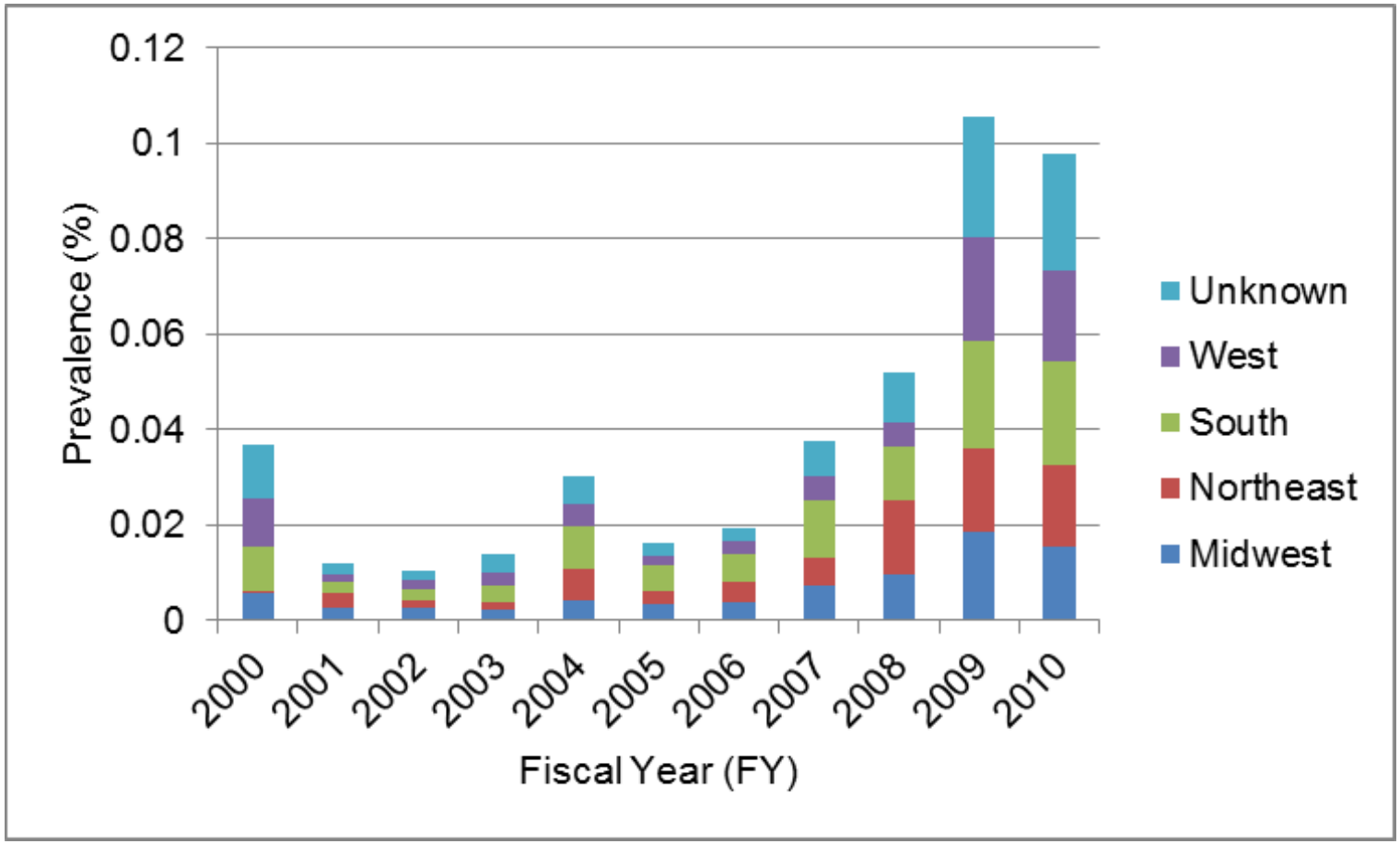

Figure 3.15g. Age-adjusted prevalence of CIRCADIAN RHYTHM DISORDERS by region among all Veterans seeking care through the Veterans Health Administration (VHA), FY2000 – 2010 (N= 9,786,778)

Chapter 4

The National Veteran Sleep Disorder Study II: Determinants of New Sleep Disorders among United States Veterans[2]

[2]Alexander M, Ray MA, Hébert JR, Youngstedt SD, Zhang H, Steck SE, Bogan RK, Burch JB. Submitted to *Sleep Medicine.*

4.1 Abstract

Introduction: Individuals with untreated or poorly managed sleep disorders are vulnerable to an increased risk of chronic disease, a poor quality of life, and earlier mortality. Sleep disturbances are common among veterans but few studies have examined factors influencing sleep disorder diagnoses in this population. This study identified risk factors for sleep disorder diagnoses among United States veterans seeking care in FY2010 through the Veterans Administration (VA) (N=5,492,156). **Materials and Methods**: De-identified electronic medical record data were accessed, and incident sleep disorder diagnoses were defined using American Academy of Sleep Medicine specified ICD-9 codes. Unconditional multiple logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) that identified demographic characteristics or pre-existing clinical conditions associated with a new sleep disorder diagnosis, after adjustment for potential confounding. **Results**: There were 128,679 new sleep disorder diagnoses in the study population (2.3% of all patients). Conditions that resulted in the greatest adjusted odds of sleep disorder onset included a pre-existing history of post-traumatic stress disorder (OR: 2.56 95% CI: 2.51-2.62), and obesity (OR: 2.41, 95% CI: 2.35-2.47). Patients with a sleep disorder diagnosis also were more likely to have a pre-existing diagnosis of: asthma, cancer, cardiovascular disease, chronic obstructive pulmonary disease, diabetes, fibromyalgia, gastrointestinal illness, renal disease, or a mental disorder compared to those without sleep disorders. **Discussion**: Results

from this study can assist health care providers in identifying high risk patients and in developing effective prevention and control strategies for sleep disorders.

4.2 Introduction

Sleep disorders arise due to complex circumstances that can include genetic, environmental, behavioral, or psychosocial risk factors. They often co-occur with chronic diseases,[179] but due to the interplay between sleep disorders and other comorbidities, the directionality of the relationship is often unclear. For example, sleep disorders can act as independent predictors of psychiatric disorders,[55, 57] but it also has been reported that psychiatric disorders are predictive of sleep disturbances.[177-179] The number of outpatient visits that occur due to sleep complaints has been increasing over time.[65] However, few studies have examined the role of pre-existing diseases on sleep disorder development while accounting for other known risk factors. Also, the use of hypnotics to treat sleep disorders is a common practice, although this practice may have deleterious health or safety consequences.[209] Thus, alternative strategies for sleep disorder prevention or treatment that target modifiable risk factors may be prudent for health care providers to consider. The identification of predictive factors also may allow clinicians to target high-risk groups that could benefit from timely diagnosis and treatment.

Despite the presence of symptoms, a large proportion of those afflicted with a sleep disorder can remain undiagnosed or untreated. For example, more than 80% of individuals with clinically significant sleep apnea may remain

undiagnosed.[48] Under-diagnosis or poor sleep disorder management can have adverse health consequences. Previous studies have linked sleep disorders with higher health care utilization[183] and increased risks for: obesity,[53, 54] type 2 diabetes,[53, 54] cardiovascular disease,[52] cancer,[59, 60, 147-150] or psychiatric disorders.[55-58] Alterations in circadian rhythms, neuroendocrine function, the immune system and inflammation, or energy utilization and metabolism have been suggested as biological processes altered by sleep disruption that can influence chronic disease risks.[159-162] The co-occurrence of a sleep disorder with another comorbid chronic disease can predict a poor quality of life[62, 63] and earlier mortality relative to those with the comorbid disease but without a sleep disorder.[49, 144, 158, 176, 195, 196] These findings underscore the importance of identifying independent sleep disorder risk factors, particularly in vulnerable populations.

Sleep disruption is common among veterans and they tend to be susceptible to the onset of sleep disorders.[155, 156, 163, 165, 167, 168] However, etiological models for new sleep disorder diagnoses have not been previously described in a national sample of veterans or others in the United States (US). To address this issue, this study identified independent risk factors for new sleep disorder diagnoses, including an assessment of pre-existing chronic diseases or disorders, by studying US veterans who utilized health services in FY2010 through the Veterans Health Administration (VHA), which is the largest integrated health care system utilizing electronic medical records (EMRs) in the US.[180] The approach allowed for an examination of objectively determined pre-existing

diseases that occur at higher rates among veterans relative to the general population (e.g., PTSD, other mental health disorders), as well as demographic characteristics that may influence sleep disorder onset.

4.3 Materials and Methods

Study Population

The study population consisted of all US veterans seeking care through VHA system in FY2010, along with data extracted from EMRs since FY2000. Following regulatory approvals, veteran's outpatient records were accessed from the MedSAS Dataset files and the VHA Corporate Data Warehouse (CDW) in the Veterans Administration Informatics and Computing Infrastructure (VINCI) system. Requested data elements from different VHA system files were linked by social security number, and each patient's social security number was then scrambled and replaced with a unique subject identifying number. Thus, personal identifying information was inaccessible to the study investigators;[65, 179, 209] only the VINCI data manager was able to access or link social security numbers with unique subject identifiers. Patients <18 years old on January 1 as of FY2010, spouses of veterans receiving care, non-veterans, or veterans who died in FY2010 were excluded.

Case Ascertainment

De-identified outpatient sleep disorder diagnoses were grouped according to the American Academy of Sleep Medicine's International Classification of

Sleep Disorders (see Table 4.1 for ICD-9 codes defining: sleep apnea, insomnia, hypersomnia, circadian rhythm disorder, parasomnia, sleep disruption movement disorder, and other sleep disorders).[181] Veterans were assigned a specific sleep disorder diagnosis if the diagnostic ICD-9 code appeared two or more times in their medical record, and at least two codes in the record occurred thirty or more days apart.[182] The first occurrence of the diagnostic code was used to define the date of the primary diagnosis. No previous sleep disorder diagnoses were associated with these veterans dating back to October 1, 1999. Sleep disorders that were neither sleep apnea nor insomnia were collapsed into one category (other sleep disorders). The comparison group was comprised of all other eligible veteran patients seeking care in FY2010 without a sleep disorder diagnosis.

Covariates

Covariate data (age, sex, smoking status, body mass index [BMI], income, pre-existing diagnoses) were retrieved from VHA databases (Vital Status File and MedSAS, Table 1). Annual income was grouped into three categories (less than $20,000, $20,000 or more, or unknown) because a large proportion of veterans had an annual income less than $20,000. Age in years was calculated by subtracting the year of birth from 2010.

BMI was calculated by dividing weight in pounds (lbs) by height in inches (in) squared and multiplying by a conversion factor of 703 to obtain BMI in kilograms/meter^2. Biologically implausible heights and weights were excluded (height was restricted to 48-84 inches [122-213 cm], and weight was restricted to

75-500 pounds [34-227 kg].[184] If height was missing, any appearance of height across FY2010 was used. Multiple entries for biologically plausible values of weight were averaged. Those in the underweight BMI category (<1% of study population) were merged into the normal BMI category, and the following categories were used in the analysis: low-to-normal: <25 kg/m^2, overweight: ≥25 to <30 kg/m^2, and obese: ≥30 kg/m^2.

The following ICD-9 codes dating back as far as October 1, 1999 were used to categorize pre-existing disease: cancer (150-151, 153-155, 157, 162, 172, 174, 183, 185, 188-189, 191, 200, 202, 204-208.9), diabetes (249-250), mental disorder or mental retardation (290-319, except for 309.81), cardiovascular disease (CVD) (390-459), post-traumatic stress disorder (PTSD, 309.81), chronic obstructive pulmonary disease (COPD, 496.0), asthma (493), fibromyalgia (729.1), gastrointestinal (GI) disease (520-579), human immunodeficiency virus (HIV) infection (042-044), and renal disorder (580-589) using the same criteria as described for sleep disorders. Smoking status (current, former, never) was assessed as described previously.[185] To validate smoking status, data extracted from the EMRs was compared to self-reported smoking data found in the Veterans Aging Cohort Study (VACS-8) and VACS Virtual Cohort (VACS-VC) studies, which yielded kappa (κ) statistics of 0.66 and 0.61, respectively.

Statistical Analysis

All analyses were conducted using SAS 9.3 statistical software (Cary, NC). Patients with a pre-existing sleep disorder diagnosis or without at least one visit prior to FY2010 were excluded from the analysis. Veterans with a new sleep disorder diagnosis concurrent with a first-time diagnosis of a chronic disease or disorder of interest also were excluded; thus only pre-existing diseases and disorders were evaluated.

Unconditional multivariable logistic regression was used to determine whether pre-existing diseases or other personal or lifestyle factors were associated with a first time sleep disorder diagnosis in 2010, after adjusting for other covariates. Odds ratios (ORs) with corresponding 95% confidence intervals (CIs) were used to assess the relationship between each variable of interest and any sleep disorder diagnosis, or a specific sleep disorder subtype (sleep apnea, insomnia, all other sleep disorders).

Initially, covariates listed in Table 4.2 were evaluated separately in relation to each sleep disorder type, and those associated with a new diagnosis (p-value ≤0.20) were further evaluated in multivariable models. Models were further reduced via manual backward selection, and all variables with a p-value ≤0.05 were retained in the final adjusted model.

Due to the high rate of missing race/ethnicity data (>50%), sensitivity analyses were conducted to determine if estimates for the various risk factors

differed among the following models: a.) race/ethnicity excluded entirely from the model, b.) race/ethnicity included except those with missing race/ethnicity data, and c.) including all those with race/ethnicity data; those with missing race/ethnicity data were coded was as a separate category. Estimates in the model did not appreciably change for each of these scenarios; therefore, race/ethnicity was left out of the final models and not reported.

Because 8.4% of individuals with a new sleep disorder diagnosis had multiple new sleep disorder subtypes, sensitivity analyses also were carried out to examine any changes in estimates for models that included or excluded these individuals. Among those with sleep apneas, 9% had some other sleep disorder diagnosis, 3% had an insomnia diagnosis, and <0.1% had both insomnia and some other sleep disorder diagnosis. Among those with insomnia, 5% had a sleep apnea diagnosis, 6% had some other sleep disorder diagnosis, and approximately 2% had sleep apnea and some other sleep disorder diagnosis. Among those with 'other' primary sleep disorder diagnoses (hypersomnia, circadian rhythm disorder, parasomnia, sleep disruption movement disorder, other), 19% also had comorbid sleep apnea, 7% had comorbid insomnia, and 2% had comorbid sleep apnea and insomnia. When sensitivity analyses were performed among those with more than one sleep disorder either included or excluded from the analysis, no major differences in the effect estimates (>10%) were observed. Results presented below were based on data from individuals with more than one sleep disorder.

4.4 Results

In FY2010, 5,484,890 veterans were eligible for evaluation. The majority of this analytical sample was male (93%) and married (55%) (Table 4.2), and had an average age (± standard deviation) of 63±16 years. A total of 128,679 incident sleep disorder cases were identified (2.3% of the study population).

Those with a new sleep disorder diagnosis were more likely to be male, married, have a higher income, and a higher BMI compared to those without sleep disorders (Table 4.2). Veterans with a new sleep disorder diagnosis were six years younger than those without sleep disorders, on average (57±15 years vs. 63±16 years, respectively). Additionally, those with a new sleep disorder had a higher proportion of the following pre-existing diseases compared to those without a sleep disorder: asthma, cancer, CVD, COPD, diabetes, fibromyalgia, GI, or renal disease, or a mental health disorder. Similar differences were found when comparing only veterans with sleep apnea, insomnia, or other sleep disorders relative to those without a sleep disorder, except for patients with HIV (see Tables 4.3, 4.4, and 4.5, respectively).

Table 4.6 presents crude and adjusted ORs for associations between variables of interest and new sleep disorder diagnoses. Veterans with a new sleep disorder diagnosis were more likely to be overweight or obese (OR: 1.36, 95% CI: 1.32, 1.39; OR: 2.41 95% CI: 2.35, 2.47, respectively) relative to those without a sleep disorder. Veterans who developed a new sleep disorder also were more likely to have a pre-existing diagnosis of PTSD or another type of

mental health disorder compared to those without a sleep disorder (PTSD OR: 2.56, 95% CI: 2.51, 2.62; other mental health disorder OR: 2.16, 95% CI: 2.12, 2.20, Table 4.6). The next largest risk estimates for sleep disorders among those with pre-existing diseases were: CVD (OR: 1.71, 95% CI: 1.68, 1.74), fibromyalgia (OR: 1.69, 95% CI: 1.56, 1.83), COPD (OR: 1.67, 95% CI: 1.62, 1.72), asthma, (OR: 1.66, 95% CI: 1.59, 1.74), GI disorder (OR: 1.46, 95% CI: 1.44, 1.49), renal disorder (OR: 1.24, 95% CI: 1.19, 1.30), diabetes (OR: 1.06, 95% CI: 1.04, 1.08), and cancer (OR: 1.06, 95% CI: 1.02, 1.10) (Table 4.6). After adjustment, male veterans and those who were married also had increased odds of a new sleep disorder diagnosis (Table 4.6).

Veterans who developed sleep apnea had a two- or greater than six-fold odds of being overweight or obese, respectively (Table 4.7), relative to veterans without a sleep disorder. The following pre-existing diseases were each associated with increased odds of a new sleep apnea diagnosis, in order of decreasing magnitude of the effect estimates: PTSD (OR: 2.00, 95% CI: 1.94, 2.05), COPD (OR: 1.95, 95% CI: 1.88, 2.03), CVD (OR: 1.86, 95% CI: 1.82, 1.90), asthma (OR: 1.76, 95% CI: 1.66, 1.86), other mental health disorders (OR: 1.67, 95% CI: 1.63, 1.71), fibromyalgia (OR: 1.58, 95% CI: 1.41, 1.78), GI disease (OR: 1.42, 95% CI: 1.38, 1.45), renal disorder (OR: 1.30, 95% CI: 1.23, 1.37), and diabetes (OR: 1.14, 95% CI: 1.12, 1.17) (Table 4.7). Veterans diagnosed with sleep apnea were almost one-third less likely to have an HIV infection (OR: 0.70, 95% CI: 0.58, 0.86) (Table 4.7).

The odds of pre-existing PTSD or another mental health disorder were four times or three times greater among those with new onset of insomnia, respectively, relative to those without insomnia (Table 4.8). The next largest association between a pre-existing disease and an insomnia diagnosis was fibromyalgia (OR: 1.63, 95% CI: 1.42, 1.87), followed by: GI disease (OR: 1.50, 95% CI: 1.45, 1.55), CVD (OR: 1.46, 95% CI: 1.41, 1.51), asthma (OR: 1.27, 95% CI: 1.15, 1.40), COPD (OR: 1.24, 95% CI: 1.17, 1.31), renal disorder (OR: 1.23, 95% CI: 1.13, 1.34), and cancer (OR: 1.16, 95% CI: 1.09, 1.25) (Table 4.8). Unlike the association between diabetes and any new sleep disorder, those with diabetes were less likely to be diagnosed with insomnia (OR: 0.84, 95% CI: 0.81, 0.88, Table 8).

Finally, veterans with other sleep disorders were nearly two times more likely to be overweight or obese (Table 4.9). Similar to insomnia, pre-existing PTSD had the largest association with other sleep disorder diagnoses (OR: 3.16, 95% CI: 3.04, 3.29), followed by: other mental health disorders (OR: 2.44, 95% CI: 2.36, 2.53), fibromyalgia (OR: 1.89, 95% CI: 1.65, 2.17), asthma (OR: 1.66, 95% CI: 1.53, 1.81), CVD (OR: 1.65, 95% CI: 1.60, 1.71), COPD (OR: 1.61, 95% CI: 1.52, 1.71), GI diseases (OR: 1.54, 95% CI: 1.49, 1.60), and renal disorder (OR: 1.19, 95% CI: 1.09, 1.29) (Table 4.9).

