Essential Nutrients for Serene Nights: Exploring the Role of Iron and Vitamin D on Sleep Across Life Stages and Demographics

by

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Dedication

In loving memory of my grandfather, Salim Al Hinai, who departed during the early stages of my PhD journey. His lessons of resilience, hard work, and compassion resonate throughout these pages as a tribute to his enduring spirit.

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Abstract

Vitamin D deficiency and iron deficiency are the most common nutritional deficiencies worldwide, posing significant health challenges across diverse populations. Suboptimal levels of these essential micronutrients have been implicated in a myriad of adverse health outcomes, including compromised sleep health. The primary objective of this dissertation is to provide a comprehensive understanding of the relationships between iron, vitamin D, and sleep health. Aiming to fill critical gaps in existing literature and offer a holistic perspective, this dissertation explores the sex and racial variations in these associations at different life stages, encompassing adolescence (aim 1), reproductive stages among women (aim 2), and older adulthood (aim 3).

For aim 1, we used data from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohort to examine the associations of serum ferritin with both objective sleep measures and the DNA methylation of key circadian genes during adolescence. Results showed sex-specific associations, indicating that lower ferritin levels were associated with higher sleep fragmentation among females, whereas males exhibited delayed sleep timing. There was no difference in sleep duration or sleep movement index among adolescents with lower ferritin, compared to those with higher levels. Beyond sleep patterns, this study uncovers significant associations between ferritin and DNA methylation in some circadian genes, providing valuable insights into the role of iron in the epigenetic regulation of sleep/circadian rhythms during adolescence.

For aim 2, to examine the effect of vitamin D and iron deficiencies, we analyzed a nationally representative samples of women of reproductive age enrolled in the National Health and Nutritional Examination Survey (NHANES) from the 2005 to 2018 cycles. We found that iron deficiency and iron deficiency anemia were associated with poor sleep quality, whereas vitamin D deficiency and vitamin D inadequacy were associated with short sleep duration. Moreover, poor sleep quality may be exacerbated when individuals simultaneously experience both iron and vitamin D deficiencies, with a potential synergistic interaction of iron and vitamin D deficiency that warrants further assessments.

For aim 3, using data from the Health and Retirement Study (HRS), we investigated the sex and racial variations in the longitudinal association between iron and vitamin D with sleep disturbance in older adults. In addition, we explored the potential mediating role of sleep in the association between these micronutrients and cognitive function. Our findings identified the protective associations of vitamin D3 against sleep disturbance in White and Black individuals, with the strongest association observed among White males. Conversely, higher levels of vitamin D2 increased the risk of sleep disturbance among White individuals, both males and females, while paradoxically presenting a protective association among Black males. Notably, we found evidence that sleep may partially mediate the association between vitamin D3 and cognitive function in older Black females.

Overall, our findings underscore the significance of iron and vitamin D in sleep across the lifespan, yet additional research is needed to understand the mechanism by which these micronutrients affect sleep in different populations.

Chapter 1 Introduction

Sleep across the lifespan

Sleep plays a central role in human development and maintenance of mental and physical health. It has been hypothesized that sleep improves learning, memory, and brain plasticity, restores energy and macromolecular synthesis, and is involved in neural detoxification (1). Although the recommendations for sleep duration associated with optimal health are controversial, it is widely acknowledged that sleep patterns change across the lifespan, with an inverse relationship with age (2). Based on these changes, the National Sleep Foundations has established age-specific sleep duration recommendations that includes 9-10 hours/day for adolescents, 7-9 hours/day for young adults, and 7-8 hours/day for older adults (3). Unfortunately, most people do not meet these recommendations. The sleep trends from self-reported sleep duration data show a steady decrease in the past two decades, with 34.8% of adults reporting short sleep duration (< 7 hours/day) in 2020 (4,5). Short sleep duration is a major public health concern. A plethora of studies have shown that short sleep duration is independently and significantly associated with increased risk of all-cause mortality, obesity, hypertension, cardiovascular diseases, stroke, diabetes mellitus, and mood disorders such as depression and anxiety (6–8). Likewise, long sleep duration is associated with adverse health outcomes suggesting that the relationship between sleep duration and health outcomes is U-shaped (9,10). Nevertheless, sleep duration is only one piece of the puzzle. In fact, healthy sleep is a multidimensional concept that includes sleep duration, quality, efficiency, timing, and alertness (11). Although these dimensions are correlated, epidemiological evidence has

suggested that each sleep dimension is independently associated with certain health outcomes. For instance, it has been shown that sleep quality is associated with higher odds of cardiovascular risk and obesity, an association that significantly persists even after adjusting for sleep duration (12). Moreover, as Chandola and colleagues reported from a large longitudinal cohort study, the effect of short sleep duration on increasing coronary heart disease risk is stronger among subjects with poor sleep quality (13). Therefore, considering the different dimensions of sleep health is warranted.

Sleep dimensions vary across age, sex, and race/ethnicity groups. Of particular interest is the transition of sleep pattern from childhood to adolescence. National and international evaluation of sleep patterns using both subjective and objective sleep measurements provide evidence that sleep worsens during puberty as indicated by dramatic shifts in sleep onset, a linear decline in sleep duration and efficiency, and more night sleep variability (14–16). According to the National Sleep Foundation, about 45% of adolescents reported sleeping less than recommended during school nights, with higher prevalence of sleep insufficiency among older adolescents (17). Sleep insufficiency is defined by International Classification of Sleep Disorders (ICSD-3) as "a curtailed sleep pattern that has persisted for at least three months for most days of the week, along with complaints of sleepiness during the day" (18). Several biological and environmental factors such as adolescent growth spurts, school and extracurricular activities, electronic devices, part-time jobs, and consumption of caffeinated beverages, all contribute to the shorter sleep duration and the shifts in the sleep-wake cycle seen during adolescence (19,20). Sleep insufficiency and delayed sleep timing are particularly alarming in this period, not only because of the associated adverse metabolic outcomes, but also the negative cognitive and

behavioral consequences such as low academic performance, mood disturbance, impaired memory and attention, depression, drowsy driving, and substance use (21).

In addition, adolescence is a period of emerging sleep sex differences. Males and females tend to have a similar sleep pattern until puberty, where sex differences start to become more pronounced, as females frequently report sleeping less, having poorer sleep quality and efficiency, and difficulties falling asleep (22–24). In general, women are more prone to sleep disturbance as their sleep can be influenced by hormonal changes, menstrual cycle, pregnancy, and menopause (22,25). For instance, Sharkey and colleagues reported a positive association between the rise in progesterone level and sleep fragmentations observed during the menstrual cycle in women at childbearing age (26). Moreover, the differences in sleep interruption among women may be explained by the variability in level of progesterone rise during the menstrual cycle (26). In addition, cyclic change in ovarian hormones is associated with premenstrual syndrome that can cause mood disturbances, which may interfere with women's sleep. Indeed, sleep disorders such as insomnia and restless leg syndrome are more common among females, which could be related to the higher prevalence of depression and anxiety in females (27,28). Animal studies in rats show that unlike males, females' sleep-wake cycle is more sensitive to changes in sex hormones. Interestingly, castration in male rats does not significantly change their sleep-wake cycle; however, ovariectomized rats show a reduction or elimination of the sleep-wake cycle differences between males and females (29,30). The exogenous replacement of steroid hormones restores these differences (29,30). Another contributing factor is circadian timing. The timing of the circadian rhythms may differ by sex with women showing an earlier chronotype, as they tend to go to bed earlier and wake up earlier than men (31). A polysomnography study compared the diurnal and circadian variation of sleep-wake cycle

between men and women, controlling for oral contraceptive use and menstrual cycle phase (32). The results indicated that women have advanced core body temperature rhythms and subjective sleep alertness, suggesting that the higher complaints of sleep disturbance in women is related to the variation in the circadian rhythm between the sexes (32). Similarly, Duffy and colleagues found that the intrinsic circadian period that drives melatonin peak and core body temperature rhythms was significantly shorter in healthy women than healthy men (33). Paradoxically, accelerometer data does not always support the subjective evidence of women having worse sleep health than men. The objective assessment of sleep indicates that females sleep longer, have earlier midpoint sleep, and sleep more efficiently than their male counterparts (15,24). It is not clear what is causing these contradictory results, but it could be related to methodological issues such as study design, sample size, variability in study populations, and confounding bias. Thus, further investigations of sex differences in sleep are necessary.

As with sex, a paradox exists between subjective and objective sleep data in older adults, but in opposite directions. Along with many physiological and psychological changes that occur with aging, sleep health changes as well. Sleep in older adults is characterized by earlier sleep timing, longer sleep-onset, shorter night sleep duration, more daytime naps, more sleep disturbances, and lower ability to maintain sleep (34). Although polysomnography data show that older adults' sleep worsens with age, they are less likely to report or complain about their sleep problems compared to younger adults (35,36). This could be related to the alterations in the perception of "acceptable health status" with aging, as Brouwer and colleagues found that the acceptability of health problems increases with age (37). Therefore, older adults perceive their sleep problems as a part of normal aging and are thus less likely to report sleep health problems. Moreover, many factors influence older adults' sleep disturbance including pain, health

conditions and comorbidities, hormonal changes, depression, loneliness, and quality of life (35). Consequently, poor sleep in the elderly can lead to significant cognitive impairments and dementia (38,39). A recent systematic review and meta-analysis of case–control studies that objectively measured sleep in older adults with mild cognitive impairment (MCI) and age-matched healthy controls found that disruption of sleep quality and quantity in elderly with MCI can further exacerbate cognitive decline and increase risk of dementia, beyond the cognitive decline occurring with normal aging (40). Given the rapid growth of the elderly population and the absence of treatments or cures for cognitive decline, understanding modifiable risk factors such as sleep has the potential to improve or reduce the risk of cognitive impairment and dementia in older adults.

Another factor that may affect sleep is race/ethnicity. The National Longitudinal Study of Adolescent to Adult Health, a large ongoing longitudinal study of a nationally representative sample of US adolescents followed up since 7-12 grades, reveals that sleep duration significantly differs by race/ethnicity across the three developmental period (13-18 years adolescence, 19-22 years emerging adulthood, and 23-32 years early adulthood) (16). White and Hispanic participants consistently reported longer sleep duration compared to African Americans and individuals of other race/ethnicities across the three developmental periods (16). In a review of the literature, Johnson and colleagues reported that in general Black, Hispanic/Latino, Asian, Native Hawaiian/Pacific Islander, and American Indian/Alaska Native have shorter sleep duration compared to non-Hispanic White adults (41). In 2020, the crude prevalence of short sleep duration was highest among non-Hispanic Native Hawaiian and Other Pacific Islander (47.0%), and non-Hispanic Black or African American (43.5%) (5). However, the evidence regarding other sleep health dimensions is inconsistent. Moreover, racial/ethnic minorities are

more likely to experience health disparities, given their limited access to health promoting services and higher exposure to disadvantaged physical and social environments, which may affect their sleep (41). Collectively, differences in sleep by age, sex, and race/ethnicity exist, and accounting for these differences is important when examining the association between sleep and health. Given the physiological and psychological consequences of poor sleep, understanding the risk factors that contribute to poor sleep health such as diet is warranted.

Iron and Sleep

Iron is vital for the proper production of hemoglobin, myoglobin, and iron-containing enzymes that are involved in energy production and DNA synthesis (42). The staggering prevalence of anemia, primarily attributable to iron deficiency (ID), exerts a profound impact on a global scale, impacting approximately 2.2 billion individuals worldwide (43). Untreated ID with or without anemia can affect physical performance, work productivity, and cognitive function, and can increase the risk of morbidity and mortality in mothers and infants as well as people with chronic inflammatory diseases (44,45). Aside from these consequences, animal and epidemiological studies have proposed ID and iron deficiency anemia (IDA) as potential risk factors for poor sleep health. An experimental study in male juvenile monkeys investigated the effect of prenatal ID and monoamine oxidase A (MAOA) genotype on sleep patterns (46). At the age of two years, monkeys with prenatal ID and low-MAOA showed higher night sleep fragmentation and higher sleepiness during the day compared to monkeys with prenatal iron sufficiency and low-MAOA (46). Likewise, postnatal dietary iron deprivation in mice showed a marked increase in time spent awake in the dark period with reduction in both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep, compared to controls fed an adequate iron diet (47). In humans, analysis of NHANES data from 2005 to 2016 showed that short sleep

duration was associated with inadequacy of several dietary nutrients, including iron (48). However, assessing dietary iron intake does not reflect iron status in the body due to low bioavailability; thus, biomarkers such as ferritin and transferrin receptors adjusted for inflammation are more accurate measures for iron status than iron intake.

Assessing iron status using blood biomarkers showed that ID and IDA are frequently associated with sleep disorders such as restless leg syndrome (RLS), periodic limb movements, and sleep disordered breathing as well as with sleep disturbances observed in children with attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) (49). Allen and colleagues estimated that one in four patients with IDA suffer from clinically significant RLS symptoms that cause shorter sleep duration and poorer sleep quality (50). Moreover, a growing body of evidence suggests that iron therapy may be effective in improving sleep disorder symptoms and be particularly beneficial for RLS (49). A randomized, double-blind, placebo-controlled study in adults with RLS and low to normal ferritin levels (15–75 µg/l) found a significant improvement in RLS symptoms in the treatment group (325 mg ferrous sulfate twice daily for 12 weeks) compared to controls (51). However, oral iron treatment found no effect in improving sleep in adults with higher ferritin levels (> 75 µg/l) (52). Even with adequate peripheral iron stores, it has been found that patients with RLS have lower brain iron compared to normal controls, a finding that might help in better understanding the mechanism underlying the role of ID/IDA in RLS (53).

Although the link between RLS and ID/IDA is well-established, limited research has been conducted in healthy individuals with most of the investigations focusing on infancy and early childhood. Analysis of sleeping patterns using electroencephalographic (EEG) in six-month-old infants with IDA showed alteration in sleep spindle patterns, a key characteristic of

NREM sleep stages that are involved in memory consolidation and normal brain function (54). Moreover, a longitudinal small cohort study assessing sleep patterns in infants at 6, 12, and 18 months of age revealed that IDA infants fell asleep faster at night but spent more time awake and less time in quiet sleep compared to non-IDA infants; however, these differences disappeared after iron treatment (55). Subjective assessment of sleep showed similar results in infants. Kordas and colleagues assessed maternal reports of sleep in 6–18 months old infants and found that infants with IDA had higher night waking and shorter total sleep duration relative to infants without IDA (56). In addition, sleep improved in the infants group randomized to 12 months of iron-folic acid supplements compared to the control group, suggesting that iron and/or folate can improve sleep in infants with ID and IDA (57). However, a follow-up study found that former IDA infants may have long-term changes in the sleep architecture at four-years of age regardless of the IDA treatment in infancy and absence of anemia at age of four (58). A longer follow-up period up to ten years showed no statistical differences on overall sleep between former ID children and age-matched controls, although former ID had slightly higher but significant limb movement compared to controls (59). Nevertheless, a case-control study in adolescents 15-16 years of age shows that adolescents with former IDA in infancy had significantly shorter REM latency compared to healthy controls (60). Given the contradicting results and the methodologic limitations including study design and small sample size, it is not clear yet if ID and IDA early in life can have a persistent effect on sleep into childhood and adolescence.

In adults, large cross-sectional studies have shown that short sleep and sleep disturbances were associated with lower hemoglobin levels, and the odds of having anemia was higher among adults with sleep problems (61,62). However, given the cross-sectional design, it is uncertain whether poor sleep is a risk factor for anemia or a consequence of anemia. Moreover, these

studies did not differentiate between IDA and other types of anemia, though it is estimated that 50% of anemia cases are related to ID. Only one study specifically examined the effect of IDA on sleep in adults clinically diagnosed with IDA but healthy otherwise and found that adults with IDA reported worse sleep quality compared to age- and gender-matched controls (63). Moreover, the authors concluded that IDA affects sleep quality regardless of depression and anxiety, given that anxiety and depression were weakly and inversely correlated with poor sleep. However, they failed to account for important confounders that may affect the association between IDA and sleep. Moreover, they did not find association between blood biomarkers of IDA such as hemoglobin and ferritin with sleep quality (63). Additionally, the controls were healthy volunteers who were regular blood donors, which could cause selection bias as frequent blood donation may be associated with IDA (63). Taken together, more research is warranted to assess the relationship between sleep and suboptimal iron status in otherwise healthy individuals.

Vitamin D and Sleep

Vitamin D, the sunshine vitamin, is a fat-soluble micronutrient that plays a crucial role in supporting bone health and immune function. Vitamin D deficiency (VDD; <12 ng/mL) and vitamin D insufficiency (VDI; <20 ng/mL) are highly prevalent, affecting an estimated 1 billion people worldwide (64). The implication of VDD and VDI extend across a spectrum of chronic and acute health conditions, including mortality, cardiovascular disease, diabetes, cancer, lung diseases, musculoskeletal diseases, and depression (65). In addition, low vitamin D levels have been associated with compromised sleep health.

An analysis of NHANES data found that low vitamin D intake from both food and supplements was associated with short sleep duration (66). Moreover, a higher intake of vitamin D was associated with less difficulty maintaining sleep (67). These results are supported by

studies using serum 25-hydroxyvitamin D, the most reliable biomarker for vitamin D status. A meta-analysis of observational studies suggested that VDD is associated with short sleep duration, poor sleep quality, and daytime sleepiness (68). Similar results are observed across various age groups. In children and adolescents, both VDD and VDI are associated with short sleep duration, poor sleep efficiency, delayed sleep timing, and excessive daytime sleepiness (69–71). In adults, a large cross-sectional study of 21,083 individuals revealed that subjects with insufficient serum vitamin D levels had significantly shorter sleep duration compared to subjects with sufficient vitamin D levels (72). In addition, sufficient vitamin D levels were associated with lower odds of insomnia, in females only (72). In community-dwelling men aged 65 or older, Massa and colleagues examined the relationship between serum vitamin D and sleep actigraphy data including sleep duration, wake time after sleep onset, and sleep efficiency. The results revealed that low levels of serum vitamin D were associated with shorter sleep duration and poorer sleep efficiency (73).

Research also suggests that low levels of vitamin D may increase the risk of sleep disorders such as obstructive sleep apnea syndrome, insomnia, and restless leg syndrome (74). A polysomnography study showed that VDD was more prevalent among patients with obstructive sleep apnea syndrome (75). Furthermore, patients with severe sleep apnea symptoms were two times more likely to have VDD (75). Likewise, patients with severe RLS symptoms had significantly lower serum vitamin D levels compared to patients with moderate RLS symptoms (76). Remarkably, several studies have shown that vitamin D treatment can improve sleep disorders symptoms.

In a double-blind controlled trial, participants aged 20-50 years with sleep disorders were randomized to receive either a placebo or 50,000 IU vitamin D for eight weeks (77). The authors

reported a significant improvement in sleep duration, sleep quality, and sleep latency in the group receiving vitamin D treatment. Moreover, providing a similar dose of vitamin D supplements for nine weeks improved daytime sleepiness, insomnia symptoms, and cognitive abilities in adolescent girls (78). VDD treatment through food fortification has also been shown to be effective. A parallel blind randomized controlled clinical trial examined the efficacy of low-fat milk and yogurt fortified by 1,500 IU vitamin D3 in reducing insomnia symptoms in middle-aged adults with abdominal obesity (79). The results showed that vitamin D fortification significantly improved insomnia symptoms in the intervention group (79). However, clinical trials in people without sleep disorders are scarce and the results from available trials are inconsistent. Larsen and colleagues reported no significant effect of vitamin D supplementation on sleep duration and daytime sleepiness in individuals with vitamin D insufficiency (defined as <42 nmol/L) (80). Another randomized controlled trial showed that randomization of overweight postmenopausal women to either 12 months of weight loss and 2000 IU oral vitamin D3 or 12 months of weight loss and placebo resulted in worse sleep quality in the intervention group (81). The inconsistent results from randomized controlled trials warrant further assessment. Furthermore, most of the studies that link vitamin D with sleep are cross-sectional studies, and hence unable to establish temporality between vitamin D levels and sleep. Therefore, there is a need for more robustly designed, rigorous longitudinal studies to unravel the relationship between vitamin D and sleep across the lifespan.

Potential Mechanisms

Although the underlying mechanisms that link iron and vitamin D with sleep are not well understood, several hypothetical pathways could explain this link. Sleep is regulated independently by homeostatic (process S) and circadian rhythm (process C), yet these two

processes can influence each other. In process S, sleep-inducing substances (e.g., adenosine) build up in the brain during wakefulness which increase sleep pressure and the need to sleep (82). In process C, circadian rhythm is generated by a transcription-translation negative feedback loop of a set of circadian genes located in the suprachiasmatic nucleus (SCN) in the hypothalamus (82). Circadian rhythm is synchronized by zeitgebers, external environmental cues such as light, temperature, eating patterns, and exercise. For instance, in response to light, *BMAL1* and *CLOCK* in the cytoplasm form BMAL1: CLOCK heterodimer, which enters the nucleus and binds E-box, a specific DNA element in the target genes including cryptochrome genes (*CRY1* and *CRY2*), period genes (*PER1, PER2*, and *PER3*), as well as nuclear receptor *RAR* and *REV-ERB*α (83). This process activates the expression of these genes, and their proteins start to accumulate in the cytoplasm. As these proteins accumulate, *PER* and *CRY* proteins form PER: CRY heterodimer, which relocates into the nucleus and suppresses their transcription by inhibiting BMAL1: CLOCK activity (83). Moreover, *BMAL1* transcription is regulated by *RORα* (activator) and *REV-ERB*α (repressor) that compete to bind to their shared DNA element in the Bmal1 promoter (83). Suppression of *BMAL1* transcription by *REV-ERB*α decreases *BMAL1* protein available for dimerization with Clock protein, thus decreasing the transcription of downstream circadian genes, whereas the opposite occurs when *BMAL1* transcription is activated by *RORα*. Several studies have found that suboptimal levels of iron and vitamin D can alter the regulation and expression of these genes (84–86).

