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ORIGINAL ARTICLE

Experimental methods to study sleep disruption and immune balance in urban children with asthma

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Abstract

Study Objectives: We describe research methods developed to examine effects of sleep disruption on changes in immune balance, lung function, and cognitive performance in a sample of urban, ethnically diverse children with persistent asthma. Two case examples (8- and 10-year-old males) are presented to highlight methods of the current study and illustrate effects of experimentally disrupted sleep on the immune balance profile (Th1/Th2 cytokines), key sleep variables from polysomnography data, and lung function in our sample.

Methods: Children follow an individualized structured sleep schedule consistent with their habitual sleep need (≥9.5 hours' time in bed) for six days before a laboratory-based experimental sleep protocol. Children then spend two successive nights in the sleep lab monitored by polysomnography: a baseline night consisting of uninterrupted sleep, and a disruption night, during which they are awoken for 2 minutes between 20-minute intervals of uninterrupted sleep. Evening and morning blood draws bracket baseline and disruption nights for immune biomarker assessment.

Results: A shift towards immune imbalance following the sleep disruption protocol was observed in these illustrative cases.

Statement of Significance

This experimental approach is an early step in addressing a lack of research into the effects of sleep disruption on immune function and asthma outcomes in urban pediatric samples. Results may be clinically useful and could serve as a launchpad for future research into the mechanisms of poor sleep and immune imbalance in urban children with asthma to inform targeted interventions that address sleep disruption and nocturnal asthma.

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Conclusions: Data from these case examples provide evidence that the experimental protocol caused disruptions in sleep as observed on polysomnography and had the hypothesized downstream effects on immune balance associated with clinical asthma control. Documenting the effects of sleep disruption on immune function in children with persistent asthma is a crucial step towards understanding associations between sleep, immune balance, and asthma outcomes and provides important information for developing novel interventions for youth with asthma and suboptimal sleep.

Clinical Trials: Not applicable.

Key words: behavioral sleep medicine; biomarkers; child/children; immune function; pediatrics-behavior; pediatrics-pulmonary; pediatrics-sleep and arousal

Introduction

Asthma morbidity is prominent in urban minority children

Childhood asthma is a chronic disease that affects approximately 7.5% of youth in the US [1], and rates > 20% in urban US cities [2]. Black/African-American (B/AA) and Latino children have greater disease prevalence and asthma morbidity compared to non-Latino Whites [3–6] and tend to live in or near urban centers [7]. These youth have higher levels of asthma severity [3–8] and are exposed to urban poverty [9], environmental triggers [10, 11], and acculturative stressors [12, 13]. Any one or a combination of these factors can increase asthma morbidity [9, 14, 15]. Poorly controlled asthma [9, 16], which is more prevalent in urban minority youth, negatively affects daily functioning and behaviors, including sleep [9, 17–19].

Disrupted sleep in urban children with asthma

In healthy children, experimental studies show that shorter sleep duration is linked to inattention, academic difficulties, behavioral problems, and mood disturbance [20–28]. Children with asthma are at increased risk for poorer sleep outcomes [29]. For example, recent research demonstrates a correspondence between lung function decline, more nighttime awakenings, shorter sleep duration, and poorer sleep efficiency [30]. Nocturnal asthma symptoms, common in children with persistent asthma [31, 32], can delay sleep onset and disrupt children's sleep continuity [29, 33, 34], particularly for those whose asthma symptoms are poorly controlled [35, 36]. This is a concerning trend, given urban minority children tend to have low levels of adherence to daily asthma controllers [13] that are prescribed to those with persistent disease [13]. Greater exposure to environmental triggers can also increase nocturnal asthma symptoms and awakenings [37, 38]. This group of children also faces higher levels of urban stressors (e.g., suboptimal sleep environments, elevated environmental noise, and stress levels) that can challenge optimal sleep behaviors (e.g., maintaining consistent bedtimes and wake up times, consistent bedtime routines) [19, 39]. Thus, both urban living and asthma status may place these children at increased risk for sleep disruption, which can impact all areas of children's functioning and health in the short and long term [40].

Most studies that include urban school-aged children with asthma have been observational and cross-sectional in nature and conducted in the home environment. Although it is postulated that the association between asthma and sleep may be bi-directional, most research to date has emphasized the impact of asthma on sleep outcomes rather than the reverse. No published studies to date have used tightly controlled, in-lab experimental approaches including polysomnography (PSG) to assess the effects of sleep disruption on asthma activity in urban, school-aged children with asthma. This is a critical question, given the risk for poor sleep in this group. Focusing on sleep behaviors that enhance sleep continuity may be an important target for intervention for urban minority youth with asthma, in addition to supporting optimal asthma management strategies such as trigger control, medication adherence, and symptom monitoring.