4.5 Discussion

This study identified several determinants of new sleep disorder diagnoses in a national sample of veterans. Non-disease determinants of a new

sleep disorder diagnosis included: male sex, being married, and being overweight or obese. Pre-existing diseases that were associated with a new sleep disorder diagnosis included: asthma, cancer, CVD, COPD, diabetes, fibromyalgia, GI disease, renal disorder, and mental health disorders, especially PTSD. Several disease risk factors identified in this study were consistent with findings from prior investigations among veterans,[179, 210, 211] namely the link between PTSD and sleep disorders.[170, 191, 212] Knowledge of risk factors for new sleep disorder diagnoses, particularly pre-existing diseases, provides an opportunity to improve the quality of life among veterans who may be at a higher risk for developing long-term sequelae of sleep disorders. This may include several types of chronic disease or psychiatric disorder,[52-58] and early mortality[49, 59, 144, 158, 176, 195, 196] relative to those without sleep disorders.

There were some unexpected findings in this study. First, the development of a new sleep disorder was less likely with increasing age, which is contrary to some reports,[213] although there have been inconsistencies. For example, older age was a risk factor for sleep apnea in one previous study,[204] whereas results from another study indicated that sleep apnea prevalence peaked among middle-aged rather than older men.[205] Another study among veterans over 65 years of age found that increasing age was protective of new sleep apnea diagnoses.[183] Results from the present study were consistent with a previous study that examined correlates of prevalent insomnia among veterans in FY2010 after adjusting for several confounders. A lower prevalence of insomnia among older veterans was observed, and multivariable analyses did not identify

an association between age and insomnia.[58] Because those with prevalent sleep disorders were excluded from the present study, it is possible that older veterans who were retained in the analysis tended to be healthier and less likely to be diagnosed with a sleep disorder relative to younger veterans. An alternative explanation for this observation may be a tendency for medical providers to overlook sleep problems as symptoms of another comorbid disease rather than a separate disorder requiring diagnosis, or because older veterans are less likely to discuss sleep complaints with their physician. Although the relatively small reduction in odds with increasing age was statistically significant, the clinical significance of this observation is uncertain. Finally, middle age veterans may have other attributes that differ from older veterans, such as a greater likelihood of participation in the recent Middle East war effort.

Another unexpected finding was the inverse association of tobacco consumption with sleep disorder diagnosis. Nicotine acts as a stimulant and it interferes with the release of neurotransmitters that play a key role in regulation of the sleep-wake cycle, and thus may be involved in the onset of sleep disturbances.[214, 215] Because smoking status was assessed within the same time period as the sleep disorder diagnosis, these associations could be attributed to reverse causation. That is, individuals who were diagnosed with a new sleep disorder may have avoided smoking in an effort to improve their sleep and health.

An unexpected protective association between HIV infection and sleep apnea also was observed. In an era where HIV patients with antiretroviral

therapy are living longer, this subpopulation may be experiencing increased morbidity from conditions unrelated to HIV infection. Few large-scale studies have examined this issue. A positive association between HIV infection and sleep apnea was recently observed in a large cohort study.[216] However, a prior study among veterans observed a three-fold lower prevalence of sleep apnea among those with HIV compared to those without HIV.[217] The investigators reported that veterans with HIV had fewer sleep apnea risk factors, but noted that sleep apnea also may be underdiagnosed in this population.

Veterans have elevated rates of mental health disorders compared to the general population. The prevalence of pre-existing PTSD in the current study population (5.7%) falls within the range of previously reported rates among veterans (2-17%).[201] Results from the current study suggest that pre-existing PTSD or other mental health disorders may have a considerable role in the development of a new sleep disorder, more so than other pre-existing medical conditions. Those with a new diagnosis of any sleep disorder, sleep apnea, insomnia, or other sleep disorders were two to four times more likely to have pre-existing PTSD; one of the strongest associations observed. These observations are consistent with results reported in a prospective study of incident chronic insomnia, which was defined as not having insomnia at baseline and subsequently developing insomnia that lasted at least one year.[218] Associations between psychiatric disturbances, especially anxiety disorders (e.g., PTSD), and sleep disorders have been described previously,[55, 57, 58, 177-179, 191, 212] although the inter-related nature of these conditions may have produced inconsistencies in

terms of cause and effect.[219] Relationships between sleep and psychiatric disturbances may be bi-directional, and also may be subject to misclassification bias if symptomatic patients remain undiagnosed. Through careful selection of veterans with and without pre-existing diseases, the design of this study allowed for sleep disorder risk factors to be assessed while preserving the temporality of the relationship. Previous investigators have observed that anxiety disorders tended to precede an insomnia diagnosis rather than co-occur or occur as a consequence of insomnia.[177-179] For example, anxiety appeared prior to insomnia in 44% of people diagnosed with insomnia, which is higher than the proportion of those who developed an anxiety disorder after insomnia diagnosis (20%), or those who had co-occurring diagnoses (40%).[179] Also, sleep disturbances have persisted despite successful treatment of PTSD,[220] suggesting that although disturbed sleep is a core PTSD symptom, it also may be a pre-disposing factor, or may develop as a separate disorder during the course of PTSD.

While there is a growing literature on the role of anxiety disorders in the development of insomnia, little is known about their role in the development of sleep apnea. A recent study found an association between higher PTSD symptom severity and risk for obstructive sleep apnea (OSA) among Iraq and Afghanistan veterans presenting at a VHA outpatient PTSD clinic.[221] In the current study, veterans with a new sleep apnea diagnosis were nearly twice as likely to have pre-existing PTSD after controlling for several other known predictors including BMI. The underlying mechanism has yet to be fully elucidated, but it has been suggested that those afflicted with PTSD may lack the

ability to suppress anxiety while dreaming, resulting in an increase in the frequency of sleep disordered breathing events.[222] This may have clinical implications since PTSD reduces adherence to continuous positive airway pressure (CPAP) therapy,[223] and untreated sleep apnea has been associated with numerous adverse sequelae including all-cause mortality and cardiovascular disease.[208] Longitudinal studies are needed to confirm this association while accounting for sleep apnea treatments.

While it is possible for underlying disease processes to influence subsequent sleep disorder development, there are other explanations for the observed relationships. First, veterans with a chronic disease or mental health disorder are more likely to use VHA care, thus increasing their likelihood of being diagnosed with a sleep disorder. A study of utilization of VHA non-mental health services indicated that veterans with PTSD or mental disorders other than PTSD had 91% and 55% higher utilization, respectively, than those without a mental disorder. The increased utilization may be related to combat exposure, which has been associated with both mental and physical ailments, and the combination of these conditions increases the likelihood of a veteran seeking services in a primary care setting.[224] It also is possible that VHA clinicians in primary care settings are more likely to look for sleep issues when evaluating veterans with mental health disorders. In 2005, the VHA expanded its capacity for mental health services, which included the integration of mental health providers into primary care settings.[225] However, one study found that mental health status and

primary care visits had only a modest effect on utilization of non-mental health services.[224]

Veterans are experiencing an obesity epidemic similar to the general population,[165, 184] and results from the present study suggest that weight management may provide an opportunity to prevent sleep disorders associated with this condition. Obese veterans in the present study were about twice as likely to receive a diagnosis of any sleep disorder, sleep apnea, or other sleep disorders relative to non-obese individuals. However, the ORs for insomnia marginally decreased as BMI increased, which is inconsistent with previous reports.[206, 218] This suggests that current BMI may not predict insomnia or that other factors, such as unstudied comorbid conditions, or their treatment, could influence the relationship between BMI and insomnia. For example, psychiatric disorders increase the risk of obesity,[226-228] and both factors increase the likelihood of sleep disturbances. Explaining the nature of these complex relationships requires further examination using either prospective or experimental study designs.

Strengths of this study included a large sample size, the use of validated measures for outcomes and important variables of interest, and a medical care system that ensured equal access to care. The robust sample size provided ample statistical power and tended to reduce standard errors of the effect estimates (inflated standard errors can arise due to multicollinearity when multiple risk factors with overlapping effects are evaluated). Since the objective was to examine multiple sleep disorder risk factors, steps were taken to reduce

the likelihood of multicollinearity, including careful *a priori* consideration of variables to be included in the analyses. For example, combat exposure is associated with the onset of mental health disorders; therefore, combat and mental health disorders were not simultaneously included in the analyses. Similarly, analyses of hypertension, metabolic syndrome, and stroke were avoided since they have risk factors that overlap with CVD.

Some limitations are also noteworthy. Information on medication use or substance abuse was not included in the analysis, which may have impacted the strength or direction of the reported associations. Data on treatment efficacy or management of specific pre-existing diseases also was not available, which may have impacted sleep disorder onset. Caution is advised when interpreting small, unanticipated effects since residual confounding may have been present, and since statistical significance does not always correspond to clinical significance. Finally, this investigation was limited to veterans seeking care through the VHA system, who tend to be older males with multiple comorbidities.[207] This limits the potential for generalizability to all veterans, or to the general US population. It is also possible that multiple synchronous sleep disorder diagnoses could have influenced the results (~8% of new sleep disorder diagnoses in FY2010). However, no appreciable change in the risk estimates was observed after removal of individuals with multiple sleep disorder diagnoses from the analysis.

In summary, sleep disorders are reportedly on the rise and these conditions can have long-term deleterious health consequences.[65] In this study, a predictive analysis of factors influencing sleep disorder onset was performed

among US veterans seeking care in the VHA system in FY2010. The findings suggest that early identification and targeting of high-risk groups, such as veterans with PTSD or elevated BMI, may lead to more focused sleep disorder prevention or management strategies that may convey lower health care utilization, improved quality of life, and reduced mortality risk to those individuals.

Table 4.1. International Classification of Sleep Disorders[181]

Sleep Disorder	**ICD-9 codes**
Sleep Apnea	Organic Sleep Apnea (327.20-29) Unspecified Sleep Apnea (780.57)
Insomnia	Organic Insomnias (327.00-09) Insomnias, unspecified (780.51-780.52) Non Organic Insomnias (307.41-307.42)
Hypersomnia	Organic Hypersomnias (327.10-19) Non Organic Hypersomnias (307.43-.44) Hypersomnias, unspecified (780.53-780.54) Other Hypersomnias (347.00-347.01, 347.10-347.11)
Circadian Rhythm Disorder	Organic Circadian Rhythm Sleep Disorders (327.30-39) Circadian Rhythm Sleep disorder of Non Organic (307.45) Disruption of 24 hour sleep wake cycle, unspecified (780.55)
Parasomnia	Organic Parasomnias (327.40-327.49) Non Organic Parasomnias (307.46-307.47)
Sleep Disruption Movement Disorders	Organic sleep related movement disorders (327.51-327.59) Sleep Related Movement Disorder, unspecified (780.58) Restless Legs Syndrome (333.94)
Other Sleep Disorders	Other Organic Sleep Disorder (327.80) Specific disorders of sleep of Non Organic origin (307.40-307.49) Other Sleep disturbances, unspecified (780.50, 780.56, 780.59) Other Sleep Disorders (291.82, 292.85)

Table 4.2. Characteristics of United States veterans with and without a primary sleep disorder diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	Classification	Total Population (N = 5,484,890)[1]	Sleep Disorder (N = 128,679)[1]	No Sleep Disorder (N = 5,356,211)[1]	p-value[2]
Age (years)	Mean (SD)	63 (16)	57 (15)	63 (16)	<0.01
Sex		N (%)	N (%)	N (%)	
	Male	5,119,403 (93)	120,560 (94)	4,998,843 (93)	<0.01
	Female	365,487 (7)	8,119 (6)	357,368 (7)	
Marital Status	Not Married	2,205,522 (40)	50,710 (39)	2,154,812 (40)	<0.01
	Married	3,014,017 (55)	73,601 (57)	2,940,416 (55)	
	Unknown	265,351 (5)	4,368 (3)	260,983 (5)	
Annual Income	<$20,000	3,012,254 (55)	71,837 (56)	2,940,417 (55)	<0.01
	≥$20,000	2,144,375 (39)	56,711 (44)	2,087,664 (39)	
	Unknown	328,261 (6)	131 (0.1)	328,130 (6)	
BMI (kg/m^2)	Normal	964,244 (18)	15,087 (12)	949,157 (18)	<0.01
	Overweight	1,712,320 (31)	36,999 (29)	1,675,321 (31)	
	Obese	1,705,000 (31)	74,119 (58)	1,630,881 (30)	
	Unknown	1,103,326 (20)	2,474 (2)	1,100,852 (21)	
Tobacco Use	Never	920,168 (17)	30,458 (24)	889,710 (17)	<0.01
	Former	1,249,110 (23)	37,395 (29)	1,211,715 (23)	
	Current	956,402 (17)	30,192 (23)	926,210 (17)	
	Unknown	2,359,210 (43)	30,634 (23)	2,328,576 (43)	
Asthma	No	5,409,347 (99)	123,611 (96)	5,285,736 (99)	<0.01
	Yes	54,461 (1)	2,961 (2)	51,500 (1)	
Cancer	No	5,260,565 (96)	122,332 (95)	5,138,233 (96)	<0.01
	Yes	163,904 (3)	4,100 (3)	159,804 (3)	
CVD	No	3,371,891 (61)	52,173 (41)	3,319,718 (62)	

Table 4.2. Characteristics of United States veterans with and without a primary sleep disorder diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	Classification	Total Population (N = 5,484,890)[1]	Sleep Disorder (N = 128,679)[1]	No Sleep Disorder (N = 5,356,211)[1]	p-value[2]
	Yes	1,820,644 (33)	58,582 (46)	1,762,062 (33)	
COPD	No	5,246,370 (96)	116,863 (91)	5,129,507 (96)	<0.01
	Yes	177,253 (3)	7,160 (6)	170,093 (3)	
Diabetes	No	4,547,121 (83)	95,089 (74)	4,452,032 (83)	<0.01
	Yes	813,430 (15)	26,189 (20)	787,241 (15)	
Fibromyalgia	No	5,458,590 (99)	126,501 (98)	5,332,089 (99)	<0.01
	Yes	13,243 (0.2)	924 (0.7)	12,319 (0.2)	
GI disease	No	4,606,681 (84)	86,897 (68)	4,519,784 (84)	<0.01
	Yes	653,361 (12)	26,831 (21)	626,530 (12)	
HIV Infection	No	5,465,179 (99)	63,016 (49)	5,336,966 (99)	0.28
	Yes	17,921 (0.3)	49,688 (39)	17,518 (0.3)	
Mental disorder	None	4,083,480 (75)	55,400 (43)	4,028,080 (75)	<0.01
	PTSD[3]	310,470 (6)	16,723 (13)	293,747 (5)	
	Other[4]	748,787 (14)	29,587 (23)	719,200 (13)	
Renal disorder	No	5,338,066 (97)	123,048 (96)	5,215,018 (97)	<0.01
	Yes	95,334 (2)	3,107 (2)	92,227 (2)	

Note: All numbers are presented as counts and percentages unless otherwise indicated. [1]Cells may not add up to total for column due to missing values; [2]Chi-square test for differences in proportions for categorical variables or Student's t-test for differences in means for continuous variables; [3]Any diagnosis of PTSD, does not exclude other pre-existing mental health diagnoses; [4]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders). Abbreviations: BMI: body mass index; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; HIV, human immunodeficiency virus; PTSD, post-traumatic stress disorder

Table 4.3. Characteristics of United States veterans with and without a primary SLEEP APNEA diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	Classification	Total Population (N = 5,426,333)[1]	Apneas (N = 70,122)[1]	No Apneas (N = 5,356,211)[1]	p-value[2]
Age (years)	Mean (SD)	63 (16)	58 (13)	63 (16)	<0.01
Sex		**N (%)**	**N (%)**	**N (%)**	
	Male	5,065,833 (94)	66,990 (96)	4,998,843 (93)	<0.01
	Female	360,500 (7)	3,132 (4)	357,368 (7)	
Marital Status	Not Married	2,178,818 (40)	24,006 (34)	2,154,812 (40)	<0.01
	Married	2,984,256 (55)	43,840 (63)	2,940,416 (55)	
	Unknown	263,259 (5)	2,276 (3)	260,983 (5)	
Annual Income	<$20,000	2,977,794 (55)	37,377 (53)	2,940,417 (55)	<0.01
	≥$20,000	2,120,340 (39)	32,676 (47)	2,087,664 (39)	
	Unknown	328,199 (6)	69 (0.1)	328,130 (6)	
BMI (kg/m^2)	Normal	952,469 (18)	3,312 (5)	949,157 (18)	<0.01
	Overweight	1,690,661 (31)	15,340 (22)	1,675,321 (31)	
	Obese	1,681,033 (31)	50,152 (72)	1,630,881 (30)	
	Unknown	1,102,170 (20)	1,318 (2)	1,100,852 (21)	
Tobacco Use	Never	906,634 (17)	16,924 (24)	889,710 (17)	<0.01
	Former	1,232,979 (23)	21,264 (30)	1,211,715 (23)	
	Current	939,838 (17)	13,628 (19)	926,210 (17)	
	Unknown	2,346,882 (43)	18,306 (26)	2,328,576 (43)	
Asthma	No	5,352,660 (99)	66,924 (95)	5,285,736 (99)	<0.01
	Yes	53,316 (1)	1,816 (3)	51,500 (1)	
Cancer	No	5,205,036 (96)	66,803 (95)	5,138,233 (96)	0.41
	Yes	161,911 (3)	2,107 (3)	159,804 (3)	
CVD	No	3,344,548 (62)	24,830 (35)	3,319,718 (62)	<0.01

Table 4.3. Characteristics of United States veterans with and without a primary SLEEP APNEA diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	Classification	Total Population (N = 5,426,333)[1]	Apneas (N = 70,122)[1]	No Apneas (N = 5,356,211)[1]	p-value[2]
	Yes	1,796,450 (33)	34,388 (49)	1,762,062 (33)	
COPD	No	5,192,395 (96)	62,888 (90)	5,129,507 (96)	<0.01
	Yes	174,210 (3)	4,117 (6)	170,093 (3)	
Diabetes	No	4,500,153 (83)	48,121 (69)	4,452,032 (83)	<0.01
	Yes	804,090 (15)	16,849 (24)	787,241 (15)	
Fibromyalgia	No	5,401,182 (99)	69,093 (99)	5,332,089 (99)	<0.01
	Yes	12,754 (0.2)	435 (0.6)	12,319 (0.2)	
GI disease	No	4,567,647 (84)	47,863 (68)	4,519,784 (84)	<0.01
	Yes	640,749 (12)	14,219 (20)	626,530 (12)	
HIV Infection	No	5,406,918 (99)	69,952 (99)	5,336,966 (99)	<0.01
	Yes	17,659 (0.3)	141 (0.2)	17,518 (0.3)	
Mental disorder	None	4,063,737 (75)	35,657 (51)	4,028,080 (75)	<0.01
	PTSD[3]	302,482 (6)	8,735 (12)	293,747 (5)	
	Other[4]	733,366 (14)	14,166 (20)	719,200 (13)	
Renal disorder	No	5,281,718 (97)	66,700 (95)	5,215,018 (97)	<0.01
	Yes	94,106 (2)	1,879 (3)	92,227 (2)	

Note: All numbers are presented as counts and percentages unless otherwise indicated
[1]Cells may not add up to total for column due to missing values; [2]Chi-square test for differences in proportions for categorical variables or Student's t-test for differences in means for continuous variables; [3]Any diagnosis of PTSD, does not exclude other pre-existing mental health diagnoses; [4]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders). Abbreviations: BMI: body mass index; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; HIV, human immunodeficiency virus; PTSD, post-traumatic stress disorder

Table 4.4. Characteristics of United States veterans with and without a primary INSOMNIA diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	Classification	Total Population (N = 5,394,500)[1]	Insomnia (N = 38,289)[1]	No Insomnia (N = 5,356,211)[1]	p-value[2]
Age (years)	Mean (SD)	63 (16)	55 (17)	63 (16)	<0.01
		N (%)	**N (%)**	**N (%)**	
Sex	Male	5,033,736 (93)	34,893 (91)	4,998,843 (93)	<0.01
	Female	360,764 (7)	3,396 (9)	357,368 (7)	
Marital Status	Not Married	2,173,430 (40)	18,618 (49)	2,154,812 (40)	<0.01
	Married	2,958,722 (55)	18,306 (48)	2,940,416 (55)	
	Unknown	262,348 (5)	1,365 (4)	260,983 (5)	
Annual Income	<$20,000	2,963,517 (55)	23,100 (60)	2,940,417 (55)	<0.01
	≥$20,000	2,102,812 (39)	15,148 (40)	2,087,664 (39)	
	Unknown	328,171 (6)	41 (0.1)	328,130 (6)	
BMI (kg/m^2)	Normal	957,541 (18)	8,384 (22)	949,157 (18)	<0.01
	Overweight	1,690,139 (31)	14,818 (39)	1,675,321 (31)	
	Obese	1,645,260 (31)	14,379 (38)	1,630,881 (30)	
	Unknown	1,101,560 (20)	708 (2)	1,100,852 (21)	
Tobacco Use	Never	898,691 (17)	8,981 (23)	889,710 (17)	<0.01
	Former	1,222,063 (23)	10,348 (27)	1,211,715 (23)	
	Current	937,535 (17)	11,325 (30)	926,210 (17)	
	Unknown	2,336,211 (43)	7,635 (20)	2,328,576 (43)	
Asthma	No	5,322,840 (99)	37,104 (97)	5,285,736 (99)	<0.01
	Yes	52,166 (1)	666 (2)	51,500 (1)	
Cancer	No	5,174,528 (96)	36,295 (95)	5,138,233 (96)	<0.01
	Yes	161,055 (3)	1,251 (3)	159,804 (3)	
CVD	No	3,338,285 (62)	18,567 (48)	3,319,718 (62)	<0.01