Vitamin D has been shown to be involved in the regulation of circadian genes. Gutierrez-Monreal and colleagues found that the active form of vitamin D synchronizes *BMAL1* and *PER2* expression in adipose-derived stem cells (87) In rats, VDD significantly increased the expression of *PER2* (88). In contrast, vitamin D3 treatment significantly decreased the expression of *PER2*

(89). Moreover, *CLOCK* gene has been shown to interact with the vitamin D receptors (90). As for iron, animal studies provide evidence that heme, an iron-containing compound, is a vital ligand for nuclear hormone receptors *REV-ERB*α that are involved in suppression of *BMAL1* (91). An *in vitro* study showed that disruption of iron status altered the circadian rhythm and circadian gene expressions, as low iron status caused less production of heme, which leads to less heme binding with *REV-ERB*α, thus increasing the activation of *BMAL1* expression by *RORα*, and consequently increasing BMAL1: CLOCK heterodimers and subsequent circadian gene expression (92). In animal studies, both lower and higher iron status caused lower circadian gene expression of *CRY1, CRY2, PER1, PER2*, and *BMAL1* in the liver (93). Furthermore, feeding iron-deficient piglets a low-iron diet can lead to higher expression of *CRY1, CRY2,* and *PER2* genes in the duodenum (94). While circadian genes expression was measured in different tissues in these studies, it is conceivable that similar effects could be observed in the hypothalamus where sleep is regulated.

Another pathway that links iron and vitamin D with sleep is through neurotransmitters involved in the sleep-wake cycle. Vitamin D is involved in the synthesis of melatonin, a hormone secreted by the pineal gland to regulate the sleep-wake cycle where it peaks at night and decreases during the day (95). Both iron and vitamin D are vital in the synthesis of serotonin, a precursor of melatonin (82,95). Moreover, iron is a co-factor for tyrosine hydroxylase, a rate-limiting enzyme in the production of dopamine that is involved in the regulation of the sleep-wake cycle (96). Emerging evidence in RLS pathophysiology suggests that low iron status may lead to dopamine abnormalities and consequently to RLS (97). In addition, ID and IDA may influence adenosine that acts as a neuromodulator for sleep as it accumulates during wakefulness, increasing sleep propensity, and recedes with sleep. Animal models of RLS have

found that the brain ID can downregulate adenosine receptors in the cortex and hypothalamus, inducing a hypoadenosinergic state that causes hyperarousal and sleep disturbances (96,98).

The effect of low iron and vitamin D levels on neurotransmitters can not only lead to sleep disturbances but o cognitive impairments and dementia. A growing body of evidence shows that deficiencies in iron and vitamin D have a significant influence on cognitive development (99,100). A longitudinal study found that higher vitamin D3 levels in the brain was associated with better cognitive function in older adults (101). On the other hand, the relation between iron and cognition is U-shaped, as both low and high iron status can impair cognitive function (102,103). Likewise, both short and long sleep duration are associated with global cognitive decline in older adults. The joint effect of suboptimal micronutrients status and poor sleep health can have a detrimental impact in cognitive function in older adults. Xie and colleagues reported that sufficient levels of vitamin D3 were associated with 33% reduction in the risk of cognitive impairment, whereas good sleep quality was associated with 34% reduction in the risk of cognitive impairments in older adults (104). Furthermore, older adults with VDD and shorter sleep duration have higher odds of cognitive impairments compared to older adults with VDD and normal sleep duration (104). These interrelated associations between vitamin D, sleep, and cognitive function in older adults warrant further assessment, particularly regarding the potential pathways through which these factors interplay. Thus, in our study, we hypothesized that sleep may partially mediate the association between iron and vitamin D and cognitive function in older adults. To our knowledge, only one human study has attempted to examine this hypothesis but did not find supportive evidence for the mediation effect of sleep (105). However, the cross-sectional design and the small sample size of that study may have

affected the results, highlighting the need for further, well-designed studies to provide more conclusive insights.

Specific Aims

The overarching objective of this dissertation is, therefore, to provide a comprehensive understanding of the relationships between iron, vitamin D, and sleep across the lifespan.

Aim 1: to evaluate the longitudinal sex-specific associations between iron, measured by serum ferritin, and objective sleep outcomes (sleep duration, sleep midpoint, fragmentation index, and movement index) in a cohort of healthy adolescents enrolled in the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study. In addition, we examine the relationship between iron and blood leukocyte DNA methylation of selected circadian genes.

Aim 2: to investigate whether micronutrient deficiencies (iron deficiency, iron deficiency anemia, vitamin D deficiency, and vitamin D inadequacy) are associated with self-reported sleep measures (sleep duration, sleep latency, and sleep quality) in a nationally representative sample of women of reproductive age using data from the National Health and Nutrition Examination Survey (NHANES).

Aim 3: to examine the sex and racial variations in the longitudinal association between iron and vitamin D with sleep disturbance in a nationally representative sample of older adults using data from the Health and Retirement Study (HRS). Additionally, we evaluate whether sleep disturbance mediates the association between iron, vitamin D, and cognitive function.

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Chapter 2 Sex-Specific Associations Between Serum Ferritin, Sleep, and Blood Leukocyte DNA Methylation of Circadian Genes During Adolescence

Abstract

Background: Social jetlag and the natural delayed sleep shift in adolescents are associated with many adverse metabolic and behavioral outcomes. Previous studies suggest that iron may affect sleep and epigenetic regulation of circadian genes; however, studies in humans are scarce. This study aims first, to evaluate the longitudinal sex-specific association between iron status and objective sleep outcomes; second, to explore the cross-sectional relationship between iron status and blood leukocyte DNA methylation of key circadian genes during adolescence. **Methods:** This study includes 371 adolescents from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohort. Iron status was estimated using serum ferritin levels measured at baseline and were categorized into tertiles. Sleep duration, sleep midpoint, sleep fragmentation, and movement index during both weekdays and weekends were measured objectively using 7-day wrist actigraphy collected at two timepoints, two years apart. The Infinium MethylationEPIC BeadChip was used to measure DNA methylation of circadian genes at baseline, and data was extracted from 525 CpG sites within 12 key circadian genes. Sex-stratified linear mixed-effects regression analyses with random intercepts were used to assess the relationship between ferritin tertiles and each of the weekday and weekend sleep outcomes, adjusted for age, puberty, household socioeconomic status, body mass index z-score, smoking, alcohol, physical activity, and sedentary time. The association between ferritin tertiles and DNA methylation was evaluated by linear regression with further adjustment for batch effect and cell types. The Cauchy

combination test was used to combine results from individual CpG sites for each gene. False discovery rate was used to account for multiple comparisons. **Results:** Females with ferritin levels in the first tertile had significantly 1.35% (95% CI: 0.21, 2.50) higher sleep fragmentation during weekdays compared to females with ferritin levels in the third tertile; in contrast, males with ferritin levels in the first tertile had later sleep timing during the weekends compared to males with ferritin levels in the third tertile (β: 0.59 decimal hour, 95% CI: 0.05, 1.13). No significant associations between ferritin tertiles and sleep duration or movement index were observed in the adjusted models. Among females, ferritin levels in the first tertile were associated with DNA methylation in *CLOCK*, *PER1*, and *RORC* (FDR= 0.0392). No significant associations were observed between ferritin tertiles and DNA methylation in circadian genes among males. **Conclusion:** Differences in ferritin levels are associated with sleep fragmentation and sleep timing among adolescents. Additionally, these differences are linked to epigenetic modifications in some circadian genes, especially in females.

Introduction

Adolescence is a critical period of rapid physical and physiological development. During this period, the circadian rhythm, the body's internal biological clock, also undergoes significant changes characterized by a natural shift in the timing of sleep onset and wakefulness, which can cause sleep deprivation and disturbance. Along with this natural shift in the biological clock, social jetlag, the mismatch between sleep patterns during weekdays and weekends, can lead to adverse outcomes such as metabolic diseases, cognitive problems, mood disorders, substance misuse, and behavioral outcomes (1,2). Understanding the genetic and environmental factors that regulate the circadian rhythm during adolescence are essential for addressing the unique sleep needs and challenges associated with the circadian rhythm disruptions.

The circadian rhythm is generated internally by a transcription-translation negative feedback loop of a set of circadian genes that is synchronized by zeitgebers, external environmental cues such as light, temperature, eating patterns, sleep timing, and exercise (3). For instance, during the day, the positive transcription factors of the circadian rhythm, *BMAL1* and *CLOCK* genes, activate the expression of the negative arm of the feedback loop including cryptochrome genes (*CRY1* and *CRY2*) and period genes (*PER1, PER2,* and *PER3*), as well as nuclear receptor genes *ROR and REVERB* (aka *NR1D*) (4). During the night, PER:CRY heterodimers inhibit *BMAL1* and *CLOCK* expression. Moreover, *BMAL1* transcription is regulated by *ROR* (activator) and *REVERB* (repressor) (4). Disruption of this exquisite feedback cycle (i.e., dysregulation of genes in this pathway) by environmental factors or nutritional imbalance can lead to sleep dysregulation.

A growing body of evidence suggests that the disruption of iron status alters the circadian rhythm and circadian gene expression, consequently affecting sleep health. Heme, an iron-containing compound, is a vital ligand for the nuclear receptor *REVERBα* that is involved in the suppression of *BMAL1* (5). An *in vitro* study showed that low iron status results in reduced heme availability, leading to decreased heme binding with *REVERBα* (6). This in turn may increase the activation of *BMAL1* expression by *RORα*, consequently increasing BMAL1:CLOCK heterodimers and the transcription of downstream circadian genes (6). Furthermore, iron can affect epigenetic regulation, specifically through DNA methylation, which involves the addition of methyl groups to DNA molecules, affecting gene expression without altering the underlying DNA sequence. Schachtschneider et al. (2016) reported that neonatal iron deficiency (ID) in pigs can alter DNA methylation and gene regulation in the hippocampus, including regulation of hypoxia, angiogenesis, permeability of blood-brain barrier, and cognitive function (7). While no previous studies have examined the role of iron status in DNA methylation of circadian genes, it is plausible to consider that a

similar mechanism may occur in the suprachiasmatic nucleus in the hypothalamus, where sleep is regulated by circadian genes. Hence, we embarked on an exploratory examination, hypothesizing that iron status may affect DNA methylation of circadian genes, potentially leading to sleep dysregulation in adolescence. Prior investigations exploring the relationship between iron status and sleep have predominantly focused on infancy and young childhood or subjects with sleep disorders such as restless leg syndrome (RLS). Moreover, known differences in sleep patterns and iron requirements between sexes during adolescence merit investigation. Therefore, to mitigate the gap in comprehending the role of iron in sleep and circadian rhythm during this sensitive period of growth and maturation, this study aims to first, investigate the longitudinal sex-specific associations between iron status, measured by serum ferritin, and sleep in adolescents; second, to explore the cross-sectional associations between iron status and DNA methylation of key circadian genes.

Methods

Study Population

This study used data from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT), a mother–child pregnancy and birth cohort. The study design and data collection procedure has been published previously (8). Briefly, the study recruited 1,079 pregnant women in their first trimester from maternity hospitals in Mexico City between 1997-2004. The mothers and their offspring have been followed since gestation, through childhood and adolescence. Starting in 2015, a subset of 550 offspring between 9 and 18 years of age were re-recruited for a follow-up study, during which blood samples were collected at baseline. Participants were interviewed at two time points, two years apart, during which data about sociodemographic characteristics, anthropometry, diet, sleep, sexual maturation, toxicants exposure, and metabolic health were collected. The analytic sample for

this study included 371 adolescents who had available data on serum ferritin levels and blood leukocyte DNA methylation in key circadian genes at baseline as well as sleep outcomes from the two visits.

Ferritin level

Blood samples were collected from adolescents and immediately centrifuged and frozen at −70°C. For further analysis, serum plasma samples were shipped to the University of Michigan School of Public Health and stored at −80°C. The serum ferritin levels were determined from the blood samples collected in the first visit using angiotensin converting enzyme (ACE) immunoturbidimetric assay (Sekisui Diagnostics, USA). For our analyses, we adjusted serum ferritin by inflammation using a modified version of the regression correction approach developed by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project that calculate the adjusted ferritin levels using C-reactive protein (CRP) concentration (9). The adjustment equation is,

$$\text{Ferritin}_{\text{adjusted}} = \text{ferritin}_{\text{unadjusted}} - \beta_1 (\text{CRP}_{\text{obs}} - \text{CRP}_{\text{ref}}),$$

where β_1 is the CRP regression coefficient, obs is the observed value, and ref is the reference value, which was used to define low inflammation and is equal to the maximum value of the lowest decile determined from log-transformed CRP. To avoid overadjustment, the correction of ferritin was applied only to individuals with ln CRP greater than ln CRP_{ref}. To examine the effect of suboptimal iron status, we used tertiles of ferritin to compare those with lower ferritin levels (first tertile) to those with higher levels (third tertile).

Sleep outcomes

In the week following each clinic visit, adolescents were instructed to wear an actigraphy (ActiGraph GT3X-BT; ActiGraph LLC, Pensacola, Florida) continuously for seven days on their non-dominant wrist. The actigraphy provides an objective estimate of

sleep duration, timing, and fragmentation by measuring activity levels over each 24-hour period. A validated method against polysomnography was used to estimate the bed and wake times of each night, where the actigraphy data was analyzed with 60-second epoch lengths using a pruned dynamic programming (PDP) algorithm developed in R (R Foundation for Statistical Computing) (10). This algorithm incorporates self-reported sleep and wake time data to improve accuracy, > 95% correlation with self-reported data. Naps were excluded from the sleep parameters due to the inability of the algorithm to distinguish between naps and periods of daytime sedentary behavior.

The primary sleep outcomes for this study included sleep duration, sleep midpoint, and sleep quality measured separately for weekdays (Sunday through Thursday night) and weekends (Friday and Saturday). Sleep duration is the mean sleep duration over the actigraphy wearing period. Sleep midpoint is the average median of bedtime and wake time. We used the sleep fragmentation index and movement index to measure sleep quality. The sleep fragmentation index is the percentage of sleep periods lasting ≤ one-minute out of the total number of sleep bouts of any length, in which a higher percentage indicates higher sleep fragmentation, thus poorer sleep quality. Movement index is a percentage of minutes with movement count> 0 out of the total number of minutes in the nightly sleep duration, with higher movement index indicating poorer sleep quality.

DNA methylation

DNA was extracted from blood leukocytes collected from the first visit using the Flexigene kit (Qiagen, Valencia, CA). First, DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracils leaving methylated cytosines unchanged. Next, the DNA methylation profiles were evaluated using Infinium MethylationEPIC BeadChip (Illumina) that probes methylation at >850,000 cytosine-guanine dinucleotides (CpG) sites. Samples were randomly distributed across chips, then processed and read by expert personnel

at the University of Michigan Advanced Genomics Core. Raw data was read and processed using the minfi package in R, as previously described (11). Quality control and normalization of the data was performed. In brief, poorly detected probes (in at least 5% of samples), cross-reactive probes, and those with polymorphisms in the CpG sites or the single base extension site were excluded. Background noise was corrected via noob, and dye bias correction via RELIC, as previously described (11,12). Given the heterogeneous cellular composition of blood leukocytes, cell type proportions were estimated based on data from tissue-specific differentially methylated regions included on the BeadChip. The ratios between the intensity of methylated probes and the overall probe intensity (which includes both methylated and unmethylated signals) were used to estimate methylation levels or beta values at each CpG site, which can range from 0 to 1. Beta values for a total of 525 CpG sites were extracted, which represent 12 selected key circadian genes that are involved in establishing the circadian rhythms: *BMAL1*, *CLOCK*, *CRY1*, *CRY2*, *PER1*, *PER2*, *PER3*, *NR1D1 NR1D2*, *RORA*, *RORB*, *RORC*.

Covariates

Covariates were chosen based on *priori* knowledge and measured at the baseline including age, pubertal stage, socioeconomic status (SES), body mass index (BMI) z-score, smoking, alcohol, physical activity, and sedentary time. Using standardized protocols, pubertal stage was assessed by a trained physician based on the Tanner stages of sexual maturation for development of genitals in boys, breasts in girls and pubic hair in both. In addition, testicular volume was assessed for boys and menarche onset was self-reported by girls. For the present analysis we considered puberty as a dichotomous variable for earlier vs later pubertal stage, which was operationalized as testicular volume ≥15 mL for boys and onset of menarche for girls. SES was assessed using two maternal-reported proxy variables, which are maternal education and Mexican Association of Marketing Research and Public

Opinion Agencies (AMAI). Maternal education data were collected at baseline in the original cohort enrollment visit, and we categorized it as <12 years (some high school), 12 years (completed high school), or >12 years of schooling. The AMAI is a validated scale variable that was created based on the results of the National Survey of Household Income and Expenditure 2014, Mexico (ENIGH, Encuesta Nacional de Ingresos y Gastos delos Hogares 2014) (13). This variable classifies SES to seven categories based on household characteristics and item ownership (in descending order: A/B, C+, C, C-, D+, D, E). We grouped A/B, C+, and C in one group due to low distribution in these categories. Weight (in kg) and height (in cm) were measured by research assistants and used to calculate the BMI z-score accounting for age and sex. Smoking behavior was self-reported with a single question, "Have you ever tried smoking?" and was categorized dichotomously. Likewise, alcohol consumption was dichotomized as if they ever drank alcohol. Sedentary time and physical activity (sum of moderate and vigorous activity) were estimated from the actigraphy using the Chandler algorithm as average of minutes per day (10). Given that iron intake and dietary requirements differ by sex during adolescence, we conducted stratified analyses to explore whether there were sex-specific associations between ferritin levels and sleep as well as between ferritin levels and DNA methylation in circadian genes.

Statistical Analysis

First, we examined the distribution of the sociodemographic and lifestyle characteristics in the whole sample and by ferritin tertiles stratified by sex. Next, we assessed the differences in the mean of sleep outcomes between the two visits, on weekdays and weekends. We used sex-stratified linear mixed-effects regression analyses with random intercepts to evaluate the relationship between serum ferritin tertiles and each of the weekdays and weekends sleep outcomes. Furthermore, we repeated the analyses adjusting for the key confounders including age, puberty, SES, BMI z-score, smoking, alcohol, physical

activity, and sedentary time. A p-value of <0.5 was indicative of a statistically significant ferritin-sleep association.

For the second aim of this study, we used the limma package in R to perform linear regression analyses to explore the sex-stratified associations between the ferritin tertiles and DNA methylation at individual CpG sites. In the multivariable model, in addition to the previously mentioned confounders, we further adjusted for technical factors related to DNA methylation measurement including batch effect and some cell types (estimated proportions of monocytes, granulocytes, natural killer cells, CD4+ and CD8+ T cells, and relative abundance of CD8pCD28nCD45Ran cells). Since multiple CpG sites were included from each gene and can contribute to the regulation of that gene, we used the Cauchy combination test (CCT) to combine individual p-values from a single CpG site within a gene into one p-value for each gene. The CCT is known for its enhanced robustness and higher statistical power compared to alternative tests, making it particularly effective in accounting for dependencies among the p-values generated by various models (14). We then applied the false discovery rate (FDR) to control for multiple comparisons.

Results

Table 2.1 shows the descriptive statistics of the study sample stratified by sex. The mean±SD age was 13.9±1.9 years; 52.0% of the sample were females. About half of mothers had less than 12 years of education and approximately eighty percent of the adolescents had experienced puberty. The average ferritin level was 51.6±25.7 ng/mL with males having higher ferritin levels compared to females (60.6±24.7 vs 43.4±23.7 ng/mL, respectively). The characteristics of the study population according to ferritin tertiles are displayed in **Table 2.2**. Males in the higher ferritin tertiles were more likely to be older, born to mothers with less education, in the latter stages of puberty or post-pubertal, and less active. On the other hand,

females in the higher ferritin tertiles were more likely to be younger, in the earlier stages of puberty, never tried smoking or drank alcohol, and more active.

Ferritin level and sleep

In both visits, males and females each slept about 8.5 hours during the weekdays (**Figure 2.1**). However, females slept longer hours compared to males on the weekends (9.3 vs 8.9 hours, respectively, p-value= 0.0005). As participants got older, females maintained similar sleep duration during the weekends whereas males slept about 18 minutes less. No difference was observed in sleep midpoint between sex; however, participants had a later sleep midpoint during the weekends compared to weekdays. For instance, in the first visit, the average sleep midpoint was 3.7 decimal hours (approximately 3:42 AM) during the weekdays, and 4.6 decimal hours (approximately 4:36 AM) during the weekends. Moreover, females had significantly lower percentage of sleep fragmentation during weekdays compared to males but similar percent during the weekends in the first visit. Two years later, sleep fragmentation was still lower in females compared to males in the whole week (weekdays: 11.8 vs 13.0 %, respectively, p-value= 0.0182; weekends: 11.5 vs 12.8 %, respectively, p-value= 0.0593). Likewise, females had significantly lower percentage of movement index compared to males during weekdays and weekends in both visits.

The results of the longitudinal analyses between ferritin tertiles and sleep outcomes are shown in **Table 2.3**. The findings mostly indicated null associations between ferritin tertiles and sleep outcomes except for sleep midpoint in males and sleep fragmentation in females. In the multivariable adjusted models, males with ferritin levels in the first tertile slept significantly 35 minutes later than males with ferritin levels in the third tertile during the weekends (β: 0.59 decimal hours, 95% CI: 0.05, 1.13 comparing tertile 1 to tertile 3). For sleep fragmentation, females with ferritin levels in the first tertile had significantly 1.52 % (95% CI: 0.38, 2.65) higher fragmentation during weekdays compared to females with

ferritin levels in the third tertile. This association remained significant after controlling for confounders (β: 1.35 %, 95% CI: 0.21, 2.50). Similar evidence was found among females with ferritin levels in second tertile 2 compared to third tertile (β: 1.28 %, 95% CI: 0.04, 2.53). Conversely, the association between ferritin tertiles and sleep fragmentation was weak during weekends.

