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INSOMNIA SYMPTOMS AND SYSTEMIC INFLAMMATION IN ADOLESCENTS: A POPULATION-BASED STUDY

A Thesis in

Anatomy

by

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Abstract

Sleep is a behavior essential to healthy development. Insomnia, which is characterized by difficulty falling and/or staying asleep and associated daytime functioning impairment, is the most prevalent sleep disorder. Insomnia is not just a disruption of an individual's normal sleep but rather impacts both physical and mental health and, in adults, insomnia with objective short sleep duration has shown to be a more biologically severe form of the disorder. This insomnia phenotype has been associated with activation of the stress system, inflammation and adverse cardiometabolic, neurocognitive and psychiatric outcomes. It is also known that this insomnia phenotype is highly persistent throughout adulthood; therefore, it is important for clinicians to identify this phenotype early during development. Our primary aim in this project was to test whether objective short sleep duration, as measured by polysomnography (PSG), played a key role in predicting the association of insomnia symptoms with inflammation in adolescents. Given that PSG is not readily available for clinicians, we examined in secondary analyses whether the persistence of insomnia symptoms since childhood also helped predict the association of insomnia symptoms with inflammation in adolescents. Subjects were participants in the Penn State Child Cohort (PSCC), a population-based sample of 421 adolescents who underwent 9-h PSG followed by a single fasting blood draw to assess plasma levels of C-reactive protein (CRP) and other inflammatory markers via ELISA. Insomnia symptoms were defined by a self-report of difficulty falling and/or staying asleep on the Pediatric Sleep Questionnaire, while objective sleep duration was defined as ≥ 8 hours, 7-8 hours, ≤ 7 hours of sleep based on the quartiles of PSG-measured total sleep time. Multivariable-adjusted general linear models tested the association between insomnia symptoms, objective sleep duration and their interaction on CRP levels. Adolescents reporting insomnia symptoms had significantly higher CRP levels compared to controls and a significant interaction ($p<0.01$) showed that objective sleep duration modified this association. Elevated CRP was present in adolescents with insomnia symptoms who slept ≤ 7 hours (1.8 mg/L) as compared to controls or adolescents with

insomnia symptoms who slept ≥ 8 hours (0.9 mg/L and 1.0 mg/L, respectively) or controls who slept ≤ 7 hours (0.74mg/L; all p-values <0.01). These findings indicated that, as in adults, objective short sleep duration identifies a phenotype of insomnia at greater risk of morbidity via chronic low-grade inflammation. Secondary analyses based solely on self- and parent-reported data revealed that adolescents whose parents reported persisting insomnia symptoms since childhood had significantly higher CRP levels (1.2 mg/L, p=0.046) compared to controls or to adolescents with new-onset insomnia symptoms (1.0 and 0.8 mg/L, respectively). This finding suggests that a childhood onset of insomnia symptoms may be a useful tool to screen for those adolescents at greater risk of inflammation. Together, our findings indicate that chronic low-grade inflammation may be a common final pathway towards morbidity in adulthood in the insomnia with objective short sleep duration phenotype as early as adolescence.

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Abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ARAS	Ascending Reticular Activating System
AVP	Arginine Vasopressin
C/ABCL	Child/Adults Behavior Checklist
CNS	Central Nervous System
CRF	Corticotropin-releasing factor
CRP	C - reactive protein
DFA	Difficulty Falling Asleep
DSA	Difficulty Staying Asleep
EDTA	Ethylenediamine Teraacetic Acid
EEG	Electroencephalographic
ELISA	Enzyme-linked Immunosorbent Assay
EMG	Electromyogram
EOG	Electrooculogram
GLM	General Linear Model
HPA	Hypothalamic-pituitary-adrenal
IL-6	Interleukin-6
MEQ	Morningness-Eveningness Questionnaire
MSLT	Multiple Sleep Latency Test
NSAIDS	Non-steroidal Anti-inflammatory Drugs
PLMI	Periodic Limb Movement Index
PSCC	Penn State Child Cohort
PSG	Polysomnography
PSQ	Pediatric Sleep Questionnaire
PVN	Paraventricular Nucleus
REM	Rapid Eye Movement
SAM	Sympatho-adrenal-medullary
SE	Sleep Efficiency
SEM	Standard Error of the Mean
SOL	Sleep Onset Latency

SpO2	Hemoglobin Oxygen Saturation
SWS	Slow Wave Sleep
TNF-α	Tumor Necrosis Factor Alpha
TST	Total Sleep Time
WASO	Wake After Sleep Onset
3P	Spielman's Three Factor Model

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I would like to acknowledge Dr. Fernandez-Mendoza for his permission to use the below publication for the purposes of partial fulfillment of this master's thesis, and fantastic mentoring throughout my graduate career. Additionally, I would like to thank the staff of the Sleep Research and Treatment Center at the Pennsylvania State University College of Medicine; they have all been an integral part of this work.

Chapter 1. Insomnia in Adolescence

1.1 Introduction

Sleep is a fundamental human behavior, which is shortened or rendered ineffective in individuals with insomnia. Insomnia occurs with a high prevalence within the population; while about 20-40% report symptoms of difficulty falling or staying asleep or early morning awakening at any given time, 8-10% report insomnia as a chronic and persistent condition. (Bixler et al., 2002; Buysse, 2013) Insomnia is considered a disorder of 24-h hyperarousal as demonstrated by central nervous system (CNS) activation and hyperactivity of both limbs of the stress system, i.e., hypothalamic-pituitary-adrenal (HPA), sympatho-adrenal-medullary (SAM) axes and sympathetic / parasympathetic balance. These physiological markers of 24-hour hyperarousal are primarily found in an emerging phenotype of insomnia characterized by objective short sleep duration, as measured by polysomnography (PSG). In addition, emerging evidence supports that this insomnia phenotype is associated with increased cardiovascular, metabolic and psychiatric morbidity and proposed mechanisms of such associations are stress system activation mentioned above as well as, potentially, chronic low-grade inflammation. However, this latter hypothesis still needs to be tested. The Penn State Child Cohort (PSCC) affords a unique opportunity to examine the association of insomnia and objective short sleep duration on circulating markers of inflammation during a critical developmental period in which the onset of sleep disorders is greatest: adolescence.

1.2 Etiology and Pathophysiology of Insomnia

Insomnia disorder is described as difficulty initiating or maintaining sleep or early morning awakening occurring three or more nights a week, despite adequate opportunity for sleep, for more than three months and associated with significant daytime impairment (AASM, 2014; APA, 2013; Perlis et al., 2016). Spielman's three factor (3P) model is a behavioral proposal describing how acute insomnia can become chronic (Spielman, 1987). This perspective describes predisposing factors (e.g., genetic, environmental, psychological) that put an individual at risk for developing insomnia, which

manifests acutely with certain precipitating factors (e.g., stressful life events) (Healey et al., 1981). Thereafter, acute insomnia becomes chronic when certain perpetuating factors (e.g., keeping an irregular sleep-wake schedule, spending excessive time in bed, sleep incompatible behaviors such as watching TV or reading in bed, or daytime napping) lead to an inability to return to baseline after the precipitating factor has resolved. The 3Ps perspective is a useful model of insomnia to understand its progression from an acute, transient phenomenon to a chronic condition based on behavioral mechanisms (i.e., classical and instrumental conditioning as a result of the perpetuating factors listed above). Another, yet not mutually exclusive model focuses on the physiological and neural mechanisms of insomnia.

The physiologic hyperarousal model focuses on the role of specific brain mechanisms that regulate arousal as a key-perpetuating factor of insomnia in adults. Wakefulness results from activity of the ascending reticular activating system (ARAS), which is modulated by projections from the hypothalamus and thalamus in response to circadian and homeostatic factors (Saper, Scamell & Lu, 2005). From this perspective, "insomnia is a disorder of sleep-wake regulation characterized by persistent wake-like activity in neural structures during sleep, resulting in simultaneous and regionally specific waking and sleeping neuronal activity patterns" (Buysse, 2012). Hyperarousal in insomnia is characterized by CNS, HPA and SAM axes activation, as indicated by increased levels of cortisol, norepinephrine, increased brain and whole-body metabolic rate as well as impaired heart rate variability, among other markers (Adam, Tomney & Oswald 1986; Vgontzas et al., 1998; Vgontzas et al., 2001; Rodenbeck et al., 2002; Shaver et al., 2002; Rodenbeck et al., 2003; Irwin, et al., 2003). Thus, contrary to the common belief that insomnia is a disorder of sleep loss (i.e., reduced sleep as a result of homeostatic sleep dysfunction), evidence supports 24-h hyperarousal as the principal underlying mechanism for the chronicity of insomnia. This dysregulation, in turn, may result in activation of the immune system, which is modulated by the HPA axis and sympathetic nervous system (Chrousos, 2007). In fact, activity of specific neurons results in the accumulation of sleep-promoting pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor alpha

(TNF-α) (Krueger, 2008; Chrousos, 2007). If this were to be true, individuals with insomnia should also show marked increases in inflammatory markers, which could be an underlying mechanism by which insomnia can result in increased risk of morbidity. With this in mind, current models of insomnia are still in need of objectively measurable physiologic markers to support the underlying mechanisms they propose. Such an objective marker will have important implications for diagnosis and treatment of the disorder.