	Yes	1,776,727 (33)	14,665 (38)	1,762,062 (33)	
COPD	No	5,165,002 (96)	35,495 (93)	5,129,507 (96)	<0.01
	Yes	171,897 (3)	1,804 (5)	170,093 (3)	
Diabetes	No	4,483,727 (83)	31,695 (83)	4,452,032 (83)	<0.01
	Yes	792,417 (15)	5,176 (14)	787,241 (15)	
Fibromyalgia	No	5,369,607 (99)	37,518 (98)	5,332,089 (99)	<0.01
	Yes	12,622 (0.2)	303 (0.8)	12,319 (0.2)	
GI disease	No	4,545,038 (84)	25,254 (66)	4,519,784 (84)	<0.01
	Yes	634,473 (12)	7,943 (21)	626,530 (12)	
HIV Infection	No	5,375,047 (99)	38,081 (99)	5,336,966 (99)	<0.01
	Yes	17,696 (0.3)	178 (0.5)	17,518 (0.3)	
Mental disorder	None	4,039,287 (75)	11,207 (29)	4,028,080 (75)	<0.01
	PTSD[2]	298,893 (6)	5,146 (13)	293,747 (5)	
	Other[3]	729,498 (14)	10,298 (27)	719,200 (13)	
Renal disorder	No	5,251,903 (97)	36,885 (96)	5,215,018 (97)	<0.01
	Yes	92,990 (2)	763 (2)	92,227 (2)	

Note: All numbers are presented as counts and percentages unless otherwise indicated
[1]Cells may not add up to total for column due to missing values; [2]Chi-square test for differences in proportions for categorical variables or Student's t-test for differences in means for continuous variables; [3]Any diagnosis of PTSD, does not exclude other pre-existing mental health diagnoses; [4]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders). Abbreviations: BMI: body mass index; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; HIV, human immunodeficiency virus; PTSD, post-traumatic stress disorder

Table 4.5. Characteristics of United States veterans with and without a primary OTHER SLEEP DISORDER diagnosis who sought care in the Veterans Health Administration (VHA) in FY2010

Characteristic	**Classification**	**Total Population (N = 5,387,885[1]**	**Other Sleep Disorders (N = 31,674)[1]**	**No Other Sleep Disorders (N = 5,356,211)[1]**	**p-value[2]**
Age (years)	Mean (SD)	63 (16)	57 (15)	63 (16)	<0.01
Sex		**N (%)**	**N (%)**	**N (%)**	
	Male	5,028,180 (93)	29,337 (93)	4,998,843 (93)	<0.01
	Female	359,705 (7)	2,337 (7)	357,368 (7)	
Marital Status	Not Married	2,167,417 (40)	12,605 (40)	2,154,812 (40)	<0.01
	Married	2,958,404 (55)	17,988 (57)	2,940,416 (55)	
	Unknown	262,064 (5)	1,081 (3)	260,983 (5)	
Annual Income	<$20,000	2,958,211 (5)	17,794 (56)	2,940,417 (55)	<0.01
	≥$20,000	2,101,511 (39)	13,847 (44)	2,087,664 (39)	
	Unknown	328,163 (6)	33 (0.1)	328,130 (6)	
BMI (kg/m^2)	Normal	953,480 (18)	4,323 (14)	949,157 (18)	<0.01
	Overweight	1,685,317 (31)	9,996 (32)	1,675,321 (31)	
	Obese	1,647,666 (31)	16,785 (53)	1,630,881 (30)	
	Unknown	1,101,422 (20)	570 (2)	1,100,852 (21)	
Tobacco Use	Never	897,234 (17)	7,524 (23)	889,710 (17)	<0.01
	Former	1,220,969 (23)	9,254 (29)	1,211,715 (23)	
	Current	934,175 (17)	7,965 (25)	926,210 (17)	
	Unknown	2,335,507 (43)	6,931 (21)	2,328,576 (43)	
Asthma	No	5,316,166 (99)	30,430 (96)	5,285,736 (99)	<0.01
	Yes	52,269 (1)	769 (2)	51,500 (1)	
Cancer	No	5,168,338 (96)	30,105 (95)	5,138,233 (96)	<0.01

	Yes	160,896 (3)	1,092 (3)	159,804 (3)	
CVD	No	3,333,044 (62)	13,326 (42)	3,319,718 (62)	<0.01
	Yes	1,776,691 (33)	14,629 (46)	1,762,062 (33)	
COPD	No	5,158,291 (96)	28,784 (91)	5,129,507 (96)	<0.01
	Yes	171,953 (3)	1,860 (6)	170,093 (3)	
Diabetes	No	4,475,845 (83)	23,813 (75)	4,452,032 (83)	<0.01
	Yes	793,626 (15)	6,385 (20)	787,241 (15)	
Fibromyalgia	No	5,363,084 (99)	30,995 (98)	5,332,089 (99)	<0.01
	Yes	12,619 (0.2)	300 (1)	12,319 (0.2)	
GI disease	No	4,540,794 (84)	21,010 (66)	4,519,784 (84)	<0.01
	Yes	633,798 (12)	7,268 (23)	626,530 (12)	
HIV Infection	No	5,368,505 (99)	31,539 (99)	5,336,966 (99)	0.10
	Yes	17,643 (0.3)	125 (0.4)	17,518 (0.3)	
Mental disorder	None	4,040,261 (75)	12,181 (38)	4,028,080 (75)	<0.01
	PTSD[2]	298,571 (6)	4,824 (15)	293,747 (5)	
	Other[3]	727,050 (13)	7,850 (25)	719,200 (13)	
Renal disorder	No	5,245,383 (97)	30,365 (96)	5,215,018 (97)	<0.01
	Yes	92,978 (2)	751 (2)	92,227 (2)	

Note: All numbers are presented as counts and percentages unless otherwise indicated
[1]Cells may not add up to total for column due to missing values; [2]Chi-square test for differences in proportions for categorical variables or Student's t-test for differences in means for continuous variables; [3]Any diagnosis of PTSD, does not exclude other pre-existing mental health diagnoses; [4]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders). Abbreviations: BMI: body mass index; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; HIV, human immunodeficiency virus; PTSD, post-traumatic stress disorder

Table 4.6. Crude and adjusted odds ratios for a sleep disorder diagnosis in FY2010 among United States veterans who sought care in the Veterans Health Administration (VHA).

Characteristic	Classification	Crude	Adjusted[1]
		OR (95% CI)	OR (95% CI)
Age	Continuous years	0.98 (0.98, 0.98)	0.97 (0.97, 0.97)
Sex	Female	ref	ref
	Male	1.06 (1.04, 1.09)	1.29 (1.25, 1.33)
Marital Status	Not Married	ref	ref
	Married	1.06 (1.05, 1.08)	1.26 (1.24, 1.28)
Annual Income	<$20,000	ref	ref
	≥$20,000	1.11 (1.10, 1.12)	0.95 (0.94, 0.97)
BMI (kg/m^2)	Normal	ref	ref
	Overweight	1.39 (1.36, 1.42)	1.36 (1.32, 1.39)
	Obese	2.86 (2.81, 2.91)	2.41 (2.35, 2.47)
Tobacco Use	Never	ref	ref
	Former	0.90 (0.89, 0.92)	0.91 (0.89, 0.93)
	Current	0.95 (0.94, 0.97)	0.74 (0.73, 0.76)
Asthma	No	ref	ref
	Yes	2.46 (2.39, 2.55)	1.66 (1.59, 1.74)
Cancer	No	ref	ref
	Yes	1.08 (1.04, 1.09)	1.06 (1.02, 1.10)
CVD	No	ref	ref
	Yes	2.12 (2.09, 2.14)	1.71 (1.68, 1.74)
COPD	No	ref	ref
	Yes	1.85 (1.80, 1.89)	1.67 (1.62, 1.72)
Diabetes	No	ref	ref
	Yes	1.56 (1.54, 1.58)	1.06 (1.04, 1.08)
Fibromyalgia	No	ref	ref
	Yes	3.16 (2.96, 3.38)	1.69 (1.56, 1.83)
GI disease	No	ref	ref
	Yes	2.23 (2.20, 2.26)	1.46 (1.44, 1.49)
Mental disorder	None	ref	ref
	PTSD[2]	4.14 (4.07, 4.21)	2.56 (2.51, 2.62)
	Other[3]	2.99 (2.95, 3.03)	2.16 (2.12, 2.20)
Renal disorder	No	ref	ref
	Yes	1.43 (1.38, 1.48)	1.24 (1.19, 1.30)

[1] Each Odds ratio is adjusted for all other variables; [2]Any diagnosis of PTSD, but may have other mental health disorders; [3]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, mood disorders); ORs for unknown categories were not reported for marital status (n = 265,619), income (n = 328,267), BMI (n = 1,103,374), or tobacco use (n = 2,361,066). Abbreviations: BMI: body mass index; OR, odds ratio; CI, confidence interval; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; PTSD, post-traumatic stress disorder

Table 4.7. Crude and adjusted predictors of primary SLEEP APNEA (Cases) relative to subjects without sleep apnea, United States Veterans Health Administration (VHA) patients, FY2010

Characteristic	Classification	Crude	Adjusted[1]
		OR (95% CI)	OR (95% CI)
Age	Continuous years	0.98 (0.98, 0.98)	0.97 (0.97, 0.97)
Sex	Female	ref	ref
	Male	1.53 (1.48, 1.59)	1.69 (1.62, 1.77)
Marital Status	Not Married	ref	ref
	Married	1.34 (1.32, 1.36)	1.43 (1.40, 1.46)
Annual Income	<$20,000	ref	ref
	≥$20,000	1.23 (1.21, 1.25)	0.99 (0.97, 1.01)
BMI (kg/m^2)	Normal	ref	ref
	Overweight	2.62 (2.53, 2.73)	2.28 (2.17, 2.39)
	Obese	8.81 (8.51, 9.13)	6.13 (5.86, 6.41)
Tobacco Use	Never	ref	ref
	Former	0.92 (0.90, 0.94)	0.93 (0.90, 0.95)
	Current	0.77 (0.76, 0.79)	0.69 (0.67, 0.71)
Asthma	No	ref	ref
	Yes	2.79 (2.66, 2.92)	1.76 (1.66, 1.86)
CVD	No	ref	ref
	Yes	2.61 (2.57, 2.65)	1.86 (1.82, 1.90)
COPD	No	ref	ref
	Yes	1.98 (1.91, 2.04)	1.95 (1.88, 2.03)
Diabetes	No	ref	ref
	Yes	1.98 (1.95, 2.02)	1.14 (1.12, 1.17)
Fibromyalgia	No	ref	ref
	Yes	2.73 (2.48, 3.00)	1.58 (1.41, 1.78)
GI disease	No	ref	ref
	Yes	2.14 (2.10, 2.19)	1.42 (1.38, 1.45)
HIV Infection	No	ref	ref
	Yes	0.62 (0.52, 0.73)	0.70 (0.58, 0.86)
Mental disorder	None	ref	ref
	PTSD[2]	3.35 (3.28, 3.44)	2.00 (1.94, 2.05)
	Other[3]	2.23 (2.19, 2.27)	1.67 (1.63, 1.71)
Renal disorder	No	ref	ref
	Yes	1.59 (1.52, 1.67)	1.30 (1.23, 1.37)

[1] Estimates are mutually adjusted for all other variables; [2]Any diagnosis of PTSD, but may have other mental health disorders; [3]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral

disorders, and mood disorders); ORs for unknown categories were not reported for marital status (n = 265,619), income (n = 328,267), BMI (n = 1,103,374), and tobacco use (n = 2,361,066). Abbreviations: BMI: body mass index; OR, odds ratio; CI, confidence interval; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; HIV, human immunodeficiency virus; PTSD, post-traumatic stress disorder

Table 4.8. Crude and adjusted predictors of new INSOMNIA (Cases) relative to subjects without insomnia, United States Veterans Health Administration (VHA) patients, FY2010

Characteristic	Classification	Crude	Adjusted[1]
		OR (95% CI)	OR (95% CI)
Age	Continuous years	0.98 (0.98, 0.98)	0.98 (0.98, 0.98)
Annual Income	<$20,000	ref	ref
	≥$20,000	0.92 (0.91, 0.94)	0.87 (0.84, 0.89)
BMI (kg/m^2)	Normal	ref	ref
	Overweight	1.00 (0.98, 1.03)	1.03 (0.99, 1.07)
	Obese	0.99 (0.97, 1.02)	0.90 (0.87, 0.94)
Tobacco Use	Never	ref	ref
	Former	0.85 (0.82, 0.87)	0.87 (0.84, 0.90)
	Current	1.21 (1.17, 1.25)	0.77 (0.74, 0.80)
Asthma	No	ref	ref
	Yes	1.84 (1.71, 1.99)	1.27 (1.15, 1.40)
Cancer	No	ref	ref
	Yes	1.11 (1.05, 1.17)	1.16 (1.09, 1.25)
CVD	No	ref	ref
	Yes	1.49 (1.46, 1.52)	1.46 (1.41, 1.51)
COPD	No	ref	ref
	Yes	1.53 (1.46, 1.61)	1.24 (1.17, 1.31)
Diabetes	No	ref	ref
	Yes	0.92 (0.90, 0.95)	0.84 (0.81, 0.88)
Fibromyalgia	No	ref	ref
	Yes	3.50 (3.12, 3.92)	1.63 (1.42, 1.87)
GI disease	No	ref	ref
	Yes	2.27 (2.21, 2.33)	1.50 (1.45, 1.55)
Mental disorder	None	ref	ref
	PTSD[2]	6.30 (6.09, 6.51)	4.20 (4.03, 4.37)
	Other[3]	5.15 (5.01, 5.29)	3.52 (3.40, 3.64)
Renal disorder	No	Ref	ref
	Yes	1.17 (1.09, 1.28)	1.23 (1.13, 1.34)

[1] Estimates are mutually adjusted for all other variables; [2]Any diagnosis of PTSD, but may have other mental health disorders; [3]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders); ORs for unknown categories were not reported for marital status (n = 265,619), income (n = 328,267), BMI (n = 1,103,374), and tobacco use (n = 2,361,066). Abbreviations: BMI: body mass index; OR, odds ratio; CI, confidence interval; CVD, cardiovascular disease;

COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; PTSD, post-traumatic stress disorder

Table 4.9. Crude and adjusted predictors of new OTHER SLEEP DISORDER (Cases) relative to subjects without other sleep disorders, United States Veterans Health Administration (VHA) patients, FY2010

Characteristic	Classification	Crude	Adjusted[1]
		OR (95% CI)	OR (95% CI)
Age	Continuous years	0.98 (0.98, 0.98)	0.98 (0.98, 0.98)
Marital Status	Not Married	ref	ref
	Married	1.05 (1.02, 1.07)	1.27 (1.23, 1.31)
Annual Income	<$20,000	ref	ref
	≥$20,000	1.10 (1.07, 1.12)	0.94 (0.91, 0.97)
BMI (kg/m^2)	Normal	ref	ref
	Overweight	1.31 (1.26, 1.36)	1.31 (1.25, 1.38)
	Obese	2.26 (2.19, 2.34)	1.95 (1.86, 2.04)
Tobacco Use	Never	ref	ref
	Former	0.90 (0.88, 0.93)	0.92 (0.88, 0.95)
	Current	1.02 (0.99, 1.05)	0.76 (0.73, 0.80)
Asthma	No	ref	ref
	Yes	2.59 (2.41, 2.79)	1.66 (1.53, 1.81)
Cancer	No	ref	ref
	Yes	1.17 (1.10, 1.24)	1.11 (1.03, 1.19)
CVD	No	ref	ref
	Yes	2.07 (2.02, 2.12)	1.65 (1.60, 1.71)
COPD	No	ref	ref
	Yes	1.95 (1.86, 2.04)	1.61 (1.52, 1.71)
Diabetes	No	ref	ref
	Yes	1.52 (1.48, 1.56)	1.05 (1.01, 1.09)
Fibromyalgia	No	ref	ref
	Yes	4.19 (3.74, 4.70)	1.89 (1.65, 2.17)
GI disease	No	ref	ref
	Yes	2.50 (2.43, 2.56)	1.54 (1.49, 1.60)
Mental disorder	None	ref	ref
	PTSD[2]	5.43 (5.25, 5.62)	3.16 (3.04, 3.29)
	Other[3]	3.61 (3.51, 3.71)	2.44 (2.36, 2.53)
Renal disorder	No	ref	ref
	Yes	1.40 (1.30, 1.50)	1.19 (1.09, 1.29)

[1] Estimates are mutually adjusted for all other variables; [2]Any diagnosis of PTSD, but may have other mental health disorders; [3]Any mental health diagnosis other than PTSD (e.g., anxiety disorders, emotional and behavioral disorders, and mood disorders); ORs for unknown categories were not reported for marital status (n = 265,619), income (n = 328,267), BMI (n = 1,103,374), and tobacco use (n = 2,361,066). Abbreviations: BMI: body mass

index; OR, odds ratio; CI, confidence interval; CVD, cardiovascular disease; COPD, chronic obstructive pulmonary disease; GI, gastrointestinal; PTSD, post-traumatic stress disorder

Chapter 5

Case-Control Study of Candidate Gene Methylation and Adenomatous Polyp Formation[3]

[3]Alexander M, Burch JB, Steck SE, Chen C-F, Hurley TG, Cavicchia P, Shivappa N, Guess J, Zhang H, Youngstedt SD, Creek KE, Lloyd S, Jones K, Hébert JR. Submitted to *International Journal of Colorectal Diseases*.

5.1 Abstract

Introduction: Colorectal cancer (CRC) is one of the most common and preventable forms of cancer, yet it remains the second leading cause of cancer-related death. Colorectal adenomatous polyps (adenomas) are precursor lesions that develop in 70-90% of all CRC cases. Identification of peripheral biomarkers for adenomas would help to enhance efficiency of screening efforts. This exploratory study examined the methylation status of 20 candidate markers in peripheral blood leukocytes to determine whether epigenetic modification of these genes was associated with adenoma case status. **Materials and Methods**: Patients recruited from a local endoscopy clinic provided informed consent, and completed an interview to ascertain demographic, lifestyle, and adenoma risk factors. Cases were defined as individuals with a histopathologically confirmed adenoma, and controls included patients with a normal colonoscopy, or those with histopathological findings not requiring heightened surveillance (normal biopsy, hyperplastic polyp). Methylation-specific polymerase chain reaction was used to characterize candidate gene promoter methylation. Odds ratios and 95% confidence intervals (OR, 95% CI) were calculated using unconditional multivariable logistic regression to test the hypothesis that candidate gene methylation differed between cases and controls, after adjustment for potential confounding factors. **Results**: Complete data were available for 107 participants; 36% had adenomas (men: 40%, women: 31%). Hypomethylation of the *MINT1* locus (OR: 5.3, 95% CI: 1.0-28.2), and the *PER1* (OR: 2.9, 95% CI: 1.1-7.7) and *PER3* (OR: 11.6, 95% CI: 1.6-78.5) clock gene

promoters was more common among adenoma cases. While specificity was moderate to high for the three markers (71-97%), sensitivity was relatively low (18-45%). **Discussion**: Follow-up of these epigenetic markers is suggested to further evaluate their utility for adenoma screening or surveillance.