Ferritin level and DNA methylation

We examined 525 CpG sites that annotated to the circadian genes as following: 59 sites for *ARNTL*, 42 sites for *CLOCK*, 26 sites for *CRY1*, 29 sites for *CRY2*, 21 sites for *NR1D1*, 22 sites for *NR1D2*, 19 sites for *PER1*, 39 sites for *PER2*, 34 sites for *PER3*, 186 sites for *RORA*, 25 sites for *RORB*, and 23 sites for *RORC*. **Table 2.4** shows the top ten CpG sites ordered by raw p-values resulting from the multivariable adjusted associations between ferritin tertiles and DNA methylation among females. **Table 2.5** illustrates the gene-level p-values from the CCT and FDR tests among females. At FDR <0.05, ferritin levels in the first tertiles were significantly associated with DNA methylation of *CLOCK, PER1*, and *RORC* genes compared to ferritin levels in the third tertile. Additionally, alongside these genes, *PER2* and *CRY2* were marginally significant with 10% FDR. **Figure 2.2** displays the beta values difference for the association between ferritin (tertile 1 vs tertile 3) and DNA methylation at individual CpG sites representing *CLOCK, PER1*, and *RORC* genes among females. In general, comparing ferritin in the lowest tertile to ferritin in the highest tertile, ferritin in the lowest tertile appeared to be positively associated with DNA methylation in 26 of the 42 CpG sites that represent the *CLOCK* gene. For the *PER1* gene, the direction of the associations was positive at position 8044256 to 8054688 and became negative afterwards. The direction of effect was inconsistent for the *RORC* gene. Detailed results about these genes are available in **Supplementary Table 2.1.** No significant associations were observed

between ferritin tertiles and DNA methylation of circadian genes among males (**Supplementary Table 2.2-2.3**).

Discussion

In this study of 371 adolescents in Mexico City, we explored the sex-stratified relationships of serum ferritin tertiles with objective sleep measures and DNA methylation in key circadian genes. We found that females with ferritin levels in the first tertile had significantly higher sleep fragmentation compared to females with ferritin levels in the third tertile, suggesting that low iron stores can lead to poor sleep quality in females. Aligned with these results, we determined that ferritin levels in the first tertile were significantly associated with DNA methylation of *CLOCK*, *PER1*, and *RORC* genes. On the other hand, males in the first ferritin tertile had significantly later sleep timing compared to males in the third ferritin tertile.

Our primary results of the associations between ferritin and sleep fragmentation corroborate conclusions from prior studies in different populations. In animals, juvenile monkeys with prenatal iron deficiency and low monoamine oxidase A (MAOA) genotype have higher night sleep fragmentation and higher sleepiness during the day compared to monkeys with prenatal iron sufficiency and low-MAOA (15). In humans, one in four patients with iron deficiency anemia suffer from clinical symptoms of restless leg syndrome that cause shorter sleep duration and poorer sleep quality; however, treating patients with iron therapy has been shown to improve these symptoms, particularly in women (16,17). Assessment of sleep patterns in infants revealed that infants with iron deficiency anemia fell asleep faster at night but spent more time awake and less time in quiet sleep compared to infants without iron deficiency anemia, but these differences disappeared after iron treatment (18). However, a follow-up study found that infants with iron deficiency anemia may have long-term changes in the sleep architecture at four-years of age regardless of anemia

treatment in infancy and absence of anemia at age of four (19). Moreover, we recently showed that both iron deficiency and iron deficiency anemia are associated with poor sleep quality in healthy women of reproductive age, though not with sleep duration (20). In this study, we did not identify any significant associations with sleep duration. However, it is estimated that 78% of Mexican adolescents, living in rural agricultural settings, and up to 94% living in urban settings, sleep less than the recommended amount of sleep for their age during school days (21). This alarming trend warrants further exploration into the factors that contribute to insufficient sleep in this population. While these studies focused on iron deficiency (low ferritin levels is the first hallmark of iron deficiency), a low percentage of the participants of this study had iron deficiency. Nevertheless, our results show that ferritin can be associated with poor sleep quality even with apparently normal ferritin levels, in particular among females.

Prior human studies examining ferritin and DNA methylation in circadian genes are rare. Our exploratory results showed that iron status in females were significantly associated with *CLOCK*, *PER1*, and *RORC* and marginally associated with *CRY2* and *PER2*. These results are in line with some previous studies in animals. An animal study randomized 24 neonatal piglets to receive either two doses of 1mL saline, one dose of 1mL iron and one dose of 1mL saline, or two doses of 1mL iron (22). The results showed a significant difference in gene expression of *CRY1, CRY2, PER1, PER2*, and *BMAL1* genes in the liver between the randomized groups, where both lower and higher iron status caused lower circadian gene expression compared to moderate iron status, with the lowest gene expression found in the group with the lowest iron levels. Furthermore, Dong et al. (2020) showed that feeding iron-deficient piglets a low iron diet leads to higher expression of *CRY1, CRY2,* and *PER2* genes in the duodenum compared to piglets a fed high iron diet, with no significant difference observed in the expression of these genes in the liver (23). The variation in circadian gene

expression direction is likely influenced by the tissue-specific gene-expression patterns. While these studies focused on circadian gene expression, DNA methylation is an epigenetic modification that regulates gene expression. In our study we used blood leukocytes to measure DNA methylation in circadian genes.

To the best of our knowledge, only one recent study has examined the role of iron in epigenetic modification of circadian genes expression. In that study, the real-time polymerase chain reaction (PCR) analysis for six core clock genes, *BMAL1*, *CLOCK*, *PER1*, *PER2*, *CRY1*, and *CRY2* in the livers of old mice and iron-overload mice shows changes in the *PER1* and *PER2* expression compared to the control group (24). Interestingly, phlebotomy, which reduces iron levels, and iron injection, which increases iron levels, can both affect *PER1* and *PER2* expression in mice. Moreover, the authors demonstrated that the association between iron status and circadian gene expression is modulated by AMP histone methylation. In the present study, we found that low iron levels were associated with DNA methylation of these genes. Although DNA methylation and histone methylation are separate epigenetic modifications, both are important and work in concert along with other histone modifications to regulate gene expression. However, numerous studies have shown that exposure to early-life iron deficiency can alter the global DNA methylation pattern (25). Lien et al. (2019) demonstrated that fetal-neonatal iron deficiency can induce changes in DNA methylation in the rat hippocampus, contributing to neural gene dysregulation (26). Similarly, in piglets, iron deficiency caused hippocampal epigenetic modification that affected neurodevelopment and function involving learning and memory (7). Based on these results, we hypothesized that iron may affect sleep through epigenetic modification of core circadian genes. However, due to the exploratory nature and the cross-sectional design of this part of the study, we were unable to explore whether DNA methylation in circadian genes mediates the association between ferritin and sleep. Nonetheless, these results provide some preliminary evidence

suggesting that ferritin levels may be associated with DNA methylation of some circadian genes. However, it is important to note that our analysis has limited capacity to estimate the effect size and direction of association at the gene level. Therefore, further research with larger sample size and longitudinal study design is warranted to comprehensively understand the role of iron in sleep and circadian gene regulation.

To the best of the authors' knowledge, this is the first human study to examine the role of iron status in sleep and DNA methylation in circadian genes in healthy adolescents. We examined sex-differences during a sensitive period of human development. This study utilized serum ferritin, adjusted for inflammation, as an indicator of iron status. In addition, we used actigraphy to objectively measure sleep outcomes in two time points allowing for longitudinal analyses of the association between ferritin and sleep. In addition, we measured sleep on weekdays and weekends, and we assessed DNA methylation of 12 core circadian genes. However, this study has several limitations. The small sample size may limit our ability to detect significant associations. While we established the temporality of the association between ferritin and sleep outcomes, causal inference between ferritin and DNA methylation in circadian genes cannot be established given the cross-sectional design. Moreover, DNA methylation is tissue specific, but we used blood leukocytes to measure DNA methylation in circadian genes. However, measuring DNA methylation in the brain where sleep is regulated is not feasible in humans. Lastly, unmeasured confounders could potentially cause bias and the results of this study may not be generalizable to other populations.

Conclusion

Collectively, the results of this study suggest that low iron stores may pose a risk factor associated with sleep and circadian disturbances during adolescence, especially in females. Further research with larger sample size and longitudinal study design are warranted to comprehensively understand the role of iron in sleep and circadian rhythm.

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Figures and Tables

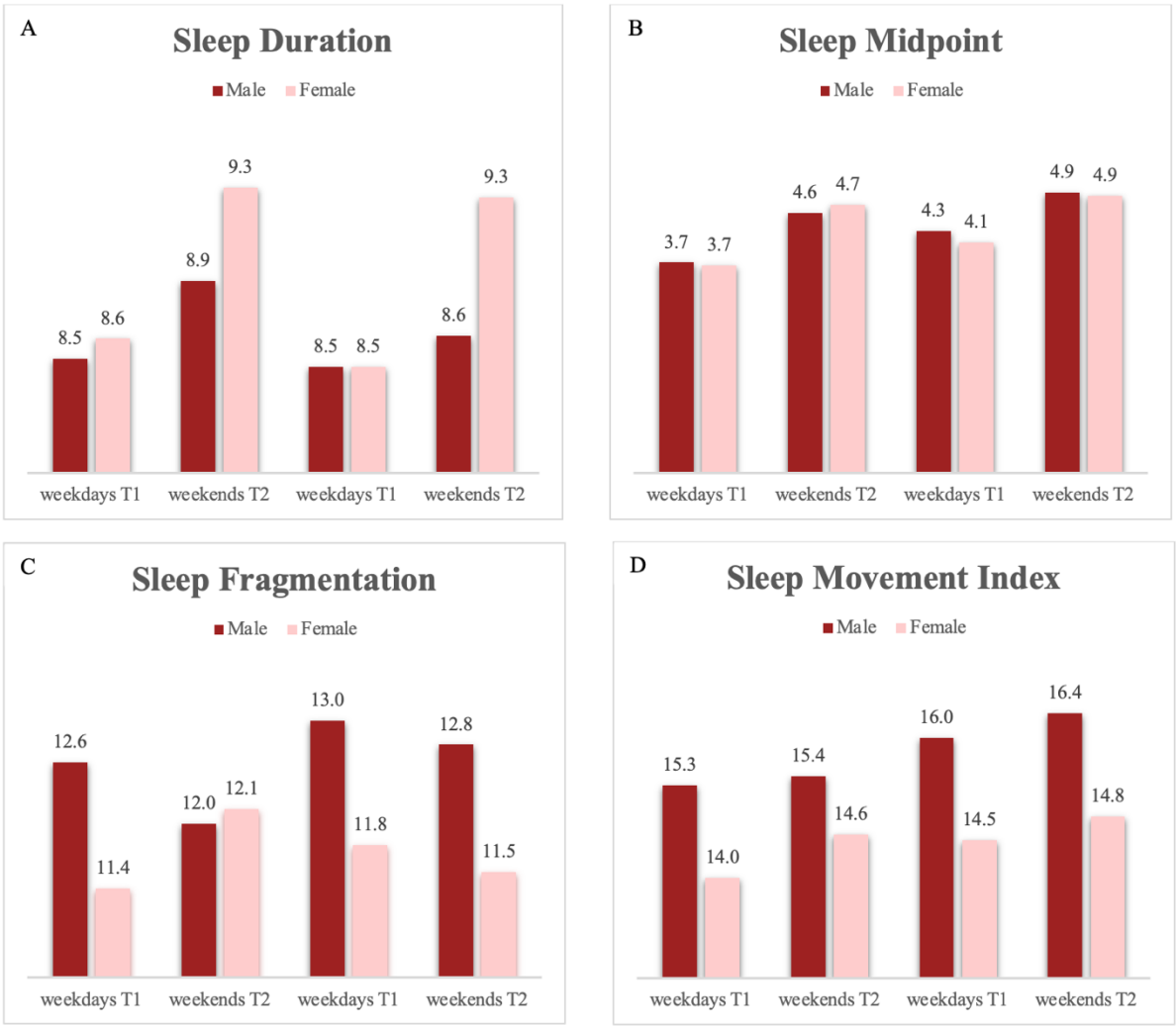


Figure 2.1 mean of sleep outcomes during the weekdays and weekends in both visits. A. sleep duration hours/day; B. sleep midpoint decimal hour; C. sleep fragmentation %; D. movement index %; T1= first visit; T2=second visit

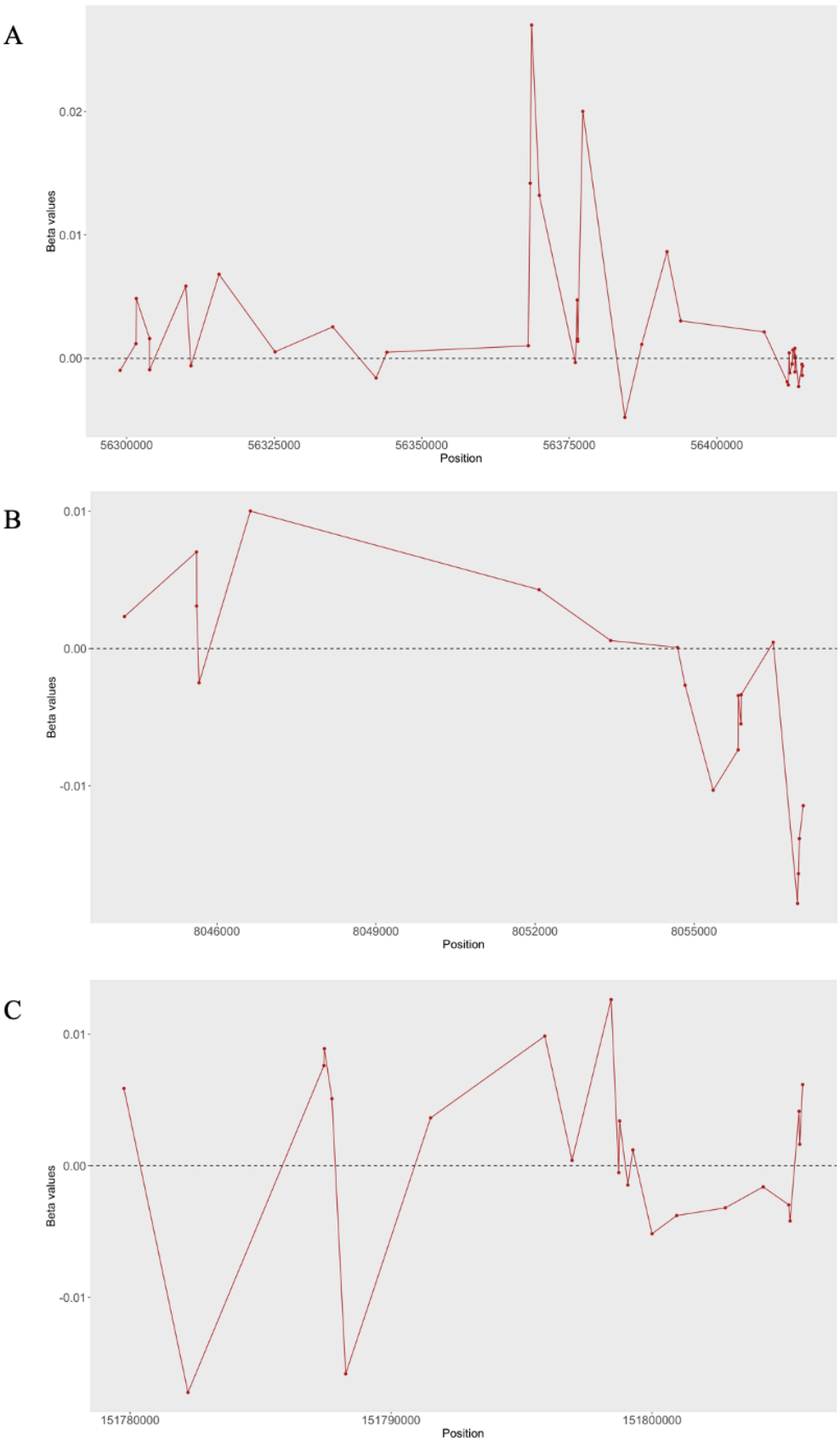


Figure 2.2 Associations between ferritin (tertile 1 vs tertiles 3) and DNA methylation at individual CpG sits among females. A. *CLOCK*, B. *PER1*, C. *RORC*

Table 2.1 The characteristics of adolescents in the ELEMENT analytic sample.

Variable	All (N=371)	Males (N=178)	Females (N=193)
Age (years)	13.86 (1.94)	13.89 (1.87)	13.83 (2.00)
Socioeconomic status (%)			
1 (highest)	25.34	26.41	24.35
2	24.52	29.21	20.21
3	25.07	22.47	27.46
4 (lowest)	25.07	21.91	27.98
Maternal education (%)			
<12 y	50.13	47.75	52.33
12 y (completed high school)	35.58	37.64	33.68
>12 y	14.29	14.61	13.99
Pubertal stage (%)			
Earlier	20.49	21.91	19.17
Later	79.51	78.09	80.83
Ever smoked cigarettes (%)			
No	83.02	82.02	83.94
Yes	16.98	17.98	16.06
Ever drink alcohol (%)			
No	27.49	30.34	24.87
Yes	72.51	69.66	75.13
Sedentary activity (minutes/day)	593.77 (72.20)	602.29 (70.79)	585.92 (72.79)
Moderate-to-vigorous physical activity (minutes/day)	80.72 (29.62)	80.24 (31.51)	81.15 (27.84)
BMI-for-age (z-scores)	0.56 (1.25)	0.51 (1.30)	0.60 (1.21)
Adjusted ferritin (ng/mL)	51.64 (25.66)	60.64 (24.69)	43.35 (23.72)

Socioeconomic status is a validated scale variable that was created based on the results of the National Survey of Household Income and Expenditure 2014, Mexico

Table 2.2 The characteristics of adolescents by Ferritin Tertiles

Variable	Males Ferritin Level (ng/mL)			Females Ferritin Level (ng/mL)		
	Tertile 1 (median 28.7)	**Tertile 2 (median 40.9)**	**Tertile3 (median 55.6)**	**Tertile 1 (median 24.9)**	**Tertile 2 (median 40.1)**	**Tertile 3 (median 57.3)**
Age (years)	13.29 (1.66)	13.68 (1.69)	14.30 (2.03)	14.22 (1.92)	13.39 (1.99)	13.56 (2.09)
Socioeconomic status (%)						
1 (highest)	32.14	21.92	28.57	20.21	29.82	26.19
2	28.57	34.25	24.68	20.21	22.81	16.67
3	17.86	23.29	23.38	29.79	21.05	30.95
4 (lowest)	21.43	20.55	23.38	29.79	26.32	26.19
Maternal education (%)						
<12	39.29	38.36	59.74	51.06	52.63	54.76
12 y (completed high school)	35.71	43.84	32.47	34.04	29.82	38.10
>12 y	25.00	17.81	7.79	14.89	17.54	7.14
Pubertal stage (%)						
Earlier	32.14	20.55	19.48	11.70	26.32	26.19
Later	67.86	79.45	80.52	88.30	73.68	73.81
Ever smoked cigarettes (%)						
No	85.71	80.82	81.82	77.66	91.23	88.10
Yes	14.29	19.18	18.18	22.34	8.77	11.90
Ever drink alcohol (%)						
No	25.00	30.14	32.47	17.02	35.09	28.57
Yes	75.00	69.86	67.53	82.98	64.91	75.13
Sedentary activity (minutes/day)	587.20 (53.43)	596.32 (73.67)	613.42 (72.60)	597.63 (70.41)	569.98 (66.08)	581.34 (83.15)
Moderate-to-vigorous Physical activity (minutes/day)	91.91 (31.18)	83.93 (35.06)	72.86 (26.31)	78.38 (30.06)	82.89 (24.19)	85.00 (27.25)
BMI-for-age (z-scores)	0.04 (1.23)	0.65 (1.32)	0.54 (1.28)	0.46 (1.16)	0.81 (1.26)	0.63 (1.24)

Socioeconomic status is a validated scale variable that was created based on the results of the National Survey of Household Income and Expenditure 2014, Mexico

Table 2.3 Associations between tertiles of ferritin (ng/mL) and sleep outcomes

	Males β (95 % CI)			Females β (95 % CI)		
Outcome	**Ferritin Tertile 1**	**Ferritin Tertile 2**	**Ferritin Tertile 3**	**Ferritin Tertile 1**	**Ferritin Tertile 2**	**Ferritin Tertile 3**
Sleep Duration minutes, weekdays						
Crude	-8.37 (-33.41, 16.67)	12.67 (-5.65, 30.98)	ref	16.74 (-8.06, 41.54)	**28.92 (1.66, 56.19)**	ref
Adjusted	-10.52 (-34.91, 13.88)	11.42 (-5.95, 28.78)	ref	18.95 (-3.81, 41.71)	22.94 (-1.81, 47.69)	ref
Sleep Duration minutes, weekends						
Crude	12.41 (-15.39, 40.21)	-11.87 (-32.42, 8.67)	ref	-2.68 (-25.22, 19.86)	-5.65 (-30.50, 19.20)	ref
Adjusted	6.61 (-23.10, 36.32)	-15.12 (-36.48, 6.25)	ref	-1.11 (-22.95, 20.73)	-10.05 (-33.76, 13.66)	ref
Midpoint Sleep decimal hour, weekdays						
Crude	0.13 (-0.40, 0.65)	-0.01 (-0.39, 0.38)	ref	**0.42 (0.03, 0.81)**	0.37 (-0.06, 0.80)	ref
Adjusted	0.38 (-0.17, 0.94)	0.06 (-0.34, 0.45)	ref	0.28 (-0.09, 0.65)	0.37 (-0.03, 0.77)	ref
Midpoint Sleep decimal hour, weekends						
Crude	0.29 (-0.23, 0.81)	0.01 (-0.37, 0.39)	ref	0.20 (-0.17, 0.57)	-0.004 (-0.41, 0.40)	ref
Adjusted	**0.59 (0.05, 1.13)**	0.08 (-0.31, 0.47)	ref	0.06 (-0.30, 0.42)	0.07 (-0.32, 0.46)	ref
Fragmentation Index percentage, weekdays						
Crude	0.25 (-1.49, 1.99)	0.92 (-0.36, 2.20)	ref	**1.52 (0.38, 2.65)**	1.04 (-0.21, 2.29)	ref
Adjusted	0.28 (-1.60, 2.17)	1.01 (-0.33, 2.38)	ref	**1.35 (0.21, 2.50)**	**1.28 (0.04, 2.53)**	ref
Fragmentation Index percentage, weekends						
Crude	-1.19 (-3.23, 0.86)	-0.36 (-1.87, 1.15)	ref	0.33 (-1.08, 1.74)	0.84 (-0.72, 2.40)	ref
Adjusted	-0.97 (-3.21, 1.26)	-0.23 (-1.84, 1.38)	ref	0.001 (-1.45, 1.46)	1.08 (-0.50, 2.66)	ref
Movement Index percentage, weekdays						
Crude	0.54 (-0.87, 1.96)	0.58 (-0.46, 1.62)	ref	**1.20 (0.16, 2.25)**	0.55 (-0.60, 1.70)	ref
Adjusted	0.50 (-1.02, 2.03)	0.58 (-0.52, 1.67)	ref	1.02 (-0.04, 2.08)	0.69 (-0.46, 1.84)	ref
Movement Index percentage, weekends						
Crude	0.34 (-1.17, 1.84)	-0.20 (-1.31, 0.91)	ref	0.80 (-0.27, 1.88)	-0.37 (-1.55, 0.82)	ref
Adjusted	0.59 (-1.07, 2.24)	-0.03 (-1.22, 1.16)	ref	0.66 (-0.45, 1.76)	-0.36 (-1.56, 0.84)	ref

Adjusted for age, maternal education, SES, BMI z-score, ever smoker, ever drink alcohol, moderate-to-vigorous physical activity, sedentary, pubertal stag

Table 2.4 The top ten CpG site for the association between ferritin tertiles and DNA methylation of circadian genes among females, ordered by raw p-values.