1.3 Insomnia, immune system and chronic low-grade inflammation

Accumulating evidence indicates that insomnia is associated with adverse health outcomes such as cardiometabolic morbidity, neurocognitive impairment and psychiatric disorders (Buysse, 2010; Fernandez-Mendoza & Vgontzas, 2013; Hall et al., 2016). Among others, inflammation has already been mentioned above as a potential mechanism by which insomnia can affect health (Vgontzas et al., 2002; Irwin et al., 2015). It is reasonable to posit that the characteristic hyperarousal of insomnia may lead to disruption in CNS regulation of the peripheral immunologic apparatus with resultant chronic elevation of circulating pro-inflammatory cytokines such as TNF-α, interleukin-1 (IL-1) and IL-6. Conversely, IL-6 along with TNF-α and IL-1 have also been shown to stimulate the HPA axis and it has been proposed that this occurs through stimulation of central noradrenergic pathways in the ARAS. Specifically, IL-6 is a strong activator of parvocellular corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) neurons in the paraventricular nucleus (PVN) of the hypothalamus, and also stimulates secretion of adrenocorticotropic hormone (ACTH), a downstream target of CRH and AVP, in the anterior pituitary (Chrousos, 2007). ACTH subsequently regulates the release of glucocorticoids, specifically cortisol from the zona fasciculate of the adrenal gland (Chrousos, 2007). Furthermore, IL-6 is of particular interest as it influences production of C-reactive protein (CRP) by the liver. In fact, CRP has been used as a clinical marker for evaluating risk for cardiovascular disease (Ridker, 2016), and an increasing field of research supports that disturbed sleep may play a role in modulation of these molecules (Kapsimalis et al., 2008; Chrousos, 2007;

Irwin, Olmstead & Carrol, 2015) and that CRP may be a more stable inflammatory biomarker to predict risk of morbidity (Irwin, Olmstead & Carrol, 2015; (Volpato et al., 2001; Ferrucci et al., 1999).

IL-6 and TNFα have been shown to exhibit a circadian rhythm, which can become shifted in disorders like insomnia. Individuals with insomnia have been shown to exhibit both higher levels of these circulating cytokines, along with a circadian shift in their release, which was not seen in healthy controls (Vgontzas et al., 2002). Importantly, sleep restriction studies in healthy good sleepers show that sleep restriction results in a significant increase in these circulating cytokines without a subsequent increase in cortisol and deeper sleep and shorter sleep latencies during sleep, post-restriction (Vgontzas et al., 2004). In individuals with insomnia, this compensatory mechanism is not seen, which suggests that there is something unique about elevated markers of inflammation paired with the characteristic HPA-axis activation of insomnia that results in nighttime short sleep duration and daytime fatigue (Vgontzas et al., 2004).

In fact, a recent meta-analysis of 72 studies comprising more than 50,000 individuals, demonstrated a significant relationships between short sleep duration, subjectively measured in the vast majority of studies, and elevated levels of CRP and IL-6 (Irwin, Olmstead & Carrol, 2015). Overall, shorter sleep duration was associated with significantly increased CRP (ES =0.09); however, it was clear that the association of shorter sleep duration was stronger (ES = 0.18) in studies using objective sleep measures (Larkin et al., 2005; Lee et al., 2009; Matthews et al. 2010; Rief et al., 2010; Taheri et al., 2007), as compared to studies using subjective sleep measures (ES= 0.04). Similarly, shorter sleep duration was associated with increased IL-6 (ES=0.11), with a stronger association when sleep duration was measured objectively (ES=0.29) vs. subjectively (ES=0.03) (Burgos et al., 2006; Friedman et al., 2005; Hong et al., 2005; Lee et al., 2009; Motivala et al., 2005; Rief et al., 2010; Vgontzas et al., 1997; Von Kanel et al., 2006). Together, these studies showed that objective sleep measures of sleep duration are a stronger predictor of inflammatory markers.

Interestingly, this same meta-analysis found a significant association between subjective

reports of insomnia symptoms and elevated levels of CRP and IL-6 (Irwin, Olmstead & Carrol, 2015). Specifically, questionnaire-based studies showed a significant association of insomnia symptoms with elevated levels of CRP (ES=0.12) and IL-6 (ES=0.20). In contrast, this meta-analysis showed that studies using in-lab conditions and diagnostic criteria for insomnia were not significantly associated with increased levels of IL-6 despite the fact that the degree of association (ES=0.41) was stronger than that seen in questionnaire-based studies using a symptomatic definition. However, when one examines the individual studies included in the meta-analysis one can see that they tell a slightly different story. An early study by Song and colleagues (1998) found elevated levels of IL-6 in a small group of individuals (n=10) with primary sleep disorders (IL-6=18.1pg/mL, p=<0.05), however the sleep disordered group included individuals with sleep disordered breathing, which is known to be associated with elevated levels of circulating inflammatory markers (Vgontzas et al., 2000) and may have driven the association with IL-6 rather than those with a diagnosis of insomnia. A later study by Vgontzas and colleagues (2002) that used stringent criteria to identify participants with insomnia and assessed inflammatory biomarkers using 24-hour sampling did find a significant elevation in IL-6 levels in insomniacs during the time period 14:00-09:00 as compared to controls, which indicated a shift in the secretion of pro-inflammatory cytokines. These results may have not been captured in the meta-analysis, as it reported that the study by Vgontzas and colleagues was negative with a null effect size (ES = 0.00). Another study found significant increases in serum levels of IL-6 collected serially over a single night during a PSG sleep study in individuals with well-defined insomnia as compared to controls (Burgos et al., 2006). Finally, a recent study in a large group of older adults, some in bereavement, did not find a significant difference between insomniacs (defined based upon sleep diary data) and controls in terms of levels of IL-6 or TNF-α (Okun et al., 2011).Thus, it appears that the non-significant but robust effect size reported in the meta-analysis was affected by the heterogeneity among these study designs, definitions and age groups as well as by the fact that some of the studies were taken as negative when they were actually positive once time of the sampling were to be taken into account (Vgontzas et al, 2002). Furthermore, there are no studies that have assessed

levels of CRP, which may be a more stable molecule not affected by circadian secretion when examining overall systemic inflammation. A critical gap in the literature, and that Irwin and colleagues (2015) could not examine or account for in their meta-analysis, is the lack of inclusion of rigorous objective measures of sleep in insomnia studies, which did not allow examining whether objective short sleep duration moderates the association of insomnia with inflammation. Thus, the presence of insomnia may be associated with increased inflammation and this relationship may be modified by the presence of objective short sleep duration; however, due to the current state of the literature the relationship has remained unclear.

1.4 Insomnia with objective short sleep duration: A more severe phenotype

As discussed above, insomnia is associated with central and peripheral markers of hyperarousal. However, it has been shown that this physiologic hyperarousal does not extend universally to all individuals with insomnia. Rather, it is those adults who complain of insomnia and exhibit objectively short sleep duration that demonstrate (**1**) increased cortisol, norepinephrine and catecholamine metabolite levels (Adam, Tomney & Oswald 1986; Vgontzas et al., 1998; Vgontzas et al., 2001; Rodenbeck et al., 2002; Shaver et al., 2002; Rodenbeck et al., 2003; Irwin et al., 2003), (**2**) longer daytime sleep latencies on the multiple sleep latency test (MSLT), a measure of physiologic sleep propensity / sleepiness (Stepanski et al., 1988; Sugerman, Stern & Walsh, 1985; Dorsey & Bootzin, 1997; Roehrs et al., 2011), (**3**) increased heart rate and lower heart rate variability during wake and sleep and faster pre-ejection period at sleep onset (Bonnet & Arand, 1998; Spiegelhalder et al., 2011; de Zambotti et al., 2011; 2013; 2014), (**4**) increased whole-body and brain metabolic rate, as measured by VO2 and functional neuroimaging (Bonnet & Arand, 1998; Nofzinger et al., 2006; Winkelman et al., 2008), and (**5**) increased high-frequency dynamics in the beta (15-35 Hz) range during sleep, as measured by electroencephalographic (EEG) spectral analysis (Hall et al., 2000; Hall et al., 2007; Perlis et al., 2001; Krystal et al., 2002; Corsi-Cabrera et al., 2012; Spiegelhalder et al., 2012).

The fact that physiologic indices of hyperarousal are primarily present in adult insomniacs with objective short sleep duration, as measured by PSG, and that these indices are known to be associated with increased morbidity led Penn State researchers, and others thereafter, to systematically study the association of this insomnia phenotype with adverse health outcomes. Specifically, insomnia with objective short sleep duration has been shown to be associated with a significant increased risk of hypertension, (Vgontzas et al., 2009a; Fernandez-Mendoza et al., 2012; Bathgate et al., 2016) impaired nocturnal blood pressure dipping (Lanfranchi et al., 2009), increased risk of type 2 diabetes and impaired glucose or insulin metabolism (Vgontzas et al., 2009b; Knutson et al., 2011; Vasisht et al., 2013), neurocognitive impairment (Fernandez-Mendoza et al., 2010; Shekleton et al., 2013), depression (Fernandez-Mendoza et al., 2015) and mortality (Vgontzas et al., 2010). Together, these findings have led Vgontzas, Fernandez-Mendoza, Liao and Bixler (2013) to propose that insomnia with objective short sleep duration is a more biologically severe form of the disorder than insomnia with normal sleep duration. The increased risk of medical morbidity is likely related to a combination of underlying mechanisms, including the characteristic physiologic hyperarousal (e.g., hypercortisolemia) and, potentially, chronic low-grade inflammation. However, the association of this specific insomnia phenotype with inflammation has not been extensively examined.