5.2 Introduction

Despite recent decreases in colorectal cancer (CRC) incidence and associated mortality, CRC remains one of the most common and deadly forms of cancer in the United States, with a lifetime risk for diagnosis of 5%.[1] Colonoscopies not only present an opportunity screen for CRC, but to also remove colorectal adenomas (precursor lesion for CRC), thus facilitating primary CRC prevention. However, in 2012, the Centers for Disease Control and Prevention reported that CRC screening rates among eligible men and women fell far short of the *Healthy People 2020* target of 70.5%.[123] Only ~25-60% of eligible adults undergo CRC screening due to socioeconomic, racial, geographic, or other barriers that contribute to a lack of compliance,[229-232] highlighting a need for accessible screening methods.

Stool-based tests (fecal occult blood test [FOBT] or the fecal immunochemical test [FIT]) have been developed as a less invasive alternative to colonoscopies. Despite high sensitivity for CRC, this screening modality has reduced adenoma detection sensitivity (FOBT: 7-23%; FIT: 13-26%) compared to a colonoscopy, which has the ability to detect 73-94% of adenomas.[233-236] A recent meta-analysis of stool-based DNA, another alternative CRC screening

strategy, found that these tests may provide a diagnostic benefit for CRC or advanced adenoma detection in high-risk, but not average-risk, populations,[237] and is a cost-prohibitive screening tool.[238] Interestingly, methylation markers provided better diagnostic performance for adenoma detection than did mutations (67% sensitivity vs. 10% sensitivity, respectively),[237] FOBT, or FIT (≤26%).[235, 236] Because blood-based measures can increase compliance with CRC screening and surveillance, genetic and epigenetic biomarkers in circulation are under active investigation.[239, 240]

The polyp→carcinoma sequence within the colon has been widely studied, and both genetic[87] and epigenetic[241] alterations have been observed at different stages of CRC development. An extensive literature describes the genetic lesions involved in this process, and an estimated 30% of CRC cases can be attributed to heritable factors.[242] However, highly penetrant genetic markers account for only ~5% of CRC cases.[242] Epigenetic modification represents an important and early event in the adenoma→CRC sequence. Methylation within gene promoter regions can lead to gene silencing and reduced gene expression.[241, 243] Promoter methylation of genes responsible for tumor suppression, DNA repair, or other critical pathways involved in the carcinogenesis process may reduce their expression and thereby promote tumor development. Aberrant methylation of several CRC-related loci (e.g., *MLH1*, CDKN2A [*p16*], *MINT1*, *MINT2*, *MINT31*) corresponds to a specific CRC subtype, the CpG island methylator phenotype (CIMP), [132] which occurs in 0-44% of adenomas,[133, 134] and up to 50% of CRCs.[244] However, associations between

aberrant gene methylation and cancer have not always been consistent, possibly due to differences in the various molecular pathways leading to CRC development.[245] For example, *MINT1*, *MINT2,* and *MINT12* are putative tumor suppressors, but were less methylated in adenomas from individuals with multiple polyps relative to those with at least one polyp and high microsatellite instability (MSI-H) in their adenomas.[245] Also, hypermethylation of *MINT31* has been associated with increased CRC risk,[246] but also has been associated with longer disease-free survival among CRC patients.[247] These findings highlight the need for more research on the relationship between promoter gene methylation and adenoma development or progression.

Few studies have examined epigenetic markers in peripheral blood leukocytes (PBLs) for early detection of adenomas or CRC. Global hypomethylation of retrotransposable (LINE-1) elements in PBLs has been associated with increased odds of adenoma formation in some studies,[248-251] and other studies targeting candidate genes also have had some success identifying patients with adenomas or CRC, although the results among these investigations have been mixed.[240, 252-256] Epigenetic changes in PBLs may reflect those observed in target tissues[257] or may serve as biologically relevant indicators of behavioral, environmental, psychosocial, or lifestyle factors that contribute to adenoma formation.[241, 258]

This exploratory case-control study tested the hypothesis that adenoma case status was associated with aberrant methylation of candidate gene promoters in PBL DNA relative to control colonoscopy patients. A panel of twenty

candidate gene markers was selected for evaluation following a literature review based on their potential role in adenoma formation or CRC risk: *APC*, *BRCA1*, *CDKN2AP16*, *CYP24A*, *CYP27B1*, *ER-alpha*, *IGF2*, *MGMT*, *MINT1*, *MLH1*, *NGFR*, *PER1*, *PER2*, *PER3*, *SEPT9*, *SFRP4*, *SFRP5*, *TIMP3*, *TMEFF2*, and *WIF1*.[105, 240, 241, 259-264]

5.3 Materials and Methods

The Epigenetics and Diet in the Carcinogenesis Process (EDCaP) study was performed among patients undergoing a colonoscopy procedure at a community endoscopy center in Columbia, SC.[265] Prior to their clinic visit, 138 participants provided informed consent, including permission to access medical records, in accordance with the University of South Carolina's Institutional Review Board approval process. Participants completed a questionnaire to provide information on: demographic (sex, marital status, income, race/ethnicity), lifestyle (smoking history, diet, physical activity), and occupational (employment status, job industry, type of shift, history of shiftwork) factors, as well as personal and family medical history (ever being diagnosed with other diseases, personal cancer history, family CRC history).

A total of 256 subjects scheduled for a screening or surveillance colonoscopy were eligible to participate, and 154 were enrolled. Among those subjects, 138 had complete questionnaire data, and 107 of those participants provided biospecimens for methylation analysis (Figure 5.1). Information on cecal intubation, visualization of the appendix, and bowel preparation were

abstracted from the patient's medical record to evaluate colonoscopy quality. Cases were defined as individuals with a complete colonoscopy procedure and at least one histologically confirmed adenoma based on review by a consulting clinical pathology laboratory. Controls included patients with a complete procedure and normal findings, or histopathological findings not requiring heightened surveillance (normal biopsy, hyperplastic polyp).[266]

Biological sample collection and laboratory assays

An on-site nurse drew 40cc of blood from each participant for DNA and RNA extraction. DNA samples obtained from blood were aliquoted and archived for future studies in genetic and epigenetic factors in colorectal polyp formation. Normal gastrointestinal (GI) tissue and a polyp samples (if available) were also extracted during the colonoscopy procedure. All biological samples were stored at -80^{o}C after collection.

Methylation assays

A peripheral whole blood sample was collected from each participant using vacutainers containing EDTA anticoagulant. Genomic DNA was isolated using the PUREGENE genomic DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol from peripheral whole blood, normal GI tissue, and polyp tissue. Sodium bisulfite conversion of DNA was performed using EpiTect kits (QIAGEN, Germantown, MD, USA). Briefly, DNA (2 µg) was added to the reaction buffer and bisulfite DNA conversion was

conducted in a Bio-Rad S1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the manufacturer's suggested program for the conversion reaction. Converted DNA was eluted from a spin column in TE buffer and either used immediately or stored at –20°C. DNA oligonucleotides for primer pairs were purchased from Integrated DNA Technologies (Coralville, IA, USA, Table 5.1). To ensure that the polymerase chain reaction (PCR) primers were specific for the detection of methylated DNA, a control human DNA set (EpiTect Control DNA, QIAGEN Sciences, Germantown, MD) containing positive (bisulfite converted, 100% methylated) and negative (bisulfite converted, 0% methylated) control DNA was used. To quantify methylation specific PCR (MSP) results, the QIAxcel system was used (QIAGEN Sciences, Germantown, MD), which utilizes a multiplexed fluorescence detection method with a resolution of 3-5 bp for DNA fragments. The detection sensitivity of QIAxcel system was 0.1 ng/µl DNA in undiluted amplification reactions. The QIAxcel system was equipped with the BioCalculator software that produces a tabular display of a variety of peak properties, including number of peaks as well as the height, width, and area of each peak. The height of the peak was used for separation of the results into three methylation categories: high (peak height >0.30), medium (peak height 0.29-0.05), and low (peak height <0.05), or none (no methylation detected). Results were categorized as either unmethylated or methylated (combination of medium/low, and high methylation categories). The methylation status of each sample was normalized via a two-step process. First, a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to

measure concentrations of the DNA templates. Subsequent experiments used the same amount of DNA template for each MSP reaction. To facilitate quantification, equal volumes of the MSP products were loaded on the QIAxcel system for analysis. Second, BRCA1 gene methylation was used as an internal standard since the same amount of DNA template had extremely high methylation in each sample. The measured peak height of BRCA1 methylation was then used to normalize the methylation of the genes of interest for each sample. All methylation assays were performed blinded to case status.

Real-time quantitative PCR (qPCR)

RNA was extracted from PAXgene tubes using PAXgene Blood RNA Kit from QIAGEN (Germantown, MD, USA). RNA quality was checked via Agilent Bioanalyzer 2100 and concentration was quantified on NanoDrop. cDNA was synthesized from total RNA using gene-specific primers according to the Verso cDNA Kit (Abgene, Rochester, New York). Reverse transcriptase reactions contained 1 µg of total RNA (1-5 µl of total RNA), 4 µl 5X cDNA synthesis buffer, 1 µl 5 µM dNTP mix, 1 µl 400 ng/µl random hexamers, 1 µl RT Enhancer, 1 µl Verso Enzyme Mix, and a variable amount of PCR grade water to have a total of a 20 µl. The 20 µl reactions were incubated in an MJ Research PTC-225 Thermo Cycler in a 96-well plate for 30 min at 42°C, 2 min at 95°C, and then held at 4°C. cDNAs were stored at -20 ^{o}C.

Gene-specific primers were created using Primer3 software. Forward and reverse sequences were confirmed for their presence within exons and lack of

single nucleotide polypmorphisms using NCBI's Basic Local Alignment Search Tool (BLAST). PCR cycling information is found in Table 5.2. All reactions were performed in singleplex mode. Each reaction contained 10 µl 2x SYBR Green PCR master mix (Bio-Rad Laboratories, Hercules, CA), 2 µl of each primer, 0.5-2 µl of cDNA template, and a variable amount of PCR grade water to have a total reaction amount of 20 µl. The standard curve method was employed and β-actin was used as the endogenous control to determine levels of expression of cDNA in study participants. A two-fold 10-point serial dilution was created through the use of diluted PCR products of *β-actin* and markers who presented an association with polyp status. The standard curve produces a linear relationship between number of threshold cycles (Ct) and the initial known amounts of cDNA, allowing the determination of the cDNA concentration of unknown samples based on their Ct value, thereby also giving the number of mRNA copies for each gene present in unknown samples. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Each sample was analyzed in duplicate, with a negative template control included in each run to check for DNA contamination.

Statistical analysis

Statistical analyses were performed using SAS® version 9.2 (Cary, NC). Frequency distributions of study variables by case status were examined using the chi-squared test for differences between proportions. Student's t-test was used for comparisons among cases and controls for normally distributed

continuous variables. In instances of non-normally distributed continuous variables, the Wilcoxon–Mann–Whitney test was used to compare cases and controls. Unconditional multiple logistic regression was used to estimate odds ratios (OR) with 95% confidence intervals (CIs), comparing candidate gene methylation among adenoma cases relative to controls, after adjustment for the effects of potential confounding factors. Variables considered for inclusion in adjusted models were known or suspected adenoma or CRC risk factors (age, sex, race, body mass index [BMI], CRC family history, smoking history, work-related factors, diet, vitamin and supplement use, physical activity, sociodemographic characteristics [income, education], personal history of inflammatory bowel disease), and variables that differed among participants and non-participants (fruit and vegetable intake). Final models included variables that were statistically significant ($p \leq 0.05$) in the saturated model, or whose inclusion produced ≥10% change in the parameter estimate for the methylation marker of interest. Each candidate gene was selected *a priori*, thus no correction for multiple testing was performed.[267] Due to sparse data, individuals who received a colonoscopy due to symptoms (presence of gastrointestinal bleeding, fecal occult blood, iron deficiency, or constipation, n=3) were grouped with patients undergoing a screening colonoscopy. Sensitivity analyses were conducted to determine whether associations were driven by data from people with a right-sided polyp, as right-sided hyperplastic polyps have been linked with the serrated adenoma, MSI-H CRC pathway.[268] Sensitivity and specificity, and positive and negative predictive values (PPV, NPV, respectively) were calculated for

methylation markers with a statistically significant association with adenoma case status.

To evaluate agreement of DNA methylation levels found in PBLs, normal colon tissue, and polyp tissue, the Kappa agreement statistic was used, which takes on a value of 0 to 1, ranging to no agreement to complete agreement. Also, linear regression was used to assess how much variation in gene expression was attributable to methylation status.

Regarding gene expression and adenoma formation, unadjusted logistic regression was also used to examine the crude association between expression levels in PBLs and adenoma formation. To detect the true association between expression levels in PBLs of these genes and adenoma formation, multiple logistic regression techniques were used and confounders were included into the model. Potential confounders were screened for in a manner similar to the protocol for determining the relationship between methylation levels and adenoma formation. Spearman correlations were used to assess the relationship between gene expression levels across PBLs, normal tissue, and polyp tissue, where values range from -1 to +1, where a negative sign indicates an inverse relationship, and a positive sign signifies a positive relationship. All associations were considered statistically significant at the $p \leq 0.05$ level.

5.4 Results

Complete data were obtained from 107 of the 154 patients (70%) who consented to participate (Figure 5.1). The mean age among participants was 60 years old (standard deviation [SD]: ±9 years). European Americans (EAs) comprised the majority of subjects (73%), and 46% of the population was female (Table 5.3). With regard to colonoscopy quality, most participants (96%) had a bowel preparation that was rated as fair or better, and the cecal intubation rate was 100%. No cases of advanced adenoma (>10 mm in size in any dimension, high-grade dysplasia, villous histology), serrated adenomatous polyps, or CRC were identified. The detection rate for any polyp was 65%, and the adenoma detection rate was 36% (male: 40%, female: 31%), with a mean of 0.7 (SD: ±0.5) adenomas per person. All procedures with a right-sided hyperplastic polyp (n=7) were synchronous with adenoma detection in the same patient. Cases were more likely to be smokers compared to controls (68% vs. 45%, p=0.02), and were less likely to ever regularly take vitamin D supplements (5% vs. 29%, p <0.01). Though not statistically significant, cases also had a tendency to be ≥65 years of age (p=0.07), married (p=0.16), to currently drink alcohol (p=0.13), or to ever regularly take a multivitamin (p=0.07), or vitamin C (p=0.15) compared to controls.

Effect of methylation in PBLs on adenoma formation

Figures 5.2 and 5.3 present agarose gel images of MSP products for *PER1, PER2, PER3* and *MINT1*, respectively, in a subsample of participants.

After adjustment for potential confounding factors, hypomethylation (i.e., no methylation detected) in the *MINT1* (methylated in tumor1; OR: 5.3, 95% CI: 1.0-28.2) locus, and the *PER1* (Period 1; OR: 2.9, 95% CI: 1.1-7.7), and *PER3* (Period 3; OR: 11.1, 95% CI: 1.6-78.5) clock gene promoters was associated with an increased odds of adenoma detection (Table 5.4). When seven subjects with right-sided hyperplastic polyps were excluded, sensitivity analyses yielded the following results for hypomethylation of *MINT1* (OR: 5.0, 95% CI: 0.9-27.9), *PER1* (OR: 2.8, 95% CI: 1.1-7.5) and *PER3* (OR: 9.8, 95% CI: 1.4-70.6, data not shown). The time of day of sample collection or working at least one year of shift work did not modify the relationship between clock gene (*PER1, PER3*) promoter methylation and adenoma case status (data not shown).

The sensitivity, specificity, PPV, and NPV for hypomethylation of *MINT1*, *PER1*, *PER3*, and their combinations are presented in Table 5.5. *PER1*, *PER3*, or *MINT1* hypomethylation correctly identified 16% to 45% of individuals with adenomas. Among patients without adenomas, specificity ranged from 71% to 97%. When evaluated in combination, hypomethylation of these genes yielded sensitivities ranging from 53% to 74%, and specificities ranging from 9% to 36% (Table 5.5). Among the possible combinations, *PER3*/*MINT1* yielded the highest sensitivity (74%), and the *PER1*/*PER3*/*MINT1* combination had the highest specificity (36%).

Concordance of methylation in PBLs, normal GI tissue, and polyp tissue

MINT1, *PER1*, and *PER3* did not exhibit significant agreement within PBLs (Table 5.6). No significant concordances were observed when comparing PBLs with polyp tissue and normal tissue with polyp tissue.

Effect of methylation on mRNA expression in PBLs, normal GI tissue, and polyp tissue

Table 5.7 presents information on *PER1* and *PER3* mRNA expression by their respective methylation status. Although *PER1* expression in normal GI tissue was lower in methylated samples compared to hypomethylated samples (1.04 fold change in PER1 expression vs 1.01 fold change in PER1 expression), this finding was only marginally statistically significant (p-value: 0.06). *PER1* methylation in normal GI tissue also explained the largest amount of variance (approximately 26%) across the three tissue types and among the three markers. No significant differences in expression by methylation status were found in PBLs, normal GI tissue, and polyp tissue.

Effect of mRNA expression in PBLs on adenoma formation

Fold change in expression for *PER1* and *PER3* by case status are found in Table 5.8. Adjusted analyses show that for a one-unit increase in expression in *PER1* and *PER3*, there was reduction in the odds of having an adenoma; however, these findings were not statistically significant.

Correlation of mRNA expression in PBLs, normal GI tissue, and polyp tissue

Table 5.9 presents Spearman correlations in expression levels across PBLs, normal GI tissue, and polyp tissue. Significant correlations were seen only between normal GI and polyp tissues, with correlation coefficients ranging from 0.62 – 0.93. No significant correlations within PBLs and between PBLs and any tissue were detected.

5.5 Discussion

Identification of novel genes associated with adenoma risk may lead to a better understanding of carcinogenesis pathways and subsequently improve CRC screening and prevention efforts. Several studies have targeted blood-based epigenetic biomarkers to identify CRC cases, and only a few have used this approach to evaluate the formation of colorectal adenomas, which serve as precursor lesions for 70-90% of CRC cases. This exploratory case-control study found that hypomethylation in the *MINT1* locus and the *PER1* and *PER3* clock gene promoters was more common among adenoma cases relative to controls. Additional analyses showed minimal agreement between PBLs and both normal tissues and polyp tissues, though few paired samples were available. However, strong, positive correlations were observed across normal tissues and polyp tissues, suggestive that genetic/epigenetic changes that occur during the carcinogenesis process can also be observed in normal tissue. Furthermore, promoter methylation did not fully explain the variation in expression of their respective gene, nor did gene-specific expression yield an association with case status.

The *MINT1* locus is one of several markers used to characterize CIMP status among CRC cases.[132, 269] Aberrant DNA methylation is considered an early event in the adenoma→carcinoma sequence,[241] and changes in CIMP markers have been identified in aberrant crypt foci and in colorectal adenomas.[134, 270-272] *MINT* methylation in adenomas or associated CRC tissue has been linked with down regulation of mismatch repair (MLH1) protein expression, microsatellite instability, and BRAF (V600E) mutation relative to normal tissue.[273] However, prevalence of the CIMP phenotype is variable in adenomas (0-44%)[133, 134] as well as normal gastrointestinal tissues.[274] To our knowledge, the *MINT1* locus has not been examined as a circulatory marker for adenoma risk and thus results obtained in the present study require confirmation.