In the few small studies that have used in-lab PSG in non-minority and non-urban children with asthma, children were shown to have more disrupted sleep (awakenings) [33], poorer efficiency [41], shorter duration [34], longer sleep onset latency [31, 36], and less slow wave sleep (SWS) [41, 42], than controls. In these studies, physiologic changes during sleep disturbance (e.g., reduction in lung volume) adversely affected respiration and arousal responses in those with asthma [43, 44]. Further, the few experimental studies including individuals with asthma have focused on the effects of sleep restriction. In a small study of adolescents with asthma, sleep restriction resulted in an overnight decrease in lung function (7–8% FEV_1, on average) [45]. In adults with asthma, experimental sleep restriction increased bronchoconstriction [46, 47]. Experimental studies that simulate the effects of sleep disruption that may occur when asthma is poorly controlled may capture what urban children with asthma experience during the night, whether those awakenings are due to urban stressors or asthma symptoms [43]. In the current study, we focus on a biological-based mechanism of influence *central both to sleep disruption and asthma*, such as immune function, which may be affected by sleep disruption and may reflect potential short- or long-term changes in children's asthma status [48].

Asthma, inflammation, and sleep

Increased inflammation in the airways at night due to circadian variation cause children with asthma to be more prone to sleep disruption [29, 49, 50]. Inflammatory mediators and cells play a role in the chronic processes of asthma, resulting in a clinically heterogeneous phenotypic expression. Inadequate allergic asthma control is associated with increased immune imbalance—that is, an altered Th1/Th2 (defined below) balance [51, 52] that has progressed to a Th2 dominant disorder. Allergic airway inflammation is induced by increased expression of Th2 cytokines (e.g., IL-4, 5, 13) and decreased expression of Th1 cytokines (e.g., IL-12, and IFNγ) [53]. A heightened proallergic cytokine profile has been found in low-income children with asthma (e.g., increased IL-5, 13) [54] and in children with asthma exposed to acute and chronic stress (e.g., increased IL-4, 5, decreased IFNγ) [55]. A higher IL-12/IL-10 ratio (suggesting greater immune balance) was also found to be related to better quality sleep in children with asthma [56].

Sleep and the immune system

Healthy sleep is associated with a predominant T helper 1 (Th1) cytokine balance, with a relative increase in antiallergic Th1 cytokines (IL-12, IFNγ), and a decrease in proallergic T helper 2 (Th2) cytokines (IL-4, IL-5, IL-13) [50, 57, 58]. Data from animal and human studies show that proinflammatory cytokines (TNFα, IL-1β, IL-6) impact sleep. Sleep fragmentation studies have shown an increase in proinflammatory cytokines, such as daytime serum IL-6 [59–64] and nocturnal plasma IL-6 levels [65, 66], associated with increased daytime somnolence [57, 61, 67–69]. Thus, sleep disruption can adversely affect the adaptive immune system, increase daytime sleepiness [58, 61, 70], impair cognition [71], and increase inflammation [72, 73].

In sum, disrupted sleep can increase IL-6 levels in healthy adults [64]. Increased Th2-type cytokines correlate with decreased asthma control, and this Th2-associated immune imbalance can affect sleep quality [50, 74]. Sleep disruption also may play a role in the immune-inflammatory cascades that contribute to altered asthma control [50, 72], as supported by results showing that sleep deprivation increases Th2 cytokines in healthy adults [75]. Although the reciprocal link between sleep and immune balance is shown in animal and adult studies [57, 72, 76–78], to our knowledge, no published work to date has considered the extent to which disruptions in sleep can affect immune balance changes in urban children with asthma. Ascertaining those components of immune balance that predictably change as a function of disrupted sleep would offer innovative opportunities for intervention in this high-risk group.

The primary goal of this paper is to describe the methods developed and in use for a study involving a laboratory-based protocol to examine experimental effects of sleep disruption on immune balance changes and lung function in a sample of urban children with persistent asthma. We describe how our approach has been informed by prior experimental work with healthy adults and children [79, 80] and by research with urban children who have persistent asthma involving objective monitoring of asthma and sleep [19, 30]. A secondary goal of the paper involves the presentation of the effects of our sleep disruption protocol on key sleep variables from the PSG data, immune balance profile (Th1/Th2 cytokines), and lung function using data from illustrative examples of two children who completed our laboratory-based protocol, thus far. We also present key clinical and sociocultural characteristics to contextualize these data.

Methods

The methods and data for the current paper are derived from Project KIDS (Kids, Immune Function and Disrupted Sleep), a study involving an examination of the effects of experimentally disrupted sleep on immune balance and lung function in urban children with persistent asthma. Participants are recruited from four urban school districts in and near a large metropolitan city in southern New England, ambulatory pediatric clinics, and an asthma educational program at a local children's hospital in these same targeted areas. Consent to Contact forms are distributed within schools, enabling research staff to contact families to determine eligibility and interest in study participation. Participating families complete a phone screening to establish preliminary study eligibility. Criteria for study entry require that the child (a) be between 8 and 10 years old; (b) live and attend

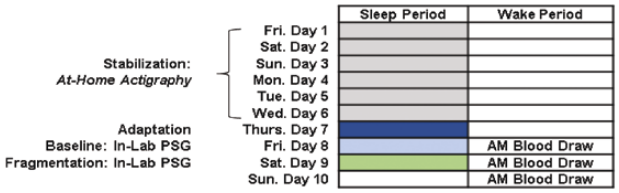

Figure 1. Study protocol. This figure illustrates the study protocol, which includes 6 nights of at-home sleep monitoring with actigraphy followed by 3 consecutive in-lab assessments, the third of which includes sleep disruption.