1.5 Adolescence: An opportunity to understand insomnia throughout the lifespan

The prevalence of insomnia symptoms peaks with the onset of puberty (Calhoun et al, 2013) reaching the adult figures reported above of about 40% during adolescence (Schmidt & Van der Linden, 2015). Adolescence is an important period for normal healthy development but is also a critical period for the onset of adult forms of morbidity, such as sleep disorders. Indeed, insomnia in adolescents is associated with adverse behavioral outcomes such as internalizing (i.e., anxiety, depression, somatic complaints) and externalizing (i.e., opposition, impulsivity, hyperactivity) problems and poor academic performance. (Schmidt & Van der Linden, 2015) However, little is

known about the pathophysiology of insomnia during this developmental period.

Adolescent insomnia, particularly difficulty falling asleep, is typically seen as the result of poor sleep habits (i.e., reading, using electronics in bed) and/or the result of the developmental delay in the circadian regulation of sleep (i.e., increased eveningness). (Schmidt & Van der Linden, 2015) In other words, adolescent sleep may be affected by the development and practice of poor sleep hygiene due to social and developmental pressure in this age group. However, there is accumulating evidence that the main pathophysiologic mechanisms of insomnia may already be present during childhood or adolescence (Calhoun et al, 2013; Fernandez-Mendoza, Vgontzas & Calhoun, 2014; Fernandez-Mendoza et al., 2016). These preliminary data indicate that early prevention of insomnia should focus on these more vulnerable groups, particularly those adolescents that are already showing markers of objective short sleep duration and physiologic hyperarousal. Furthermore, adolescence represents a unique opportunity to study highly prevalent sleep disorders before multiple other confounding morbidities have developed, such as depression, hypertension, or type-2 diabetes, known to be associated with systemic inflammation.

Needless to say, the literature examining the association of sleep duration or insomnia with inflammatory markers in adolescents is sparse. A study on sleep disordered breathing in 143 adolescents aged 13-18y found that shorter average sleep duration, as measured by actigraphy, was significantly correlated with increasing levels of CRP (Larkin et al., 2005). However, only 31 adolescents in this study had an average sleep duration of < 7 hours, which precluded any interpretation of the clinical significance of the correlation found ($r < 0.29$), which was modest at best. Another recent and slightly larger study conducted in 188 healthy adolescents found a significant inverse association between sleep duration and CRP levels when controlling for age, sex and pubertal status. (Martinez-Gomez et al., 2011) However, sleep duration in this study was obtained by self-report as part of a physical activity study and did not query about insomnia complaints, which is important given that insomniacs are more likely to self-report short sleep duration and there is great discrepancy between self-reported and objective sleep duration in insomniacs (Fernandez-Mendoza et

al, 2011). Actigraphy, an objective measure of sleep behavior, was utilized by Hall and colleagues (2015) in a recent study of sleep duration and levels of CRP in healthy adolescents. In a sample of 244 adolescents there were n=33 adolescents with CRP levels >3mg/L, and there was a significant association between short weeknight sleep duration and levels of CRP greater than 3mg/L. A unique aspect of this study was the specific examination of weekday vs. weeknight sleep duration, the results determined that averaging sleep duration across the entire week, a common practice in the current literature, may miss essential sleep characteristics of this specific age group related to CRP levels.

In fact, there are no studies examining the association of insomnia with inflammation in adolescents, despite the preliminary data mentioned above on the association of actigraphy and self-reported sleep duration with increased CRP levels. Furthermore, there are no studies that have examined the role of objective sleep duration in the association of insomnia with inflammation in adolescents.

Chapter 2. Specific Aims & Hypotheses

In this project, we seek to examine the association of insomnia and objective short sleep duration with markers of inflammation in a random, general population sample of adolescents, the PSCC. Previous evidence suggests that insomnia and short sleep duration are associated with increased inflammatory markers in adulthood. It is not known whether it is insomnia, short sleep or the combination of both that is associated with a chronic low-grade inflammatory state. Even less is known in early stages of development such as adolescence. In light of this gap in the field, the present study will be the first one to examine the association of adolescent insomnia symptoms and objective short sleep duration with markers of systemic inflammation. In order to achieve this overarching goal we plan to test the following specific aims:

Specific Aim 1: To evaluate markers of systemic inflammation associated with the presence of subjective complaints of insomnia in an adolescent sample. **Hypothesis 1:** Subjective complaints of insomnia are associated with increased markers of inflammation in adolescents. We anticipate that adolescents with subjective insomnia symptoms (i.e., self-reported difficulty falling and/or staying asleep) will have elevated markers of inflammation compared to participants without complaints of insomnia symptoms. Our primary outcome will be CRP levels. Secondary outcomes will be IL-6 and TNF-α levels, while leptin and adiponectin levels will serve as exploratory outcomes.

Specific Aim 2: To evaluate markers of systemic inflammation associated with the presence of objective short sleep duration, as measured by PSG, in an adolescent sample. **Hypothesis 2:** Objective short sleep duration (i.e., ≤ 7 hours) is associated with increased markers of inflammation in adolescents. We anticipate that adolescents with objective short sleep duration will have elevated levels of CRP when compared to adolescents with normal sleep duration (i.e., ≥ 8 hours). Our primary and secondary outcomes will be the same as in Specific Aim 1 above.

Specific Aim 3: To evaluate markers of systemic inflammation associated with the synergistic effect between the presence of subjective complaints of insomnia and objective short sleep duration in an adolescent sample. **Hypothesis 3:** Objective sleep duration acts as an effect modifier

between subjective complaints of insomnia and increased markers of inflammation in adolescents. We anticipate that adolescents with insomnia symptoms and objective short sleep duration will have significantly elevated levels of CRP when compared to controls with short sleep duration or insomniacs with normal sleep duration. Our primary and secondary outcomes will be the same as in Specific Aim 1 above.

Based upon the findings in Chapter 3 we took an additional step in secondary analyses. We sought to examine the potential of parent-reported insomnia symptoms as a screener for those adolescents with elevated levels of CRP. Given that PSG data may not be available in all clinical scenarios, it may be beneficial to examine a clinical tool to screen those adolescents at greater risk of inflammation that could benefit from a PSG study. In order to answer this question we plan to test the following secondary aims:

Secondary Aim 4: To evaluate whether persisting parent-reported insomnia symptoms since childhood are associated with increased CRP levels in adolescence. **Hypothesis 4**: Persisting parent-reported insomnia symptoms since childhood are associated with greater CRP levels as compared control or new-onset insomnia symptoms in adolescence. We anticipate that adolescents with persisting insomnia symptoms since childhood will have greater CRP levels than adolescents who have never experienced insomnia symptoms or developed insomnia symptoms in adolescence.

Chapter 3. Insomnia Symptoms with Objective Short Sleep Duration are Associated with Systemic Inflammation in Adolescents

This chapter includes a multi-author publication in which I was a key contributing author. This project was proposed and completed in partial fulfillment of my Master's thesis, and subsequently published. My role in this publication included development of a scientific hypothesis, creation of specific aims and preliminary analysis of data for model selection. In addition, I completed the final statistical analyses described in the methods section, interpreted the results and wrote a significant portion of the manuscript under the supervision of my thesis advisor, Dr. Fernandez-Mendoza. I subsequently assisted in the submission, review and revision process before its final acceptance by the journal *Brain, Behavior and Immunity*. Dr. Fernandez-Mendoza has granted permission for me to include this paper as part of my thesis given my significant role as a second co-author. Please refer to the appendix for details.

Chapter 4. Tracking Insomnia Symptoms Back to Childhood: A Clinically Useful Screen for Insomnia Severity?

4.1 Introduction

There is a distinct need for clinicians to accurately phenotype adolescents suffering from insomnia to make informed decisions about a specific treatment plan. As we have seen in Chapter 3, adolescents who report insomnia symptoms are already displaying high levels of the inflammatory marker CRP. This inflammatory profile belonged specifically to a group of adolescents experiencing insomnia symptoms and objective short sleep duration. Although this solidifies objective sleep studies such as in-lab PSG as a tool in the evaluation of the biological severity of insomnia symptoms, it may not be available, practical or cost-effective in all clinical scenarios. Although it appears that CRP levels could potentially serve as a marker of the severity of insomnia, it is unspecific when compared to PSG, which is a direct physiologic measure of sleep. Therefore, there is a need to identify other markers of insomnia severity that can guide clinicians in identifying those adolescents with the more biologically severe form of insomnia symptoms.

Given the critical developmental period under study, one potentially important clinical factor that is readily available to all clinicians may be whether insomnia symptoms had a persistent course since childhood or developed during adolescence itself. It is likely that adolescents who report insomnia symptoms and have had a history of these symptoms since childhood, as identified by clinical history or actual parent report, may be a group with a more severe form of insomnia symptoms and greater risk of association with inflammation and adverse health outcomes. In these secondary analyses, we hypothesized that adolescents with persisting insomnia symptoms since childhood will show significantly elevated CRP levels as compared to controls or adolescents with new-onset insomnia symptoms.