Two of the three candidate genes associated with adenomas in this study (*PER1, PER3)* are members of the clock gene family. Clock gene expression is involved in the maintenance of endogenous circadian rhythms, as well as the regulation of cellular processes that are considered hallmarks of carcinogenesis (cell cycle control, DNA damage response, apoptosis).[275-277] In colon and other cancer cell lines, *PER1* insertion can activate cell cycle checkpoint proteins, sensitize cells to ionizing radiation-induced apoptosis, and exert an antiproliferative effect.[116] A hypomethylation zone in the *PER1* promoter has been associated with uncoupling of *PER1* transcription from promoter methylation in cervical cancer cells,[243] and may have played a role in the lack of association between methylation and expression in *PER1* in the present study. Structural or epigenetic variability in E-boxes, particularly the E-box closest to the

promoter, may play a role in these processes, and mutations within this element may lead to a reduction of *PER1* promoter activity.[243, 278] *PER1* and *PER2* mutations occur in human colorectal tumors,[18] and *PER* expression is reduced in colorectal adenomas or tumors relative to normal tissue.[104, 279-283] In mice, a mutation in the *PER2* gene (*PER2*$^{m/m}$) results in altered diurnal rhythms, deregulated expression of cell cycle control genes, tissue hyperplasias and tumors,[94, 284] and increased colon polyp formation.[285] Polymorphisms in the *PER3* gene have been associated with increased odds of adenoma formation,[283] and aberrant methylation of *PER* genes has been observed among leukemia and other cancer patients.[30, 33, 113, 114, 286] However, to the authors' knowledge, no studies have examined epigenetic modifications in the *PER* or other clock genes in relation to adenoma formation.[277, 283]

Clock genes facilitate the circadian expression of ~5-10% of the mammalian transcriptome, and clock-controlled genes include known tumor suppressors (e.g., *p21, Chk2, XPA, ATM*) and oncogenes (e.g., *β-catenin, c-Myc, WEE-1*).[276] Circadian patterns of clock-controlled gene expression occur via rhythmic epigenetic modification of histones and chromatin.[276, 277, 287] Histone modification may play a role in tumorigenesis,[288] although few studies have examined the role of circadian histone modifications in relation to adenoma or CRC development.[277] Animal model studies have suggested that gene regulation in the mouse liver is mediated by clock gene interactions with the histone methyltransferase, MLL3 (mixed lineage leukemia 3), a tumor suppressor that has been associated with CRC and other cancers.[287, 289] *PER* complexes recruit

histone-modifying proteins that help orchestrate the circadian expression of core clock genes, although it is not known whether this function extends to clock-controlled, cancer-related genes.[277, 290]

This study had several noteworthy strengths and limitations. Strengths included the valid ascertainment of adenoma cases and statistical adjustment for known and suspected adenoma risk factors. The sample size limited power and precluded examination of the relationship between methylation patterns and adenoma status within factors that may influence epigenetic processes and detecting differences in expression by both methylation and case status. Although each candidate gene was selected *a priori* following a literature review, this study was exploratory in nature and the potential for spurious or false positive findings cannot be eliminated. Also, methylation among specific white blood cell subsets was not quantified. Methylation patterns do not always vary among T-cell subsets, and the extent to which methylation differs among PBL subtypes for *PER1, PER3*, or *MINT1* is not known. This cellular heterogeneity could play a role in the poor agreement in methylation between PBLs and tissues within subject and lack of a relationship between methylation and expression found in the present study, phenomena that have been previously observed.[291-293] It is further supported by the strong correlations in expression between normal and polyp tissues, but not between PBLs and normal and/or polyp tissues. Nonetheless, a lack of standardization for these potential differences represents a study uncertainty.[258, 294, 295] The pattern of promoter hypomethylation that was observed for *PER1* and *PER3* in this study was not

consistent with that expected for a tumor suppressor. Other studies that targeted clock genes in PBLs indicated that DNA hypomethylation was associated with breast cancer diagnoses (*CLOCK*),[296] or with advanced stage (II-IV) breast cancer (*TIMELESS*),[297] whereas promoter hypermethylation was associated with postmenopausal breast cancer (*CRY2*),[296] and with chronic myeloid leukemia (*PER3*)[33] relative to controls. The mechanism through which *PER1 or PER3* gene methylation may impact adenoma formation is unknown and may depend on tissue context, timing of expression relative to other cellular processes, location of epigenetic changes within the gene, or other factors that may disrupt clock gene expression, such as shift work.[276, 277] Time of day of sample collection in the present study did not modify the relationship between *PER1* or *PER3* promoter methylation and adenoma case status (data not shown). However, more detailed genetic and epigenetic studies of the duration, phase and amplitude of *PER1* and *PER3* expression in conjunction with cancer-related, clock-controlled genes are suggested to help elucidate their role in adenoma formation. Methylation of non-target tissue (i.e., PBLs) can be influenced by psychosocial or environmental stressors, or lifestyle risk factors (e.g., diet, smoking). For example, cancer risk factors that can alter clock gene expression, such as shiftwork or sleep disturbances, also may exert epigenetic effects.[298-302] Global DNA hypomethylation in PBLs was recently observed among night workers relative to those working days, and among the clock genes examined, the largest differences in hypomethylation were observed within *PER3* loci.[300] In the present study, neither the proportion of current shiftworkers nor those who

engaged in at least one year of shiftwork (3% and 42% of the study population, respectively, Table 1) differed by adenoma case status, or by *MINT1, PER1*, or *PER3* methylation status (data not shown). Future studies would need to consider the role of these factors when examining the independent effect of promoter methylation status on health outcomes.

In summary, the identification of peripheral epigenetic markers that are associated with adenoma risk may lead to a deeper understanding of colorectal carcinogenesis and facilitate CRC prevention. Results from this study suggest the need for follow-up blood-based monitoring of *MINT1, PER1*, and *PER3* to examine their role in adenoma formation and CRC risk, along with an examination in the relationship between their corresponding normal and polyp tissues.

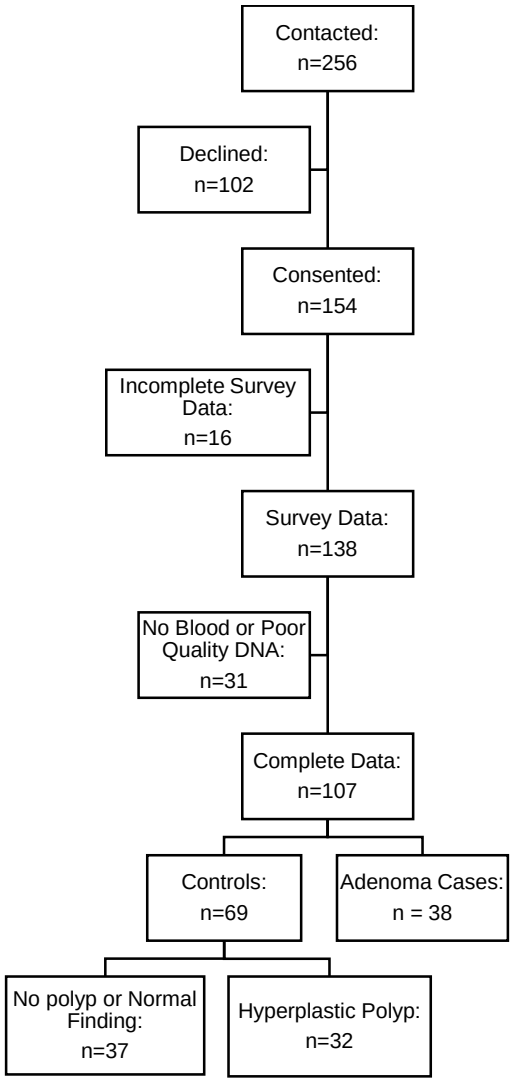

Figure 5.1. Flow chart of participant recruitment and participation in the Epigenetics and Diet in the Carcinogenesis Process (EDCaP) study.

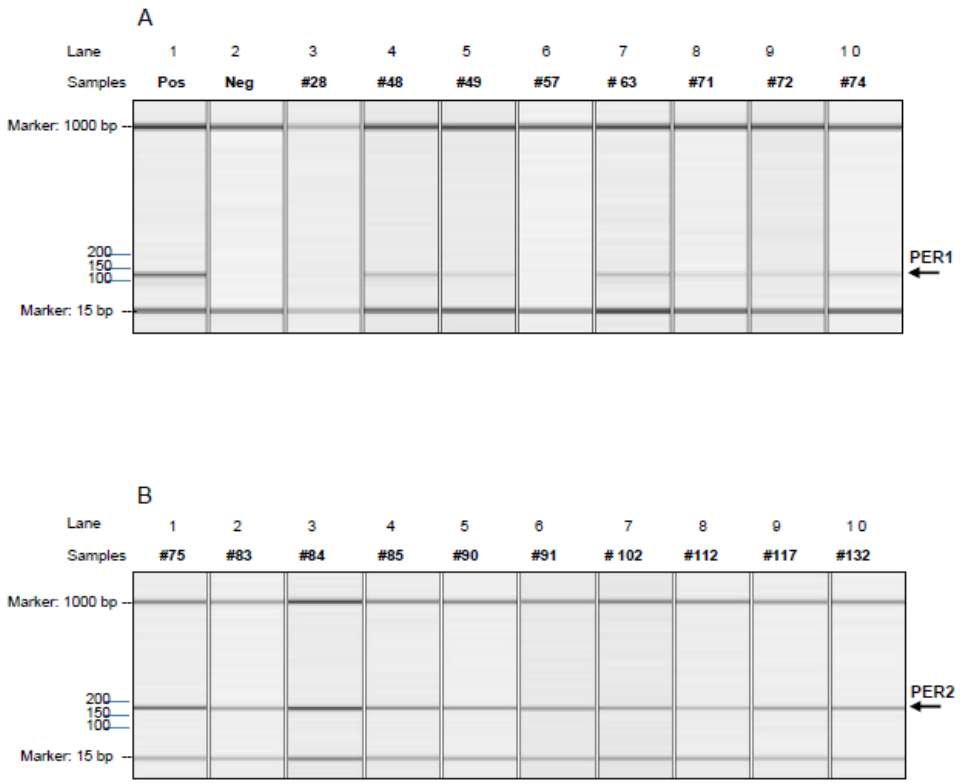

Figure 5.2. QIAxel gel analysis for the methylation-specific polymerase chain reaction (MSP) of PER1 (A) and PER2 (B). Black arrows indicate MSP products of PER1 and PER2. (A) Lanes 1 and 2 represent a positive control and negative control, respectively. Lanes 3 through 10 represent a subsample of participants and PER1 methylation. Subjects #28 and #48 exhibited no methylation; subjects #49, #63, #71, #72, and #74 exhibited low/medium methylation; and subject #48 exhibited high methylation. (B) Lanes 1 through 10 represent a subsample of participants and PER2 methylation. All subjects with the exception of subjects #75 and #84 exhibited low/medium methylation. Subjects #75 and #84 had high methylation.

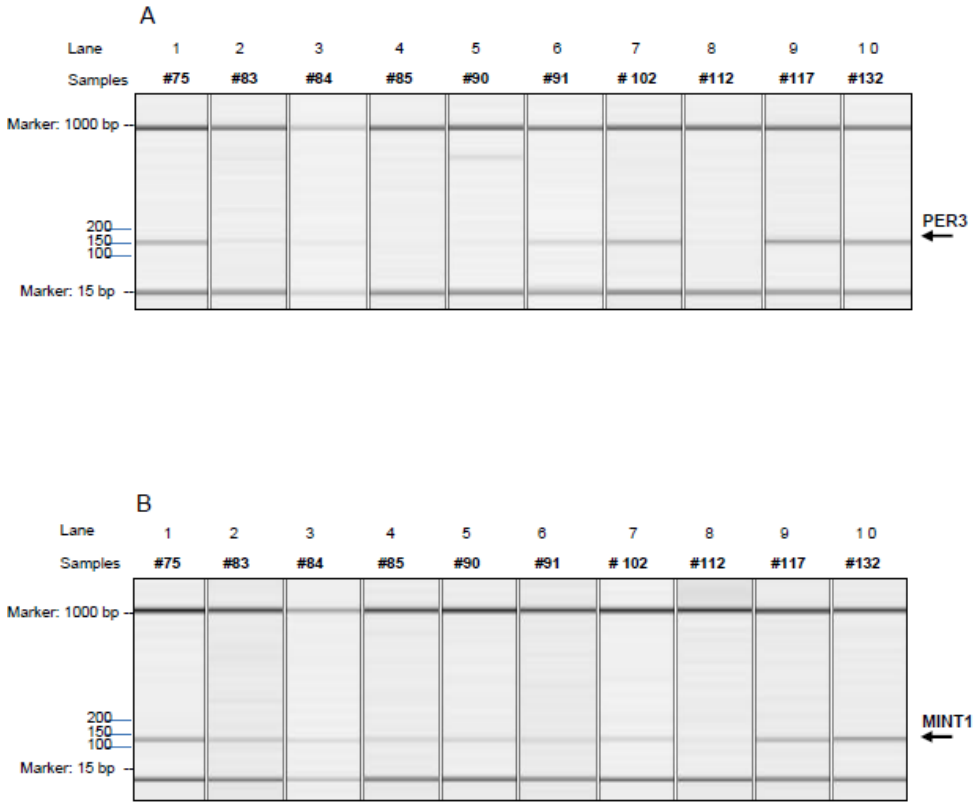

Figure 5.3. QIAxel gel analysis for the methylation-specific polymerase chain reaction (MSP) of PER3 (A) and MINT1 (B). Black arrows indicate MSP products of PER3 and MINT1. (A) Lanes 1 through 10 represent a subsample of participants and PER3 methylation. Subjects #85 and #112 exhibited no methylation; subjects #83, #84, #90, #91 exhibited low/medium methylation; and subjects #75, #102, #117, and #132 exhibited high methylation. (B) Lanes 1 through 10 represent a subsample of participants and MINT1 methylation. Subject #112 had no methylation; subjects #83, #84, #85, #90, #91, and #102 exhibited low/medium methylation; and subjects #75, #117, and #132 exhibited high methylation.

Table 5.1. Primers for Methylation-Specific PCR

Gene Name	Forward (5’ -> 3’)	Reverse (5’ -> 3’)	Amplicon (bp)
APC	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98
BRCA1	TCGTGGTAACGGAAAAGCGC	AAATCTCAACGAACTCACGCCG	86
CDKN2A (p16)	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150
CYP24	TGTTGATTTTGTGAGTTTAATTCGA	AAACTTTAATATCCGCCCTACGTA	142
CYP27B1	GGTAGTATTTTTCGTTTGTTTGC	TCAAAACCTCTCTAATCATCTCGAT	139
ER-alpha	ACGAGTTTAACGTCGCGGTC	ACCCCCCAAACCGTTAAAAC	110
IGF2	TAAGTTTGGTTTAGATTCGG	AAACCCCTAAACTCGAACGA	197
MGMT	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81
MINT1	GTATTTGTTATTTGGTTTTTGTCGT	GCTCCTCAATTCTAATTAATCGAA	121
MLH1	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	115
NGFR	GAGGGTTTATGTAGATACGA	TATAATCGTACACTTACGTA	176
PER1	ATTTAGGTTTACGTGCGTTC	GATTAACTAAAAATCTCTTCCCGAC	95
PER2	GTTTTTGGTCGGTGGTCGG	CGAAACTCCTACGCACCTCC	92
PER3	CGTCGGTTTGTTGGGATTG	AAACCGACTCCGCAAACC	160
SEPT9	TTTTTTTATTATTGTTTGCGTTATTC	CAAATAATCATTTATTCTCCCTACG	112
SFRP4	AAGATTTGGCGTTGGGCGGGACGTTC	ACTCCAACCCGAACCTCGCCGTACG	120
SFRP5	TCGAGGATTTAGCGGTAAGTATC	GAAACCGAAAACAAAAAATAACG	136
TIMP3	TTCGTTATGTCGTTTGTTTTTTTC	AATAACCCTACGTTCCTTACTCGA	137
TMEFF2	GTATTATCGGATAGTCGAGTTTCGA	AATAATAACTCCTATTCCTCCTCCG	142
WIF1	TCGTGGTAACGGAAAAGCGC	AAATCTCAACGAACTCACGCCG	176

Table 5.2. Gene-specific primers for *PER1*, *PER3*, and *β-Actin* and qPCR cycling information

Gene	**Forward sequence**	**Reverse sequence**	**Annealing temperature (ºC)**	**Number of cycles**	**Amplicon (bp)**
PER1	ATTTGGCTGCTCATGGCCAATGCT	ATCCTGCTTCAGCACAGAGGTCAT	63	40	90
PER3	TATGCAGGGCATCCTCCCTTTGAA	TATGCAGGGCATCCTCCCTTTGAA	60	40	121
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCAGGCACGAT	60	40	250

Table 5.3. Characteristics of EDCaP study participants

Variable	Total population (n = 107)	Controls (n = 69)	Cases (n = 38)	Controls vs. Cases p-value[1]
	n (%)	n (%)	n (%)	
Gender				0.33
Male	58 (54)	35 (51)	23 (61)	
Female	49 (46)	34 (49)	15 (39)	
Race				0.55
European American	78 (73)	49 (71)	29 (76)	
African American	29 (27)	20 (29)	9 (24)	
Married				0.16
Yes	85 (79)	52 (75)	33 (87)	
No	22 (21)	17 (25)	5 (13)	
Education				0.62
Up to High School	32 (30)	21 (30)	11 (29)	
Some College	28 (26)	16 (23)	12 (32)	
College Undergraduate or Post-Graduate Degree	47 (44)	32 (46)	15 (40)	
Income Level				0.86
Under $50,000	33 (33)	21 (33)	12 (33)	
≥$50,000 to $100,000	44 (44)	29 (46)	15 (42)	
≥$100,000	22 (22)	13 (21)	9 (25)	
Age Group (Years)				0.07
<54	28 (26)	20 (29)	8 (21)	
55-64	46 (43)	33 (48)	13 (34)	
≥65	33 (31)	16 (23)	17 (45)	
Body Mass Index (kg/m^2)				0.57
Underweight or normal	20 (19)	14 (20)	6 (16)	
Overweight or obese	87 (81)	55 (80)	32 (84)	
History of Smoking				0.02
Ever	57 (53)	31 (45)	26 (68)	
Never	50 (47)	38 (55)	12 (32)	
Current alcohol consumption				0.13
Yes	46 (43)	26 (38)	20 (53)	
No	61 (57)	43 (62)	18 (47)	
Ever regularly taken a multivitamin				0.07
Yes	68 (64)	40 (58)	28 (76)	
No	38 (36)	29 (42)	9 (24)	
Ever regularly taken a vitamin C supplement				0.15
Yes	31 (29)	17 (25)	14 (38)	

Table 5.3. Characteristics of EDCaP study participants

Variable	Total population (n = 107)	Controls (n = 69)	Cases (n = 38)	Controls vs. Cases p-value[1]
	n (%)	n (%)	n (%)	
No	75 (71)	21 (75)	23 (62)	
Ever regularly taken a vitamin D supplement				<0.01
Yes	22 (21)	20 (29)	2 (5)	
No	84 (79)	49 (71)	35 (95)	
Hemorrhoids				0.18
Yes	67 (63)	40 (58)	27 (71)	
No	40 (37)	29 (42)	11 (29)	
Procedure reason				<0.01
Routine screening/Symptoms[2]	34 (32)	30 (43)	4 (11)	
Elevated Risk[3]	73 (68)	39 (57)	34 (89)	
Time of day blood was collected				0.23
Morning	83 (78)	56 (81)	27 (71)	
Afternoon	24 (22)	13 (19)	11 (29)	
Worked >1 year of shift work over lifetime				0.99
Yes	45 (42)	29 (42)	16 (42)	
No	62 (58)	40 (58)	22 (58)	
Current Work Shift				0.31
Days	53 (47)	33 (48)	20 (53)	
Other	4 (3)	4 (6)	0 (0)	
Unemployed/Retired	50 (50)	32 (46)	18 (47)	

[1]X^2 test for differences in proportions between cases and controls.; [2]Three subjects exhibited symptoms (presence of gastrointestinal bleeding, hematochezia, melena, fecal occult blood, iron deficiency, or constipation) and were collapsed into screening category; [3]Subject had a previous adenomatous polyp detected (n = 59) and/or a family history of colorectal cancer (n = 20)

Table 5.4. Unadjusted and adjusted odds Ratios for leukocyte DNA promoter methylation relative to adenoma case status