Ferritin Tertile	Probe	Chromosome	Position	gene	Relation to CpG Island	Gene Region	Beta value	Error	P-value
Tertile 1 vs Tertile 3	cg23114183	chr1	151798432	*RORC*	Open sea	Body	0.0126	0.0034	0.0002
	cg12893431	chr4	56315641	*CLOCK*	Open sea	Body	0.0068	0.0019	0.0006
	cg24004414	chr4	56369919	*CLOCK*	Open sea	5'UTR	0.0132	0.0039	0.0008
	cg06997028	chr4	56368613	*CLOCK*	Open sea	5'UTR	0.0270	0.0081	0.0010
	cg13433366	chr17	8057058	*PER1*	S. Shore	TSS1500	-0.0114	0.0035	0.0011
	cg11254550	chr15	61472541	*RORA*	Open sea	Body	-0.0116	0.0035	0.0011
	cg04310348	chr2	239186648	*PER2*	Open sea	5'UTR	0.0119	0.0037	0.0013
	cg12340454	chr15	61519486	*RORA*	N. Shore	Body	-0.0128	0.0040	0.0014
	cg13284983	chr17	8046631	*PER1*	Open sea	Body	0.0100	0.0032	0.0020
	cg24796870	chr4	56377288	*CLOCK*	Open sea	5'UTR	0.0200	0.0065	0.0023
	cg10767365	chr15	61520097	*RORA*	S. Shore	Body	-0.0076	0.0025	0.0030
Tertile 2 vs Tertile 3	cg08099431	chr15	61346347	*RORA*	Open sea	Body	-0.0061	0.0021	0.0050
	cg16757984	chr17	38257936	*NR1D1*	S. Shore	TSS1500	-0.0114	0.0041	0.0061
	cg09578018	chr15	60883649	*RORA*	Island	Body	-0.0131	0.0049	0.0083
	cg19620383	chr15	60860767	*RORA*	Open sea	Body	-0.0104	0.0039	0.0083
	cg11254550	chr15	61472541	*RORA*	Open sea	Body	-0.0099	0.0038	0.0098
	cg20855344	chr3	23986090	*NR1D2*	N. Shore	TSS1500	0.0086	0.0033	0.0106
	cg23905308	chr2	239196947	*PER2*	Island	5'UTR	0.0015	0.0005	0.0108
	cg20146792	chr2	239168496	*PER2*	Open sea	Body	0.0095	0.0037	0.0121
	cg04130427	chr9	77113915	*RORB*	Island	Body	0.0108	0.0043	0.0122
	cg22322535	chr11	13299888	*BMAL1*	Island	5'UTR	-0.0034	0.0013	0.0129
	cg03952578	chr15	61509038	*RORA*	Open sea	Body	0.0082	0.0033	0.0141

Adjusted for age, maternal education, SES, BMI z-score, ever smoker, ever drink alcohol, moderate-to-vigorous physical activity, sedentary, pubertal stage, batch effect, and estimated blood cell type proportions (Gran, CD4T, CD8T, Mono, and NK), or abundance (CD8pCD28nCD45RAn)

Table 2.5 Cauchy combination test for gene-level DNA methylation of circadian genes among females

Circadian Gene	Chromosome	Position	Ferritin Tertile 1 vs Tertile 3		Ferritin Tertile 2 vs Tertile 3	
			CCT	FDR	CCT	FDR
BMAL1	chr11	13298033-13397054	0.1996	0.3422	0.5257	0.8172
CLOCK	chr4	56298893-56414511	0.0087	0.0392**	0.7334	0.8801
CRY1	chr12	107388741-107488596	0.2586	0.3517	0.5448	0.8172
CRY2	chr11	45867485-45903754	0.0334	0.0922*	0.9644	0.9904
PER1	chr17	8044256-8057058	0.0098	0.0392**	0.2839	0.6814
PER2	chr2	239153865-239198437	0.0384	0.0922*	0.1621	0.6814
PER3	chr1	7843515-7904830	0.9924	0.9924	0.4816	0.8172
NR1D1	chr17	38249266-38257936	0.3162	0.3794	0.0939	0.6814
NR1D2	chr3	23985575-24020542	0.2638	0.3517	0.2433	0.6814
RORA	chr15	60786924-61522112	0.0647	0.1294	0.9904	0.9904
RORB	chr	77111502-77282233	0.9229	0.9924	0.2035	0.6814
RORC	chr1	151779765-151805773	0.0050	0.0392**	0.6828	0.8801

CCT: Cauchy combination test, FDR: false discovery rate
* FDR <0.1
**FDR <0.05

Appendix

Supplementary Table 1: Associations between ferritin (tertile 1 vs tertile 3) and DNA methylation in CpG sites representing CLOCK, PER1, and RORC genes, ordered by genomic location, among females.

Probe	Position	Chromosome	gene	Relation to Island	Gene Region	Beta values	Error	P-Value
cg14567142	151779765	chr1	*RORC*	Open Sea	3'UTR	0.0059	0.0034	0.0810
cg08566367	151782221	chr1	*RORC*	Open Sea	Body	-0.0172	0.0142	0.2231
cg09760189	151787430	chr1	*RORC*	Open Sea	Body	0.0076	0.0039	0.0531
cg09363447	151787446	chr1	*RORC*	Open Sea	Body	0.0089	0.0038	0.0211
cg01748473	151787734	chr1	*RORC*	Open Sea	Body	0.0051	0.0035	0.1516
cg25206113	151788262	chr1	*RORC*	Open Sea	Body	-0.0158	0.0092	0.0859
cg15313608	151791516	chr1	*RORC*	Open Sea	Body	0.0036	0.0041	0.3800
cg16646916	151795893	chr1	*RORC*	Open Sea	Body	0.0098	0.0052	0.0583
cg07038418	151796936	chr1	*RORC*	Open Sea	Body	0.0004	0.0033	0.9000
cg23114183	151798432	chr1	*RORC*	Open Sea	Body	0.0126	0.0034	0.0002
cg10269078	151798724	chr1	*RORC*	Open Sea	TSS200	-0.0005	0.0039	0.8935
cg03638686	151798760	chr1	*RORC*	Open Sea	TSS1500	0.0034	0.0032	0.2865
cg21784096	151799073	chr1	*RORC*	Open Sea	TSS1500	-0.0015	0.0025	0.5557
cg14204738	151799266	chr1	*RORC*	Open Sea	TSS1500	0.0012	0.0030	0.6853
cg10111759	151800000	chr1	*RORC*	Open Sea	TSS1500	-0.0052	0.0086	0.5464
cg11390381	151800946	chr1	*RORC*	Open Sea	Body	-0.0038	0.0053	0.4754
cg22228337	151802807	chr1	*RORC*	Open Sea	Body	-0.0032	0.0065	0.6199
cg25112191	151804260	chr1	*RORC*	Open Sea	1stExon	-0.0016	0.0058	0.7807
cg03005293	151805241	chr1	*RORC*	Open Sea	TSS1500	-0.0030	0.0039	0.4423
cg22223419	151805290	chr1	*RORC*	Open Sea	TSS1500	-0.0042	0.0045	0.3505
cg18149207	151805634	chr1	*RORC*	Open Sea	TSS1500	0.0041	0.0030	0.1686
cg03984502	151805661	chr1	*RORC*	Open Sea	TSS1500	0.0016	0.0039	0.6745
cg23454735	151805773	chr1	*RORC*	Open Sea	TSS1500	0.0062	0.0039	0.1188
cg02683371	56298893	chr4	*CLOCK*	Open Sea	3'UTR	-0.0010	0.0014	0.4859
cg25530661	56301576	chr4	*CLOCK*	Open Sea	3'UTR	0.0012	0.0029	0.6837
cg05826175	56301631	chr4	*CLOCK*	Open Sea	Body	0.0048	0.0036	0.1779
cg08407670	56303880	chr4	*CLOCK*	Open Sea	Body	0.0016	0.0015	0.2870
cg19429538	56303887	chr4	*CLOCK*	Open Sea	Body	-0.0009	0.0018	0.6034
cg15048607	56310021	chr4	*CLOCK*	Open Sea	Body	0.0059	0.0023	0.0120
cg10515766	56310888	chr4	*CLOCK*	Open Sea	Body	-0.0006	0.0022	0.7770
cg12893431	56315641	chr4	*CLOCK*	Open Sea	Body	0.0068	0.0019	0.0006
cg26535070	56325135	chr4	*CLOCK*	Open Sea	Body	0.0005	0.0016	0.7474
cg08935434	56334911	chr4	*CLOCK*	Open Sea	Body	0.0025	0.0019	0.1925
cg19825305	56342262	chr4	*CLOCK*	Open Sea	Body	-0.0016	0.0018	0.3684
cg18470649	56344092	chr4	*CLOCK*	Open Sea	Body	0.0005	0.0015	0.7397

Probe	Position	Chromosome	gene	Relation to Island	Gene Region	Beta values	Standard Error	P-Value
cg04200137	56368046	chr4	*CLOCK*	Open Sea	5'UTR	0.0010	0.0029	0.7288
cg18539169	56368375	chr4	*CLOCK*	Open Sea	5'UTR	0.0142	0.0052	0.0072
cg06997028	56368613	chr4	*CLOCK*	Open Sea	5'UTR	0.0270	0.0081	0.0010
cg24004414	56369919	chr4	*CLOCK*	Open Sea	5'UTR	0.0132	0.0039	0.0008
cg05960024	56376020	chr4	*CLOCK*	Open Sea	5'UTR	-0.0003	0.0020	0.8673
cg24000108	56376331	chr4	*CLOCK*	Open Sea	5'UTR	0.0047	0.0016	0.0048
cg04485603	56376410	chr4	*CLOCK*	Open Sea	5'UTR	0.0016	0.0021	0.4506
cg02613926	56376440	chr4	*CLOCK*	Open Sea	5'UTR	0.0014	0.0016	0.4044
cg24796870	56377288	chr4	*CLOCK*	Open Sea	5'UTR	0.0200	0.0065	0.0023
cg06764795	56384408	chr4	*CLOCK*	Open Sea	5'UTR	-0.0048	0.0046	0.2983
cg25181236	56387275	chr4	*CLOCK*	Open Sea	5'UTR	0.0011	0.0024	0.6392
cg09847994	56391558	chr4	*CLOCK*	Open Sea	5'UTR	0.0086	0.0059	0.1413
cg07327568	56393876	chr4	*CLOCK*	Open Sea	5'UTR	0.0030	0.0022	0.1628
cg08772927	56408023	chr4	*CLOCK*	N_Shelf	5'UTR	0.0021	0.0018	0.2404
cg11591729	56411904	chr4	*CLOCK*	N_Shore	1stExon	-0.0019	0.0017	0.2611
cg27132802	56412113	chr4	*CLOCK*	Island	TSS200	-0.0022	0.0013	0.0932
cg22523077	56412259	chr4	*CLOCK*	Island	5'UTR	0.0004	0.0006	0.4834
cg18928811	56412338	chr4	*CLOCK*	Island	5'UTR	-0.0012	0.0020	0.5525
cg11774413	56412743	chr4	*CLOCK*	Island	5'UTR	-0.0004	0.0007	0.5606
cg07500410	56412748	chr4	*CLOCK*	Island	5'UTR	-0.0005	0.0010	0.6524
cg09744584	56412880	chr4	*CLOCK*	Island	TSS1500	0.0007	0.0006	0.2635
cg23506964	56413211	chr4	*CLOCK*	Island	TSS1500	0.0002	0.0005	0.7748
cg11569219	56413215	chr4	*CLOCK*	Island	TSS1500	0.0008	0.0023	0.7261
cg08033122	56413227	chr4	*CLOCK*	Island	TSS1500	0.0002	0.0007	0.8076
cg07236904	56413229	chr4	*CLOCK*	Island	TSS1500	-0.0011	0.0005	0.0462
cg19095271	56413338	chr4	*CLOCK*	Island	TSS1500	0.0000	0.0008	0.9691
cg06085476	56413854	chr4	*CLOCK*	S_Shore	TSS1500	-0.0023	0.0020	0.2586
cg16125666	56414406	chr4	*CLOCK*	S_Shore	TSS1500	-0.0005	0.0013	0.7108
cg19298990	56414485	chr4	*CLOCK*	S_Shore	TSS1500	-0.0014	0.0016	0.3732
cg15096653	56414511	chr4	*CLOCK*	S_Shore	TSS1500	-0.0006	0.0023	0.7783
cg05893602	8044256	chr17	*PER1*	Open Sea	3'UTR	0.0023	0.0027	0.3888
cg17314998	8045615	chr17	*PER1*	Open Sea	Body	0.0070	0.0037	0.0598
cg27073561	8045617	chr17	*PER1*	Open Sea	Body	0.0031	0.0042	0.4593
cg02359589	8045663	chr17	*PER1*	Open Sea	Body	-0.0025	0.0033	0.4456
cg13284983	8046631	chr17	*PER1*	Open Sea	Body	0.0100	0.0032	0.0020
cg03301481	8052075	chr17	*PER1*	N_Shelf	Body	0.0043	0.0033	0.1977
cg00693172	8053426	chr17	*PER1*	N_Shore	Body	0.0006	0.0038	0.8789
cg08521677	8054688	chr17	*PER1*	Island	5'UTR	0.0001	0.0014	0.9529
cg10028884	8054829	chr17	*PER1*	Island	5'UTR	-0.0027	0.0011	0.0216

Probe	Position	Chromosome	gene	Relation to Island	Gene Region	Beta values	Standard Error	P-Value
cg27004243	8055360	chr17	*PER1*	Island	5'UTR	-0.0103	0.0054	0.0549
cg14158601	8055831	chr17	*PER1*	Island	TSS200	-0.0074	0.0032	0.0214
cg21093368	8055834	chr17	*PER1*	Island	TSS200	-0.0034	0.0027	0.2083
cg20503576	8055884	chr17	*PER1*	S_Shore	TSS200	-0.0055	0.0029	0.0631
cg16545079	8055888	chr17	*PER1*	S_Shore	TSS200	-0.0034	0.0041	0.4114
cg08189088	8056492	chr17	*PER1*	S_Shore	TSS1500	0.0005	0.0037	0.9004
cg10708955	8056950	chr17	*PER1*	S_Shore	TSS1500	-0.0186	0.0071	0.0098
cg04658021	8056967	chr17	*PER1*	S_Shore	TSS1500	-0.0164	0.0114	0.1512
cg13759852	8056985	chr17	*PER1*	S_Shore	TSS1500	-0.0138	0.0047	0.0034
cg13433366	8057058	chr17	*PER1*	S_Shore	TSS1500	-0.0114	0.0035	0.0011

Adjusted for age, maternal education, SES, BMI z-score, ever smoker, ever drink alcohol, moderate-to-vigorous physical activity, sedentary, pubertal stage, batch effect, and estimated blood cell type proportions (Gran, CD4T, CD8T, Mono, and NK), or abundance (CD8pCD28nCD45RAn)

Supplementary Table 2: The top ten CpG site for the association between ferritin tertiles and DNA methylation of circadian genes among males, ordered by raw p-values

Ferritin Tertile	Probe	Chromosome	Position	gene	Relation to CpG Island	Gene Region	Beta values	Error	P-Value
Tertile 1 vs Tertile 3	cg05956150	chr15	61491665	*RORA*	Open sea	Body	-0.0130	0.0034	0.0002
	cg10435282	chr11	13303971	*BMAL1*	S. Shelf	5'UTR	0.0109	0.0035	0.0022
	cg08106764	chr15	61266830	*RORA*	Open sea	Body	-0.0193	0.0067	0.0041
	cg16426858	chr15	61487998	*RORA*	Open sea	Body	-0.0172	0.0060	0.0047
	cg16456870	chr1	7844471	*PER3*	Island	TSS200	0.0049	0.0017	0.0060
	cg16252131	chr17	38257290	*NR1D1*	S. Shore	TSS1500	0.0040	0.0015	0.0073
	cg03853227	chr12	107487365	*CRY1*	Island	1stExon	-0.0136	0.0051	0.0079
	cg09404790	chr11	13310386	*BMAL1*	Open sea	5'UTR	-0.0119	0.0044	0.0080
	cg15048607	chr4	56310021	*CLOCK*	Open sea	Body	-0.0444	0.0175	0.0118
	cg01400040	chr15	61091220	*RORA*	Open sea	Body	-0.0165	0.0065	0.0122
	cg24831107	chr2	239197139	*PER2*	Island	1stExon	-0.0073	0.0029	0.0122
Tertile 2 vs Tertile 3	cg01400040	chr15	61091220	*RORA*	Open sea	Body	-0.0146	0.0047	0.0021
	cg05956150	chr15	61491665	*RORA*	Open sea	Body	-0.0076	0.0024	0.0022
	cg21860398	chr11	13379485	*BMAL1*	Open sea	Body	0.0071	0.0024	0.0033
	cg24598906	chr15	61319340	*RORA*	Open sea	Body	-0.0047	0.0019	0.0119
	cg02254327	chr9	77111502	*RORB*	N. Shore	TSS1500	-0.0161	0.0064	0.0121
	cg16252131	chr17	38257290	*NR1D1*	S. Shore	TSS1500	0.0027	0.0010	0.0132
	cg11619602	chr11	13360707	*BMAL1*	Open sea	5'UTR	-0.0161	0.0068	0.0183
	cg03300961	chr15	61055949	*RORA*	Open sea	Body	-0.0052	0.0022	0.0184
	cg11129701	chr1	7862851	*PER3*	Open sea	5'UTR	0.0049	0.0021	0.0200
	cg10992253	chr2	239181120	*PER2*	Open sea	Body	0.0052	0.0023	0.0228
	cg20041802	chr15	61521674	*RORA*	Island	TSS200	0.0020	0.0009	0.0255

Adjusted for age, maternal education, SES, BMI z-score, ever smoker, ever drink alcohol, moderate-to-vigorous physical activity, sedentary, pubertal stage, batch effect, and estimated blood cell type proportions (Gran, CD4T, CD8T, Mono, and NK), or abundance (CD8pCD28nCD45RAn)

Supplementary Table 3: Cauchy combination test for gene-level DNA methylation of circadian genes, among males

Circadian Gene	Chromosome	Position	Ferritin Tertile 1 vs Tertile 3		Ferritin Tertile 2 vs Tertile 3	
			CCT	FDR	CCT	FDR
BMAL1	chr11	13298033-13397054	0.1147	0.5511	0.1587	0.9446
CLOCK	chr4	56298893-56414511	0.1837	0.5511	0.7260	0.9446
CRY1	chr12	107388741-107488596	0.7748	0.8916	0.7248	0.9446
CRY2	chr11	45867485-45903754	0.3232	0.6464	0.6599	0.9446
PER1	chr17	8044256-8057058	0.7474	0.8916	0.4367	0.9446
PER2	chr2	239153865-239198437	0.9982	0.9982	0.9446	0.9446
PER3	chr1	7843515-7904830	0.2546	0.6110	0.8942	0.9446
NR1D1	chr17	38249266-38257936	0.1456	0.5511	0.9387	0.9446
NR1D2	chr3	23985575-24020542	0.7579	0.8916	0.5701	0.9446
RORA	chr15	60786924-61522112	0.0299	0.3588	0.1408	0.9446
RORB	chr	77111502-77282233	0.8173	0.8916	0.2406	0.9446
RORC	chr1	151779765-151805773	0.5328	0.8916	0.4352	0.9446

CCT: Cauch combination test, FDR: false discovery rate
* FDR <0.1
**FDR <0.05

Chapter 3 Iron Deficiency and Vitamin D Deficiency Are Associated with Sleep in Women of Reproductive Age: An Analysis of NHANES 2005-2018 Data

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Abstract

Background: Iron and vitamin D deficiencies have been implicated in sleep disturbance. Although females are more susceptible to these deficiencies and frequently report sleep-related issues, few studies have examined these associations in females. **Objective:** This study investigates the association of iron and vitamin D deficiencies on sleep in a nationally representative sample of women of reproductive age. **Methods:** We used two samples of 20-49 years old non-pregnant females from NHANES 2005-2008 (N=2,497) and NHANES 2005-2010 and 2015-2018 (N=6,731) to examine the associations of iron deficiency (ID), iron deficiency anemia (IDA), vitamin D deficiency (VDD), vitamin D inadequacy (VDI), and the joint association of both deficiencies with sleep duration, latency, and quality. Sleep outcomes were measured using a self-reported questionnaire. We used the body iron model based on serum ferritin and serum soluble transferrin receptor to identify ID, along with hemoglobin to identify IDA cases. Additionally, 25-hydroxyvitamin D levels were used to determine VDD and VDI cases. Logistic regression was used to evaluate these associations, adjusting for potential confounders. Additionally, we assessed the multiplicative and additive interactions of both

deficiencies. **Results:** ID and IDA were associated with poor sleep quality, with 1.42 (95%CI: 1.02, 2.00) and 2.08 (95%CI; 1.29, 3.38) higher odds respectively, whereas VDD and VDI were significantly associated with short sleep duration, with 1.26 (95%CI: 1.02, 1.54) and 1.22 (95%CI: 1.04, 1.44) higher odds respectively. Subjects with both nutritional deficiencies had significantly higher odds of poorer sleep quality compared to subjects with neither condition. For sleep quality, a significant multiplicative interaction was observed between ID and VDD (p-value= 0.0005). No associations were observed between study exposures and sleep latency. **Conclusions:** Among women of reproductive age, iron and vitamin D deficiencies are associated with sleep health outcomes. The potential synergistic effect of both deficiencies warrants further assessment.