4.2 Methods

Subjects

The sample composition of the Penn State Child Cohort and the methods used are described

in detail in Chapter 3 under the Methods section of the paper published in Brain Behavior and immunity (see page 12).

Onset of insomnia symptoms

We retrospectively identified whether adolescents with self-reported insomnia symptoms at follow-up exhibited a persistent course of insomnia symptoms since childhood as reported by their parents at baseline. At baseline, all parents completed the Pediatric Behavior Scale (PBS) and Pediatric Sleep Questionnaire (PSQ). The presence of insomnia symptoms at baseline was defined as a parent report of "trouble falling asleep" and/or "wakes up often in the middle of the night" rated as "often" or "very often" on the PBS. For those 3 participants with missing data on the parent-reported PBS, the presence of insomnia symptoms was defined as a positive response to either "difficulty falling asleep" and/or "difficulty staying asleep" on the parent-reported PSQ. Based on these data, a 3-level insomnia symptoms variable was retrospectively identified: those with self-reported insomnia symptoms in adolescence and parent-reported insomnia symptoms in childhood (i.e., persistent, childhood-onset insomnia symptoms), those with self-reported insomnia symptoms in adolescence but without parent-reported insomnia symptoms in childhood (i.e., incident, new-onset onset insomnia symptoms), and those without insomnia symptoms in adolescence (the same reference group as in Chapter 3).

Statistical analysis

A general linear model with polynomial contrast examined the association between the 3-level insomnia symptoms variable (i.e., controls, adolescent-onset, and childhood-onset) with CRP levels. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corp., Armonk, NY).

4.3 Results

As shown in Figure 2, adolescents with childhood-onset insomnia symptoms had significantly higher levels of CRP compared to those with adolescent-onset insomnia symptoms and to controls (1.2 mg/L, 1.0 mg/L and 0.8 mg/L, respectively, p=0.046).

Figure 4.1 C-reactive protein for insomnia symptoms groups based on parent report of onsent in childhood or adolescence

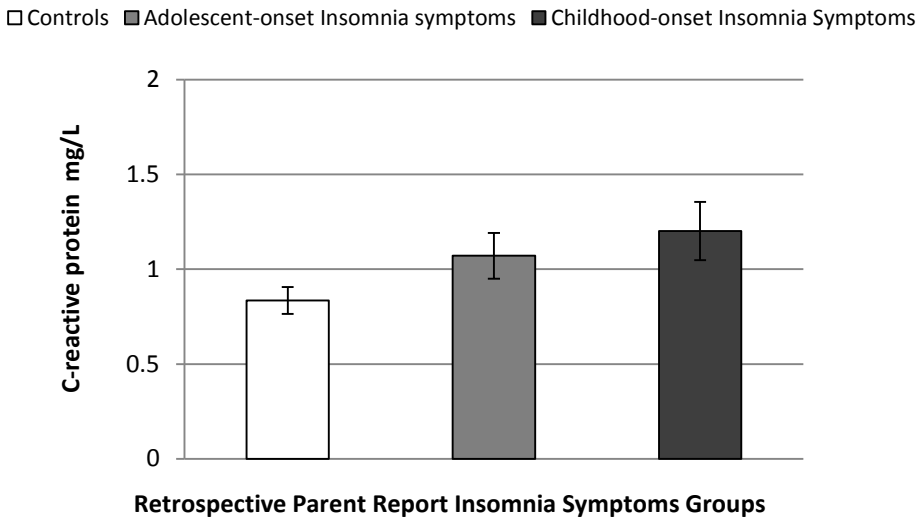

Figure 4.1- Data are mean and standard errors of CRPlevels of the sample (mg/L) stratified by insomnia symtpoms groups . CRP levels were highest for childhood onset insomnia (1.2 mg/L, p=0.046).

However, the strength of association observed using childhood parent reports was smaller than that in our primary aim in Chapter 3, which indicated that objective short sleep duration played a more significant role in the association of insomnia symptoms with CRP levels. As shown in Figure 3, indeed CRP levels were highest in adolescents with childhood-onset insomnia symptoms (1.7 mg/L) followed by those with adolescent-onset insomnia symptoms (1.6 mg/L) who slept objectively ≤ 7 hours, while adolescents with childhood-onset or adolescent-onset insomnia symptoms who slept > 7 hours did not show significantly elevated CRP levels as compared to controls (e.g., 0.82 mg/L, 0.82 mg/L, 1.09 mg/L and 0.8 mg/L for controls with > 8 hours and 7-8 hours, and adolescent-onset insomnia symptoms with > 8 hours and 7-8 hours, respectively).

Figure 3. C-reactive protein for insomnia symptoms groups based on parent report of onset in childhood or adolescence by objective sleep duration

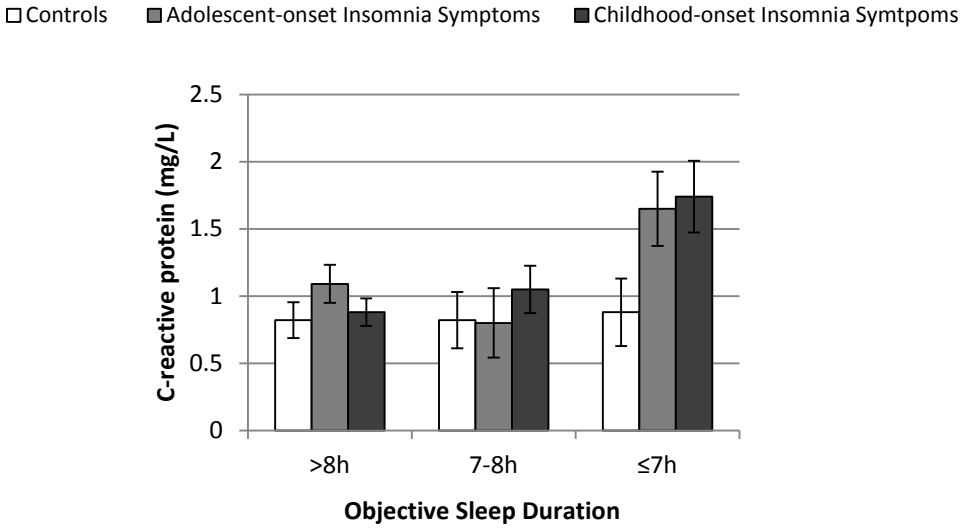

Figure 4.2- Data are means and standard errors of C-reactive protein levels of the sample stratified by objective sleep duration and insomnia symptoms groups . CRP Levels were highest for adolescents short sleep duration and adolescent- and childhood-onset insomnia symptoms (1.6 and 1.7 mg/L respectivelty).

4.4 Discussion

In this secondary analysis, we examined whether parent reports of insomnia symptoms in childhood may be a useful tool to evaluate which adolescents are at greatest risk of inflammation, given that this will indicate a chronic, persisting course. Indeed, we found that adolescents with persistent insomnia symptoms since childhood had significantly higher CRP levels as compared to adolescents with new-onset insomnia symptoms or controls. Based on these data, it appears that obtaining clinical information from parents on the presence of insomnia symptoms during childhood may help screen those adolescents with increased risk of inflammation and potentially associated morbidity when objective sleep data is not readily available for clinicians. Increasing and validating the number of tools to phenotype individuals with insomnia in the transition from childhood to adolescence will help us guide treatment plans for specific phenotypes. Accurate diagnosis and personalized treatment is crucial, as the biological repercussion of insomnia in adulthood is well

defined and there is evidence that untreated insomnia is likely to persist throughout adulthood (Fernandez-Mendoza et al., 2012; Vgontzas et al., 2012). However, the observed statistically significant differences in CRP levels between the childhood-onset insomnia symptoms group and controls and adolescent-onset insomnia symptoms were rather small interms of effects size and particularly as compared to our findings in Chapter 3 in respect to the role of objective short sleep duration. Indeed, upon stratification by objective sleep duration, the childhood- and adolescent-onset groups sleeping ≤ 7 hours had similarly elevated CRP levels. These data demonstrated the moderating role of objective sleep duration in the association between insomnia symptoms and inflammation, regardless of the time of onset of insomnia symptoms. This provides support for parent report of insomnia symptoms as a potential screener for insomnia phenotypes, however, objective sleep duration as measured by PSG remains the most powerful measure to phenotype insomnia in adolescents as it pertains to its potential effect on adverse health outcomes.

In summary, this secondary analysis demonstrated that in the aim of preventing the downstream biological and behavioral morbidity as a result of insomnia symptoms and chronic low-grade inflammation persisting throughout the lifespan, childhood history of insomnia symptoms may be useful for screening purposes in adolescents with potentially greatest severity. However, to truly identify those adolescents in which systemic inflammation may be already present, objective sleep measures seem to be needed and will provide the most rigorous phenotyping.