school in one of the targeted urban districts; (c) have a parent- and physician-reported asthma diagnosis; (d) have persistent asthma as indicated by having a prescription for an asthma controller medication; (e) and have a primary caregiver who speaks English or Spanish and self-identifies as Black/African American, Latino, or Non-Latino White. The caregiver provides permission for their child to participate in the laboratory-based protocol (described below and in Figure 1); the caregiver can opt to accompany the child during the overnight visits. The child also must meet criteria for "typical sleep," defined as caregiver report of ≥9.5 hours' time-in-bed (TIB) in the previous month (from lights out to time awake). This requirement is based on our prior work with this population and aged-based sleep recommendations [81–83]. We did not include those with <9.5h TIB, to ensure children were not sleep deprived prior to the laboratory-based experimental sleep protocol. We also confirmed that children followed their typical sleep schedule for 6 days prior to the experimental sleep protocol with a monitored lead-in period (see below).

Exclusionary criteria include (a) poorly controlled asthma by parent report; (b) comorbid pulmonary diseases or medical conditions other than asthma; (c) craniofacial abnormalities; (d) sleep-disordered breathing; (e) immune deficiency disorders diagnosed by a physician; (f) chronic use of stimulant and/or systemic corticosteroid medications; (f) having an asthma-related emergency department visit in the month prior to screening; (g) >2 confirmed bacterial pneumonia infections in the year prior to screening; (h) significant developmental delay; (i) severe psychiatric condition; and (j) physician-diagnosed ADHD or sleep disorders. Children are also excluded if they report being afraid of the dark, have a recent history of frequent enuresis, or have never previously slept away from home. Finally, we confirm that child enrollees do not have poorly controlled asthma at screening, at each study home visit, and prior to participating in the experimental sleep protocol by re-screening them for current symptoms and emergency healthcare utilization.

The enrollment goal for the larger study is 45 urban and ethnically diverse children (8–10 years old) with mild to moderate persistent, allergic asthma and their primary caregivers [84]. We target this age range because during this time children become more independent with their own asthma management [85]. Sample size was determined based on analyses demonstrating this number of participants would be sufficient to support adequate power (>.80) to detect expected effects. Data collection for this study occurs during the academic year, when children's sleep schedules tends to be more regulated by the school routine. This study was approved by the Institutional Review Board at the institution where the study took place. The current paper presents two illustrative child enrollees who completed all components of the protocol. Data from this study will be available on

written request to the study PI once enrollment is closed and all data have been processed and reported.

Research protocol: procedures for monitoring/lead-in period

Day 0: enrollment. Once children and caregivers are eligible and express interest in participating, an enrollment visit is scheduled at the family's home. Informed consent, child assent, and release forms are reviewed and signed by caregivers. Releases enable study staff to obtain key medical information and history from the child's primary care provider regarding asthma and severity status, to confirm persistent asthma. Children's caregivers also answer questions about perceived neighborhood safety, stressful life events, demographic information and information about the child's asthma symptoms, control status, and medication use and adherence.

Day 1: session 2 and 6-day at-home sleep schedule. Ten days to 2 weeks after the enrollment session, another home visit occurs to review procedures for the use of actigraphy and daily diary (Day 1, Friday), which assess and confirm the child's sleep schedule. This lag between Enrollment and Day 1 allows time for study staff to obtain the completed Physician Query form from the child's asthma healthcare provider. Following Session 2, children are asked to follow an individualized structured sleep schedule that is consistent with their habitual sleep need (≥9.5-hour TIB) for 6 days leading up to the laboratory-based experimental sleep protocol. This stable sleep schedule (consistent bed/wake times) is (a) based on children's average TIB and bedtimes/waketimes over the previous 2 weeks; (b) allows for confirmation of sleep eligibility criteria; and (c) supports circadian entrainment and optimal sleep duration/quality prior to the laboratory-based sessions [58, 61, 63, 75, 86].

If necessary, a participant's sleep schedule is adjusted by up to 30 minutes to align with the schedule they will follow during the laboratory-based protocol. Participants are instructed to wear wrist-worn actigraphy (Actiwatch) for the 6 days, and to complete a sleep diary every morning and evening using standard procedures [19, 30]. Participants are also trained using standard procedures [19, 30] to use a handheld electronic spirometer twice daily (3 blows each morning and evening) to provide real-time measurements of lung function. Study staff call the caregiver daily to verify adherence to the sleep schedule. At Day 6, another home visit occurs and Actiwatch data are downloaded to confirm the child's sleep schedule. If children are not adherent on most nights, they discontinue participation in the study. Non-adherence to the prescribed schedule is defined as less than 9.5 hours TIB on more than 1 night. In the event of an adherence violation, they discontinue participation in the study [87–89].

Day 7: laboratory adaptation visit. On Day 7 the child and caregiver visit the sleep laboratory for a tour and orient to the sleep lab environment, equipment, and procedures that take place on the subsequent overnight visits. The child and family complete three brief sleep-related questionnaires to assess bed and rise times, the child's sleep and wake behaviors, and level of alertness on that day. Study staff remind caregivers to bring all of the child's medications (asthma rescue medications, EpiPen (if child has food allergies), and any topical skin emollients for eczema) for the subsequent laboratory-based research visits.