Introduction

Sleep plays a central role in human development and maintenance of mental and physical health. A plethora of studies have shown that short sleep duration, defined as ≤ 6 hours/day, is independently and significantly associated with increased risk of all-cause mortality, obesity, hypertension, cardiovascular diseases, stroke, diabetes mellitus, and mood disorders such as depression and anxiety (1,2). The age-adjusted prevalence of adults reporting short sleep duration (< 7 hours/day) has remained constant from 2013 to 2020 with approximately 34.8% of adults reporting getting less than the recommended amount of sleep in 2020 (3). While the trends have been steady recently, short sleep duration remains a major public health concern. It is only one aspect of healthy sleep behavior, which is a multidimensional concept encompassing sleep duration, quality, efficiency, timing, and alertness (4). Although these dimensions are correlated, epidemiological evidence suggested that each sleep dimension can be independently associated with certain health outcomes. For instance, a meta-analysis of randomized controlled trials

showed that sleep duration, sleep quality, and sleep timing have a detrimental effect on markers of insulin sensitivity (5). Additionally, sleep dimensions can differ by sex. Males and females tend to have a similar sleep pattern until puberty, when sex differences start to become more pronounced, as females frequently report sleeping less, having poorer sleep quality and efficiency, and difficulties falling asleep (6,7). Throughout the lifespan, women are more prone to sleep disturbance as their sleep can be influenced by menstrual cycle, pregnancy, and menopause (6).

Iron deficiency (ID) and vitamin D deficiency (VDD) are also more prevalent in women than in men. Women, particularly at childbearing age, are predisposed to iron deficiency and iron deficiency anemia (IDA) given the high iron requirements during this age period along with menstrual blood loss and inadequate consumption of dietary iron (8). Untreated ID with or without IDA can affect physical performance, work productivity, and cognitive function, and can increase the risk of morbidity and mortality in mothers and infants as well as people with chronic inflammatory diseases (9). Aside from these consequences, ID and IDA are proposed as potential risk factors for poor sleep health. ID and IDA are frequently associated with sleep disorders such as restless leg syndrome (RLS), periodic limb movements, and sleep disordered breathing as well as sleep disturbances observed in children with attention-deficit/ hyperactivity disorder and autism spectrum disorders (10). It has been estimated that one in four patients with IDA suffer from clinically significant RLS symptoms that cause shorter sleep duration and poorer sleep quality (11). However, limited research has been conducted in healthy individuals, as most of the research examining the link between ID/IDA and sleep has focused on people with sleep disorders or on infancy and early childhood.

In contrast to research on ID/IDA and sleep, vitamin D deficiency and vitamin D inadequacy (VDI) have been extensively studied, and a well-established association with sleep health has been examined in different age groups and clinical conditions (12). Interestingly, it is not uncommon for ID and VDD to co-exist. Emerging evidence from recent studies show that vitamin D is associated with iron and anemia. While most of the evidence emerged from animal studies, in humans the results are inconsistent but in favor of a positive association between vitamin D and iron status (13,14). Despite the inconsistent literature, the link between vitamin D and iron is plausible and warrants further assessment in relation to women's sleep. The current study aims to examine the association between ID, IDA, VDD,VDI, and the joint association of both deficiencies with sleep in a nationally representative sample of the U.S. women of reproductive age.

Methods

Study Population

This study used nationally representative data from the serial cross-sectional National Health and Nutrition Examination Surveys (NHANES) initiated in the early 1960s to provide data about the health and nutritional status of the US population. NHANES uses a multistage cluster sampling design and collects data via interviews, self-administered questionnaires, physical examinations, and laboratory data. Details of the design and the data from each survey are available online (https://www.cdc.gov/nchs/nhanes.htm). For this study, we used two samples from different NHANES cycles that met the inclusion criteria of non-pregnant females ages 20-49 years and include measures of exposures (ID, IDA, VDD, VDI) and outcomes (sleep duration, sleep quality, and sleep latency) (**Figure 3.1**). A longer, more extensive sleep questionnaire was

used in NHANES 2005-2008 cycles, and a shorter version was used in NHANES 2009-2018 cycles. The first sample included participants from NHANES 2005-2008 cycles only and used for sleep quality and sleep latency outcomes, due to the lack of these two outcomes in the subsequent cycles. The second sample included participants from NHANES 2005-2010 and 2015-2018 cycles, excluding participants from NHANES 2011-2014 cycles due to lack of iron data, and used to assess sleep duration (**Figure 1**). The final sample size that met the inclusion criteria was 2,497 females for the first sample and 6,713 females for the second sample.

Iron and vitamin D measures

Blood samples were collected at mobile examination centers (MECs) by trained personnel under controlled environmental conditions. Frozen specimens were shipped to the NHANES biorepository or directly to the analyzing lab. Complete blood counts (CBC) were performed in duplicate for all samples using Beckman Coulter MAXM. Hemoglobin was measured as part of the CBC. Serum ferritin and serum soluble transferrin receptor were used as iron deficiency indicators, as these tests can be used as an indicative measure for iron storage and transportation in the body, respectively. Different assay methods were used during NHANES 2005-2018 to measure serum ferritin and serum soluble transferrin receptor. In 2005-2008, Roche /Hitachi 912 clinical analyzer was used to measure serum ferritin concentrations which was changed to the Roche Elecsys 170 clinical analyzer in 2009-1010 and then to Roche e601 instrument in 2015-2018. NHANES conducted crossover studies to compare the data due to method changes and a weighted Deming regression was provided to adjust for method differences. For serum soluble transferrin receptor concentration, Hitachi 912 instrument was used in 2005-2008, then Hitachi Mod P in 2009-2010, and lastly Roche c501 in 2015-2018; however, the three methods give similar results, thus no adjustment was required. As ferritin is a positive acute-phase protein, we

used a regression correction approach recommended by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) group to calculate adjusted ferritin levels by C-reactive protein (CRP) as follows (15):

$$\text{Ferritin}_{\text{adjusted}} = \text{Ferritin}_{\text{unadjusted}} - \beta 1\ (\text{CRP}_{\text{obs}} - \text{CRP}_{\text{ref}})$$

Where β_1 is the CRP regression coefficient, obs is the observed value, and ref is the reference value used to define low inflammation and is equal to the maximum value of the lowest decile determined form log-transformed CRP. The correction of ferritin was applied only to individuals with ln CRP greater than ln CRP_{ref} to avoid overadjustment. Next, we used the body iron model to identify subjects with ID which have been verified and used in previous NHANES research (16). This model uses the ratio of the serum soluble transferrin receptor to serum ferritin as in the following formula:

$$\text{Body iron (mg/kg)} = \frac{-[\log 10\ (\text{soluble transferrin receptor [mg/L]} \times 1000/\text{ferritin [ug/L]} - 2.8229}{0.1207}$$

A negative value of <0 mg/kg is indicative of ID. Before we applied this model, we adjusted serum ferritin for inflammation using C-reactive protein Anemia was defined according to the WHO criterion as having hemoglobin <12 g/dL, and the body iron was used to determine if anemic subjects have IDA.

As with iron measures, vitamin D was analyzed using different methods. In 2005-2006, serum 25-hydroxyvitamin D (25(OH)D) was measured using the DiaSorin RIA kit, which then changed to standardized liquid chromatography-tandem mass spectrometry (LC-MS/MS) in 2007-2018. NHANES later converted vitamin D levels measured before 2007 to LC-MS/MS method equivalent levels due to bias and imprecise measurements from DiaSorin RIA kit (17). We added an indicator for the vitamin D method in the multivariable models. Given the absence of a universal definition for cutoff value of vitamin D deficiency, we used two cutoff points adapted

from the Institute of Medicine (18). We used vitamin D levels <12 ng/mL as indicative of vitamin D deficiency (VDD) and we used higher cutoff point of <20 ng/mL to include subjects with vitamin D inadequacy (VDI).

Sleep measures

Our main outcomes were sleep duration, sleep latency, and sleep quality. A self-reported questionnaire related to sleep habits and disorders was used to collect sleep data. Sleep duration was assessed by asking "How much sleep do you usually get at night on weekdays or workdays?". We categorized this variable as short sleep duration (≤6 hours/night) or normal sleep duration (>6 hours/night). Sleep latency was assessed by asking "How long does it usually take you to fall asleep at bedtime?" and we categorized the responses as normal (≤30 minutes/night) or prolonged (>30 minutes/night). For sleep quality, subjects were asked several questions about the past month's frequency of trouble falling asleep, waking up during night, waking up too early in morning, feeling unrested during the day, feeling overly sleepy during the day, and not getting enough sleep. We categorized the variable as poor sleep quality if the response was often or almost always for any question and good sleep quality if otherwise.

Covariates

Covariates selection was based on a *priori* knowledge of risk factors that affect sleep and include age, race/ethnicity, poverty income ratio (PIR), education level, marital status, season, smoking status, alcohol status, physical activity, sedentary level, body mass index (BMI), depression, and health conditions. Self-reported race/ethnicity was categorized as non-Hispanic white, non-Hispanic black, Mexican American Hispanic, other Hispanic, and other. We used PIR and education level as indicators of family socioeconomic status. PIR was calculated by dividing

family income by the poverty guidelines specific to the survey year accounting for inflation and family size. The PIR ranged from 0-5 with a ratio of <1 considered below the poverty threshold; we categorized this variable as poor if PIR <1, near poor if PIR 1 - <2, middle income if PIR 2 - <4, and high income if PIR ≥4 (19). Education level was categorized as less than high school, high school, and more than high school. Marital status was categorized to three groups which were married/partner, widowed/divorced/separated, or never married. Seasons refer to the six-month period when the examination was performed, where the period from November through April 30 indicates Winter/Spring and the period from May 1 through October 31 indicates Summer/Fall.

Smoking and alcohol status was obtained from associated questionnaires. Smoking was categorized as never smoker (smoke <100 cigarettes ever), former smoker (not currently smoking, but has smoked ≥100 cigarettes ever), and current smoker (smoked ≥100 cigarettes ever & currently smoking). Alcohol status was categorized as never drinker (drink < 12 alcohol drinks ever), former drinker (not currently drinking, but has drunk ≥12 alcohol drinks ever), and current drinker (drink ≥12 alcohol drinks ever & currently drinking). The Global Physical Activity Questionnaire was used to collect data about sedentary time and physical activity levels (20). We converted the reported minutes of sedentary activity to hours/per day and then categorized the variable to <4, 4-<8, 8-<11, and >11 hours of sedentary time. For physical activity, we summed the total Metabolic Equivalents (METs) from vigorous and moderate physical activity levels. METs were calculated by multiplying the weekly physical activity volume (duration x frequency) of each activity level by its corresponding METs value (8 for vigorous and 4 for moderate). Afterwards, we categorized METs as no METPA (0 MET min/wk), insufficient METPA (1-499 MET min/wk), and sufficient METPA (≥500 MET

min/wk). Body measures data were taken during the MECs visit by trained health technicians. Height was measured in centimeters using a stadiometer with a fixed vertical backboard and an adjustable head piece. Weight was measured in kilograms using a digital weight scale. BMI was calculated as weight (kg)/ height^2 (m^2) and then categorized normal weight (<25 kg/m^2), overweight (≥25-<30 kg/m^2), and obese (>30 kg/m^2).

Depression was assessed by self-reported Patient Health Questionnaires (PHQ-9), a validated screening instrument to assess depression in the past two weeks. The questionnaire contains nine questions with possible frequency response of 0 indicating not at all, 1 indicating several days, 2 indicating more than half the days, and 3 indicating nearly all days with a total possible score ranging from 0 to 27. Depression was categorized to none or minimum (0–4), mild (5–9), moderate (10–14), and moderately severe to severe (15–27) (21). Health conditions including hypertension, high cholesterol, diabetes, heart disease, and kidney were defined using self-reported questionnaires, physical examinations, or laboratory tests. Hypertension was defined as responding yes to "Have you been told on two or more different visits that you had hypertension?", or "Are you taking prescribed medicine to lower blood pressure" or having a mean diastolic blood pressure ≥80 mmHg, or a mean systolic blood pressure ≥130 mmHg. Participants were considered as having hypercholesterolemia if they were told by a physician that they had high cholesterol, or if the total cholesterol level was ≥240 mg/dL. Diabetes was defined as responding yes to "Have you ever been told by a doctor that you have diabetes?", or "Are you now taking diabetic pills to lower your blood sugar?", or a fasting glucose concentration ≥126 mg/dl, or HbA1c ≥6.5%. Subjects responding yes to "Have you ever been told by a doctor that you have prediabetes?" or having a fasting glucose concentration ≥100-<126 mg/dl, or HbA1c ≥5.7-<6.5% were considered prediabetics. Participants were categorized

as having heart disease if they were told by a doctor or other health professional that they had congestive heart failure, angina, heart attack, or coronary heart disease. Subjects have kidney disease if the calculated glomerular filtration rate (GFR) was <60 ml/min per 1.73 m^2 or urinary albumin-creatinine ratio was ≥ 30 mg/g. GFR was calculated using the CKD-Epidemiology (CKD-EPI) (22)

Statistical Analysis

All statistical analyses were performed in SAS 9.4 (Cary, NC, USA) using procedures for sample survey data to account for the complex survey design used in NHANES and using appropriate sampling weights and design variables (clustering and stratification). Before the analysis, we checked for missing values in outcome, exposure, and covariate variables, finding 1,590 missing values in sample 1 and 2,798 missing value in sample 2. To preserve sample size, improve precision, and increase statistical power, we performed multiple imputations by fully conditional specifications (FCS MI) (23). The FCS MI is a validated and robust statistical method that involves imputing the dataset multiple times using a sequence of regression models, where each incomplete variable is regressed on the other observed variables, allowing for accurate imputation. Afterwards, we assessed the distribution of sleep outcomes, ID/IDA, and VDD/VDI across categories of baseline sociodemographic and lifestyle characteristics. For continuous variables, we reported means and standard deviations and for categorical variables we reported percentages with Rao-Scott chi-square tests. In the bivariate analysis, we ran logistic regression to estimate odd ratios and 95% confidence intervals considering survey data complexity. In the multivariable analysis, we adjusted for potential confounders that were associated with exposures in the bivariate analysis. Given the role of vitamin D on iron metabolism, we evaluated the odds of having both deficiencies on sleep by combining the main exposures into one exposure variable

as iron deficiency and vitamin D deficiency (IDVDD), and as iron deficiency and vitamin D inadequacy (IDVDI). For each variable we created four categories which are ID and VDD/VDI, non-ID and VDD/VDI, ID and non-VDD/VDI, non-ID and non-VDD/VDI. Similar categories were created for iron deficiency anemia and vitamin D deficiency (IDAVDD), and for iron deficiency anemia and vitamin D inadequacy (IDAVDI). To test the interaction of ID and VDD/VDI, we assessed the multiplicative interaction by including the product term of ID and VDD/VDI in the model. We assessed additive interactions using relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP), and synergy index (S). Lastly, we conducted several sensitivity analyses to assess the robustness of our results, including the exclusion of subjects who exhibited symptoms of sleep disorders, exclusion of subjects with missing pregnancy data, the omission of sleep outcomes from the multiple imputation process, and performing a complete case analysis. Diagnostic-checking of the model assumptions was performed to check for any violation that could affect the results.

Results

Sociodemographic characteristics of the two samples were comparable. The mean age was 34.9 (0.1) years for the sleep duration sample (NHANES 2005-2010 and 2015-2018) and 35.3 (0.2) years for the sleep latency and quality sample (NHANES 2005-2008). About 12.8% of females had ID, 6.0% had IDA, 7.6% had VDD, and 30.1% had VDI. For sleep outcomes, 25.9% reported short sleep duration, 20.2% reported long sleep latency, and more than half reported poor sleep quality.

Table 3.1 shows the results of the bivariate analysis between sleep outcomes and baseline sociodemographic and lifestyle characteristics. Black females slept fewer hours and took longer to fall asleep compared to other races, whereas White females had poorer sleep quality even

though they slept longer hours. Shorter sleep duration was common among females with obesity, less education and less income, were currently smoking, reported no physical activity, and had depression, high cholesterol, or heart disease. Similar trends were observed among females with long sleep latency and poorer sleep quality. For the exposure-covariate associations (**Table 3.2**), racial differences were more pronounced as Black and Mexican American females had higher prevalence of ID/IDA and VDD/VDI compared to White females. Season was associated with VDD/VDI. Nutrient deficiencies were more common among current drinkers, while females who never smoked seemed to have more ID/IDA. Depression and hypertension were more common among females with VDD/VDI, while kidney disease was more prevalent among females with ID/IDA.

The results of the multivariable analysis are shown in **Table 3.3-3.5**. The odds of short sleep duration were 1.22 (95%CI: 1.01, 1.49) times significantly higher among those with ID compared to those who did not have ID. However, adjusting for confounders attenuated the association to 1.13 (95%CI: 0.92, 1.38), such that it was no longer significant. In contrast, significant associations were detected between VDD and VDI with shorter sleep duration that persisted after adjustment for confounders: 1.26 (95%CI: 1.02, 1.54) and 1.22 (95%CI: 1.04, 1.44), respectively. This association was consistent with the results shown in **Table 3.4-3.5,** where we assessed the joint association of iron and vitamin D. Moreover, females who had both deficiencies, had significantly higher odds of sleeping fewer hours compared to females who had neither deficiency. However, this association attenuated after adjustment.

None of the exposures were significantly associated with sleep latency. However, we found significant adjusted associations between ID/IDA with sleep quality. The odds of having poor sleep quality were 1.42 (95%CI:1.02, 2.00) times higher among females with ID compared to not

having ID and 2.08 (95%CI: 1.29, 3.38) times higher among females with IDA compared to not having IDA (**Table 3.3**). The odds significantly increased by 39% when females had both iron and vitamin D deficiencies (1.42 vs 1.98) and increased by 19% when females have iron deficiency anemia and vitamin D deficiency (2.08 vs 2.47) (**Table 3.4-3.5**). Females with both IDA and VDI had significantly higher odds of poor sleep quality compared to those with neither condition (**Table 3.5**). However, this association was not significant among females who had ID and VDI (**Table 3.4**). The post hoc analysis indicated that not getting enough sleep was the main characteristics driving the association between ID/IDA and sleep quality. Notably, results from sensitivity analyses closely aligned with the initial findings, indicating that our results were robust. Moreover, for sleep quality, we found a significant multiplicative interaction between ID and VDD (p-value: 0.0005). No statistical multiplicative or additive interactions for other sleep outcomes were observed with ID and VDI.

Discussion

In this study, we examined the cross-sectional association of iron and vitamin D deficiencies and inadequacies with sleep duration, latency, and quality in a representative sample of the US women of reproductive age. We found that ID/IDA was associated with sleep quality while VDD/VDI was associated with sleep duration, which emphasizes the importance of assessing different sleep dimensions when studying sleep health. Moreover, having both deficiencies may increase the odds of poorer sleep quality more than the expected multiplicative association, suggesting a synergistic effect of ID and VDD, which warrants further investigation. These results contribute to the literature on sleep and health, specifically targeting the two most common dietary deficiencies worldwide that are predominant in women of reproductive age.

While the research in women's sleep and ID/IDA is scarce, our results are in line with some previous studies in infants and young children. An analysis of sleeping patterns using electroencephalographic (EEG) in six-month-old infants with IDA showed alteration in sleep spindle patterns, a key characteristic of NREM sleep stages that is involved in memory consolidation and normal brain function (24). Moreover, a longitudinal cohort study assessing sleep patterns in infants at 6, 12, and 18 months of age revealed that IDA infants fell asleep faster at night but spent more time awake and less time in quiet sleep compared to non-IDA infants; and these differences disappeared after iron treatment (25). Subjective assessment of sleep showed similar results in infants. Maternal reports of sleep in infants aged 6–18 months show that infants with IDA had higher night waking and shorter total sleep duration compared to infants without IDA (26). In addition, sleep improved in the infants group randomized to 12 months of iron-folic acid supplements compared to the control group, suggesting that iron and/or folate can improve sleep in infants with ID and IDA (27).

In adults, large cross-sectional studies have shown that short sleep and sleep disturbance were associated with lower hemoglobin levels, and the odds of having anemia was higher among adults with sleep problems (28,29). However, given the cross-sectional design, it is uncertain whether poor sleep is a risk factor for anemia or a consequence of anemia. Moreover, these studies did not differentiate between IDA and other types of anemia, though it is estimated that 50% of anemia cases are related to ID. To our knowledge, only one study specifically examined the association between IDA and sleep in adults clinically diagnosed with IDA, but healthy otherwise, and found that adults with IDA reported worse sleep quality compared to age and gender matched controls (30). However, they did not find an association between blood biomarkers of IDA, such as hemoglobin and ferritin, with sleep quality. In addition, iron therapy

was shown to be effective in treating sleep disorders symptoms, in particular RLS. A randomized, double-blind, placebo-controlled trial in adults with RLS and low to normal ferritin levels (15–75 µg/l) found a significant improvement in RLS symptoms in the treatment group (325 mg ferrous sulfate twice daily for 12 weeks) compared to controls (31). In a more recent trial, IV iron therapy significantly improved sleep quality in patients with RLS and IDA (32). Regardless of having RLS or other sleep disorders symptoms, our study demonstrates a significant association between ID/IDA and sleep quality in healthy women, suggesting that adequate iron intake may be an important component in women's sleep health.