Chapter 5. Discussion & Conclusion

In this project, we have shown that adolescents with insomnia symptoms and objective short sleep duration have increased indices of low-grade inflammation as measured by CRP levels. Neither controls with objective short sleep duration or adolescents with insomnia and objective normal sleep duration were associated with increased inflammation, which indicates a synergistic effect of insomnia symptoms and objective short sleep duration on inflammation. Although we did not find elevated levels of IL-6 in our primary aims (Chapter 3), despite its role as an upstream regulator of CRP, it is possible that the circadian rhythm of this inflammatory marker (Vgontzas et al, 2002) and the lack of serial blood draws was responsible for these null findings. Still, elevation of CRP in those individuals representing the short sleeping insomnia phenotype would strongly support the proposed relationship between short sleep duration being a moderator of insomnia and inflammation. Despite this, further work to isolate more specific biomarkers would aid in designing an effective treatment to combat the negative effects of chronic-low grade inflammation. Currently, the best treatment is early intervention to prevent incident insomnia symptoms from becoming chronic, therefore, avoiding the host of morbidity associated with this insomnia phenotype.

With this in mind, there was a need to investigate potential screeners for the inflammatory profile associated with the more severe insomnia phenotype. This would allow for easy determination of what adolescents would benefit form a sleep study when the option is available. The present study is strengthened by the availability of baseline data during childhood in respect to insomnia symptoms; however, inflammatory markers were not measured in the whole sample at baseline (n=58). Nevertheless, a further secondary analysis demonstrated insomnia symptoms with an onset during childhood are more strongly associated with increased CRP levels in adolescence than new-onset insomnia symptoms. Again, early prevention of the insomnia symptoms to prevent the development of a chronic and persistent course into adulthood is the best course of treatment to avoid the host of morbidity and mortality associated with chronic insomnia in adulthood. However, the association of childhood-onset and adolescent-onset insomnia symptoms with increased inflammation was similar

when the role of objective short sleep duration was taken into account, which indicates that objective sleep duration remains the most powerful measure to phenotype insomnia in adolescents as it pertains to its potential effect on adverse health outcomes, including systematic inflammation.

Potential future directions may include an analysis of the adverse outcomes associated with insomnia and elevated inflammation such as blood pressure, insulin resistance or neurocognitive functioning. In addition, it will be important to expand on Chapter 4, to determine the demographic, clinical and behavioral profiles of adolescents who newly-developed, persisted with or remitted from insomnia symptoms during the transition from childhood to adolescence. Understanding the childhood predictors of the more biologically severe phenotype of insomnia symptoms, will enhance treatment and prevention of the disorder. Finally, the PSCC has also a detailed assessment of self-reported and objective sleep data (e.g., actigraphy) as well as physical activity data, and there is an increasing body of research to support that exercise could mediate the effects of inadequate sleep on adverse health outcomes. Thus, there is ample opportunity to continue to more fully define factors that may relate to insomnia, short sleep and inflammation.

Appendix

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Full-length Article

Insomnia symptoms with objective short sleep duration are associated with systemic inflammation in adolescents

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ABSTRACT

Inflammation has been suggested as a potential pathway by which insomnia and short sleep can affect risk of morbidity in adults. However, few studies have examined the association of insomnia with inflammation in adolescents, despite accumulating evidence that pathophysiologic changes may already occur during this critical developmental period. The present study sought to examine the association of insomnia symptoms with systemic inflammation and the role of objective sleep duration in this association. Participants were 378 adolescents (16.9 ± 2.3 y, 45.8% female) from the Penn State Child Cohort, a population-based sample who underwent 9-h polysomnography (PSG) followed by a single fasting blood draw to assess plasma levels of C-reactive protein (CRP) and other inflammatory markers. Insomnia symptoms were defined by a self-report of difficulties falling and/or staying asleep, while objective sleep duration groups were defined as a PSG total sleep time ⩾8, 8–7, and ⩽7 h. We assessed the association of insomnia symptoms, objective sleep duration, and their interaction with inflammatory markers, while adjusting for multiple potential confounders. Adolescents reporting insomnia symptoms had significantly higher levels of CRP compared to controls and a significant interaction ($p < 0.01$) showed that objective sleep duration modified this association. Elevated CRP was present in adolescents with insomnia symptoms and ⩽7 h of sleep (1.79 mg/L) as compared to controls or adolescents with insomnia symptoms and ⩾8 h of sleep (0.90 mg/L and 0.98 mg/L, respectively) or controls with ⩽7 h of sleep (0.74 mg/L; all p-values <0.01). In sum, insomnia symptoms with objective short sleep duration are associated with systemic inflammation as early as adolescence. This study suggests that chronic low-grade inflammation may be a common final pathway towards morbidity in adulthood in this insomnia phenotype.

1. Introduction

Insomnia, particularly in adults with objective short sleep duration, has been associated with increased physiologic indices of hyperarousal, ranging from increased cortisol and norepinephrine levels, whole-body metabolic rate and impaired heart rate variability to increased high-frequency cortical dynamics, cerebral glucose metabolism and decreased gamma-aminobutyric acid levels (Bonnet and Arand, 2010; Riemann et al., 2010; Vgontzas et al., 2013). Furthermore, adults with insomnia and objective short sleep duration have been found to be at increased risk of hypertension, type 2 diabetes, neurocognitive impairment, depression and mortality (Bathgate et al., 2016; Fernandez-Mendoza et al., 2015; Vgontzas et al., 2013). Thus, there is a need to identify biomarkers to target the diagnosis and treatment of these adverse health outcomes early in the life span. Chronic low-grade inflammation has been proposed as one of the potential paths by which insomnia can lead to adverse health outcomes (Irwin, 2015; Vgontzas et al., 2002, 2013). However, little is known about the association of adolescent insomnia with inflammation, despite accumulating evidence that the main pathophysiologic mechanism of insomnia, i.e., hyperarousal, may already be present during this critical developmental period (Fernandez-Mendoza et al., 2014, 2016; Ly et al., 2015; Zhang et al., 2014). This period is also when sleep disorders develop and, therefore, marks a point for potential interventions; indeed, the prevalence of insomnia symptoms increases with the onset of puberty (Calhoun et al., 2014), reaching a peak of 40% in adolescence, similar to the prevalence of insomnia symptoms in adults (Ohayon, 2002).

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An increasing field of research supports that disturbed sleep may play a role in the modulation of circulating inflammatory molecules such as C-reactive protein (CRP) and interleukin-6 (IL-6). A recent meta-analysis by Irwin et al. (2016) of 72 studies comprising 50,000 primarily middle-aged individuals demonstrated that insomnia symptoms were significantly associated with increased levels of CRP (effect size [ES] = 0.12) and IL-6 (ES = 0.20). To a lesser extent, shorter sleep duration was associated with significantly increased CRP (ES = 0.09) and IL-6 (ES = 0.11) levels. Interestingly, the association of shorter sleep duration with CRP and IL-6 was stronger in studies using objective sleep measures (ES = 0.18 and ES = 0.29, respectively) as compared to studies using subjective sleep measures (ES = 0.04 and ES = 0.03, respectively). Tumor necrosis factor alpha (TNF-α) levels were not significantly associated with either insomnia symptoms or shorter sleep duration in the studies covered by this meta-analysis. Despite this promising evidence, these effect sizes are modest and a potential explanation may be the lack of studies examining the joint effect of insomnia symptoms and objective sleep duration on inflammatory markers, as pointed out by Irwin et al. (2016). Furthermore, few studies have examined these associations across the lifespan, which will provide a better understanding of the association of insomnia symptoms and short sleep duration with inflammation early in the development of sleep disorders and other morbidity.

Indeed, the literature examining the association of insomnia symptoms or short sleep duration with inflammatory markers in adolescents is sparse. For example, a study in 143 adolescents aged 13–18 y found that shorter average sleep duration, as measured by actigraphy, was significantly correlated ($r = -0.29$) with increasing levels of CRP (Larkin et al., 2005). Another study conducted in 188 healthy adolescents found that shorter sleep duration, as measured by self-report, was significantly correlated ($\beta = -0.17$) with increasing CRP levels only after controlling for age, sex and pubertal status (Martinez-Gomez et al., 2011). Another recent study in 244 healthy high school students found that shorter sleep duration on school nights, as measured by actigraphy, was not significantly associated with increasing CRP levels ($\beta = -0.04$) but rather with greater likelihood (odds ratio = 0.62) of having CRP levels >3 mg/L (Hall et al., 2015). However, there are no studies examining the association of insomnia symptoms with inflammation in adolescents and the role of objective sleep duration in this age group.

The overall aim of the present study is to gain a better understanding of the association between adolescent insomnia symptoms and objective short sleep duration with systemic inflammation. CRP was the primary outcome in the present study, while we also examined IL-6, TNF-α, leptin and adiponectin levels as secondary outcomes. We hypothesized that adolescents with insomnia symptoms, as measured by self-report, would have elevated CRP levels compared to adolescents without insomnia symptoms. We also hypothesized that adolescents with objective short sleep duration, as measured by polysomnography (PSG), would have elevated CRP levels compared to adolescents with normal sleep duration. Finally, we tested whether objective sleep duration modifies that association between insomnia symptoms and increased CRP levels in adolescents.