Day 8: morning home visit. Research staff and a phlebotomist visit the family's home to conduct a blood draw for the first immune biomarker assessment (two 10ml tubes of heparinized blood), within 1 hour after the child's scheduled waketime. Blood draws occur at the same time of day for each study visit.

Day 8: baseline laboratory-based evening session. Children and their primary caregivers return to the sleep lab for the baseline overnight condition involving PSG. Upon their arrival, children are re-screened for severe asthma symptoms, and if none are endorsed assessments involving asthma control, sleepiness, and bedtime and risetimes for that day are administered. Asthma-related lung function and symptoms are then assessed via desktop spirometry and symptom reports, respectively. To allow for a naturalistic assessment of sleep disruption on asthma and for safety reasons, children are not asked to withhold any medications on the day of each PSG; they use asthma controllers and albuterol as prescribed and as needed. Children are hooked up to the PSG (see below) by study staff and follow their sleep schedule in the sleep lab in their own bedroom. Bedtimes and waketimes follow each child's individualized sleep stabilization schedules and sleep is monitored via PSG.

Day 9: baseline laboratory-based morning session. Children are awakened at their scheduled time, and the second blood draw for immune biomarker assessment is conducted by a phlebotomist. Lung function is then assessed.

Day 9: sleep disruption laboratory-based evening visit. Children and their caregivers return to the sleep lab for the overnight sleep disruption protocol. Lung function and asthma symptoms/control are assessed as described for the baseline condition. The child is hooked up to the PSG by study staff prior to their bedtime, and the sleep disruption protocol begins (see below for description of procedures). TIB follows the at-home schedule prescribed to each participant, and sleep is monitored via the PSG and video.

Day 10: sleep disruption laboratory-based morning session. Children are awoken during their scheduled wake up time, after which the third blood draw for immune biomarker assessment is conducted by a phlebotomist. Lung function, symptoms, and asthma control are assessed.

Day 10: evening check-in. Participants receive a phone call from study stuff, who administer a questionnaire related to children's daytime performance and answer any questions the family may have about the laboratory-based sleep protocol. Participation is complete after this contact.

Measures and Assessments

Demographic and descriptive information. Poverty status is determined by comparing the family's annual per capita income to the US federal poverty threshold for a family of that size during the calendar year of their study participation [30, 90], Table 1. Caregivers answer questions evaluating children's risk for sleep disordered breathing at screening using the Sleep-related

Table 1. Demographic and descriptive information

	Participant 1	Participant 2
Participant characteristics		
Age	8	10
Sex	Male	Male
Race/Ethnicity	Latino	Black/African American
Neighborhood Risk Summary Score	20 out of 28	12 out of 28
Body Mass Index	21	15.6
Asthma characteristics		
Controllermedication adherence via caregiver report	Miss occasionally	Miss occasionally
Asthma Control Questionnaire	1.85(Not Well Controlled Asthma)	1.14(Not Well Asthma Control)
Sleep characteristics		
Sleep duration at screening	9–10 hours	11 hours

Breathing Disorders scale of the Pediatric Sleep Questionnaire, a well-validated and reliable questionnaire [65].

Asthma diagnosis and severity classification. Participants' asthma status and asthma medication prescriptions are collected by caregiver-report during the eligibility screening process and verified by the RA during the enrollment session. Once enrolled, the family signs a release enabling study personnel to contact the child's primary healthcare and/or asthma healthcare provider who completes a Physician Query form to confirm asthma status and medications, and to rule out any exclusionary physical or psychological/behavioral conditions. A study clinician classifies the participant's asthma severity based on NHLBI EPR-3 guidelines [91] by reviewing the child's asthma medications, pulmonary function tests, and responses to the asthma control questionnaire at enrollment.

Physician query. Participants' primary healthcare providers or asthma specialists complete a form detailing the date of the child's last office visit, asthma diagnosis, asthma triggers, relevant past medical history, and current asthma regime. Information from the Physician Query is used to evaluate asthma status.

Sleep duration and sleep schedule. To confirm participants' daily sleep duration and sleep schedule prior to the in-lab protocol, participants wear a Phillips Respironics Actiwatch Spectrum (Pittsburgh, PA, USA) on their non-dominant wrist during the 6-day home monitoring period. One-minute epochs estimate whether the participant is awake or asleep using Actiware-Sleep software V 2.53. A scoring algorithm is applied to portions of the record identified as sleep through a combination of diary reports, and Actiwatch event markers set by participants at "lights-off" and "lights-on" [18].

Asthma-related lung function. Each participant's lung function is measured twice daily, morning and evening, via the AM1 hand-held spirometer (AM1; eResearchTechnology GmbH, Estenfeld, Germany; data not included in this paper). Participants use the device at home during the 6-day sleep stabilization period and each evening and morning of the laboratory-based sleep study protocol. Participants are instructed to perform three successive forced expirations into the device at each time point. The blow yielding the highest FEV1 value at each assessment point is retained for data analysis. Previously established standardized procedures are used for data cleaning and reduction [92, 93]. Participants and their caregivers are trained by research staff on the proper use of the device.