Vitamin D, in contrast, is well known to contribute to different dimensions of sleep health. A meta-analysis of observational studies suggested that VDD is associated with short sleep duration, poor sleep quality, daytime sleepiness, and increased risk of sleep disorders (33). In a large cross-sectional study of 21,083 individuals, sleep duration was positively associated with serum vitamin D levels in females, but not in males (34). Moreover, females with insufficient vitamin D have significantly shorter sleep duration compared to females with sufficient vitamin D levels. In a double-blind control trial, participants aged 20-50 years with sleep disorders were randomized to receive either a placebo or 50,000 IU vitamin D for eight weeks (35). The authors reported a significant improvement in sleep duration, sleep quality, and sleep latency in the group receiving vitamin D treatment. However, clinical trials in people without sleep disorders are scarce and the results from available trials are inconsistent, which warrants further investigations (36,37). Larsen et al. (2021), reported no significant effect of vitamin D supplementation on sleep duration and daytime sleepiness in individuals with vitamin D insufficiency (defined as <42 nmol/L) (36). While the underlying mechanisms between iron, vitamin D, and sleep are not well understood, evidence from animal studies indicates that both

low iron and vitamin D levels can significantly affect circadian genes expression (38–40). Moreover, iron is a vital co-factor in the synthesis of neurotransmitters, dopamine and serotonin, which are involved in the sleep-wake cycle (41). likewise, vitamin D is vital in the synthesis of sleep hormone melatonin, which initiates sleep through synchronization of the sleep-wake cycle with night and day (40). Another possible mechanism is the exposure to natural light, as both vitamin D and the circadian rhythm are regulated by sunlight (40). The present study suggests a synergistic effect between ID and VDD/VDI that can increase the odds of poor sleep quality. This could be due to the role of vitamin D in iron metabolism, as emerging evidence from *in vitro* and *in vivo* studies suggest that vitamin D can directly and indirectly affect iron homeostasis. Vitamin D plays a direct role in transcriptional regulation of hepcidin through suppression of the hepcidin antimicrobial peptide (HAMP) gene (42). Hepcidin is a hormone encoded by the HAMP gene that functions as the master regulator of iron homeostasis, as it can inhibit iron absorption from the gut and sequester iron in the macrophages, preventing iron recycling. On the other hand, vitamin D can indirectly restore iron recycling and promote erythropoiesis by down-regulating pro-inflammatory cytokines which can increase iron availability for erythropoiesis and hemoglobin synthesis (42,43). This association between vitamin D and iron can provide new insights into the mechanisms that could explain the relationship between sleep and these nutrient deficiencies.

This study has several strengths, including the use of NHANES data which is based on a representative sample of the US population, accounting for various potential confounders, and using biomarkers to assess iron and vitamin D status. However, the cross-sectional design of this study limits its ability to assess temporality and causal inferences. Moreover, the self-reported sleep data without further objective or clinical assessment of sleep outcomes may cause

recall/reporting bias. While women reported their habitual sleep, their nutritional deficiencies were measured at one point in time. Yet, it is possible that transient iron deficiencies due to factors such as menstrual cycle could result in temporary changes in sleep. Therefore, since the study's sleep questions only assessed the usual or typical sleep patterns, they may not have captured the true relationship between sleep and iron deficiency during the time of the deficiency. This limitation may have led to an underestimation of the association between sleep and iron deficiency. Moreover, we have limited information regarding the intake of dietary supplements for both sleep and nutrient deficiencies which could affect our findings. Although we adjusted serum ferritin for inflammation using CRP, we could not adjust serum soluble transferrin receptor due to lack of AGP measures (44). The variation in methods used to measure vitamin D in NHANES can introduce inconsistencies in the data. We address this issue by including a vitamin D method indicator in the multivariable models. Finally, while this study controls for various confounders, residual confounding, the effects of unmeasured confounders – such as genetic predisposition or other unknown factor associated with sleep and study exposures– cannot be excluded.

In summary, we have demonstrated a significant association between ID/IDA and sleep quality as well as VDD/VDI and sleep duration among women of reproductive age. The noteworthy combined association of ID and VDD with sleep quality warrants further investigation. These findings hold potential implications for clinicians providing care to women of reproductive age, as they may consider screening for vitamin D deficiency when caring for women with ID/IDA and sleep problems.

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Figures and Tables

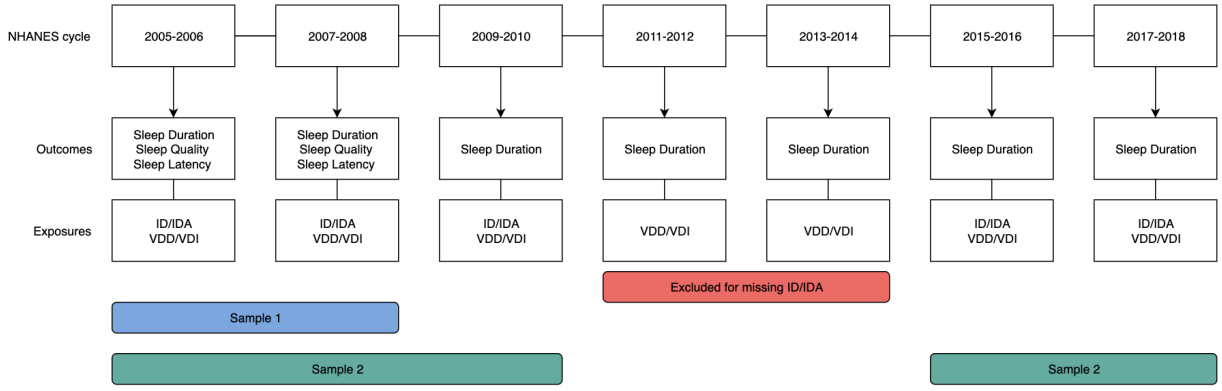


Figure 3.1 Flow chart of available outcomes and exposures from NHANES 2005-2018 cycles. ID: iron deficiency, IDA: iron deficiency anemia, VDD: vitamin deficiency, VDI: vitamin D inadequacy

Table 3.1 : Associations between sleep measures and sociodemographic and lifestyle characteristics[1]

	Sleep Duration (N=6,731)			Sleep Latency (N=2,497)			Sleep Quality (N=2,497)		
	≤6hrs	>6hrs	p-value	≤30 mins	>30 mins	p-value	poor	good	p-value
Age, years (y)	35.5 (0.2)	34.7 (0.2)		35.1 (0.3)	35.7 (0.4)		35.4 (0.3)	35.2 (0.3)	
Race/ethnicity (%)			**<0.0001**			**0.0418**			**<0.0001**
Non-Hispanic White	55.2	63		67.2	61.8		70	61.4	
Non-Hispanic Black	20.5	11		12.5	17		12.6	14.5	
Mexican American	9.3	10.3		9.1	8.7		6.3	12.3	
Other Hispanic	7	6.5		5	6		4.7	5.7	
Others	8	9.2		6.2	6.5		6.4	6.1	
PIR (%)			**<0.0001**			**<0.0001**			**0.0373**
Poor	19.9	16.2		13.8	24.3		17.4	14	
Near poor	23.1	20.5		19.1	23.1		20.4	19.2	
Middle income	30	30.1		30.5	29.4		29.5	31.4	
High income	27	33.2		36.6	23.2		32.7	35.4	
Education level (%)			**<0.0001**			**<0.0001**			**0.009**
< high school	18.4	12.8		13.9	23.6		14.4	17.7	
high school	23.1	19.3		20.3	25		22.5	19.7	
>high school	58.5	67.9		65.8	51.4		63.1	62.6	
Marital Status (%)			**<0.0001**			**<0.0001**			**0.0073**
Married, partner	58.7	63.2		64.9	58.8		62.1	65.6	
Widowed, Divorced, Separated	15.9	11.4		12.2	18.3		15.2	11.2	
Never married	25.4	25.4		22.9	22.9		22.7	23.2	
Season (%)			0.9128			0.2783			0.3215
Winter\spring	42.7	42.5		42.3	39.4		40.7	42.7	
Summer\fall	57.3	57.5		57.7	60.6		59.3	57.3	

	Sleep Duration (N=6,731)			Sleep Latency (N=2,497)			Sleep Quality (N=2,497)		
	≤6hrs	>6hrs	p-value	≤30 mins	>30 mins	p-value	poor	good	p-value
Smoking status (%)			**<0.0001**			**<0.0001**			**<0.0001**
Never smoker	56.2	65.9		63.1	45.6		53.4	67.1	
Former smoker	14.5	14.3		15.1	16		16.4	14	
Current smoker	29.3	19.8		21.8	38.4		30.2	18.9	
Alcohol status (%)			**0.0041**			0.1222			**0.0077**
Never drinker	11.8	12.2		12.4	12.2		10.4	14.8	
Former drinker	13.7	10.6		12.2	15.5		13.4	12.2	
Current drinker	74.5	77.2		75.4	72.3		76.2	73	
Physical activity (%)			**0.0003**			**<0.0001**			**0.0185**
No reported MVPA	38.9	34		20.4	33.8		25.1	20.7	
Insufficient MVPA	24.9	24		40.4	29.8		37.6	39	
Sufficient MVPA	36.2	42		39.2	36.4		37.3	40.3	
Sedentary time (%)			**0.0014**			0.1231			0.1251
<4 hrs	29	27.7		29.5	33.2		28.7	32.1	
4-<8 hrs	42.7	39.8		41.2	41.1		42.2	39.9	
8-<11 hrs	19.8	24.5		21.2	20.1		21.2	20.7	
≥11 hrs	8.5	8		8.1	5.6		7.9	7.3	
BMI (%)			**<0.0001**			**0.0001**			0.3019
Normal weight	32.7	40.6		42.3	32.3		39.6	41.3	
Overweight	26.9	24.9		25.1	27.6		25.2	25.9	
Obese	40.4	34.5		32.6	40.1		35.2	32.8	
Depression (%)			**<0.0001**			**<0.0001**			**<0.0001**
none or minimum	65.6	74.7		78	53.6		63.1	85.2	
mild	19.1	16.9		15.2	24.1		21.5	11.5	
moderate	9.4	5.5		4.2	14.3		9.5	2.3	
severe	5.9	2.9		2.6	8		5.9	1	

	Sleep Duration (N=6,731)			Sleep Latency (N=2,497)			Sleep Quality (N=2,497)		
	≤6hrs	>6hrs	p-value	≤30 mins	>30 mins	p-value	poor	good	p-value
Hypertension (%)			**0.0174**			**0.0331**			0.1508
No	73.6	77.1		76.9	71		74.3	77.6	
Yes	26.4	22.9		23.1	29		25.7	22.4	
High cholesterol (%)			**<0.0001**			**0.0001**			**0.0079**
No	76.2	81.9		79.6	69.7		75.1	80.7	
Yes	23.8	18.1		20.4	30.3		24.9	19.3	
Diabetes (%)			**0.0098**			**0.0421**			0.6579
Not diabetic	70.4	73.7		78.2	73.5		76.8	77.8	
pre-diabetic	25.5	23.1		18.8	22.2		19.6	19.3	
diabetic	4.1	3.2		3	4.3		3.6	2.9	
Heart disease (%)			**<0.0001**			**<0.0001**			**0.0015**
No	96.3	98.3		98.1	94.4		96.3	98.6	
Yes	3.7	1.7		1.9	5.6		3.7	1.4	
Kidney disease (%)			0.0566			0.6667			0.2864
No	90.8	92.4		90.8	91.4		91.6	90	
Yes	9.2	7.6		9.2	8.6		8.4	10	

[1] p-value obtained from Rao-Scott chi-square test; PIR: poverty income ratio, BMI: body mass index.

Table 3.2 Associations between exposure and sociodemographic and lifestyle characteristics (N=6,731)[1]

	ID			IDA			VDD			VDI		
	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value
Age, years	34.8 (0.2)	36.2 (0.4)		34.8 (0.2)	37.1 (0.5)		35.0 (0.2)	33.8 (0.5)		35.2 (0.2)	34.4 (0.2)	
Race/ethnicity (%)			**<0.0001**			**<0.0001**			**<0.0001**			**<0.0001**
Non-Hispanic White	63.1	46.7		62.6	35		64.6	17.3		73.7	31.4	
Non-Hispanic Black	12.3	21.3		12.3	32.4		10.5	49.4		6.2	30.3	
Mexican American	9.4	14.4		9.7	15.1		9.6	15.8		6.6	18	
Other Hispanic	6.4	7.9		6.4	9.5		6.6	6.4		5.7	8.8	
Others	8.8	9.7		9	8		8.7	11.1		7.8	11.5	
PIR (%)			**<0.0001**			**0.0001**			**<0.0001**			**<0.0001**
Poor	16.7	20.1		16.9	21.7		16.5	24.9		14.3	23.8	
Near poor	20.4	26.3		20.9	26.2		20.9	25.4		19.3	25.7	
Middle income	30.1	30		30	29.8		29.8	33.4		30.1	30	
High income	32.8	23.6		32.2	22.1		32.8	16.3		36.3	20.5	
Education level (%)			**0.0002**			**0.0001**			**<0.0001**			**<0.0001**
< high school	13.7	17.5		13.9	19		13.8	18.9		11.5	20.3	
high school	19.9	23.6		20	25.4		19.9	25.9		18.6	24.4	
>high school	66.4	58.9		66.1	55.6		66.3	55.2		69.9	55.3	
Marital Status (%)			0.2246			0.7356			**<0.0001**			**<0.0001**
Married, partner	62.2	60.6		62	62.7		63.2	47.6		65.4	54.2	
Widowed, Divorced, Separated	12.4	14		12.6	12.7		12.5	13.6		12	13.9	

	ID			IDA			VDD			VDI		
	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value
Never married	25.4	25.4		25.4	24.6		24.3	38.8		22.6	31.9	
Season (%)			0.0608			**0.0019**			**<0.0001**			**<0.0001**
Winter\spring	42	46.6		42	52.2		40.6	66.2		35.7	58.6	
Summer\fall	58	53.4		58	47.8		59.4	33.8		64.3	41.4	
Smoking status (%)			**0.0002**			**<0.0001**			**0.0001**			**<0.0001**
Never smoker	62.5	69.8		62.7	74.2		63	68.5		61.6	67.6	
Former smoker	14.5	12.9		14.7	8.8		14.9	7.7		16.5	9.3	
Current smoker	23	17.3		22.6	17		22.1	23.8		21.9	23.1	
Alcohol status (%)			**<0.0001**			**<0.0001**			**<0.0001**			**<0.0001**
Never drinker	11.4	17.5		11.6	20.6		11.6	18.7		9.9	17.3	
Former drinker	10.8	15.2		11.2	14.4		11.3	12.2		10.1	14.4	
Current drinker	77.8	67.3		77.2	65		77.1	69.1		80	68.3	
Physical activity (%)			**0.0002**			**<0.0001**			**<0.0001**			**<0.0001**
No reported MVPA	34.3	41.6		34.5	47.5		33.9	51.2		30.7	45.8	
Insufficient MVPA	24.9	19.4		24.7	16.2		24.9	16.5		25.2	22	
Sufficient MVPA	40.7	39		40.8	36.3		41.2	32.3		44.1	32.2	
Sedentary time (%)			**0.0197**			**0.0160**			0.0755			**0.0081**
<4 hrs	27.5	31.6		27.8	31.8		28.3	24.8		28	28.1	
4-<8 hrs	40.6	40		40.5	42		40.4	42.5		41.3	38.7	
8-<11 hrs	23.7	20.9		23.7	18		23.4	22.2		23.2	23.6	
≥11 hrs	8.2	7.4		8	8.2		7.9	10.5		7.5	9.6	

	ID			IDA			VDD			VDI		
	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value	No	Yes	p-value
BMI (%)			**0.0024**			**0.0090**			**<0.0001**			**<0.0001**
Normal weight	39.3	33.5		38.8	34.2		39.6	26.1		43.7	26.6	
Overweight	25.3	26.1		25.6	23		26	18		26	24	
Obese	35.4	40.4		35.6	42.8		34.4	55.9		30.3	49.4	
Depression (%)			0.2045			0.2928			**0.0469**			**0.0002**
none or minimum	72.6	70.3		72.4	70.8		72.6	69.2		73.8	69.1	
mild	17.3	18.4		17.4	18.7		17.4	18.4		16.6	19.4	
moderate	6.4	7.2		6.6	6.1		6.4	7.6		6	7.7	
severe	3.7	4.1		3.6	4.4		3.6	4.8		3.6	3.8	
Hypertension (%)			**0.0065**			**0.0302**			**0.0002**			**<0.0001**
No	76.8	72.4		76.5	71.2		76.8	68.6		78	72	
Yes	23.2	27.6		23.5	28.8		23.2	31.4		22	28	
High cholesterol (%)			0.9102			0.4321			0.7321			0.0605
No	80.4	80.3		80.3	82.2		80.4	80		79.8	81.8	
Yes	19.6	19.7		19.7	17.8		19.6	20		20.2	18.2	
Diabetes (%)			**<0.0001**			**<0.0001**			**0.0004**			**<0.0001**
Not diabetic	73.8	66.5		73.8	57.3		73.4	65.3		75.3	67.1	
pre-diabetic	23	29		22.9	37		23.3	29.4		22	27.8	
diabetic	3.2	4.5		3.3	5.7		3.3	5.3		2.7	5.1	
Heart disease (%)			0.2252			0.4245			0.1565			**0.0008**
No	97.9	97.3		97.8	97.2		97.9	96.9		98.2	96.9	
Yes	2.1	2.7		2.2	2.8		2.1	3.1		1.8	3.1	
Kidney disease (%)			**0.0041**			**0.0019**			0.2465			**0.0140**
No	92.4	89.2		92.3	87.8		92.1	90.7		92.6	90.5	
Yes	7.6	10.8		7.7	12.2		7.9	9.3		7.4	9.5	

[1] p-value obtained from Rao-Scott chi-square test; ID: iron deficiency, IDA: iron deficiency anemia, VDD: vitamin D deficiency, VDI: vitamin D inadequacy, PIR: poverty income ratio, BMI: body mass index.

Table 3.3 : OR (95% CI) from crude and adjusted associations between sleep measures and exposures (ID/IDA and VDD/VDI)

	ID[1]	IDA[1]	VDD[2]	VDI[2]
Short sleep duration				
Crude	**1.22 (1.01, 1.49)**	1.15 (0.89, 1.48)	**1.72 (1.41, 2.09)**	**1.55 (1.35, 1.79)**
Adjusted	1.13 (0.92, 1.38)	0.95 (0.73, 1.24)	**1.26 (1.02, 1.54)**	**1.22 (1.04, 1.44)**
Prolonged sleep latency				
Crude	1.12 (0.77, 1.64)	1.13 (0.69, 1.86)	1.21 (0.95, 1.55)	1.22 (0.98, 1.50)
Adjusted	1.04 (0.71, 1.55)	1.08 (0.64, 1.84)	0.91 (0.68, 1.22)	0.89 (0.65, 1.23)
Poor sleep quality				
Crude	1.23 (0.90, 1.70)	**1.62 (1.02, 2.56)**	0.96 (0.75, 1.22)	0.93 (0.77, 1.14)
Adjusted	**1.42 (1.02, 2.00)**	**2.08 (1.29, 3.38)**	1.03 (0.78, 1.37)	1.02 (0.78, 1.34)

[1] Adjusted for age, race/ethnicity, poverty income ratio, educational level, BMI, smoking, alcohol, physical activity, sedentary time, diabetes, hypertension, and kidney disease.
[2] further adjusted for season, marital status, depression, heart disease, and Vitamin D method indicator.
ID: iron deficiency, IDA: iron deficiency anemia, VDD: vitamin D deficiency, VDI: vitamin D inadequacy

Table 3.4 OR (95% CI) from crude and adjusted associations between sleep measures and exposures (IDVDD and IDVDI)[1]

	IDVDD				IDVDI			
	Both	Only VDD	Only ID	None	Both	Only VDI	Only ID	None
Short sleep duration								
Crude	**1.84 (1.34, 2.54)**	**1.73 (1.36, 2.20)**	1.20 (0.96, 1.50)	Ref	**1.81 (1.40, 2.34)**	**1.52 (1.30, 1.76)**	1.11 (0.82, 1,49)	Ref
Adjusted	1.27 (0.92, 1.75)	**1.29 (1.01, 1.64)**	1.13 (0.90, 1.42)	Ref	1.31 (1.00, 1.75)	**1.22 (1.04, 1.45)**	1.12 (0.83, 1.52)	Ref
Prolonged sleep latency								
Crude	1.19 (0.55, 2.60)	1.24 (0.94, 1.64)	1.13 (0.75, 1.69)	Ref	1.21 (0.74, 1.99)	1.24 (0.97, 1.59)	1.18 (0.65, 2.15)	Ref
Adjusted	0.82 (0.38, 1.77)	0.95 (0.66, 1.35)	1.03 (0.69, 1.56)	Ref	0.79 (0.44, 1.43)	0.94 (0.69, 1.28)	1.19 (0.65, 2.21)	Ref
Poor sleep quality								
Crude	**1.85 (1.09, 3.14)**	0.80 (0.59, 1.09)	1.14 (0.82, 1.57)	Ref	1.32 (0.91, 1.92)	0.87 (0.70, 1.08)	1.08 (0.69, 1.68)	Ref
Adjusted	**1.98 (1.04, 3.77)**	0.90 (0.64, 1.25)	1.31 (0.95, 1.83)	Ref	1.53 (0.96, 2.43)	0.96 (0.73, 1.28)	1.27 (0.80, 2.00)	Ref

[1] Adjusted for age, race/ethnicity, poverty income ratio, educational level, smoking, alcohol, physical activity, sedentary time, diabetes, kidney disease, season, marital status, body mass index, depression, hypertension, vitamin D method indicator; IDVDD: iron deficiency and vitamin D deficiency, IDVDI: iron deficiency and vitamin D inadequacy.