2. Materials and methods

2.1. Participants

The Penn State Child Cohort (PSCC) is a general population sample of 700 children between ages 5–12 years, of whom 421 were followed up 8.4 years later as adolescents (mean age 17.0 ± 2.2 years, 53.9% male, and 21.9% ethnic minority). Baseline demographic characteristics were similar between those who did and did not participate in the follow-up study (Bixler et al., 2016). The study protocol was approved by the Penn State University College of Medicine Institutional Review Board. Written informed consents were obtained from participants 18 years and older. Assent was sought for those younger than 18 years, and consent was obtained from their parents or legal guardians.

2.2. Physical assessment

During their laboratory visit, adolescents underwent a physical examination and height (stadiometer Model 242, SECA Corp.; Hanover, MD) and weight (Model 758C, Cardinal Manufacturing; Webb City, MO) were measured (CDC, 2011). Body mass index (BMI) was calculated (in kg/m^2) and converted to a percentile according to a formula based on the Centers for Disease Control's sex-specific BMI-for-age growth charts (CDC, 2009). Pubertal development (Tanner staging) was determined via a self-administered rating scale (Carskadon and Acebo, 1993). Participants identified their race/ethnicity from one of six options, as part of the clinical history. Based on the clinical history and physical examination, a composite variable of number of chronic health conditions was created, which included a history of conditions known to be associated with inflammation such as asthma, chronic sinusitis/rhinitis, arthritis/tendonitis, colitis, or hypertension. Furthermore, parents or adolescents also reported whether the subject was taking any anti-inflammatory medication, which included NSAIDS, asthma or colitis medications.

2.3. Sleep laboratory protocol

All participants underwent a single-night, 9-h PSG recording in a sound-attenuated, light- and temperature-controlled room with a comfortable, bedroom-like atmosphere. Each subject was continuously monitored from 22:00 h until 07:00 h using 14-channel recordings of electroencephalogram (EEG), electrooculogram (EOG), and electromyogram (EMG). Respiration was monitored via nasal pressure (Pro-Tech PTAF Lite; Mukilteo, WA), thermocouple (Salter Labs; Lake Forest, IL), and thoracic/abdominal strain gauges (Model 1312, Sleepmate Technologies; Midlothian, VA). Hemoglobin oxygen saturation (SpO_2) was assessed using a pulse oximeter placed on the index finger (Model 3011 Xpod, Nonin Medical, Inc.; Plymouth, MN). Snoring sounds were monitored via a sensor attached to the throat. All data were recorded using Twin Recording & Analysis software (Grass-Telefactor; West Warwick, RI). Visual sleep stage scoring was conducted by a registered polysomnography technologist according to standardized criteria (Rechtschaffen and Kales, 1968). Apnea/hypopnea index (AHI), the number of apneas and hypopneas summed per hour of sleep, was ascertained.

Sleep parameters derived from PSG included sleep continuity variables such as sleep onset latency (SOL; the number of minutes to fall asleep since lights off), wake after sleep onset (WASO; the amount of time awake after the onset of sleep), total sleep time (TST; the total number of minutes slept since lights off until lights on), and sleep efficiency (SE; the amount of time spent asleep divided by the amount of time spent in bed) as well as sleep architecture parameters defined as the amount of time spent in each sleep stage (i.e., stage 1, stage 2, slow wave sleep [SWS] and rapid eye movement sleep [REM]) divided by total sleep time. From the PSG-measured TST, we split the overall sample into 3 ordinal groups based on the population quartiles: $\geqslant$ 8 h (i.e., top 25% of the sample or 75th percentile), 7–8 h (i.e., middle 50% of the sample), and $\leqslant$ 7 h (i.e., bottom 25% of the sample or 25th percentile). Thus, we defined objective short sleep duration based on the distri-

bution of TST in the overall sample, which is a distribution similar to that reported for this age range (Ohayon et al., 2004).

2.4. Insomnia symptoms and other self-reported behaviors

Parent- and self-reported questionnaires were administered to measure behaviors including internalizing and externalizing symptoms, circadian preference, and insomnia symptoms. All participants older than 18 years competed the Adult Behavior Checklist (ABCL), while the parents of participants younger than 18 completed the Child Behavior Checklist (CBCL) (Achenbach and Rescorla, 2001). Internalizing and externalizing scales T scores from the C/ABCL were used to measure the severity of anxiety, depression, and somatic complaints and inattention, hyperactivity, rule-breaking behaviors etc., respectively. Circadian preference was measured using the Morningeness-Eveningness Questionnaire (MEQ), which has been validated in both adults and adolescents (Carskadon et al., 1993). Insomnia symptoms were considered present if participants answered "yes" to either or both of the following questions: "do you have difficulty falling asleep" (DFA) or "do you have difficulty staying asleep" (DSA) from a self-report version of the Pediatric Sleep Questionnaire (Chervin et al., 2000).

2.5. Blood draw and assay procedures

A blood sample was provided by 392 (93.1%) of the 421 participants at 7:00 following the evening PSG recording. Samples were collected in an ethylenediamine teraacetic acid (EDTA)-containing tube then centrifuged for 10 min at 3000 RPM. Plasma was aliquoted into cryotubes and stored at −80° C until assayed. Plasma high-sensitivity CRP, IL-6, TNF-α, leptin, and adiponectin were measured via enzyme-linked immunosorbent assay (ELISA; R&D Systems; Minneapolis, MN). The intra- and interassay coefficients of variation were 5.8% and 5.3%, respectively (CRP), 4.7% and 5.1% (IL-6), 4.6% and 4.9% (TNFα), 6.5% and 7.0% (leptin), and 5.6% and 5.6% (adiponectin). The lower detection limits were 0.010 ng/mL (CRP), 0.039 pg/mL (IL-6), 0.106 pg/mL (TNFα), 7.2 pg/mL (leptin), and 0.25 ng/mL (adiponectin). All samples and standards were run in duplicate.

2.6. Statistical analysis

Sociodemographic and physical characteristics of the insomnia symptoms and objective sleep duration groups were calculated using analysis of variance (ANOVA) for continuous variables and chi-square for categorical variables. A 2-way, full-factorial general linear model (GLM) was used to assess the association of insomnia symptoms, PSG sleep duration, and their interaction on the primary outcome, while controlling for the potential confounding effect of sex, age, race, SES, BMI percentile, MEQ, internalizing and externalizing behaviors, AHI, periodic limb movement index (PLMI), alcohol, caffeine, tobacco and drug use, number of chronic health problems, and use of anti-inflammatory medication, with Bonferroni correction for multiple comparisons (unless otherwise stated). Our two independent variables in the GLM were insomnia symptoms (no / yes) and objective sleep duration ($\geqslant$8/7–8/$\leqslant$7 h), while our primary outcome was CRP levels. Our secondary outcomes included IL-6, TNF-α, leptin and adiponectin levels, for which the same type of GLM was used. Descriptive data from GLMs are reported as estimated marginal means and their standard error (SEM) after adjustment for all the covariates mentioned above. The statistical confidence level selected for all analyses was $p < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Demographic and clinical characteristics of the sample

Out of the 392 adolescents who provided a fasting blood draw to assay for inflammation, a final sample of 378 had complete data on insomnia symptoms, objective sleep duration and the primary outcome (i.e., CRP). Overall, participants were 16.9 ± 2.3 years old and 45.8% were female. A total of 38% of the sample identified as fully-developed and 46% identified as late pubescent, while less than 16% of the sample identified as mid-pubescent or earlier stages. In terms of race/ethnicity, 6.5% identified as Hispanic, while 0.2% identified as American Indian, 1.0% Native Hawaiian/Asian, 9.0% Black or African American, and 79.8% White. The average BMI for the sample was at the 65.3 ± 1.4 percentile and 15% were obese, the average waist circumference was 65.7 cm ± 0.5 cm. The average AHI was 2.7 ± 0.3 and the prevalence of SDB (AHI $\geqslant$ 2) was about 38%.

More than a third (36%) of the sample self-reported insomnia symptoms and 25% exhibited objective short sleep duration (i.e., $\leqslant$7 h). As shown in Table 1, among individuals reporting insomnia symptoms there was a significantly greater proportion of girls (57.7% vs. 37.8%, respectively), evening-types (42.3% vs. 24.2%, respectively) and adolescents of low SES (73.7% vs. 60.4%, respectively) as compared to controls. Furthermore, internalizing and externalizing scores were significantly higher in the insomnia symptoms group as compared to controls. Otherwise, no other significant differences were observed between the insomnia symptoms and control groups. Similarly, there were only a few statistically significant differences between objective sleep duration groups in terms of the sociodemographic or clinical characteristics (Table 1).

3.2. Sleep characteristics of the sample

Sleep characteristics were largely similar between adolescents who reported insomnia symptoms and those who did not. As shown in Table 2, adolescents who reported insomnia symptoms had a slightly longer sleep onset latency ($p = 0.064$) as compared to those without insomnia symptoms, which was consistent with the fact that DFA was the most prevalent complaint (92.6%) among adolescents with insomnia symptoms (39.7% for DSA). These non-significant, small differences in PSG parameters further supported the existence of heterogeneity and potential subgroups among adolescents with insomnia symptoms. Furthermore, there were no significant differences in terms of number of apneas / hypopneas or periodic limb movements between the two groups. As would be expected, there were significant differences in sleep characteristics between the objective sleep duration groups in respect to sleep continuity and sleep architecture parameters. Interestingly, there was no significant difference in terms of the proportion of adolescents reporting insomnia symptoms (i.e., DFA/DSA) across the objective sleep duration groups (Table 2); specifically, there were 46, 57, and 34 adolescents with insomnia symptoms and 66, 113, and 62 without insomnia symptoms across the $\geqslant$8 h, 7–8 h and $\leqslant$7 h objective sleep duration groups, respectively.