Lab-based pulmonary function measurements including FEV1 are collected during the Adaptation, Baseline, and Disruption sleep lab visits, using the Koko incentive desktop spirometer (nSpireHealth, Longmont, CO). Children are coached to perform sustained forced exhalations while seated, before and after short-acting beta agonist administration [94].

Asthma control. Participants complete the seven-itemed Asthma Control Questionnaire [95, 96] which is a well-validated questionnaire to assess asthma control. Participants indicate how often in the past week they experienced various asthma symptoms or used their short-acting bronchodilator on a seven-point scale (0 = no impairment, 6 = maximum impartment). Additionally, research staff indicate the participants' FEV_1% predicted using the Koko incentive desktop spirometer (described above) using a similar seven-point scale. An average score across the seven items is calculated such that higher scores indicate poorer asthma control.

Polysomnography (PSG). *Sleep recording*. Continuous PSG recordings occur on two nights in the temperature-, light-, and sound-controlled sleep laboratory, scheduled at the participants' usual sleep times as for the at-home protocol. Recordings are performed using Compumedics Grael recording systems (Charlotte, NC), with signals digitized and stored using a sampling rate of 400 samples per second. Electrodes are attached to scalp for electroencephalogram (EEG) from 10 to 20 placements (Jasper et al., 1958) C3, C4, O1, O2, Fp3, and Fp4 electrodes; electro-oculogram (EOG) is obtained from electrodes taped to the skin at the left and right outer canthi; EEG and EOG leads are acquired with contralateral mastoid or ear lobe references; chin electromyogram (EMG) is acquired from electrodes taped to the skin over mentalis/submentalis muscles; electrocardiogram (ECG; modified lead II) is recorded from electrodes taped on the right sternum and left lower rib cage; finger plethysmography/oximetry is also acquired. All leads are recorded on both in-lab nights.

Sleep staging. Sleep staging is performed by visual scoring in 30-second epochs using the criteria of Rechtschaffen and Kales [97] by trained research staff who are reliable against the sleep lab director and/or co-director with a minimum rolling agreement of 85% verified every 10 recordings.

Baseline night. The PSG recording is continuously observed throughout, and the child is permitted to sleep without interruption.

Sleep disruption night and protocol. As on the Baseline night, bedtime and risetime are determined by the child's at-home fixed sleeping schedule. The arousal night procedure was developed based on the goal of disrupting sleep at the same frequency as occurs in children with poorly controlled asthma. We recognized that awakenings as a result of nocturnal asthma or urban stressors may be variable due to the heterogeneity of the illness and to environmental conditions specific to families. Further, these factors as well as individual differences among children in their thresholds for arousal to stimuli would be challenging to replicate across children. We ultimately aimed to develop a disruption protocol that simulates the *effects* of sleep disruption on this group's sleep outcomes, by producing arousals, if not full awakenings, reflected in the sleep EEG.

Given that published reports at this time showed that sleep disruption or fragmentation experimental protocols have not been employed with this population nor with healthy samples of children, we referred to studies including healthy adults [98–102]. Our approach was informed by data from a previous study evaluating sleep duration and night awakenings via actigraphy in urban children with persistent asthma [19]. Analyzing the actigraphy data from 2010, 8–9 year old urban children collected over 4 weeks revealed that the distribution of night awakenings of at least 3 minutes across each hour of the sleep period looked identical for children with well-controlled and not-well-controlled asthma. The mean frequency of arousals lasting >=3 minutes was 1.4 per hour, while the mean frequency of arousals lasting >=1 minute was 3.3 per hour. The data summary also showed that the mean duration of awakenings defined as greater than 1 minute, but less than 3 minutes was 2.4 minutes. These data informed the duration of arousals for the current study, which were set at 2 minutes in duration. The mean frequency of arousals was increased by 50% to ensure that the number of arousals would be clinically relevant for the study population, while also considering child's stress levels as a result of high frequency of awakenings.

Rather than attempt arousals by auditory stimuli, for which arousal thresholds are in a decibel range that can damage hearing in young children, we chose to arouse children by turning on a dim light, entering the bedroom, calling the child's name, and (if needed) applying light pressure to the child's shoulder. To ensure that the child is aroused by this procedure, each is asked to sit up in bed and complete a sleepiness/mood scale read to them by staff. The arousals are limited to two minutes, whether or not the scale is completed. The first arousal is timed to occur after 20 minutes of sleep, as determined by the sleep technologist who observes the PSG recording; subsequent arousals occur 20 minutes after the child returns to sleep. Thus, approximately 25 interruptions are produced if a child sleeps throughout most of the 9.5-hour window. Figures 2 and 3 each illustrate the sleep hypnogram profiles of a child on the non-disturbed baseline night and on the arousal night. These procedures are kept standard for each participant despite the differences in individual responses throughout the sleep period. Research staff take detailed notes pertaining to child's level of alertness during each arousal to supplement the PSG data.