Candidate Gene	Controls (n = 69) n (%)	Cases (n = 38) n (%)	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Hypomethylation*						
APC[a]	44 (64)	23 (61)	0.9 (0.4 – 2.0)	0.74	1.0 (0.4 – 2.5)	0.97
BRCA1[b]	31 (44)	18 (47)	1.1 (0.5 – 2.4)	0.81	1.5 (0.6 – 3.9)	0.36
MINT1[d]	4 (6)	7 (18)	3.7 (1.0 – 13.5)	0.05	5.3 (1.0 – 28.2)	0.05
PER1[e]	20 (29)	17 (45)	2.0 (0.9 – 4.5)	0.10	2.9 (1.1 – 7.7)	0.03
PER3[f]	2 (3)	6 (16)	6.3 (1.2 – 32.9)	0.03	11.1 (1.6 – 78.5)	0.02
SFRP4[e]	12 (17)	5 (29)	0.7 (0.2 – 2.2)	0.57	1.1 (0.3 – 3.8)	0.89
SFRP5[b]	5 (7)	5 (29)	1.9 (0.5 – 7.2)	0.32	3.7 (0.8 – 17.1)	0.09
TIMP3[g]	42 (61)	25 (66)	1.2 (0.5 – 2.8)	0.61	1.2 (0.4 – 3.0)	0.78
TMEFF2[h]	33 (48)	21 (55)	1.4 (0.6 – 3.0)	0.46	1.5 (0.6 – 3.4)	0.36
WIF1[i]	24 (35)	15 (39)	1.2 (0.5 – 2.8)	0.63	1.3 (0.5 – 3.2)	0.56
Hypermethylation						
CDKN2A (p16)[j]	28 (41)	17 (45)	0.8 (0.4 – 1.9)	0.68	0.8 (0.3 – 1.9)	0.54
CYP27B1[l]	58 (84)	31 (82)	1.2 (0.4 – 3.4)	0.74	0.8 (0.2 – 3.0)	0.72
ER alpha[j]	31 (45)	20 (53)	0.7 (0.3 – 1.6)	0.45	0.6 (0.3 – 1.5)	0.27
IGF2[e]	52 (75)	28 (74)	1.1 (0.4 – 2.7)	0.85	0.8 (0.3 – 2.0)	0.57
MGMT[h]	28 (41)	16 (42)	0.9 (0.4 – 2.1)	0.88	0.9 (0.4 – 2.1)	0.80
MLH1[l]	63 (91)	34 (89)	1.2 (0.3 – 4.7)	0.76	0.7 (0.2 – 3.1)	0.67
NGFR[a]	20 (29)	12 (32)	0.9 (0.4 – 2.1)	0.78	0.9 (0.3 – 2.2)	0.75
PER2[g]	62 (90)	35 (92)	0.8 (0.2 – 3.1)	0.70	0.6 (0.1 – 3.0)	0.56
SEPT9[a]	32 (46)	18 (47)	1.0 (0.4 – 2.1)	0.92	0.9 (0.4 – 2.1)	0.81

* - Odds of adenoma status given no methylation detected in the candidate gene. Note: *CYP24A* was not included in the table due to all subjects being unmethylated in the promoter. Adjusted for: a – vitamin D and multivitamin use; b – vitamin C and vitamin D use and physical activity; c – multivitamin , vitamin C, and vitamin D use, physical activity, ever smoking, currently drinking alcohol, being married, and age group; d – multivitamin use, vitamin C and D use, and ever smoking; e – vitamin C and D use and physical activity; f – multivitamin use, vitamin C use, vitamin D use, physical activity, ever smoking, age, and being married; g – multivitamin and vitamin D use, physical activity, and age group; h – vitamin D use; i – vitamin D use and age group; j - vitamin d use, physical activity and age; k - vitamin C and D use, physical activity and age; l – vitamin D use, current drinking alcohol, and age group. OR: odds ratio; CI: confidence interval

Table 5.5. Sensitivity, specificity, positive predictive value, and negative predictive value of *PER1*, *PER3*, and *MINT1*

		Controls (n)	Cases (n)	Total (n)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
PER1 Hypomethylation	Present	20	17	37	45	71	46	70
	Absent	49	21	70				
	Total	69	38	107				
PER3 Hypomethylation	Present	2	6	8	16	97	75	68
	Absent	67	32	99				
	Total	69	38	107				
MINT1 Hypomethylation	Present	4	7	11	18	94	64	68
	Absent	65	31	96				
	Total	69	38	107				
PER1/*PER3* Hypomethylation	Present	49	21	70	55	29	30	54
	Absent	20	17	37				
	Total	69	38	107				
PER1/*MINT1* Hypomethylation	Present	45	20	65	53	35	31	57
	Absent	24	18	42				
	Total	69	38	107				
PER3/*MINT1* Hypomethylation	Present	63	28	91	74	9	31	38
	Absent	6	10	16				
	Total	69	38	107				
PER1/*PER3*/*MINT1* Hypomethylation	Present	44	19	63	50	36	30	57
	Absent	25	19	44				
	Total	69	38	107				

PPV: Positive predictive value; NPV: Negative predictive value

Table 5.6. Concordance of methylation in matched PBLs, normal GI tissue, and polyp tissue

			Normal GI Tissue			
		n_{pairs} = 35	Hypomethylated	Hypermethylated	Kappa Coefficient	p-value
PBLs	*MINT1*	Hypomethylated	0	4	-0.11	0.52
		Hypermethylated	3	28		
	PER1	Hypomethylated	7	5	0.22	0.18
		Hypermethylated	8	15		
	PER3	Hypomethylated	2	2	0.12	0.40
		Hypermethylated	9	22		
			Polyp Tissue			
		n_{pairs} = 12	Hypomethylated	Hypermethylated	Kappa Coefficient	p-value
PBLs	*MINT1*	Hypomethylated	0	0	0	1.00
		Hypermethylated	1	11		
	PER1	Hypomethylated	1	1	-0.09	0.58
		Hypermethylated	7	3		
	PER3	Hypomethylated	1	1	0.25	0.37
		Hypermethylated	2	8		
			Polyp Tissue			
		n_{pairs} = 4	Hypomethylated	Hypermethylated	Kappa Coefficient	p-value
Normal GI Tissue	*MINT1*	Hypomethylated	0	1	0	1.00
		Hypermethylated	0	3		
	PER1	Hypomethylated	2	0	0.5	0.25
		Hypermethylated	1	1		
	PER3	Hypomethylated	1	2	0.2	0.51
		Hypermethylated	0	1		

Abbreviations: PBLs, peripheral blood leukocytes; GI, gastrointestinal

Table 5.7. Adjusted means of fold change expression for *PER1* and *PER3* by methylation status

PBL Marker		n	Fold Change in Expression Mean (SD)	p-value	Explained Variance (%)
PER1	Hypermethylated	57	0.99 (0.05)	0.62	0.18
	Hypomethylated	26	1 (0.02)		
PER3	Hypermethylated	77	0.99 (0.03)	0.82	0.06
	Hypomethylated	6	0.99 (0.04)		
Normal GI Tissue Marker					
PER1	Hypermethylated	8	1.01 (0.02)	0.06	25.88
	Hypomethylated	6	1.04 (0.02)		
PER3	Hypermethylated	10	1.02 (0.04)	0.29	9.18
	Hypomethylated	4	1.05 (0.06)		
Polyp Tissue Marker					
PER1	Hypermethylated	2	1.02 (0.07)	0.66	16.04
	Hypomethylated	6	0.99 (0.02)		
PER3	Hypermethylated	5	0.99 (0.05)	0.82	0.91
	Hypomethylated	3	1 (0.04)		

Note: *MINT1* expression could not be measured.
Abbreviations: SD, standard deviation; PBL, peripheral blood leukocyte; GI, gastrointestinal

Table 5.8. Unadjusted and adjusted odds ratios* for gene expression relative to adenoma case status (N = 83)

Candidate Gene	Crude OR	95% CI	Adjusted OR	95% CI
PER1[a]	0.003	<0.01 – 181.72	<0.01	<0.01 – 1.86
PER3[b]	0.06	<0.01 – >999.99	0.34	<0.01 – >999.99

Note: *MINT1* expression was not measured.

Abbreviations: OR, odds ratio; CI, confidence interval; * - Odds of adenoma status given for a one unit increase in expression in the candidate gene. Adjusted for: a – ever smoking, physical activity, and vitamin C and vitamin D use; b – ever smoking, being married, age, physical activity, ever taking a multivitamin, and vitamin C and vitamin D use

Table 5.9. Spearman's correlations of *PER1* and *PER3* expression in PBLs, normal GI tissue, and polyp tissue

			PBLs		Normal Tissue		Polyp Tissue	
			PER1	*PER3*	*PER1*	*PER3*	*PER1*	*PER3*
PBLs	*PER1*	Spearman's rho	1	-0.04	0.26	-0.05	0	0.26
		p-value		0.69	0.34	0.85	1	0.42
		n_{pairs}	83	83	16	16	12	12
	PER3	Spearman's rho	-0.04	1	0.4	-0.37	0.13	0.53
		p-value	0.69		0.12	0.16	0.70	0.08
		n_{pairs}	83	83	16	16	12	12
Normal Tissue	*PER1*	Spearman's rho	0.26	-0.05	1	0.64	0.79	0.93
		p-value	0.34	0.85		0.01	0.04	<0.01
		n_{pairs}	16	16	16	16	7	7
	PER3	Spearman's rho	0.40	-0.37	0.64	1	0.43	0.71
		p-value	0.12	0.16	0.01		0.34	0.07
		n_{pairs}	16	16	16	16	7	7
Polyp Tissue	*PER1*	Spearman's rho	0	0.26	0.79	0.93	1	0.62
		p-value	1	0.42	0.04	<0.05		0.03
		n_{pairs}	12	12	7	7	12	12
	PER3	Spearman's rho	0.13	0.53	0.43	0.71	0.62	1
		p-value	0.70	0.08	0.34	0.07	0.03	
		n_{pairs}	12	12	7	7	12	12

Note: *MINT1* expression could not be measured.
Abbreviations: PBLs, peripheral blood leukocytes; GI, gastrointestinal

Chapter 6

Case-Control Study of the *PERIOD3* Clock Gene Length Polymorphism and Colorectal Adenoma Formation[4]

[4] Alexander M, Burch JB, Steck SE, Chen CF, Hurley TG, Cavicchia P, Ray M, Shivappa N, Guess J, Zhang H, Youngstedt SD, Creek KE, Lloyd S, Yang X, Hébert JR. Accepted at *Oncology Reports.* Reprinted here with permission of publisher (Appendix B).

6.1 Abstract

Introduction: Clock genes are expressed in a self-perpetuating, circadian pattern in virtually every tissue including the human gastrointestinal tract. They coordinate cellular processes critical for tumor development, including cell proliferation, the DNA damage response, and apoptosis. Circadian rhythm disturbances have been associated with an increased risk for colon cancer and other cancers. This mechanism has not been elucidated, but may involve mutation or dysregulation of the 'period' (*PER*) clock genes, which have tumor suppressor properties. A variable number tandem repeat (VNTR) in the *PERIOD3* (*PER3*) gene has been associated with sleep disorders, differences in diurnal hormone secretion, and increased premenopausal breast cancer risk. Colorectal tumors have reduced *PER3* expression relative to normal tissue. However, gene expression or susceptibility related to *PER3* has not been examined in conjunction with adenomatous polyps. This exploratory case-control study was the first to test the hypothesis that the 5-repeat *PER3* VNTR sequence is associated with increased odds of adenoma formation among 146 colonoscopy patients. **Materials and Methods**: Information on demographics, medical history, occupation, and lifestyle was collected prior to colonoscopy. Cases (n=49) were individuals with at least one histopathologically confirmed adenoma. Controls (n=97) included patients with normal findings or hyperplastic polyps not requiring enhanced surveillance. Unconditional multiple logistic regression was used to calculate odds ratios (ORs) with 95% confidence intervals (CIs), after adjusting for potential confounding. **Results**: Adenomas were detected in 34% of participants (average age: 59±9 years; 65% European

American [EA]; 72% male). Cases were more likely to possess the 5-repeat *PER*3 genotype relative to controls (4/5 OR: 2.1, 95% CI: 0.9-4.8; 5/5 OR: 5.1, 95% CI: 1.4-18.1; 4/5+5/5 OR: 2.5, 95% CI: 1.7-5.4). Examination of the Oncomine microarray database indicated that *PERIOD* gene expression in adenomas was reduced relative to adjacent normal mucosa in several studies. **Discussion**: Results suggest a need for follow-up in a larger sample.

6.2 Introduction

According to recent estimates, over 143,000 new patients and more than 51,000 deaths occurred in 2012 in the United States due to colorectal cancer (CRC), which makes it the third most common and deadly cancer among both men and women.[1] Colorectal adenomatous polyps are the primary precursor lesions for CRC, accounting for 85-90% of cases.[244] Developing a better understanding of factors related to adenoma susceptibility and progression thus represents an important goal for CRC prevention.

Disruption of circadian rhythms or clock gene expression is emerging as a novel and potentially modifiable cancer risk factor, although the pathophysiological mechanism is incompletely understood.[275, 298] The central circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Generation of circadian rhythms is accomplished primarily via photic input from the retina, which synchronizes the reciprocal transcriptional-translational expression of at least nine core clock genes: *PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, TIM,* and *CK1ε*.[275, 298, 303] In most

tissues, this system facilitates the diurnal expression of ~10% of the entire mammalian genome via genetic and epigenetic regulation of clock-controlled genes. [89, 304-307] Various factors, such as shiftwork, late bedtimes, or poorly timed light exposure can disrupt endogenous circadian timing, thus altering clock gene expression and the cellular processes they help regulate.[298] Because clock genes regulate processes that are considered hallmarks of carcinogenesis (cell cycle control, DNA damage response, apoptosis), their dysregulation may serve as an underlying biological mechanism linking altered circadian rhythms with cancer.[298, 308-312] Clock gene polymorphisms have been associated with non-Hodgkin's lymphoma and cancers of the breast and prostate.[298] Clock gene polymorphic variation also influences sleep regulation,[303, 313, 314] which may contribute to increases in cancer susceptibility that have been observed among people who experience circadian rhythm disturbances or sleep disruption.[45, 315, 316] For example, shiftwork and sleep disturbances have been associated with increased CRC risk,[106, 316-318] and truncated sleep (<6 hours per night) has been associated with an increased odds of colorectal adenoma formation relative to adenoma-free controls.[45]

The *Period (PER)* clock genes have immunomodulatory[303, 319, 320] and tumor suppressor properties.[29, 298, 308, 315, 321] Mutation or altered expression of *PER* genes has been observed among cancer patients relative to controls, within human tumors relative to adjacent normal tissue, and in experimental cancer bioassays.[19, 29, 31, 32, 104, 105, 116, 298, 308, 322, 323] Whether differential expression of *PER* or other clock genes occurs in human adenomas versus normal tissue is

not known. The *PER3* variable number tandem repeat (VNTR, rs57875989) length polymorphism contains 4 or 5 copies of a 54-bp sequence encoding 18 amino acids. The 5-repeat variant adds several potential phosphorylation motifs to the gene, and *PER3*'s interaction with circadian processes may be enhanced among those individuals.[313, 324] The 5-repeat *PER3* allele is associated with a relatively penetrant phenotype that includes morning circadian preference,[38, 313, 325] increased cognitive decline in response to sleep deprivation,[313] differences in levels or timing of melatonin or cortisol secretion,[40, 324, 326] and a tendency towards depressive symptoms or an earlier onset of bipolar disorder.[24, 327] *PER3* is considered a candidate tumor suppressor gene, [19, 29, 321] and the 5/5 *PER*3 VNTR genotype has been associated with increased premenopausal breast cancer risk,[19] though not consistently.[328, 329] Recently, the relationship between the *PER3* VNTR and CRC risk was examined in Greece and no association was observed, although a relatively small portion of the study population was homozygous for the 5-repeat allele (<2%), and differences in genotype frequency among cases and controls were not adjusted for potential confounding.[330] The role of *PER3* or other clock genes in human adenoma formation has yet to be examined in detail. Therefore, this exploratory study tested the hypothesis that adenoma cases are hetero- or homozygous for the 5-repeat *PER3* variant relative to adenoma-free controls.

6.3 Materials and Methods

Participants and data from two different endoscopy centers in the Columbia, SC metropolitan area were pooled for this analysis; the South Carolina Medical Endoscopy Center Site 1, n=93), and the WJB Dorn Veterans Administration Medical Center (Site 2, n=53). Participants completed an on-site interview to collect information on: demographic (sex, marital status, income, race/ethnicity), lifestyle (smoking history, diet, physical activity), and occupational (employment status, job industry, type of shift, history of shiftwork) factors, as well as personal and family medical history (ever being diagnosed with other diseases, personal history of cancer, family history of CRC). Cases were defined as individuals with at least one histologically confirmed adenoma, and controls were subjects with a normal colonoscopy, or a normal biopsy not requiring heightened surveillance (e.g., hyperplastic polyp).

Genotyping procedure

Genomic DNA was extracted and genotyping for the *PER3* VNTR sequence was performed using previously described methods.[24, 331] For participants recruited from Site 1, the *PER3* VNTR sequence was amplified via polymerase chain reaction (PCR) using the following forward (5′-CAAAATTTTATGACACTACCAGAATGGCTGAC-3′ and reverse (5′-AACCTTGTACTTCCACATCAGTGCCTGG-3′) primers.[19,328] The reactions were heated to 94°C for 2 minutes followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. Finally, the reactions were extended

for 7 minutes at 72°C. PCR products were then separated by electrophoresis on a 3% agarose gel. The PCR primers used for Site 2 assays were: forward, 5'-TGGCAGTGAGAGCAGTCCT-3' and reverse 5'-AGTGGCAGTAGGATGGGATG-3'. PCR cycling conditions were as follows: 3 minutes at 94°C ; 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C; and at 72°C for 30 seconds, PCR products were extended. A 2% agarose gel stained with ethidium bromide was used to separate and visualize the PCR fragments at 220V for 30 minutes. Both primers provide valid characterization of the *PER3* VNTR[19, 24, 35, 328, 330-332] and amplicons from both methods were confirmed via Sanger sequencing. Duplicate genotyping was performed in 10% of all samples from both sites for quality control purposes, and there was 100% concordance among duplicates.[24] DNA sequences of amplicons produced by each set of primers were verified via Sanger sequencing. Hardy-Weinberg equilibrium (HWE) was examined, and gene frequencies for the *PER*3 VNTR were in HWE among the entire study population (p=0.74), and among controls from both sites (p=0.99) or within each site (Site 1: p=0.94; Site 2: p=0.82, Table 6.1).

Methylation assays

A peripheral whole blood sample was collected from each participant in Site 1 using vacutainers containing EDTA anticoagulant. Genomic DNA was isolated using the PUREGENE genomic DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol from peripheral whole

blood, normal GI tissue, and polyp tissue. Sodium bisulfite conversion of DNA was performed using EpiTect kits (QIAGEN, Germantown, MD, USA). Briefly, DNA (2 µg) was added to the reaction buffer and bisulfite DNA conversion was conducted in a Bio-Rad S1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the manufacturer's suggested program for the conversion reaction. Converted DNA was eluted from a spin column in TE buffer and either used immediately or stored at –20°C. DNA oligonucleotides for primer pairs were purchased from Integrated DNA Technologies (Coralville, IA, USA, Table 6.2). To ensure that the polymerase chain reaction (PCR) primers were specific for the detection of methylated DNA, a control human DNA set (EpiTect Control DNA, QIAGEN Sciences, Germantown, MD) containing positive (bisulfite converted, 100% methylated) and negative (bisulfite converted, 0% methylated) control DNA was used. To quantify methylation specific PCR (MSP) results, the QIAxcel system was used (QIAGEN Sciences, Germantown, MD), which utilizes a multiplexed fluorescence detection method with a resolution of 3-5 bp for DNA fragments. The detection sensitivity of QIAxcel system was 0.1 ng/µl DNA in undiluted amplification reactions. The QIAxcel system was equipped with the BioCalculator software that produces a tabular display of a variety of peak properties, including number of peaks as well as the height, width, and area of each peak. The height of the peak was used for separation of the results into three methylation categories: high (peak height >0.30), medium (peak height 0.29-0.05), and low (peak height <0.05), or none (no methylation detected). Results were categorized as either unmethylated or methylated (combination of

medium/low, and high methylation categories). The methylation status of each sample was normalized via a two-step process. First, a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to measure concentrations of the DNA templates. Subsequent experiments used the same amount of DNA template for each MSP reaction. To facilitate quantification, equal volumes of the MSP products were loaded on the QIAxcel system for analysis. Second, BRCA1 gene methylation was used as an internal standard since the same amount of DNA template had extremely high methylation in each sample. The measured peak height of BRCA1 methylation was then used to normalize the methylation of the genes of interest for each sample. All methylation assays were performed blinded to case status.