Table 3.5 OR (95% CI) from crude and adjusted associations between sleep measures and exposures (IDAVDD and IDAVDI)[1]

	IDAVDD				IDAVDI			
	Both	Only VDD	Only IDA	None	Both	Only VDI	Only IDA	None
Short sleep duration								
Crude	**1.70 (1.20, 2.41)**	**1.73 (1.39, 2.15)**	1.10 (0.82, 1.46)	Ref	**1.68 (1.24, 2.28)**	**1.53 (1.32, 1.77)**	0.95 (0.61, 1.48)	Ref
Adjusted	1.11 (0.75, 1.63)	**1.28 (1.02, 1.60)**	0.97 (0.71, 1.32)	Ref	1.17 (0.82, 1.67)	**1.22 (1.04, 1.44)**	0.90 (0.57, 1.43)	Ref
Prolonged sleep latency								
Crude	1.00 (0.47, 2.12)	1.26 (0.98, 1.62)	1.19 (0.66, 2.12)	Ref	1.05 (0.57, 1.95)	1.25 (0.99, 1.58)	1.42 (0.61, 3.30)	Ref
Adjusted	0.79 (0.38, 1.64)	0.94 (0.68, 1.30)	1.11 (0.60, 2.05)	Ref	0.72 (0.38, 1.35)	0.93 (0.67, 1.28)	1.53 (0.67, 3.50)	Ref
Poor sleep quality								
Crude	1.99 (0.88, 4.52)	0.88 (0.65, 1.18)	1.52 (0.91, 2.56)	Ref	**1.71 (1.04, 2.80)**	0.89 (0.73, 1.10)	1.41 (0.71, 2.79)	Ref
Adjusted	**2.47 (1.08, 5.66)**	0.97 (0.69, 1.36)	**1.94 (1.15, 3.29)**	Ref	**2.23 (1.26, 4.04)**	0.98 (0.74, 1.31)	1.78 (0.88, 3.59)	Ref

[1] Adjusted for age, race/ethnicity, poverty income ratio, educational level, smoking, alcohol, physical activity, sedentary time, diabetes, kidney disease, season, marital status, body mass index, depression, hypertension, vitamin D method indicator; IDAVDD: iron deficiency anemia and vitamin D deficiency, IDAVDI: iron deficiency anemia and vitamin D inadequacy.

Chapter 4 Vitamin D is Associated with Sleep Disturbance and Cognitive Function in Older Adults

Abstract

Background: Vitamin D and iron are essential for sleep health. In older adults, suboptimal levels of these micronutrients, coupled with poor sleep, have been associated with cognitive decline. Despite this connection, there are limited studies on sex and racial differences. This study aims to first examine the sex and racial variation in the association between iron and vitamin D with sleep disturbance in older adults. Second, we examine the mediating role of sleep disturbance in the association between these micronutrients and cognitive function. **Methods:** We used data from 6,371 adults enrolled in the Health and Retirement Study (HRS). Blood samples were collected between 2016 and 2017 and were used to measure serum ferritin and serum 25-hydroxyvitamin D2 and D3. The sum of these vitamin D forms was used to determine the total vitamin D level. Sleep disturbance was self-reported in the HRS core interview in 2018 and 2020. Cognitive function was assessed using a score derived from a battery of tests administered in 2018. Stratifying by sex and race/ethnicity, logistic mixed-effects models with random intercept were used to examine the longitudinal association between these micronutrients and sleep disturbance, adjusted for confounders. Causal mediation analysis with adaptive bootstrap tests was used to assess whether sleep mediates the associations between micronutrients and cognitive function. **Results:** Higher levels of vitamin D2 were associated with higher odds of sleep disturbance in White individuals (male OR: 1.535 (95%CI: 1.105, 2.133)); females OR: 1.107 (95%CI: 1.042, 1.175)), but lower odds of sleep disturbance among

Black males (OR:0.738 (95%CI: 0.586, 0.939)). In contrast, vitamin D3 and the total level of vitamin D was protective against sleep disturbance in both White and Black individuals, with the strongest association observed among White males (OR: 0.872 (95%CI: 0.826, 0.920)). No associations with ferritin were observed. Moreover, we found evidence that sleep partially mediates the associations between vitamin D3 and the total vitamin D level with cognitive function among Black females. **Conclusion:** Our study found a significant association between vitamin D and sleep disturbance, with some differences in males and females across races. Sleep partially mediates the association between vitamin D3 and the total level of vitamin D with cognitive function among Black females.

Introduction

In recent years, there has been a pronounced global shift in the demographic age structure, with a distribution shift towards older age groups. According to the World Health Organization, it is estimated that 1.2 billion people will be aged 60 years or older by 2050 (1). As the population ages, the importance of understanding and optimizing the determinants of health and well-being of older adults becomes increasingly evident. Sleep has gained considerable attention due to the age-related changes in sleep pattern. Older adults tend to sleep earlier and wake up earlier than younger adults; however, their sleep is characterized by longer sleep-onset, shorter night sleep duration, higher sleep fragmentation, more sleep disturbance, more nighttime awakening, and higher daytime naps and daytime sleepiness (2,3). Nevertheless, not all older adults experience these changes to the same extent. Factors such as sex and race/ethnicity play a role in the variability of sleep disruption associated with age, with female and Black individuals reporting worse sleep (4,5). Moreover, potentially modifiable factors including sufficient micronutrient status of vitamin D and iron has been associated with better sleep health in the elderly.

Observational studies suggest that vitamin D deficiency is associated with short sleep duration, poor sleep quality, daytime sleepiness, and increased risk of sleep disorders in different age groups (6). On the other hand, iron deficiency in infants and individuals with restless leg syndrome is correlated with poor sleep health, which improves after iron supplementation (7,8). Most of the previous studies that have examined the role of vitamin D and iron on sleep were cross-sectional studies, and few were conducted in older adults. Nevertheless, both vitamin D and iron contribute to the regulation of circadian rhythms and synthesis of neurotransmitters that are involved in the sleep-wake cycle (9–11). Notably, both factors, the suboptimal levels of vitamin D and iron, as well as sleep disruption, are associated with adverse health outcomes in older adults, especially cognitive decline (12-17).

Several meta-analyses of observational studies reveal a significant association of low vitamin D level on different domains of cognitive function including memory and learning performance, psychomotor and executive functions, mental speed, and visuospatial abilities (12,13). On the other hand, the association between iron and cognition is U-shaped with both iron deficiency and iron accumulation, especially in the brain, leading to a detrimental effect on cognitive performance in older adults (14,15). Similar associations have been observed with sleep duration, as both short (≤4 hours/night) and long (≥10 hours/night) sleep duration is associated with faster cognitive decline (16). Moreover, good sleep quality with lower nighttime restlessness and shorter sleep latency was associated with better memory and executive functions in adults 50 years or older (17). The interrelated association between vitamin D and iron, sleep, and cognition warrants further investigation, particularly the potential pathways by which micronutrients and sleep could impact cognition.

While numerous studies have provided valuable insight into the direct association of vitamin D and iron on cognitive function in older adults, there is a growing need to examine the potential mediating role of sleep on this association. Early detection of cognitive decline is crucial for intervening appropriately and preventing further progression of cognitive decline to dementia. Accordingly, this study aims, firstly, to investigate the longitudinal associations between iron and vitamin D with sleep disturbance in a representative sample of the United States older adults, considering the role of sex and race/ethnicity in modifying these associations. Secondly, we will examine whether sleep disturbance partially mediates the association between micronutrients, iron and vitamin D, and cognitive function.

Methods

Study Population

Data were drawn from waves 13 (2016) to 15 (2020) of the Health and Retirement Study (HRS), a nationally representative longitudinal study of adults aged 50 years or older living in the United States. The HRS surveys have been fielded every 2 years since 1992 and collect data about demographic characteristics, physical health, physical performance, employment history, retirement, income and wealth, cognitive status, and family connections (18). The sample was drawn using a multi-stage area probability design involving geographical stratification and clustering and oversampling of African American and Hispanic populations. The sample replenishes every six years with a younger cohort. In the 13th wave (HRS 2016), respondents who completed the HRS core interview were invited to participate in the Venous Blood Study (VBS), excluding proxy respondents and nursing home residents. A total of 9,934 subjects provided blood samples between 2016 and 2017, which were subsequently used to measure various blood biomarkers. Sleep disturbance data were obtained from waves 14 and 15 (HRS

2018 and 2020), and cognitive function data were obtained from wave 14 (HRS 2018) for those who provided blood samples. The analytical sample of this study includes 6,371 subjects with available data on ferritin, vitamin D, sleep disturbance, and cognitive function.

Ferritin and Vitamin D level

Serum ferritin was measured by turbidimetric antibody method using Roche reagents (Roche Diagnostics Corporation) on the Roche COBAS 6000 instrument (Roche Diagnostics, Indianapolis IN) (19). Given that serum ferritin is affected by inflammation, we used a modified version of the regression correction approach developed by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project to calculate the adjusted ferritin levels using C-reactive protein (CRP) concentration (20). The adjustment equation is,

$$\text{Ferritin}_{\text{adjusted}} = \text{ferritin}_{\text{unadjusted}} - \beta_1 (\text{CRP}_{\text{obs}} - \text{CRP}_{\text{ref}}),$$

where β_1 is the CRP regression coefficient, obs is the observed value, and ref is the reference value used to define low inflammation and is equal to the maximum value of the lowest decile determined from log-transformed CRP. To avoid overadjustment, the correction of ferritin was applied only to individuals with ln CRP greater than ln CRP_{ref}. We excluded subjects with extreme ferritin level higher than1000 ng/mL as it is indicative of hereditary hemochromatosis.

Serum vitamin D was measured as 25-hydroxyvitamin D2 and D3 using liquid Chromatography coupled to tandem Mass Spectrometry (LC/MS/MS) (21). Given the distinct sources of vitamin D and the relative lack of research on vitamin D2, in this study, we used both vitamin D2 and vitamin D3 as well as the sum of both values as the total vitamin D levels. Including assessments for both forms of vitamin D and their sum, allowed for more a comprehensive understanding of the role of vitamin D in sleep.

Sleep Disturbance

Insomnia-related sleep disturbance was self-reported using the physical health questionnaire, which was part of the core HRS interview in 2018 and 2020. The sleep questions were adapted from the Jenkins Sleep Scale, a validated assessment tool that measures sleep disturbance (22). These questions include: "How often do you have trouble falling asleep?", "How often do you have trouble with waking up during the night?", "How often do you have trouble with waking up too early and not being able to fall asleep again?", and "How often do you feel really rested when you wake up in the morning?". Participants' responses were rarely or never; sometimes; or most of the time. We created a binary variable for sleep disturbance where participants coded as 1 if they reported most of the time for any of these questions, and zero otherwise. The fourth question was reverse coded to ensure consistency with other questions responses.

Cognitive Function

Cognitive function was measured using a the Langa-Weir score (range 0-27) derived from a validated battery of tests as modified version of the Telephone Interview for Cognitive Status and administered in HRS 2018 core interview (23). These tests measure memory, working memory, and mental processing, and include immediate and delayed recall of 10 words from a word list randomly assigned for each participant (0–20 points), backward counting test (0–2 points), and serial seven subtraction test (0–5 points). Higher scores indicate better cognitive function. While the HRS used a proxy respondent for participants with cognitive or physical impairments that limited their ability to self-respond, it is noteworthy that none of the participants in this study required proxy respondents.

Covariates

Covariates were chosen based on *prior* knowledge and were self-reported in the 2016 HRS core interview. Covariates include age, education level (less than high school, high school, more than high school), total wealth (based on tertiles of the distribution in this sample), marital status (married/not married, divorced/separated/widowed/never married), alcohol consumption (never, former, current), smoking (never, former, current), body mass index (calculated as weight (kg)/height (m^2): <24.9, 25.0‑29.9, ≥30.0), physical activity (vigorous, moderate, light), depression (yes, no), hypertension (yes, no), diabetes (yes, no), and heart disease (yes, no).

Statistical Analysis

We first examined the distribution of sociodemographic and lifestyle characteristics across sex and race/ethnicity groups. Next, we examined the prevalence of sleep disturbance in 2018 and 2020. For the first aim, we used logistic mixed-effects models with random intercept accounting for complex survey data to examine the longitudinal association between micronutrients and sleep disturbance. This analysis was conducted using SAS 9.4 (Cary, NC, USA) on a high-performance computing system, using the GLIMMIX Procedure with quadrature algorithm to obtain the weighted likelihood and the empirical (sandwich) estimator to obtain the standard errors (SEs) for inferences on the fixed effects (24). In the multivariable model we adjusted for confounders as listed in the covariate section. In sensitivity analyses, we removed subjects who tested positive for COVID-19 in 2020 and we further adjusted for additional potential covariates such as serum calcium, pain, and the use of sleep medication.

For the second aim, we first examined the mediation criteria which included: a significant relationship between exposure and mediator (path from ferritin or vitamin D to sleep disturbance); a significant relationship between mediator and outcome (path from sleep

disturbance to cognitive function); relationship between exposure and outcome (path from ferritin or vitamin D to cognitive function) (**Figure 1**) (25). Next, we used a causal mediation approach based on the counterfactual framework to examine whether sleep disturbance partially mediated the associations between micronutrients and cognitive function, reporting the direct effect, the indirect effect, and the total effect of these associations. We adjusted for age, education, total wealth, marital status, alcohol status , smoking status, body mass index, physical activity, depression, hypertension, diabetes, and heart disease. To test whether the indirect effect (mediation effect) was significantly different from zero, we used an adaptive bootstrap test that better controls type-I errors and improves statistical power (26). Then, the false discovery rate (FDR) was used to adjust for multiple comparisons, with FDR <0.05 indicating statistical significance (27). The mediation analysis was only applied for sex and racial groups that met the mediation criteria.

Results

The mean age was 67.4 ± 0.2 years with 58.8% females, 67.6% White, 17.9% Black, and 14.5% Hispanic. **Table 4.1** shows the characteristics of the study sample stratified by sex and race/ethnicity. Among males, White males were slightly older, more educated, wealthier, more likely to drink alcohol but less likely to smoke, more likely to be overweight but physically active, and less likely to have hypertension or diabetes compared to Black or Hispanic males. Similar trends were observed among females, although White females were more likely to have a normal weight and be married or in partnership compared to Black or Hispanic females. For micronutrients distribution, males exhibited significantly higher mean levels of ferritin compared to females (p-value<0.0001); when further stratified by race, Black males and females had the highest levels of ferritin (166.9 and 129.2 ng/mL, respectively). Conversely, females had higher

mean levels of vitamin D2 compared to males, with Black males and females having the highest levels (2.3 and 3.5 ng/mL, respectively). For vitamin D3 and the total vitamin D levels, both White males and females exhibited higher mean levels compared to other races/ethnicities. As for sleep disturbance prevalence, females had more sleep disturbance compared to males across all races/ethnicities for both years (**Figure 4.1**). Hispanic females had the highest prevalence of sleep disturbance; however, their sleep appeared to have improved in 2020 compared to 2018 (45.6 vs 49.1%, respectively). During the COVID-19 pandemic, sleep disturbance became more common among Black males and White females compared to 2018 (40.0 vs 33.9% and 38.3 vs 35.5%, respectively). **Figure 4.3** displays the distribution of cognitive function scores in 2018 by sex and race/ethnicity groups. White individuals had higher mean cognitive function scores compared to Black and Hispanic individuals. Specifically, among males, Black males exhibited the lowest cognitive function score (13.2), while among females, both Black and Hispanic females exhibited the lowest score (14.4).

Micronutrients and sleep disturbance

The associations between ferritin and vitamin D and sleep disturbance are shown in **Table 4.2**. There was no association between ferritin and sleep disturbance across all sex and race/ethnicity groups. On the other hand, the adjusted mixed-effect analysis showed that one unit increase in vitamin D2 levels was significantly associated with higher odds of sleep disturbance among White individuals (males OR: 1.535, 95%CI:1.105, 2.133; females OR: 1.107, 95%CI: 1.042, 1.175). On the contrary, for every one unit increase in vitamin D2 level, the odds of sleep disturbance decreased by 26.2% (95%CI: 7.0%-31.4%) among Black males. Vitamin D3 appeared to be protective against sleep disturbance among White and Black males and females, with a stronger association observed among White males (OR: 0.872, 95%CI:0.826, 0.920).

Similar associations were detected with the total levels of vitamin D, with a stronger association observed among Black males; for instance, one unit increase in total vitamin D was significantly associated with 12.6% reduction in the odds of sleep disturbance among Black males. No significant associations were identified with Hispanic males and females. Notably, results from sensitivity analyses closely aligned with our main findings, affirming the robustness of our results.

Mediation analysis

Examining the mediation criteria listed in the methods section shows that only Black females met these criteria, and only in relation to two exposures: vitamin D3 and the total level of vitamin D. The causal mediation analysis revealed a significant direct, indirect, and total effect with sleep disturbance in the association between vitamin D3 and the total level of vitamin D with cognitive function among Black females. Sleep disturbance explained around 7% of the association between vitamin D3 and cognitive function, and around 5% of the association between total vitamin D and cognitive function (β=0.0028, FDR=0.0015 and β=0.0018, FDR=0.0059, respectively) (**Table 4.3**).

Discussion

In this study of 6,371 adults aged 50 years or older, we examined the longitudinal relationship between ferritin and vitamin D with sleep disturbance. Findings from our study demonstrate that, both forms of vitamin D (D2 and D3) were associated with sleep disturbance. In general, vitamin D3 and the total level of vitamin D was protective against sleep disturbance among White and Black males and females, whereas vitamin D2 may increase the odds of sleep disturbance among White males and females. We did not find associations between ferritin and sleep disturbance in older adults. Moreover, sleep disturbance may partially mediate the

association between vitamin D3 and the total level of vitamin D with cognitive function among Black females. This suggests that increased levels of vitamin D3 and total vitamin D are associated with lower odds of sleep disturbance, potentially contributing to better cognitive function among Black females.

Our results are aligned with previous research that has investigated the influence of vitamin D on sleep. Massa et al. (2015) reported a dose-dependent relationship between insufficient total vitamin D levels and higher odds of poor sleep efficiency, increased sleep fragmentation, and short sleep duration in older men (28). Moreover, vitamin D deficiency is associated with excessive daytime sleepiness in older adults (29). A meta-analysis of randomized control trials show that vitamin D supplementation improves sleep quality in various age populations (30). Regarding sex and racial disparities, previous studies have yielded inconsistent findings when examining the association between vitamin D and sleep. A cross-sectional study found that low concentration of the total serum vitamin D was associated with short sleep duration in men, but not women (31). Beydoun et al (2020) found that higher total serum levels of vitamin D were associated with better sleep quality and longer sleep duration over time among White adults only (32). An analysis of the Multi-Ethnic Study of Atherosclerosis found that vitamin D deficiency was associated with short sleep duration in Black adults (33). Moreover, data from the National Health and Nutrition Examination Survey reveal that higher total serum vitamin D levels were correlated with longer sleep duration among older White and Black adults but not with Mexican American and other Hispanic older adults (34). Similarly, our study found protective longitudinal association of vitamin D3 against odds of sleep disturbance in both White and Black, males and females, older adults. Additionally, we did not observe any significant

association with Hispanic individuals, who comprise diverse groups sharing cultural and social factors rather than biological characteristics.

The unexpected detrimental association between vitamin D2 and sleep disturbance among White people warrants further investigation. Previous research did not distinguish between the forms of vitamin D, employing the term "vitamin D" generically to encompass both vitamin D2 and Vitamin D3. Vitamin D2, also referred as ergocalciferol, is derived from plant sources exposed to ultraviolet B (UVB) radiation such as mushroom and yeast (35). Vitamin D3, also referred as cholecalciferol, is primarily obtained through exposure to sunlight and intake of animal-food sources such as meat, fish, and eggs (35). Other sources such as supplements and fortified food may contain both forms. It is worth noting that vitamin D3 has demonstrated greater efficacy in raising the total concentrations of 25-hydroxyvitamin D in our body; thus, most of the total vitamin D levels is derived from vitamin D3 (36). Our study emphasizes the importance of distinguishing between the different forms of vitamin D when assessing their impact on sleep patterns. While the observed potential risk associated with vitamin D2 in inducing sleep disturbance in White individuals warrants attention, it is essential to consider the possibility that this association may be due to random chance or act as a proxy for unknown variables. Unfortunately, we lack data on genetic variations and dietary habits among racial and ethnic groups. Therefore, further assessment is needed to confirm these results.

Few studies have examined the association between serum ferritin and sleep in older adults. A cross-sectional study in South Korea found that moderate level of serum ferritin was associated with lower risk of short sleep duration among older women only (37). However, ferritin levels were not adjusted for inflammation in this study. Other studies found a link between serum ferritin and sleep disorders such as restless leg syndrome and obstructive sleep

apnea (38,39). We did not find significant longitudinal associations between serum ferritin and sleep disturbance in our sample. This inconsistency could be due to methodological differences, demographic variations, or potential influences of unexplored variables. Notably, most of previous studies focused on individuals with iron deficiency or iron deficiency anemia. The mean level of ferritin in this study was 130 ng/mL (<15 ng/mL is the cutoff for iron deficiency), indicating that the majority of the participants have optimal ferritin levels. As a result, the association between ferritin and sleep disturbance might be less pronounced or obscured by other contributing factors in this sample.

The mediation analysis reveals that sleep disturbance partially mediates the association between vitamin D3 and the total vitamin D levels with cognitive function in Black females only. A cross-sectional study found that poor sleep quality mediated the association between vitamin D deficiency and cognitive function in older adults with heart failure (40). A recent study showed that subjects with sufficient serum vitamin D3 levels and good sleep quality had significantly lower odds of cognitive impairments compared to subjects with vitamin D3 deficiency and fair or poor sleep quality (41). Although this study did not explicitly investigate the mediation effect, the significant association between low vitamin D levels, poor sleep quality, and cognitive function raises intriguing possibilities. Our study provides further evidence that sleep may mediate the association between vitamin D and cognitive function using a cohort study design. Notably, we only observed this mediation effect among Black females, as they were the only subgroup meeting the criteria for mediation. This may be due to the higher variation in serum vitamin D levels and cognitive function score among Black females compared to some other subgroups. Specially, Black females have lower vitamin D3 levels, higher prevalence of sleep disturbance, and lower cognitive function scores compared to White

individuals. While Black males and Hispanic individuals exhibited similar variability to Black females, but their smaller sample sizes limited statistical power. Given the minimal magnitude of the observed mediation effect, detecting a mediation effect in subgroups with small sample sizes may not be feasible. Therefore, further investigation is needed to evaluate the mediation effect of sleep in the association between vitamin D and cognitive function in older adults.