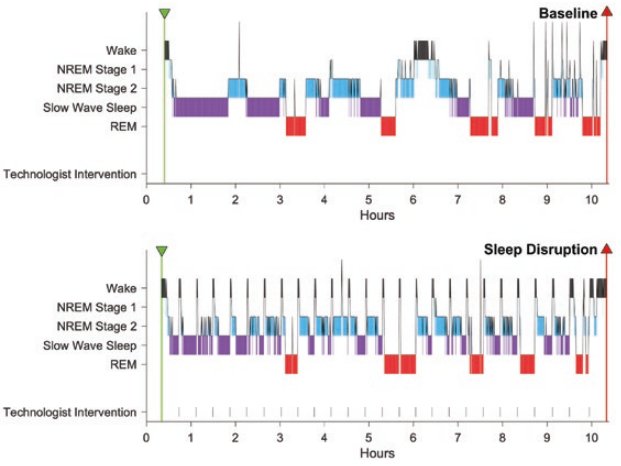

Figure 2. Baseline and sleep disruption hypnograms for participant 1. Green downward pointing triangles indicate bedtimes and red upward pointing triangles indicate risetime. Waking and sleep stages are identified on the y axis and by color in the plot: black = wake, light blue = NREM stage 1, darker blue = NREM Stage 2, lavender = slow wave sleep, and red = REM sleep. Hatch marks on the Sleep Disruption hypnogram indicate the times at which arousals occurred by technician intervention. The x axis in each hypnogram shows time in hours.

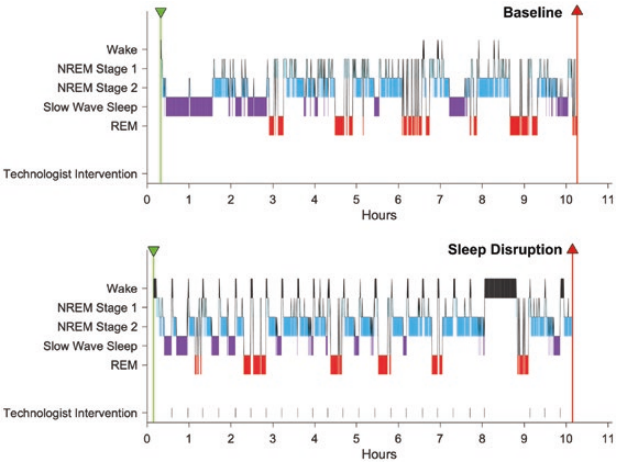

Figure 3. Baseline and sleep disruption hypnograms for participant 2. Annotations are as described for Figure 2.

If a child wakes up at any point during the sleep period, the sleep technician resets the arousal time to ensure there is 20 minutes of uninterrupted sleep prior to each experimental arousal. If the child does not want to answer questions or wakes up at the time of any given arousal, the child is allowed to sleep through that arousal. If a child expresses that s/he does not want to be woken up any more throughout the sleep period, the child is allowed to sleep uninterrupted until rise time. Finally, if an arousal is scheduled within fifteen minutes of child's rise time, that arousal is not performed.

Outcomes of PSG. We anticipated that the sleep disruptions would modify the PSG outcomes and present in Table 2 the primary variables for the same children as in Figures 2 and 3. These variables include the minutes of the entire sleep period

(SPT, initial sleep onset to final arousal); total minutes and percentages (of SPT) for wakefulness (TWT), sleep (TST); and minutes and percentages (of TST) for NREM sleep stages 1, 2, SW (slow wave sleep), and REM sleep. Several parameters of overnight SpO2 are also shown in Table 2, including overnight mean level, lowest value, and number of minutes in which SpO2 was below 90%.

Immune biomarkers assessments. Our main immune hypothesis is that immune balance is negatively affected by sleep disturbance, and this can lead to subsequent increased clinical signs and symptoms. Accordingly, the perturbed balance may manifest as decrease in Th1 cytokine (IFNγ) and/or increase in one or more Th2 cytokines (IL-4, IL-5, IL-13). Plasma IL-6 changes have been reported to increase in children and adolescents with sleep disturbance [103].

Specific immune biomarkers (CD4+IFNγ+ Th1 cells; CD4+IL4+, CD4+IL5+, CD4+IL13+ Th2 cells; plasma IL-6) relevant to allergic asthma and sleep are performed on cryopreserved peripheral blood mononuclear cells (PBMC) and frozen plasma samples. PBMC are separated within 2 hours of collection by standardized density gradient centrifugation. Cell viability is confirmed at > 90% prior to cryopreservation in 30% DMSO-based freezing liquid. Samples are stored at –80°C until transported on dry ice to the analyzing lab monthly, to be thawed to confirm viability, and for immune biomarker analysis using standard procedures [104]. Levels of Th1 and Th2 cytokines are assessed by intracellular flow cytometry, providing comparative data on populations reassessed using mean fluorescent intensity measures. Baseline samples serve to determine if the biomarker profile (Th1/Th2R, plasma levels of IL-6 [pIL-6]) and/or which biomarker changes after experimental intervention correlate with sleep disruption, changes in lung function, and/or daytime performance. The morning sample following sleep disruption will be compared to the sample following baseline sleep to assess changes to the biomarker profile and lung function as a result of sleep disruption. Thus, the baseline sample collecting during the morning following the baseline overnight will be used to establish normative values in each measure of the biomarker profile as a comparator for the samples following the sleep disruption overnight. The expected changes in direction of responses can be assessed over the sample to determine if absolute values or relative changes best correlate with altered lung function/sleep disruption. All blood draws occur within 1 hour after participant's scheduled wake up to account for effects of diurnal variation on changes in immune biomarkers.