3.3. Association of insomnia symptoms and short sleep duration with inflammation

As shown in Table 3, adolescents who reported insomnia symptoms had significantly higher levels of CRP compared to adolescents without insomnia symptoms ($p < 0.01$) and adolescents who slept objectively $\leqslant$7 h had significantly higher levels of CRP

Table 1
Demographic characteristics of the sample stratified by insomnia symptoms and objective sleep duration.

	Insomnia symptoms			Objective sleep duration			
	No	Yes	P	⩾8 h	7–8 h	⩽7 h	P
N	241	137		112	170	96	
Female (%)	37.8	57.7	<0.001	55.4	42.9	36.5	0.019
Ethnic-Minority (%)	20.3	25.5	0.241	23.2	22.9	19.8	0.801
Low SES (%)	60.4	73.7	0.009	71.2	59.4	68.8	0.091
Age (years)	16.9 ± 2.3	17.2 ± 2.3	0.182	16.7 ± 2.2	17.2 ± 2.4	17.1 ± 2.2	0.172
Tanner (%)			0.152				0.908
Prepubertal	0.9	1.5		0.9	1.2	1.1	
Early pubertal	0.9	1.5		1.9	1.2	0	
Mid puberty	16.3	7.6		10.2	13.3	16.3	
Late puberty	46.8	47.0		49.1	45.5	46.7	
Adulthood	35.2	42.4		38.0	38.8	35.9	
BMI% (percentile)	65.6 ± 27.8	68.9 ± 27.9	0.260	67.9 ± 28.6	67.5 ± 25.2	64.4 ± 31.2	0.609
BMI% ⩾ 85 (%)	33.6	39.4	0.257	38.1	32.8	35.3	0.652
Eveningness (score)	26.7 ± 4.8	24.5 ± 5.2	<0.001	25.7 ± 4.8	26.5 ± 5.0	25.1 ± 5.5	0.126
M-type	38.3	21.9	<0.001	26.8	37.6	29.5	0.258
I-type	37.5	35.8		38.4	36.5	35.8	
E-type	24.2	42.3		34.8	35.8	34.7	
Internalizing (T score)	49.4 ± 10.0	52.9 ± 11.1	0.002	50.8 ± 9.5	50.6 ± 10.9	50.7 ± 11.4	0.987
Externalizing (T score)	47.4 ± 9.3	51.8 ± 11.1	<0.001	48.7 ± 10.1	49.3 ± 10.1	49.0 ± 10.6	0.887

Data are mean ± standard deviation, unless otherwise stated. AHI = apnea hypopnea index. BMI% = body mass index percentile. E-type = evening chronotype. I-type = intermediate chronotype. M-type = morning chronotype. SES = socioeconomic status.

Table 2
Sleep characteristics of the sample stratified by insomnia symptoms and objective sleep duration.

	Insomnia symptoms			Objective sleep duration			
	No	Yes	P	⩾8 h	7–8 h	⩽7 h	P
N	241	137		112	170	96	
DFA/DSA (%)			N/A				0.753
None	100.0	0.0		58.9	66.5	64.6	
DFA/DSA	0.0	69.3		29.5	23.0	24.0	
Both	0.0	30.7		11.6	10.6	11.5	
Sleep onset latency	24.0 ± 19.3	28.8 ± 30.7	0.064	12.5 ± 6.4	24.3 ± 14.0	43.9 ± 37.0	<0.001
Awakes	36.7 ± 11.4	37.2 ± 13.9	0.703	33.5 ± 9.2	39.2 ± 12.1	36.5 ± 14.9	0.001
Wake after sleep onset	72.7 ± 45.1	67.2 ± 42.7	0.247	33.8 ± 10.3	63.7 ± 19.4	125.9 ± 47.0	<0.001
Total wake time	94.5 ± 52.1	94.1 ± 60.7	0.952	44.6 ± 11.4	86.2 ± 18.3	167.0 ± 54.8	<0.001
Total sleep time	445.6 ± 52.7	446.7 ± 61.6	0.855	496.6 ± 10.7	454.3 ± 18.0	372.1 ± 55.6	<0.001
Sleep efficiency	82.5 ± 9.7	82.5 ± 11.3	0.936	91.7 ± 2.1	84.0 ± 3.4	68.9 ± 10.2	<0.001
%Stage 1	1.1 ± 1.3	0.98 ± 1.9	0.658	0.5 ± 0.43	0.9 ± 1.1	1.9 ± 2.5	<0.001
%Stage 2	53.9 ± 9.2	53.3 ± 10.4	0.553	53.6 ± 8.6	53.7 ± 9.6	53.9 ± 10.9	0.966
%Stage 3	26.4 ± 8.7	27.1 ± 8.9	0.469	26.0 ± 7.7	26.3 ± 9.0	28.1 ± 9.5	0.191
%Stage R	18.6 ± 4.7	18.57 ± 5.8	0.969	19.9 ± 4.4	19.1 ± 4.5	16.1 ± 5.9	<0.001
PLMI	4.3 ± 6.5	3.2 ± 4.9	0.083	3.1 ± 5.4	3.8 ± 5.9	4.7 ± 6.7	0.175
<5 events/hour	73.4	80.3	0.134	80.4	76.5	69.8	0.201
⩾5 events/hour	26.6	19.7		19.6	23.5	30.2	
AHI	2.5 ± 3.1	2.8 ± 4.5	0.378	2.4 ± 3.8	2.5 ± 2.6	3.0 ± 5.0	0.420
<2 events/hour	61.0	58.4	0.620	63.4	57.1	61.5	0.539
⩾2 events/hour	39.0	41.6		36.6	42.9	38.5	

Data are mean ± standard deviation, unless otherwise stated. AHI = apnea hypopnea index. DFA/DSA = difficulty falling or staying asleep. PLMI = periodic leg movements index. Stage R = rapid eye movement sleep.

Table 3
Inflammatory characteristics of the sample stratified by insomnia symptoms and objective sleep duration.

	Insomnia symptoms			Objective sleep duration			
	No	Yes	P	⩾8 h	7–8 h	⩽7 h	P
CRP (mg/L)	0.84 ± 0.07	1.21 ± 0.09	0.002	0.94 ± 0.10	0.86 ± 0.08	1.26 ± 0.11	0.011
IL-6 (pg/mL)	1.17 ± 0.07	1.24 ± 0.09	0.554	1.14 ± 0.09	1.16 ± 0.08	1.31 ± 0.10	0.400
TNF-α (pg/mL)	1.97 ± 0.10	1.93 ± 0.13	0.839	1.85 ± 0.14	1.85 ± 0.12	2.14 ± 0.16	0.269
Leptin (ng/mL)	12.55 ± 0.69	13.73 ± 0.93	0.328	13.60 ± 0.98	11.94 ± 0.82	13.90 ± 1.11	0.276
Adiponectin (μg/mL)	7.60 ± 0.35	8.01 ± 0.48	0.496	8.42 ± 0.49	7.59 ± 0.42	7.41 ± 0.56	0.324

Data are means ± standard errors adjusted for sex, age, race, SES, BMI percentile, MEQ, internalizing and externalizing behaviors, AHI, PLMI, alcohol, caffeine, tobacco and drug use, number of chronic health problems, and use of anti-inflammatory medication. P for interaction between insomnia and objective sleep duration on CRP levels p = 0.0002 (see Fig. 1); all other Ps for interaction ⩾0.200.

compared to those with objective normal sleep duration ($p < 0.05$). Importantly, the interaction between insomnia symptoms and objective sleep duration was statistically significant ($p < 0.001$), indicating a synergistic effect on CRP levels. As shown in Fig. 1, elevated CRP levels were significantly higher (1.79 ± 0.18 mg/L) in adolescents who reported insomnia symptoms and slept objec-

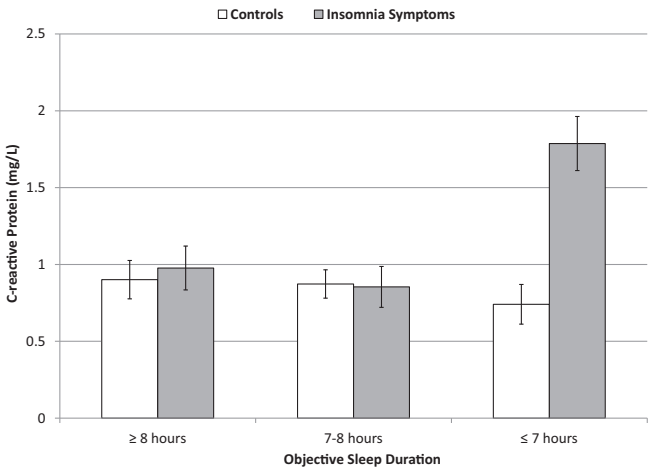

Fig. 1. Association of adolescent insomnia symptoms with plasma CRP levels across objective sleep duration groups. Adolescents who reported insomnia symptoms and slept objectively less than 7 h had increased CRP levels as compared to all other groups. In contrast, adolescents who reported insomnia symptoms and slept objectively more than 7 h did not have significantly different CRP levels as compared to controls. Data are estimated marginal means after adjustment for sex, age, race, SES, BMI percentile, MEQ, internalizing and externalizing behaviors, AHI, PLMI, alcohol, caffeine, tobacco and drug use, number of chronic health problems, and use of anti-inflammatory medication. Error bars represent the standard error of the mean (SEM).

tively ⩽7 h as compared to all other groups but, most importantly, as compared to controls who slept ⩽7 h (0.74 ± 0.13 mg/L, P = 0.00005), to adolescents with insomnia symptoms who slept ⩾8 h (0.98 ± 0.14 mg/L, P = 0.005) or to adolescents with insomnia symtpoms who slept 7–8 h (0.85 ± 0.13 mg/L, P = 0.0004). Among controls, there were no significant differences in CRP levels across the three objective sleep duration groups (all Ps for least significant difference ⩾0.374).