This study has several strengths and limitations. Strengths include using a large, representative sample of older adults with repeated measures of sleep. Moreover, we used blood biomarkers for ferritin and vitamin D, and thoroughly examined several potential confounders. However, sleep data was self-reported which possibly can cause recall bias. In addition, we only measured one aspect of sleep related to insomnia symptoms. Although the Langa-Weir cognitive score is widely used in epidemiological research, it is primarily designed for screening purposes and does not evaluate all cognitive domains. While we have sufficient overall sample size, stratifying the analysis by sex and race/ethnicity may have reduced the statistical power. Moreover, the sample size limits our ability to further stratify by age. Further assessment with a larger sample size in each sex and racial group is warranted to confirm our results. Finally, unmeasured confounders, such as genetic variation and dietary habits may bias our results.

Conclusion

Our study extends and refines the published literature on sleep and vitamin D in older adults and emphasizes the importance of assessing different forms of vitamin D. In summary, Vitamin D3 exhibited protective associations in both White and Black males and females, while vitamin D2 demonstrated detrimental associations particularly in the sleep of White individuals. Moreover, sleep disturbance may partially mediate the association between vitamin D3 and the total vitamin D levels with cognitive function among Black females.

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Figures and Tables

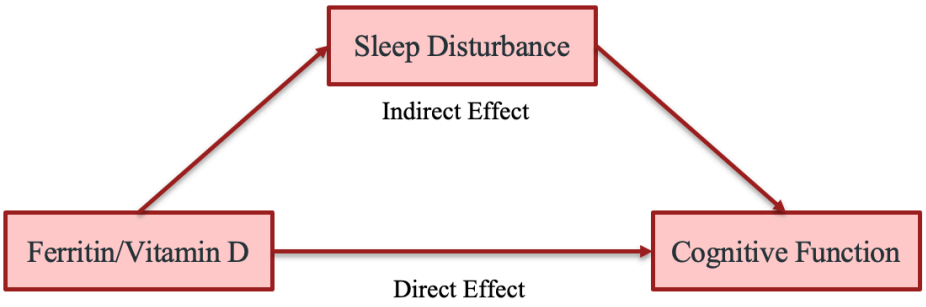


Figure 4.1 Causal mediation diagram illustrating the relationship between exposure (micronutrients), outcome (cognitive function), and mediator (sleep disturbance).

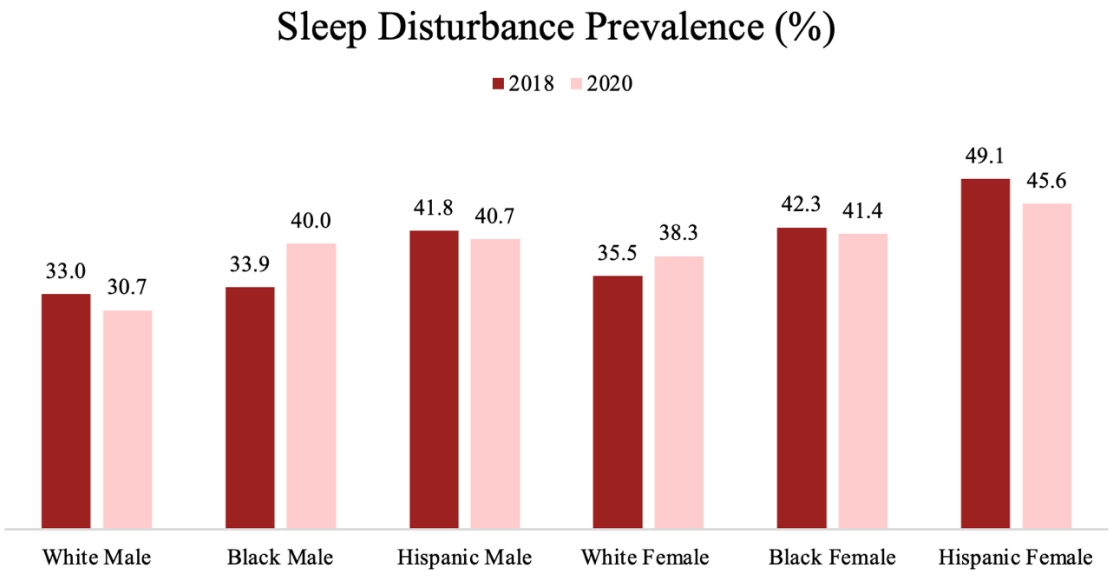


Figure 4.2 Prevalence of sleep disturbance in 2018 and 2020 by sex and race/ethnicity.

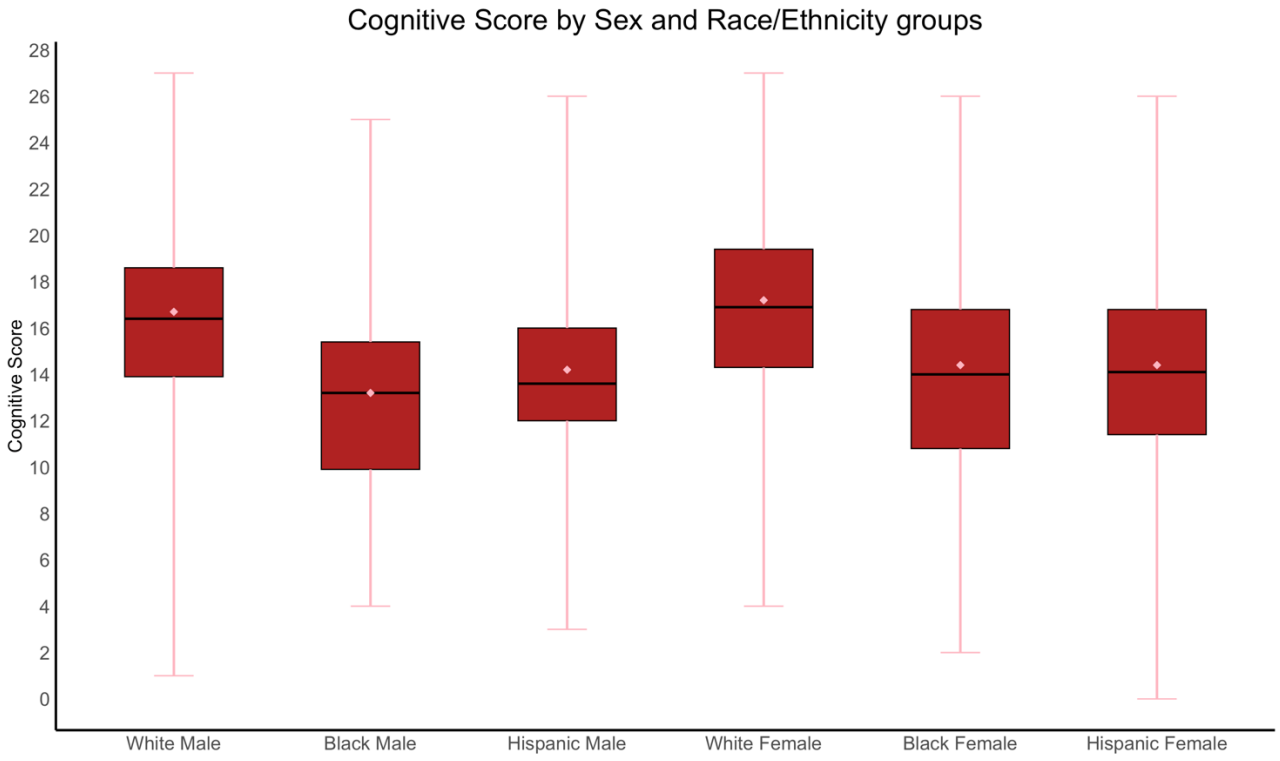


Figure 4.3 Distribution of cognitive score in 2018 by sex and race/ethnicity groups.

Table 4.1 characteristics of the study sample stratified by sex and race/ethnicity.

	Male (N=2,625)			Female (N=3,746)		
	White	**Black**	**Hispanic**	**White**	**Black**	**Hispanic**
N	1,852	402	371	2,453	738	555
Age (years)	67.26 (0.30)	65.55 (0.49)	65.88 (0.59)	68.04 (0.26)	66.62 (0.41)	66.38 (0.93)
Education (%)						
Less than high school	9.01	25.27	47.05	8.78	26.07	51.24
High school	24.75	27.09	19.45	28.65	25.83	21.99
More than high school	66.24	47.64	33.49	62.56	48.10	26.78
Total wealth (%)						
Tertile 1	19.36	63.49	53.18	22.48	65.70	56.99
Tertile 2	34.48	25.31	32.12	34.66	28.20	31.56
Tertile 3	46.17	11.21	14.70	42.85	6.10	11.45
Marital status (%)						
Married/partner	79.31	57.25	83.17	60.25	30.82	55.60
Divorced, separated, widowed, unmarried	20.70	42.75	16.84	39.75	69.18	44.40
Alcohol (%)						
Never drinker	29.32	44.38	30.94	37.96	56.50	61.13
Former drinker	12.36	12.53	22.43	18.75	18.41	19.50
Current drinker	58.32	43.09	46.64	43.29	25.09	19.38
Smoking (%)						
Never smoker	38.97	28.51	36.35	50.83	45.24	54.94
Former smoker	50.49	40.25	51.26	40.18	37.76	36.48
Current smoker	10.54	31.23	12.39	8.99	17.00	8.58
BMI (%)						
Normal weight	16.46	19.31	20.14	29.74	15.60	20.46
Overweight	44.26	40.64	39.60	33.51	29.70	35.50
Obese	39.28	40.05	40.26	36.76	54.31	44.04
Physical activity (%)						
Vigorous	37.42	31.93	28.92	25.59	16.90	21.39
Moderate	30.38	19.23	23.73	35.05	27.93	25.93
Light	32.20	48.83	47.34	39.35	55.17	52.68
Depression (%)						
No	82.34	79.01	84.43	69.60	70.24	59.09
Yes	17.66	20.99	15.57	30.40	29.77	40.91
HTN (%)						
No	45.92	25.61	38.52	48.61	19.16	37.56
Yes	54.08	74.39	61.48	51.39	80.84	62.44
DM (%)						
No	78.10	68.00	58.96	81.73	67.23	60.84
Yes	21.90	32.00	41.04	18.27	32.77	39.16

	Male (N=2,625)			Female (N=3,746)		
	White	**Black**	**Hispanic**	**White**	**Black**	**Hispanic**
Heart disease (%)						
No	73.66	78.65	78.19	79.90	75.45	79.49
Yes	26.34	21.35	21.81	20.10	24.55	20.51
Ferritin (ng/mL)	161.38 (3.27)	166.90 (5.92)	160.79 (9.89)	99.70 (2.02)	129.20 (5.46)	94.26 (4.82)
Vitamin D2 (ng/mL)	1.15 (0.11)	2.29 (0.50)	1.85 (0.43)	1.92 (0.16)	3.52 (0.51)	2.75 (0.52)
Vitamin D3 (ng/mL)	30.78 (0.35)	22.51 (0.72)	24.18 (0.56)	35.20 (0.39)	23.94 (0.66)	25.72 (1.06)
Total vitamin D (ng/mL)	31.93 (0.33)	24.79 (0.82)	26.03 (0.46)	37.13 (0.34)	27.46 (0.73)	28.47 (1.21)

Table 4.2 odds ratio (95% CI) from mixed-effect logistic associations between ferritin, vitamin D, and sleep disturbance stratified by sex and race/ethnicity.

	Male			Female		
	White	**Black**	**Hispanic**	**White**	**Black**	**Hispanic**
Ferritin						
Crude	1.001 (0.997, 1.006)	0.993 (0.985, 1.001)	0.992 (0.981, 1.002)	1.005 (1.000, 1.010)	0.998 (0.989, 1.006)	0.999 (0.990, 1.007)
Adjusted	0.998 (0.994, 1.001)	0.995 (0.988, 1.002)	0.996 (0.978, 1.014)	1.002 (0.997, 1.007)	0.998 (0.990, 1.006)	0.998 (0.990, 1.006)
Vitamin D2						
Crude	1.096 (0.968, 1.241)	0.959 (0.778, 1.182)	1.042 (0.911, 1.191)	**1.101 (1.024,1.184)**	1.121 (0.972, 1.293)	0.997 (0.876, 1.134)
Adjusted	**1.535 (1.105, 2.133)**	**0.738 (0.586, 0.930)**	1.226 (0.937, 1.546)	**1.107 (1.042, 1.175)**	1.064 (0.949, 1.194)	0.941 (0.832, 1.064)
Vitamin D3						
Crude	0.987 (0.936, 1.041)	0.952 (0.876, 1.036)	0.907 (0.820, 1.004)	**0.952 (0.917, 0.987)**	**0.906 (0.845, 0.971)**	1.069 (0.934, 1.223)
Adjusted	**0.872 (0.826, 0.920)**	**0.911 (0.836, 0.993)**	0.914 (0.800, 1.044)	**0.960 (0.931, 0.990)**	**0.912 (0.854, 0.974)**	1.074 (0.988, 1.168)
Total vitamin D						
Crude	**0.988 (0.947, 1.032)**	0.934 (0.844, 1.035)	0.991 (0.870, 1.128)	**0.959 (0.927, 0.992)**	0.953 (0.894, 1.016)	1.043 (0.952, 1.143)
Adjusted	**0.901 (0.860, 0.944)**	**0.874 (0.779, 0.981)**	0.940 (0.817, 1.081)	**0.919 (0.889, 0.950)**	**0.934 (0.876, 0.996)**	1.067 (0.969, 1.175)

Adjusted models controlled for age, education level, total wealth, marital status, alcohol, smoking, body mass index, physical activity, depression, hypertension, diabetes, and heart disease.

Table 4.3 mediation effects of sleep disturbance on the association between micronutrients and cognition score in Black females

Exposure	Direct Effect		Indirect Effect		Total Effect	
	Effect	FDR	Effect	FDR	Effect	FDR
Vitamin D3	0.0390	**0.0018**	0.0028	**0.0015**	0.0418	**0.0012**
Vitamin D Total	0.0380	**0.0018**	0.0018	**0.0059**	0.0398	**0.0012**

FDR: false discovery rate (bold FDR <0.05)

Chapter 5 Conclusion

This dissertation extends current knowledge and contributes to the understanding of the complex interplay between iron, vitamin D, and sleep health across different life stages, pointing toward critical avenues for further exploration. This chapter provides a summary of the main results for each aim and discusses future directions and public health implications of this dissertation.

From the cradle to the golden years, sleep patterns evolve across life stages, influenced by different biological, psychological, and environmental factors. The tumultuous period of adolescence brings about numerous physiological and behavioral changes, including natural shift in sleep patterns and higher iron requirements. In chapter 2 (aim 1), we evaluated the longitudinal, sex-specific associations between iron, measured by ferritin, and sleep outcomes including sleep duration, sleep midpoint, and sleep quality, measured objectively in weekdays and weekends in two visits. We found that lower serum ferritin was associated with higher sleep fragmentation in females during weekdays, suggesting that suboptimal iron levels are associated with poor sleep quality among adolescent females. On the other hand, during the weekends, lower ferritin levels were associated with later sleep timing among males. No significant association was identified with sleep duration and movement index in both males and females. These results emphasize the importance of examining sleep patterns during both weekdays and weekends. Epidemiological studies have indicated that social jetlag, the mismatch

in sleep patterns between weekdays and weekends, can disrupt the circadian rhythm, potentially leading to adverse health outcomes such as cardiometabolic disease, mood disorders, cognitive impairment, and substance abuse (1). While previous research has mostly focused on infants and young children with iron deficiency (ID) as well as on adults with sleep disorders, this study further extends the scope to include adolescents, thereby broadening our understanding of the impact of iron on sleep across different age groups (2–4). Unfortunately, vitamin D data was not available in this aim; hence, future studies may examine the role of vitamin D on adolescents' sleep.

The study further established a link between iron status and sleep at molecular levels. To our knowledge, this is the first human study to examine the role of iron on DNA methylation of key circadian genes, providing invaluable and novel insights on the potential mechanism by which iron is associated with sleep. The results showed that lower ferritin levels were significantly associated with DNA methylation of several circadian genes in females only. We hypothesized that DNA methylation of circadian genes may mediate the association between iron and sleep. Nevertheless, the small sample size and the cross-sectional design of this analysis hinder our ability to perform causal mediation analysis. Consequently, future studies must incorporate larger sample sizes and longitudinal designs to explore this potential pathway.

The observed sex variations in this aim may reflect the difference in sleep patterns and iron requirements in this age group. Adolescents, particularly girls, are predisposed to ID. The iron Recommended Dietary Allowances (RDAs) for non-vegetarian increase from 8 mg/day for both males and females at age of 9-13 years to 11 mg/day for males and 15 mg/day for females at 14-18 years of age, with 1.8 times higher iron requirements for vegetarians (5). The increased iron requirements during adolescence are caused by rapid growth spurts, the expansion of blood

volume, the increase in muscular growth in boys, and the onset of menstruation in girls (6). The requirements of iron continue to rise as females get older. Unlike males whose iron requirements decrease back to 8 mg/day at the age of 19-50 years, females' iron requirements increase to 18 mg/day and up to 27 mg/day during pregnancy in the same age group (5). These higher iron requirements and menstrual blood loss, along with the inadequate consumption of dietary iron, put females at a higher risk of ID and iron deficiency anemia (IDA).

Consequently, in chapter 3 (aim 2), we shifted the focus to non-pregnant females at reproductive age. In this chapter we examined the association between ID and IDA with self-reported sleep duration, sleep quality, and sleep latency in a nationally representative sample of females at reproductive age. Given that it is common for ID and vitamin D deficiency (VDD) to co-exist, we further assessed the role of VDD and vitamin D inadequacy (VDI) on females' sleep (7,8). Similar to the results from chapter 2, we found that suboptimal iron status was associated with poor sleep quality and not with sleep duration. On the other hand, VDD and VDI were associated with higher odds of short sleep duration. Moreover, females with both deficiencies (ID and VDD) exhibited elevated odds of poorer sleep quality compared to those without either condition. Our results also shed light on the synergistic effect of both deficiencies on poor sleep quality, which highlights the complexity of nutritional influences on sleep health in females of reproductive age. These findings emphasize the potential implications for public health initiatives targeted at females of reproductive age, emphasizing the role of nutrition in improving sleep health. Ensuring adequate intake of an iron-rich diet and adequate exposure to sunlight, or supplementation if needed, could effectively enhance sleep among females of reproductive age. Although these significant results are promising, the cross-sectional design limited our ability to

establish temporality, underscoring the need for longitudinal studies that can provide insights into whether iron and vitamin D affect sleep over time.

Accordingly, in chapter 4 (aim 3), we used a large longitudinal sample from the Health and Retirement Study (HRS), a nationally representative longitudinal study of adults aged 50 years and older. The HRS deliberately oversamples African American and Hispanic populations making it well-suited to examine racial disparities in sleep pattern among older adults, who are especially susceptible to sleep-related issues. Therefore, in this aim, we explored the sex and racial variations in the associations between serum ferritin and serum vitamin D (D2 and D3) with sleep disturbance in adults aged 50 years and older. While we did not find a significant association between serum ferritin and sleep, the association between serum vitamin D and sleep was more pronounced. Notably, we uncovered paradoxical associations between serum vitamin D forms, D2 and D3, with sleep disturbance. Particularly, we found a protective association of serum vitamin D3 against sleep disturbance, contrasting with the increased risk associated with higher levels of vitamin D2, among White individuals. Remarkably, both vitamin D2 and D3 were protective against sleep disturbance in Black individuals. While it is not clear what could cause these contradictory relationships in White people, several factors may contribute to these associations such as dietary habits, genetic predispositions, variations in vitamin D metabolism, or interactions with other environmental or lifestyle factors. Therefore, more research is needed to confirm these results and to unravel the underlying mechanisms driving these paradoxical findings.

Expanding our exploration into the realm of older adults, evidence from previous research showed that both suboptimal micronutrients status and poor sleep health may contribute to the cognitive decline in older adults. We hypothesized that sleep disturbance may partially

mediate the associations between micronutrients, ferritin and vitamin D, and cognitive function. To test this hypothesis, we used causal mediation analysis in each of the sex and race/ethnicity groups. From the six groups (White males, Black males, Hispanic males, White females, Black females, and Hispanic females), only Black females met the mediation criteria proposed by Baron and Kenny (1986) (9). The causal mediation results revealed that sleep disturbance partially mediates the association of vitamin D3 and the total level of vitamin D with cognitive function among older Black females. Although the mediation effect size was small, it was statistically significant. The small effect size may still be meaningful if it contributes to the understanding of the underlying mechanism between these micronutrients, sleep, and cognitive function. The early detection of cognitive decline is crucial for ensuring healthy aging especially with the growing population of older adults. However, we cannot exclude the effect of unmeasured confounders such as genetic variation and sun exposure. Hence, further studies are needed to explore the causal pathways between vitamin D and cognitive function through sleep.

While many questions remain about the role of iron and vitamin D on sleep, the wealth of insights generated from this dissertation contributes valuable knowledge to the broader understanding of how vitamin D and iron are associated with sleep in different life stages. Across the chapters of this dissertation, we reveal varying patterns and associations, emphasizing the importance of considering various age, sex, and race/ethnicity groups in understanding the complex multifaceted relationships between these micronutrients and sleep health. From the public health perspective, the identified associations between suboptimal iron and vitamin D status with sleep outcomes underscore the significance of nutrition in sleep health. Subclinical iron and vitamin D deficiencies may not manifest with overt symptoms and often go unrecognized. However, it can still have a detrimental impact on overall health and wellbeing,

including sleep health. Since there isn't a specific set of sleep symptoms that uniquely indicate iron or vitamin D deficiencies, common mild symptoms of these deficiencies such as weakness, fatigue, headache can overlap with symptoms of other health conditions. Consequently, sleep issues may be attributed to other factors, leading to the oversight of potential underlying suboptimal nutritional status. Moreover, suboptimal micronutrients status could serve as an indicator of the overall dietary quality. Therefore, nutritional interventions aimed at ensuring optimal diet may potentially improve sleep health.

This dissertation also emphasizes the need for holistic approaches to public health initiatives. Tailoring interventions based on life stage, sex, and race/ethnicity can enhance their effectiveness in improving sleep especially in vulnerable groups. Taken together, this dissertation emphasizes the role of iron and vitamin D in promoting sleep health, while acknowledging the unique considerations at different life stages and demographic groups.

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