An immune biomarker profile was created and consisted of levels of (1) Th2 cytokines (IL-4, IL-5, and IL-13); (2) Th1 cytokine (IFNγ); (3) Th1/Th2Ratio (IFNγ/IL-4, IL-5, or IL-13), and (4) pIL-6. For patients with asthma, optimal *immune balance* is defined as a relatively (1) higher ratio of Th1 to Th2 cytokines Th1/Th2R and (2) lower pIL-6. We expect that "greater imbalance" after disrupted sleep will show a decreased Th1/Th2R and/or increased pIL-6 when compared to individual stabilized and recovery sleep.

Results: Case Summary Descriptions

Results demonstrating effects of sleep disruption protocol on sleep variables (PSG)

We describe the effects of the sleep disruption protocol on two children enrolled in the sample. The first child is an 8-year-old Latino male with not-well-controlled asthma at study entry. The second enrollee is a 10-year-old Black male with not-well-controlled asthma. Both children have a prescription for daily controller medications, consistent with the study eligibility criteria. In these two children, the arousal protocol produced expected brief arousals (see Figures 2 and 3), increased time awake, and reduced amount of SW (see Table 2). Other sleep variables showed inconsistent modification by the arousal protocol. Finally, the SpO2 was not worsened by the arousal protocol in these exemplary children.

Table 2. PSG data

	Participant 1		Participant 2	
	Baseline	Arousal	Baseline	Arousal
SPT	582	579.5	571	570
TST	558.5	523.5	554	545
TST %	96.0%	90.3%	97.0%	95.6%
TWT	32	73	33.5	52
TWT %	5.5%	12.6%	5.9%	9.1%
Stage 1	33.5	14.5	74	97.5
Stage 1 %	6.0%	2.8%	13.4%	17.9%
Stage 2	173.5	201	268.5	256.5
Stage 2 %	31.1%	38.4%	48.5%	47.1%
SW	227	200.5	137	100.5
SW %	40.6%	38.3%	24.7%	18.4%
REM	124.5	107.5	74.5	90.5
REM %	22.3%	20.5%	13.4%	16.6%
Mean SpO2%	97.1	97.9	97.7	98.1
Lowest SpO2%	49.8	78.7	62.7	71.0
Min SpO2 <90%	8.9	1.4	2.5	1.0

SPT, minutes from initial sleep onset to final arousal; TST, minutes asleep during SPT; TST %, percent time asleep of SPT; TWT, minutes awake during SPT; TWT %, percent time awake of SPT; Stage 1, minutes of Stage 1during SPT; Stage 1 %, percent time in Stage 1 of SPT; Stage 2, minutes of Stage 2during SPT; Stage 2 %, percent time in Stage 2 of SPT; SW, minutes of slow wave sleep during SPT; Stage 2 %, percent time in slow wave sleep of SPT; REM, minutes of REM during SPT; REM %, percent time in REM of SPT; Mean SoO2%, mean level of SpO2% across the night; Lowest SpO2%, lowest recorded value of SpO2% across the night; Min SpO2 <90%, number of minutes of SpO2% lower than 90% across the night.

Results demonstrating effects of sleep disruption protocol on immune balance and lung function

As described above, both participants presented with asthma that was not-well-controlled. As illustrated in Table 3, Participant 1's sleep interruption produced a worsening of his immune imbalance—namely a decrease in Treg expression (8.67 to 2.52), a decrease in Th1 (3.81 to 2.84), and an increase in Th2 (28.64 to 33.14). Additionally, immune imbalance was heightened in all three measures (IFNg/IL4R decreased from 0.126 to 0.086; IFNg/IL-5 and IFNg/IL-16 ratios both decreased as well). The data from this individual represents the greatest immune effect of the sleep disruption.

Participant number 2 presents a mixed immune pattern to the sleep interruption. Tregs again decreased (13.1 to 10) but both Th1 (25.72 to 26.19) and Th2 (12.1 to 13.9) cytokine profiles increased. Yet there was still evidence of Th1/Th2 imbalance in this individual. IFNg/IL-4 (2.134 to 1.88) and IFNg/IL-5 (1.998 to 1.896) were decreased while IFNg/IL-13 increased 2.471 to 13.641). This represents a mixed immune pattern to the sleep stressor.

Of note, there was a mild decrease in FEV1 for both participants following sleep disruption (see Table 4). The FEV1/FVC ratio (an indicator of airway obstruction) remained essentially unchanged, suggesting that the adverse effects of the sleep disturbance-induced immune dysfunction may either need to be larger or more prolonged to see clinical changes consistent with decrements in asthma control.

Discussion

A greater understanding of the relationships between asthma control, impaired immune balance, and disturbed sleep has important clinical applications for patients with asthma. The goal of this paper is to describe a novel approach involving real-time methodologies to allow for in-depth study of effects of sleep disruption (using PSG) on immune balance and lung function in a controlled laboratory setting. Our sleep disruption protocol was developed to simulate the effects of nighttime sleep disruption on immune balance in urban children with asthma. For our overall study, we hypothesize that disrupted sleep as a result of our laboratory-based experimental protocol is associated with a biomarker profile reflective of immune imbalance, evidenced by a shift in the Th1/Th2 ratio toward Th2 dominance, and a relatively higher level of plasma IL-6. We anticipate that heightened Th2 dominance and elevated plasma IL-6 resulting from sleep disruption will be associated with worsening asthma (decreased lung function/FEV1). We also expect poorer lung function in these children, as a result of the sleep disruption protocol.