There were no statistically significant differences in the secondary outcomes of IL-6, TNF-α, leptin or adiponectin levels associated with insomnia symptoms or objective sleep duration and none of the interactions were statistically significant (Table 3).

4. Discussion

This is the first study to examine the association of insomnia symptoms with objective short sleep duration with inflammation in a population-based sample of adolescents. Consistent with the adult literature, we found that insomnia symptoms and short sleep duration are associated with systemic inflammation in adolescents. Importantly, our study showed that elevated CRP levels are primarily present in adolescents who report insomnia symptoms and slept objectively ⩽7 h in the laboratory, and that this association is independent of demographic factors or comorbid factors frequently associated with insomnia symptoms or inflammation such as depression, anxiety, evening circadian preference, substance use or medical conditions, among others. Furthermore, other inflammatory biomarkers followed a similar pattern, but did not reveal statistically significant differences across groups. Our findings provide further evidence that objective measures of sleep duration in insomnia, even as early as adolescence, may be a useful marker of the biological severity and medical impact of the disorder (Vgontzas et al., 2013).

In our study, adolescents with complaints of insomnia symptoms were associated with significantly elevated CRP levels. When we introduced the criterion of objectively-measured short sleep duration, we showed a strong and significant joint effect on the association of insomnia symptoms with increased CRP levels. Adolescents with insomnia symptoms who slept ⩽7 h had higher CRP levels than adolescents who slept >7 h and did not complain of insomnia symptoms (1.8 vs. 0.8 mg/L, respectively). In contrast, adolescents with insomnia symptoms who slept >7 h did not show increased CRP levels compared with the control group. CRP is a particularly stable biomarker that does not exhibit a circadian secretory pattern and is currently used to estimate risk of cardiovascular diseases. In adults, CRP levels <1 mg/L are considered optimal, while levels 1–3 mg/L are indicative of average cardiovascular risk and those ⩾3 mg/L indicative of high cardiovascular risk (Ridker, 2003); however, no guidelines or cut-offs have been developed yet for adolescents. The average CRP levels observed in this study for the most severe group (adolescents with insomnia symptoms and objective short sleep duration) were 1.8 mg/L and twice as high as the other groups. Given their age (mean 17 years) and the fact that all other study groups had healthy CRP levels on average (<1 mg/L), the observed differences may be indicative of potential cardiometabolic risk. Our finding on this synergistic effect of insomnia symptoms and objective sleep duration on CRP levels is consistent with previous reports that insomnia with objective short sleep duration is associated with physiologic hyperarousal and adverse health outcomes (Bathgate et al., 2016; Vgontzas et al., 2013). Indeed, insomnia with objective short sleep duration has been associated with increased 24-h activity of both limbs of the stress system (i.e., hypothalamic-pituitary-adrenal and sympatho-adrenal-medullary axes) and increased daytime secretion of pro-inflammatory cytokines (Bonnet and Arand, 2010; Irwin et al., 2003; Vgontzas et al., 1998, 2001, 2002, 2013; Zhang et al., 2014). It is likely that this 24-h physiological hyperarousal is responsible for the dissociation beyond short sleep duration observed in adolescents with insomnia symptoms, a hypothesis that should be tested in future studies. Thus, the results of the present study further support the proposal that insomnia symptoms with objective short sleep duration are a more biologically severe phenotype (Vgontzas et al., 2013), as indicated by increased systemic inflammation, and that this effect may be present as early as adolescence.

With respect to IL-6 and TNF-α levels, molecules tightly related to CRP, we did not find statistically significant differences across insomnia symptoms subgroups. These results are somewhat consistent with a previous meta-analysis in adults in which IL-6 and TNF-α, as compared to CRP, showed a weaker association with either insomnia symptoms or short sleep duration (Irwin, 2015). However, we observed that the pattern of elevated IL-6 and TNF-α levels in adolescents with insomnia symptoms with short sleep duration was similar to that of CRP. A potential explanation could be found in the well-documented circadian secretory pattern of IL-6 and TNF-α (Vgontzas et al., 2005; Keller et al., 2009) as compared to the 24-h stability of CRP. In fact, Vgontzas et al. (2002) previously reported no significant differences in mean 24-h IL-6 or TNF-α secretion between controls and short sleeping insomniacs; however, in that sample of young adults with severe insomnia, there was a significant shift in the circadian secretion of IL-6 and TNF-α from nighttime to daytime peak (e.g., insomniacs had significantly elevated IL-6 levels during the 19:00–21:00 period when compared to controls, but not in the morning) (Vgontzas et al., 2002). It is likely that in the present study this shift was not captured for IL-6 and TNF-α given that only a single morning fasting blood draw was obtained. Future studies should examine the joint effect of insomnia and short sleep duration on IL-6 and TNF-α levels using continuous 24-h sampling.

Finally, leptin and adiponectin levels did not show a statistically significant association with insomnia symptoms or short sleep duration, nor did the observed pattern indicate any abnormal levels across objective sleep duration groups in adolescents with insomnia symptoms. Leptin and adiponectin are cytokines secreted

by adipocytes (i.e., "adipokines") and have not been previously hypothesized to be associated with insomnia, although some experimental studies demonstrate altered leptin levels in response to sleep deprivation (Capers et al., 2015; Pejovic et al., 2010). However, these adipokines have been shown to be strongly associated with SDB, a disorder in which central obesity and visceral adiposity plays a key pathophysiologic role (Bixler et al., 2016; Tsaoussoglou et al., 2010). In fact, we have shown in this population-based sample that adolescent SDB is associated with significantly increased leptin and decreased adiponectin levels (Gaines et al., 2016), which suggests that the inflammatory pattern found for insomnia symptoms with objective short sleep duration appears to be different from that of SDB in adolescents. Alternatively, it is also plausible to speculate that adolescents with insomnia symptoms and short sleep duration may have increased noradrenergic activity (Irwin et al., 2003; Vgontzas et al., 1998), which is known to affect the secretion of these inflammatory markers (Chrousos, 1995). Future studies should examine the association of this insomnia phenotype with sympathetic nervous system activation in adolescents.

Some limitations should be taken into account when interpreting the results of the present study. Despite the high response rate (60%) of the Penn State Child Cohort, the present study is limited in that it may not perfectly represent the adolescent general population. However, this population-based sample overcomes the limitations of clinical samples of adolescents in which multiple comorbidities may affect the relationships examined. Second, the determination of short sleep duration was from a single night of PSG recording and, therefore, may have been susceptible to the first night effect and may not be representative of the objective sleep duration in the adolescent community. For example, adolescents may obtain insufficient sleep during schooldays and compensate for their sleep debt during weekends (Hall et al., 2015), which is something not captured by a single night of PSG. However, a recent study has shown that a single night of PSG for evaluating the relative classification of insomniacs as normal vs. short sleepers is a reliable and stable measure indicative of a subjects' physiologic sleep ability (Gaines et al., 2015). Third, inflammatory makers were assayed at one single time point and did not include 24-h sampling, which did not allow examining the circadian secretory pattern of cytokines and may explain the lack of some associations as discussed above. Fourth, insomnia status was assessed by the presence of the nighttime sleep symptoms of insomnia and did not include an assessment of duration, frequency or daytime functioning criteria that would have allowed establishing a diagnosis of an insomnia disorder. Future studies should examine the association of insomnia and objective sleep duration with inflammatory markers using a diagnostic definition of insomnia disorder in population-based, research-volunteer and clinical samples. Fifth, we did not have physical activity available in this study and, therefore, could not control for its' potential confounding effect. Finally, due to the cross-sectional nature of the study, no causal inferences can be made about the direction of the results.

In conclusion, insomnia symptoms with objective short sleep duration are associated with elevated inflammation in adolescents, and this association appears to be of a clinically significant magnitude in this general population sample. In contrast, insomnia symptoms with objective normal sleep duration did not show elevated inflammation as compared to controls. These findings are consistent with adult studies demonstrating that insomnia with objective short sleep duration is a more biologically severe phenotype of the disorder associated with activation of both limbs of the stress system and cardio-metabolic and neurocognitive morbidity. Future studies should examine the role of systemic inflammation in adolescence in predicting adverse health outcomes into adulthood in this insomnia phenotype.

Conflict of interest statement

All authors report no biomedical financial interests or any potential conflicts of interest.

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