Real-time quantitative PCR (qPCR)

RNA was extracted from PAXgene tubes using PAXgene Blood RNA Kit from QIAGEN (Germantown, MD, USA) from participants in Site 1. RNA quality was checked via Agilent Bioanalyzer 2100 and concentration was quantified on NanoDrop. cDNA was synthesized from total RNA using gene-specific primers according to the Verso cDNA Kit (Abgene, Rochester, New York). Reverse transcriptase reactions contained 1 µg of total RNA (1-5 µl of total RNA), 4 µl 5X cDNA synthesis buffer, 1 µl 5 µM dNTP mix, 1 µl 400 ng/µl random hexamers, 1 µl RT Enhancer, 1 µl Verso Enzyme Mix, and a variable amount of PCR grade water to have a total of a 20 µl. The 20 µl reactions were incubated in an MJ

Research PTC-225 Thermo Cycler in a 96-well plate for 30 min at 42°C, 2 min at 95°C, and then held at 4°C. cDNAs were stored at -20 ºC.

Gene-specific primers were created using Primer3 software. Forward and reverse sequences were confirmed for their presence within exons and lack of single nucleotide polypmorphisms using NCBI's Basic Local Alignment Search Tool (BLAST). PCR cycling information is found in Table 6.3. All reactions were performed in singleplex mode. Each reaction contained 10 µl 2x SYBR Green PCR master mix (Bio-Rad Laboratories, Hercules, CA), 2 µl of each primer, 0.5-2 µl of cDNA template, and a variable amount of PCR grade water to have a total reaction amount of 20 µl. The standard curve method was employed and β-actin was used as the endogenous control to determine levels of expression of cDNA in study participants. A two-fold 10-point serial dilution was created through the use of diluted PCR products of *β-actin* and markers who presented an association with polyp status. The standard curve produces a linear relationship between number of threshold cycles (Ct) and the initial known amounts of cDNA, allowing the determination of the cDNA concentration of unknown samples based on their Ct value, thereby also giving the number of mRNA copies for each gene present in unknown samples. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Each sample was analyzed in duplicate, with a negative template control included in each run to check for DNA contamination.

Statistical analysis

Statistical analyses were performed using the Statistical Analysis Software (SAS®) computer program (version 9.2, Cary, NC) and the R meta-analysis package (version 2.14.1, http://cran.r-project.org). Potential differences between study variables by case status within the entire study population, and within each site separately, were examined using the chi-squared test for differences between proportions. Differences in median shiftwork duration by PER3 genotype were compared using the Wilcoxon rank sum test. Unconditional multiple logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (95% CIs) for *PER3* VNTR genotype among adenoma cases relative to controls using the 4/4 genotype as referent.[19, 24] Covariates considered for inclusion in adjusted models were known or suspected adenoma or CRC risk factors (age, sex, race, body mass index [BMI], CRC family history, smoking history, work-related factors, diet, vitamin and supplement use, physical activity, sociodemographic characteristics [income, education], personal medical history) , and variables that differed among participants and non-participants (sex, personal cancer history, ulcer diagnosis, lactose intolerance, secondhand smoke exposure, recruitment site). Final models included variables that were statistically significant ($p \leq 0.05$) in the saturated model, or produced at least a 10% change in the parameter estimate for genotype (decision latitude at work, procedure reason, recruitment site). Ancillary analyses examined relationships between lifetime shiftwork and adenoma status; or *PER3* genotype and adenoma status after stratification by procedure reason (screening vs. surveillance

colonoscopy), shiftwork (none vs. any, or median split on years of lifetime shiftwork), and methylation status in *PER1*, *PER2*, or *PER3*. Random-effects meta-analysis was used to evaluate the study hypothesis since recruitment occurred at two separate sites. The I^2 and Q statistics were used to assess heterogeneity between sites. Oncomine,[333] a publically accessible gene expression microarray database, was queried to examine potential differential expression of *PER1*, *PER2*, and *PER3* clock genes in adenomas versus adjacent normal tissue. Expression levels of *PER1*, *PER2*, and *PER3* were also examined for differences by genotype in a subset of participants.

6.4 Results

The median age of the participants was 58 years (25th percentile: 53 years, 75th percentile: 64 years), and they were primarily European American (65%), and male (72%). Most (69%) participants had engaged in at least one year of shiftwork (median: 3 years; 25th percentile: 0 years; 75th percentile: 12.3 years). Adenomas were detected in 34% of participants (females: 28%, males: 36%). Compared to controls, cases were more likely to have smoked (78% vs. 56%), have higher decision latitude (57% vs. 29%), and have undergone a surveillance relative to a screening colonoscopy (59% vs. 40%, Table 6.4). Additional results on characteristics by site and by case status within each site are found in Tables 6.5 and 6.6, respectively.

Overall *PER3* VNTR genotype frequencies (4/4: 47%, 4/5: 42%, 5/5: 11%) were consistent with those reported elsewhere (4/4: 35-74%, 4/5: 25-46%, 5/5: 2-

18%).[327, 328, 334, 335] Adenoma cases were more likely than controls to possess one or two copies of the 5-repeat sequence (5/5 OR: 5.1, 95% CI: 1.4-18.1; 4/5+5/5 OR: 2.5; 95% CI: 1.7-5.4, Table 6.7). Shiftwork, or the combined effect of shiftwork and genotype was not related to adenoma case status (data not shown).

When analyses were stratified by procedure reason, the 5/5 genotype was associated with adenoma status among screening patients (OR: 10.7, 95% CI: 1.4-80.7), whereas no statistically significant relationship was observed among those with the 5/5 genotype receiving a surveillance colonoscopy (OR: 2.3, 95% CI: 0.5-11.4), or among the other genotypes for either procedure reason (Table 6.8). After stratification by methylation status of *PER1*, *PER2*, and *PER3* methylation status (hypomethylation or hypermethylation), no statistically significant relationships were observed (Table 6.9).

The distribution of lifetime shiftwork history varied by *PER3* VNTR genotype (Table 6.10). Those with the 4/4 genotype had greater median lifetime years of shiftwork (5 years) compared to those with the 4/5 (2 years, p=0.02), 5/5 (0.75 years, p=0.05), or the combined genotype (4/5+5/5: 1 year, p<0.01). When the data were evaluated using meta-analytic methods, adenoma cases were ~2-3 times more likely than controls to have at least one 5-repeat allele, although the confidence intervals were wide and did not achieve statistical significance (4/5 OR: 2.27, 95% CI: 0.43-11.62, $p_{heterogeneity}$: 0.12, I^2: 60%; 5/5 OR: 3.02, 95% CI:

0.72-12.71, $p_{heterogeneity}$: 0.84, I^2: 0%; 4/5+5/5 OR: 2.35, 95% CI: 0.60-9.21, $p_{heterogeneity}$: 0.14, I^2: 54%).

Data for *PER1*, *PER2*, and *PER3* expression in adenomas relative to normal tissue retrieved from the Oncomine microarray database are presented in Table 6.11.[336-338] A statistically significant reduction in *PER3* expression was observed in adenomas relative to normal tissue among each of the available data sets, and similar differences were noted for *PER1* and *PER2* expression (Table 6.11).

Expression levels of *PER1*, *PER2*, and *PER3* were stratified by genotype (4/4 genotype vs. 4/5 or 5/5 genotype, Figure 6.1). Those expression levels were slightly higher for those with at least one 5-repeat allele, the only statistically significant difference was observed in *PER1* expression ($p \leq 0.05$). That is, individuals with at least one 5-repeat allele had higher levels of *PER1* expression compared to individuals without a 5-repeat *PER*3 genotype.

6.5 Discussion

Few studies have examined the role of the *PER3* VNTR on cancer-related outcomes.[19, 328-330] To our knowledge, this exploratory study is the first to examine the relationship between the *PER3* VNTR and human adenoma risk. Adenoma cases were ~2-5 times more likely to possess the 5-repeat *PER3* length polymorphism compared to controls. There are several noteworthy strengths, limitations and uncertainties associated with this study. Quality criteria for genotyping and screening colonoscopy adenoma detection rates were

satisfactory,[339, 340] and adjustment for potential confounding by known or suspected adenoma risk factors did not alter the interpretation of the results. The meta-analysis indicated that the strength of association between *PER3* genotype and adenoma status was generally consistent with the main analysis and the results were not strongly impacted by heterogeneity between the sites. Some imprecise risk estimates with wide confidence intervals were observed due to a limited sample size, particularly for the stratified analyses. Thus, examination of possible effect modification by factors such as race, chronotype, procedure indication (screening versus surveillance), or methylation would benefit from a larger sample in future studies. Nonetheless, the lower bound of the confidence intervals suggest a ~40% increased risk for adenoma formation among the 5-repeat *PER3* variants. *PER* gene expression was not performed among cases and controls in the present study, thus changes in expression relative to the *PER3* VNTR genotype could not be evaluated. However, our query of the Oncomine database indicated that *PER3* and to a lesser extent *PER1* and *PER2* expression was reduced among adenomas compared to normal mucosa, which is consistent with previous studies that observed a reduction in *PER1* and *PER3* expression in human colorectal tumors relative to adjacent normal tissue.[32, 104, 341] However, findings from the Oncomine query somewhat contradict findings from Figure 6.1, which show increased *PER1* expression among those with at least one 5-repeat allele. This is an unexpected finding because those with at least one 5-repeat allele should be at an increased risk for adenoma formation, and those individuals should have lower *PER1* expression. Further investigation

in the relationships between *PER3* VNTR variation and expression of the *PER* genes is warranted.

The spectrum of known genetic susceptibility markers does not fully account for all CRC cases. For example, 10 loci identified from genome-wide association studies had population attributable risks ranging from 1.7% to 11.9%,[342] and another previous study estimates that up to 35% of CRC cases are due to heritable factors.[343] The current study mirrors previous investigations that have examined clock gene polymorphisms in conjunction with cancer susceptibility,[19, 298, 328, 330] including one that identified an association between the 5-repeat *PER3* VNTR sequence and increased odds of premenopausal breast cancer.[19] Evidence suggests that *PER*3 may function as a tumor suppressor. A recent study among *PER3* knockout mice indicated that 36% of the homozygous null variants developed chemically-induced mammary tumors compared to 12% among heterozygotes and 0% among wild-type mice.[29] Another recent study used methylation arrays and stringent selection criteria to screen >14,000 genes to identify putative tumor suppressors associated with human hepatocellular carcinoma; *PER3* was one of only three candidate tumor suppressor genes identified.[321] Chronic gastrointestinal inflammation is important for adenoma and CRC development,[244, 344] and since PERIOD genes play a role in immune system regulation, their expression may influence these processes.[303, 319, 320] Recently, another *PER3* polymorphism (rs2797685) was associated with inflammatory bowel disease, a known CRC risk factor.[345] The current study attempted to look at joint effects of the *PER3* VNTR and methylation status of *PER1*, *PER2*, and

PER3. Though no statistically significant findings were observed when examining the joint effect of genetic and epigenetic variation (Table 6.9), future larger studies should examine these interactive effects since the mechanism whereby *PER3* may exert a tumor suppressor function is currently unknown.

The clock genes exert genetic and epigenetic regulatory effects that facilitate the circadian expression of ~5-10% of the entire mammalian transcriptome,[89, 304-307] including other known tumor suppressors and oncogenes (e.g., *c-Myc*, *p53*).[26, 31, 346] Clock genes also help regulate cellular processes that are active during carcinogenesis (cell proliferation, DNA damage response, apoptosis), and clock gene dysregulation may foster adenoma formation by influencing these pathways.[298, 308, 310, 312]

Individuals with the 5-repeat *PER3* VNTR sequence tend to have relatively penetrant phenotypic characteristics including delayed sleep phase syndrome, increased susceptibility to cognitive impairment after sleep deprivation, morning circadian preference, and differences in the timing or levels of circadian hormone secretion,[40, 313, 324] although some inconsistencies have been reported.[36, 38, 347, 348] Whether alterations in sleep and other circadian processes can increase cancer susceptibility remains to be determined. Studies of shift work and cancer incidence suggest this is possible.[349] In the present study, participants with the 5-repeat allele reported less cumulative shiftwork experience relative to those with the 4/4 genotype (Table 6.10). Additional research is needed to determine whether this is a chance finding or if individuals carrying these variants are less tolerant of shiftwork compared to those with the 4/4 genotype. Individuals with

the 5-repeat allele may be more susceptible to disturbances in circadian timekeeping.[40, 313, 324, 326] For example, those carrying the 5/5 *PER3* variant were sensitive to light-induced melatonin suppression whereas 4/4 homozygotes were not responsive.[326] Because melatonin has potent antioxidant, anti-proliferative and anti-inflammatory properties in the gastrointestinal tract, a reduction in its secretion (e.g., by exposure to light at night) may facilitate physiologic changes that predispose to increased risks for CRC or other cancers.[106, 275, 298, 316, 317] Although the *PER*3 has tumor suppressor properties and its length polymorphism tends to have relatively penetrant phenotypic characteristics, the role of these factors in cancer susceptibility, if any, remains to be characterized.

In conclusion, results from this study indicate that individuals with the 5-repeat *PER3* length polymorphism may be more susceptible to adenoma formation. The results are consistent with Oncomine data indicating that *PERIOD* clock gene expression is reduced in adenomas relative to normal GI tissue. Further interrogation of interrelationships between the *PER3* VNTR and genetic or epigenetic pathways that may facilitate adenoma risk, such as changes in the expression of clock-controlled, cancer-related genes, is recommended. Further elucidation of the *PER3* VNTR genotype in relation to circadian rhythm or clock gene dysregulation may lead to development of novel, modifiable targets for adenoma and CRC prevention.

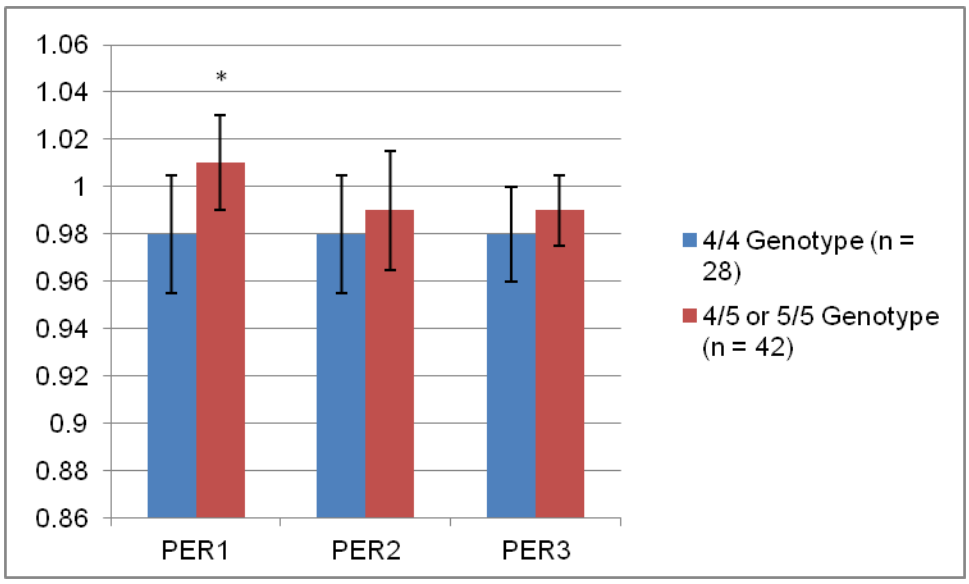

Figure 6.1. Differences in fold change expression of the *PER* gene family in blood by *PER3* VNTR genotype.
Note: *p≤ 0.05

Table 6.1. PER3 VNTR gene frequencies among study participants

	Entire Population (n = 146)		**All Controls (n = 97)**		**Recruitment Site 1 (n = 62)**		**Recruitment Site 2 (n = 35)**	
Genotype	n (%)	HWE p-value	n (%)	HWE p-value	n (%)	HWE p-value	n (%)	HWE p-value
4/4	68 (47)	0.74	52 (54)	0.99	29 (47)	0.94	23 (44)	0.82
4/5	62 (42)		38 (39)		27 (43)		11 (31)	
5/5	16 (11)		7 (7)		6 (10)		1 (3)	

Abbreviation: HWE, Hardy-Weinberg equilibrium

Table 6.2. Primers for Methylation-Specific PCR

Gene Name	Forward (5’ -> 3’)	Reverse (5’ -> 3’)	Amplicon (bp)
PER1	ATTTAGGTTTACGTGCGTTC	GATTAACTAAAAATCTCTTCCCGAC	95
PER2	GTTTTTGGTCGGTGGTCGG	CGAAACTCCTACGCACCTCC	92
PER3	CGTCGGTTTGTTGGGATTG	AAACCGACTCCGCAAACC	160

Table 6.3. Gene-specific primers for *PER1*, *PER3*, and *β-Actin* and qPCR cycling information

Gene	**Forward sequence (5' -> 3')**	**Reverse sequence (5' -> 3')**	**Annealing temperature (ºC)**	**Number of cycles**	**Amplicon (bp)**
PER1	ATTTGGCTGCTCATGGCCAATGCT	ATCCTGCTTCAGCACAGAGGTCAT	63	40	90
PER3	TATGCAGGGCATCCTCCCTTTGAA	TATGCAGGGCATCCTCCCTTTGAA	60	40	121
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCAGGCACGAT	60	40	250

Table 6.4. Demographic characteristics of study population

Variable[1]	**Total Population (n = 146)**	**Controls (n = 97)**	**Cases (n = 49)**	**Cases vs. Controls p-value[2]**
	N (%)	N (%)	N (%)	
Sex				0.32
Male	105 (72)	67 (70)	38 (78)	
Females	40 (28)	39 (30)	11 (22)	
Race				0.12
European American	94 (65)	58 (60)	36 (73)	
African American	51 (35)	38 (40)	13 (27)	
Marital Status				0.46
Unmarried	38 (26)	27 (28)	11 (22)	
Married	107 (74)	69 (72)	38 (78)	
Education				0.75
Up to High School	52 (36)	34 (35)	18 (37)	
Some College	40 (28)	25 (26)	15 (31)	
College Undergraduate or Post-Graduate Degree	53 (37)	37 (39)	16 (33)	
Income level				0.37
Under $50,000	63 (46)	40 (45)	23 (49)	
≥$50,000 to $100,000	53 (39)	38 (43)	15 (32)	
≥$100,000	20 (15)	11 (12)	9 (19)	
Body Mass Index (kg/m^2)				0.22
Underweight or normal	33 (23)	19 (20)	14 (29)	
Overweight	113 (77)	78 (80)	35 (71)	
Family History of Colorectal Cancer				0.84
Yes	25 (17)	17 (18)	8 (16)	
No	120 (83)	79 (82)	41 (84)	
Diagnosis of Diabetes				0.15
Yes	42 (29)	24 (25)	18 (38)	
No	102 (81)	71 (75)	31 (62)	
History of Smoking				0.01
Ever	91 (63)	53 (56)	38 (78)	
Never	53 (37)	42 (44)	11 (22)	
Work Decision Latitude				0.03

Table 6.4. Demographic characteristics of study population

Variable[1]	**Total Population (n = 146)**	**Controls (n = 97)**	**Cases (n = 49)**	**Cases vs. Controls p-value[2]**
	N (%)	N (%)	N (%)	
Unknown	74 (51)	51 (53)	23 (47)	
Never or Sometimes	21 (14)	18 (19)	3 (6)	
Often or Always	51 (35)	28 (29)	23 (57)	
Age Group				0.11
30-54	45 (31)	34 (35)	11 (22)	
55-65	66 (46)	44 (46)	22 (45)	
> 65	34 (23)	18 (19)	16 (33)	
Reason for Colonoscopy				0.03
Symptoms/Screening	78 (53)	58 (60)	20 (41)	
Surveillance	68 (47)	39 (40)	29 (59)	
	Median (25th percentile, 75th percentile)			
Lifetime Shiftwork (Years)	3 (0, 12.3)	5 (0, 15)	2 (0, 10)	0.10

[1]Number of subjects for each variable category may not equal total number of subjects due to missing data; [2]Chi-squared test for differences in proportions or Wilcoxon rank sum test for differences in medians between cases and controls