As described in the summary of results for our case examples, our sleep disruption protocol succeeded in accomplishing our stated goals, i.e., to disrupt sleep during the sleep period and to impact immune imbalance as a consequence of this disruption. The brief and consistent arousals produced by the experimental protocol led to increased time awake, decreased amount of time asleep, and decreased amount of slow wave sleep from the baseline night to the sleep disruption night. It is possible that the SpO2 was not affected by the arousal protocol in these case examples due to having multiple safeguards in place to ensure that participants are not

Table 3. Immune functioning data

	Participant 1			Participant 2		
	Morning before baseline (Fri. AM)	Baseline morning (Sat.AM)	Sleep disruption morning (Sun. AM)	Morning before baseline (Fri. AM)	Baseline morning (Sat. AM)	Sleep disruption morning (Sun. AM)
Treg	3.82	8.67	2.52	13.3	13.1	10
IL10 % gated	4.11	2.67	5.34	1.26	1.39	1.52
TGFb % gated	4.11	2.84	4.13	4.04	8.34	2.63
IL17A % gated	4.65	4.06	4.52	11.5	10.7	12.6
Th1 % gated	2.87	3.61	2.84	30.52	25.72	26.19
Th2 % gated	127.64	28.64	33.14	10.8	12.1	13.9
CD3+CD8-IL5+ % gated	1.14	1.02	1.02	1.1	0.99	1.06
CD3+CD8-IL13+ % gated	4.79	4.83	4.73	4.7	5.61	5.85
IFNg/IL4 Ratio	0.022	0.126	0.086	2.826	2.134	1.88
IFNg/IL5 Ratio	0.168	0.167	0.153	2.247	1.998	1.896
IFNg/IL13 Ratio	0.663	0.737	0.671	3.034	2.471	13.641

Table 4. FEV_1 data

	Participant 1		Participant 2	
	Baseline morning (Sat. AM)	Sleep disruption morning (Sun.AM)	Baseline morning (Sat. AM)	Sleep disruption morning (Sun. AM)
FEV_1 (% predicted)	111	107	89	86
FEV_1/FVC (% predicted)	102	101	105	103

experiencing any clinically significant asthma symptoms immediately prior to and during the protocol.

We observed a shift towards Th2 dominant immune imbalance following the sleep disruption protocol in these illustrative cases. This may demonstrate an early signal for changes in lung function; however, it is not clear if the mild changes in lung function would have been exacerbated if the sleep disruption were more chronic across nights or produced prolonged waking during the night. Lung function is expected to be a secondary outcome in our study, and we anticipate it may change over time with prolonged sleep disruption. We anticipate that any changes in lung function that are observed in our larger sample will not necessarily be clinically meaningful, given the short data collection period, but may be an important indicator of worsening control and/or the potential for future compromise.

When we examine data that will be generated in the larger sample, we will consider that while our urban sample will be somewhat homogenous due to study inclusion criteria, we anticipate individual differences in response to the stressor of disturbed sleep that may be affected by the lab's novel setting and to repeated arousals of the overnight protocol. We will need to contextualize study results related to our study hypotheses with the information collected from questionnaires on stressors experienced, and on additional clinical, contextual, and behavioral factors.

Our protocol was developed to simulate the effects of potential sleep disruption due to urban stresses, family and sleep environment factors, or asthma symptoms. The in-lab setting will allow us to systematically employ the same protocol for each child, thereby providing the opportunity to examine individual-differences in the effects of sleep disruption on children's immune balance and lung function. Thus, the research methods described herein hold much clinical value, as the results of this work will be clinically informative and will address the lack of ethnically and racially diverse urban pediatric samples in overnight sleep experimental research. If sleep disruption results in greater immune imbalance, which may predict a decline in lung function following disrupted sleep, this may suggest the need for targeted interventions to address sleep disruption and nocturnal asthma. Tailored strategies to address sleep disruption resulting from increased nocturnal asthma activity and/or poor sleep hygiene can be integrated with guidelines-based treatment to enhance asthma control.

Further, identifying changes in immune biomarkers as a result of sleep disruption may clarify distinct individual pathways of influence between sleep, immune balance, and asthma (e.g., are relative changes in the Th1/Th2 profile and IL-6 a consequence of disrupted sleep?) that could present clinically in similar fashions. Thus, results can serve as a launching point for future research, informing the development of clinically useful immune biomarker profiles associated with changes in sleep disruption and/or asthma in specific ethnic groups that may ultimately: a) enhance the assessment and treatment process for children with asthma, to more precisely describe their clinical needs promptly and efficiently, and b) provide basis for mechanism-based future interventions. Study of immune balance should help clarify the underlying processes linking sleep disruption and poorer asthma control in individual children, to ultimately assist in prioritizing treatments and enhancing response to treatments targeted to an individual's need(s).

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