Table 6.5. Differences in patient characteristics by site

	Recruitment Site 1 (N = 93)	**Recruitment Site 2 (N = 53)**	**p-value[2]**
Variable[1]	N (%)	N (%)	
Sex			<0.0001
Male	52 (57)	53 (100)	
Female	40 (43)	0 (0)	
Race			0.02
European American	66 (72)	28 (53)	
African American	26 (28)	25 (47)	
Marital Status			0.02
Unmarried	18 (20)	20 (38)	
Married	74 (80)	33 (62)	
Education			0.06
Up to High School	28 (30)	24 (45)	
Some College	24 (26)	16 (30)	
College Undergraduate or Post-Graduate Degree	40 (43)	13 (25)	
Income level			<0.0001
Under $50,000	26 (31)	37 (70)	
≥$50,000 to $100,000	40 (48)	13 (25)	
≥$100,000	17 (20)	3 (6)	
Body Mass Index (kg/m^2)			0.10
Underweight or normal[3]	17 (18)	16 (30)	
Overweight	76 (82)	37 (70)	
Family History of Colorectal Cancer			0.60
Yes	17 (18)	8 (15)	
No	75 (82)	45 (85)	
Diagnosis of Diabetes			0.58
Yes	28 (31)	14 (26)	
No	64 (69)	39 (74)	
History of Smoking			0.01
Ever	51 (55)	40 (77)	
Never	41 (45)	13 (23)	
Work Decision Latitude			0.27
Unknown	43 (47)	31 (58)	
Never or Sometimes	16 (17)	5 (9)	
Often or Always	34 (37)	17 (32)	
Age group			0.04
30-54	23 (25)	22 (42)	
55-65	42 (46)	24 (45)	

Table 6.5. Differences in patient characteristics by site

	Recruitment Site 1 (N = 93)	**Recruitment Site 2 (N = 53)**	**p-value[2]**
Variable[1]	N (%)	N (%)	
>65	27 (29)	7 (13)	
Reason for Procedure			<0.0001
Screening/Symptoms	25 (27)	53 (100)	
Surveillance	68 (73)	0 (0)	
Case Status[4]			0.94
Controls	62 (67)	35 (66)	
Adenomas	31 (33)	18 (34)	
	Median (25th percentile, 75th percentile)		
Lifetime Shiftwork (Years)	1 (0, 10)	6 (2, 17)	0.0004

[11]Number of subjects for each variable category may not equal total number of subjects due to missing data ; [2] Chi-squared test for differences in proportions or Wilcoxon rank sum test for differences in medians between Site 1 and Site 2; [3]Three subjects who were in the underweight BMI category (≤18.5 kg/m^2); [4]No polyps or hyperplastic polyp(s) only

Table 6.6. Differences in patient characteristics by case status and site

	Recruitment Site 1				Recruitment Site 2			
	Total population (n = 93)	**Controls[1] (n = 62)**	**Cases (n = 31)**	**p-value[3]**	**Total population (n = 53)**	**Controls[1] (n = 35)**	**Cases (n = 18)**	**p-value[3]**
Variable[2]	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
Sex				0.27				1.00
Male	52 (57)	32 (52)	20 (65)		53 (100)	35 (100)	18 (100)	
Female	40 (43)	29 (48)	11 (35)		0 (0)	0 (0)	0 (0)	
Race				0.18				0.39
European American	66 (72)	41 (67)	25 (81)		28 (53)	17 (49)	11 (61)	
African American	26 (28)	20 (33)	6 (19)		25 (47)	18 (51)	7 (39)	
Marital Status				0.25				0.90
Unmarried	18 (20)	14 (23)	4 (13)		20 (38)	13 (37)	7 (39)	
Married	74 (80)	47 (77)	27 (87)		33 (62)	22 (63)	11 (61)	
Education				0.60				0.50
Up to High School	28 (30)	20 (33)	8 (26)		24 (45)	14 (40)	10 (56)	
Some College	24 (26)	14 (23)	10 (32)		16 (30)	11 (31)	5 (28)	
College Undergraduate or Post-Graduate Degree	40 (43)	27 (44)	13 (42)		13 (25)	10 (29)	3 (17)	
Income level				0.40				0.07
Under $50,000	26 (31)	19 (35)	7 (24)		37 (70)	21 (60)	16 (89)	
≥$50,000 to $100,000	40 (48)	26 (48)	14 (48)		13 (25)	12 (34)	1 (6)	
≥$100,000	17 (20)	9 (17)	8 (28)		3 (6)	2 (6)	1 (6)	
Body Mass Index (kg/m^2)				0.85				0.11
Underweight or normal[4]	17 (18)	11 (18)	6 (19)		16 (30)	8 (23)	8 (44)	
Overweight	76 (82)	51 (82)	25 (33)		37 (70)	27 (77)	10 (56	
Family History of Colorectal Cancer				0.68				0.82

Table 6.6. Differences in patient characteristics by case status and site

	Recruitment Site 1				Recruitment Site 2			
	Total population (n = 93)	**Controls[1] (n = 62)**	**Cases (n = 31)**	**p-value[3]**	**Total population (n = 53)**	**Controls[1] (n = 35)**	**Cases (n = 18)**	**p-value[3]**
Variable[2]	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
Yes	17 (18)	12 (20)	5 (16)		8 (15)	5 (14)	3 (17)	
No	75 (82)	49 (80)	26 (84)		45 (85)	30 (86)	15 (83)	
Diagnosis of Diabetes				0.01				0.25
Yes	28 (31)	13 (22)	15 (48)		14 (26)	11 (31)	3 (17)	
No	64 (69)	39 (78)	16 (52)		39 (74)	24 (69)	15 (83)	
History of Smoking				0.03				0.14
Ever	51 (55)	29 (48)	22 (71)		40 (77)	24 (71)	16 (89)	
Never	41 (45)	32 (52)	9 (29)		13 (23)	11 (29)	2 (11)	
Missing	1	1	0		0	0	0	
Work Decision Latitude				0.08				0.26
Unknown	43 (46)	31 (50)	12 (39)		31 (58)	20 (57)	11 (61)	
Never or Sometimes	16 (17)	13 (21)	3 (10)		5 (9)	5 (14)	0 (0)	
Often or Always	34 (37)	18 (29)	16 (52)		17 (32)	10 (29)	7 (39)	
Age group								
30-54	23 (25)	16 (26)	7 (23)	0.65	22 (42)	18 (51)	4 (22)	0.03
55-65	42 (46)	29 (48)	13 (42)		24 (45)	15 (43)	9 (50)	
>65	27 (29)	16 (26)	11 (35)		7 (13)	2 (6)	5 (28)	
Reason for Procedure								
Screening/Symptoms	25 (27)	23 (37)	2 (6)	0.002	53 (100)	35 (100)	18 (100)	1.00
Surveillance	68 (73)	39 (63)	29 (94)		0 (0)	0 (0)	0 (0)	
	Median (25th percentile, 75th percentile)				Median (25th percentile, 75th percentile)			
Lifetime Shiftwork (Years)	1 (0, 10)	1 (0, 10)	0.50 (0, 7)	0.36	6 (2, 17)	10 (3, 18)	3.5 (1, 12)	0.11

Table 6.6. Differences in patient characteristics by case status and site

	Recruitment Site 1				Recruitment Site 2			
	Total population (n = 93)	**Controls[1] (n = 62)**	**Cases (n = 31)**	**p-value[3]**	**Total population (n = 53)**	**Controls[1] (n = 35)**	**Cases (n = 18)**	**p-value[3]**
Variable[2]	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	

[1]No polyps or hyperplastic polyp(s) only; [2] Number of subjects for each variable category may not equal total number of subjects due to missing data; [3]Chi-squared test for differences in proportions or Wilcoxon rank sum test for differences in medians between cases and controls; [4]Three subjects who were in the underweight BMI category (≤18.5 kg/m^2);

Table 6.7. *PER3* VNTR genotype by adenoma status

Genotype	**Controls (n = 97)**	**Cases (n = 49)**	**Crude OR**	**95% CI**	**p-value**	**Adjusted OR[1]**	**95% CI**	**p-value**
	n (%)	n (%)						
4/4	52 (54)	16 (33)	Ref	--	--	Ref	--	--
4/5	38 (39)	24 (49)	2.1	0.9 – 4.4	0.06	2.1	0.9 – 4.8	0.07
5/5	7 (7)	9 (18)	4.2	1.3 – 13.0	0.01	5.1	1.4 – 18.1	0.01
4/5 or 5/5	45 (46)	33 (67)	2.4	1.7 – 4.9	0.02	2.5	1.7 – 5.4	0.02

Abbreviations: OR, Odds ratio; CI, Confidence interval; VNTR: Variable number tandem repeat

[1]Adjusted for: decision latitude at work, recruitment site, procedure reason.

Table 6.8. Relationship between *PER3* VNTR genotype and adenoma status: stratified by procedure reason

		Controls	Cases	Crude OR[1]	95% CI	p-value	Adjusted OR[2]	95% CI	p-value
		n (%)	n (%)						
Screening (n_{cases} = 20; $n_{controls}$ = 58)	4/4	34 (59)	7 (35)	Ref	--	--	Ref	--	--
	4/5	21 (36)	9 (45)	2.1	0.7 – 6.4	0.20	2.2	0.7 – 7.1	0.19
	5/5	3 (5)	4 (20)	6.5	1.2 – 35.6	0.03	10.7	1.4 – 80.7	0.02
	4/5 or 5/5	24 (41)	13 (65)	2.6	0.9 – 7.6	0.07	2.9	0.9 – 8.7	0.07
Surveillance (n_{cases} =29; $n_{controls}$ = 39)	4/4	18 (46)	9 (31)	Ref	--	--	Ref	--	--
	4/5	17 (44)	15 (52)	1.8	0.6 – 5.1	0.29	1.9	0.6 – 5.6	0.29
	5/5	4 (10)	5 (17)	2.5	0.5 – 11.7	0.24	2.3	0.5 – 11.4	0.30
	4/5 or 5/5	21 (54)	20 (69)	1.9	0.7 – 5.2	0.21	2.0	0.7 – 5.6	0.20

Abbreviations: OR, Odds ratio; CI, Confidence interval; VNTR: Variable number tandem repeat

[1] - Unconditional logistic regression with Firth's bias correction; [2] - Adjusted for decision latitude at work, recruitment site.

Table 6.9. Relationship between *PER3* VNTR genotype and adenoma status: stratified by methylation status in *PER1*, *PER2*, and *PER3*

Methylation Marker	Methylation Status	Genotype	Controls n (%)	Cases n (%)	Crude OR[1] (95% CI)	p-value	Adjusted OR[2] (95% CI)	p-value
PER1	Hypomethylation (n_{cases} = 12; $n_{controls}$ = 13)	4/4	8	3	Ref	--	Ref	--
		4/5	4	6	3.51 (0.57 – 21.46)	0.17	2.18 (0.25 – 18.79)	0.48
		5/5	1	3	5.67 (0.48 – 69.20)	0.17	5.90 (0.35 – 98.45)	0.22
		4/5 or 5/5	5	9	4.20 (0.77 – 22.84)	0.10	2.99 (0.40 – 22.38)	0.29
	Hypermethylation (n_{cases} = 19; $n_{controls}$ = 42)	4/4	18	7	Ref	--	Ref	--
		4/5	19	9	1.20 (0.37 – 3.88)	0.76	1.72 (0.48 – 6.11)	0.40
		5/5	5	3	1.57 (0.30 – 8.29)	0.60	1.84 (0.29 – 11.59)	0.52
		4/5 or 5/5	24	12	1.26 (0.42 - 3.81)	0.68	1.75 (0.53 – 5.77)	0.36
PER2	Hypomethylation (n_{cases} = 2; $n_{controls}$ = 5)	4/4	3	1	Ref	--	Ref	--
		4/5	2	1	1.40 (0.06 – 33.29)	0.84	0.62 (0.00 – 220.41)	0.87
		5/5	0	0	9.52 (0.95 – 95.79)	0.06	2.74 (0.59 – 12.60)	0.20
		4/5 or 5/5	2	1	1.40 (0.06 – 33.29)	0.84	0.62 (0.00 – 220.41)	0.87
	Hypermethylation (n_{cases} = 29; $n_{controls}$ = 50)	4/4	23	9	Ref	--	Ref	--
		4/5	21	14	1.67 (0.60 – 4.63)	0.33	1.79 (0.59 – 5.42)	0.30

Table 6.9. Relationship between *PER3* VNTR genotype and adenoma status: stratified by methylation status in *PER1*, *PER2*, and *PER3*

Methylation Marker	Methylation Status	Genotype	Controls **n (%)**	Cases **n (%)**	Crude OR[1] (95% CI)	p-value	Adjusted OR[2] (95% CI)	p-value
		5/5	6	6	2.47 (0.63 – 9.70)	0.19	2.74 (0.59 – 12.61)	0.20
		4/5 or 5/5	27	20	1.84 (0.71 – 4.81)	0.21	2.01 (0.71 – 5.71)	0.19
PER3	Hypomethylation (n_{cases} = 5; $n_{controls}$ = 2)	4/4	1	2	Ref	--	Ref	--
		4/5	1	2	1.00 (0.04 – 27.26)	1.00	0.30 (0.00 – 32.83)	0.62
		5/5	0	1	1.98 (0.01 – 358.88)	0.80	4.34 (0.02 – 1118.84)	
		4/5 or 5/5	1	3	1.40 (0.06 – 33.27)	0.84	1.02 (0.03 – 30.00)	0.99
	Hypermethylation (n_{cases} = 26; $n_{controls}$ = 53)	4/4	25	8	Ref	--	Ref	--
		4/5	22	13	1.80 (0.63 – 5.11)	0.27	1.91 (0.60 – 6.03)	0.27
		5/5	6	5	2.54 (0.61 – 10.54)	0.20	2.34 (0.48 – 11.43)	0.29
		4/5 or 5/5	28	18	1.95 (0.73 – 0.5.22)	0.18	2.02 (0.68 – 5.98)	0.20

Abbreviations: OR, Odds ratio; CI, Confidence interval; VNTR: Variable number tandem repeat

[1] - Unconditional logistic regression with Firth's bias correction; [2] - Adjusted for decision latitude at work, recruitment site, and procedure reason.

Table 6.10. Relationship between *PER3* VNTR genotype and lifetime shiftwork exposure

		Lifetime Shiftwork in Years			
	n (%)	**Median**	**25th percentile**	**75th percentile**	**p-value[1]**
4/4	67 (47)	5	1	15	--
4/5	61 (42)	2	0	10	0.02
5/5	16 (11)	0.75	0	8.5	0.05
4/5 or 5/5	77 (53)	1	0	10	<0.01

[1]Wilcoxon rank sum test for group differences in medians by genotype compared to those with the 4/4 genotype; 2 subjects missing information on lifetime shiftwork; VNTR: Variable number tandem repeat

Table 6.11. *PERIOD* gene expression in human adenomas vs. normal tissue[1]

Referent Tissue	Pathological Tissue Type	*PER1*	p-value	*PER2*	p-value	*PER3*	p-value	Reference
Normal colon epithelium (n=22)	Colorectal adenoma epithelium (n=56)	-1.3	<0.01	-1.2	0.05	N/A	N/A	[336]
Normal colon (n=32)	Colon adenoma (n=25)	-1.7	<0.01	-1.2	<0.01	-2.3	<0.01	[337]
Normal colon (n=32)	Rectal adenoma (n=7)	-1.9	<0.01	1.3	0.88	-1.7	<0.01	[337]
Normal colon (n=10)	Colon adenoma (n=5)	-1.0	0.37	1.6	0.99	-2.1	<0.01	[338]
Normal colon epithelium (n=10)	Colorectal adenoma epithelium (n=5)	-1.2	0.05	1.2	0.94	-1.7	<0.01	[338]

[1]Fold change in mRNA expression in adenomas relative to adjacent normal tissue (number of tissue samples evaluated in parentheses). Source: www.oncomine.com

Chapter 7

Conclusion

This dissertation project aimed to accomplish two goals: 1) understand the scope of sleep disorders in a national sample, and 2) elucidate the genetic and epigenetic mechanisms of clock genes and their role in adenoma formation, a precursor lesion for CRC. Sleep disruption has been previously linked to increased cancer risk, potentially through circadian rhythm disruption; therefore it is necessary to characterize populations at risk (e.g., shift workers, people with sleep disorders).

The first project aimed at characterizing sleep disorders over time in a national sample of veterans. This project found that the prevalence of sleep disorders have increased nearly six-fold from FY2000 to FY2010. As expected, apneas were the most commonly-diagnosed sleep disorder, followed by insomnia. However, it is not known whether or not this increase in sleep disorders were a true increase due to certain factors (increasing trends in obesity, PTSD, etc.), increased awareness, or a combination of both. In this national sample of veterans, PTSD tripled over time; and those with PTSD had the highest prevalence of sleep disorders (16%). Increase in awareness could also be attributed to the surge in sleep disorder prevalence, as a national sleep medicine training and certification program was initiated in 2004-2005.[350] Using

this national sample, etiological models for sleep disorders were also created, as there is a dearth in the literature on risk factors for sleep disorders, while simultaneously accounting for other factors. Pre-existing history of PTSD and being obese resulted in a more than doubling of odds of developing a sleep disorder in FY2010. Future research will need to be conducted in large, national samples to confirm findings in the present studies.

Because people with sleep disorders may have genetic alterations or epigenetic changes that could increase their risk for cancer, the second part of this dissertation examined relationships in a set of genes involved in the regulation of circadian rhythms. Circadian rhythms are established in the central pacemaker, located in the SCN of the anterior hypothalamus, via photic input. Nine core clock genes have been found to play a role in circadian oscillation: *PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, TIM,* and *CK1ε*.[33] ~2-10% of the diurnal gene expression in most tissues is controlled by this set of genes.[89] In particular, the *Period (Per)* gene family plays a role in immunomodulation[20-25] and tumor suppression.[26, 27] However, environmental (e.g., shiftwork) [91-93] and/or genetic factors (genetic polymorphisms or epigenetic modifications)[30, 94] can confuse the master clock, affecting physiological processes involved in tumorigenesis, such as cell proliferation and apoptosis.[95] Disruption of these processes has been suspected of increasing the risk of cancer in certain populations (e.g., shiftworkers),[96] and these effects have been confirmed in animal model studies.[97] Previous research found that genetic polymorphisms,[19, 28, 29] variable expression in tumor tissues,[30-32] and aberrant methylation[33] within

the *Per* genes have shown some promise as novel biomarkers of cancer, which warranted the second project.

In a case-control pilot study where the methylation status of 20 candidate markers in PBLs were examined for their relationships with adenoma formation, individuals with hypomethylation in the promoters of three markers (*PER1*, *PER3*, and *MINT1*) were 3 to 11 times more likely to have adenomas compared to adenoma-free controls, after controlling for multiple confounders. Although aberrant methylation of *PER* genes has been observed among leukemia and other cancer patients,[30, 33, 113, 114, 286] little is currently known about the *MINT1* locus, and all three markers had not been previously examined in PBLs in relation to adenoma formation. It is suspected that clock-controlled genes, which include known tumor suppressors (e.g., p21, Chk2, XPA, ATM) and oncogenes (e.g., β-catenin, c-Myc, WEE-1),[276] may be dysregulated, thus potentially promoting a carcinogenic microenvironment.

In addition to examining epigenetic variation and adenoma risk, an additional study of the second project was undertaken, specifically focusing on the *PER3* VNTR, a tandem repeat that contains four or five copies of a 54-bp sequence encoding 18 amino acids, with the 5-repeat variant adding several phosphorylation motifs. In this study, subjects with adenomas were anywhere from 2 to 5 times more likely to possess at least one 5-repeat variant compared to controls. This association may be partially mediated by alterations in circadian processes (i.e., delayed sleep phase syndrome, cognitive impairment after sleep

deprivation, morning circadian preference, and differences in the timing or levels of circadian hormone secretion)[40, 117, 313] among those with at least one 5-variant repeat. Although this finding was not consistent with a previous study that examined *PER3* VNTR polymorphisms and CRC,[351] a prior study has observed an association between the 5-repeat variant and premenopausal breast cancer,[19] suggesting that *PER3* may act as a tumor suppressor; however, more studies will need to confirm findings observed in this study.

In closing, although it is unclear as to why sleep disorder prevalence is increasing over time, it is clear that a subset of the population may be increasing their risk for adverse health events, including cancer, and genetic and/or epigenetic mechanisms may be partially mediating this pathway. Elucidation of these mechanisms may not only allow for understanding the association between circadian disturbances and cancer, but to also develop novel biomarkers from readily-accessible sources, such as blood.

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Best regards,

Andy

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