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ORIGINAL ARTICLE

PAX8/PAX8-AS1 DNA methylation levels are associated with objective sleep duration in persons with unexplained hypersomnolence using a deep phenotyping approach

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Abstract

Study Objectives: Patients with unexplained hypersomnolence have significant impairment related to daytime sleepiness and excessive sleep duration, the biological bases of which are poorly understood. This investigation sought to examine relationships between objectively measured hypersomnolence phenotypes and epigenetic modification of candidate hypersomnolence genes to advance this line of inquiry.

Methods: Twenty-eight unmedicated clinical patients with unexplained hypersomnolence were evaluated using overnight ad libitum polysomnography, multiple sleep latency testing, infrared pupillometry, and the psychomotor vigilance task. DNA methylation levels on CpG sites annotated to 11 a priori hypersomnolence candidate genes were assessed for statistical association with hypersomnolence measures using independent regression models with adjusted local index of significance (aLIS) P-value threshold of 0.05.

Results: Nine CpG sites exhibited significant associations between DNA methylation levels and total sleep time measured using ad libitum polysomnography (aLIS *p*-value < .05). All nine differentially methylated CpG sites were annotated to the paired box 8 (*PAX8*) gene and its related antisense gene (*PAX8-AS1*). Among these nine differentially methylated positions was a cluster of five CpG sites located in the body of the *PAX8* gene and promoter of *PAX8-AS1*.

Conclusions: This study demonstrates that *PAX8/PAX8-AS1* DNA methylation levels are associated with total sleep time in persons with unexplained hypersomnolence. Given prior investigations that have implicated single nucleotide polymorphisms in *PAX8/PAX8-AS1* with habitual sleep duration, further research that clarifies the role of DNA methylation levels on these genes in the phenotypic expression of total sleep time is warranted.

Statement of Significance

Biological changes related to excessive sleep duration in central nervous system disorders of hypersomnolence are not known. This study applied a deep phenotyping approach to examine relationships between DNA methylation of candidate genes with key objectively measurable aspects of hypersomnolence. Results demonstrate DNA methylation levels of *PAX8/PAX8-AS1* are associated with sleep duration in patients with unexplained hypersomnolence. Future research will clarify the clinical applicability of *PAX8/PAX8-AS1* methylation as a marker of pathological hypersomnia, as well as the role of this gene in the cellular mechanisms that underlie sleep duration more broadly.

Key words: hypersomnolence; *PAX8*; *PAX8-AS1*; DNA methylation; polysomnography; sleepiness; hypersomnia; sleep

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Introduction

Hypersomnolence, broadly defined as excessive daytime sleepiness often accompanied by prolonged sleep duration, is one of the most common symptom constellations encountered in persons with sleep disorders. While the pathophysiology of hypersomnolence can be identified in some cases, for example orexin deficiency in type 1 narcolepsy [1], the cause of hypersomnolence cannot universally be determined for many patients despite comprehensive evaluation, resulting in ambiguous diagnoses and a lack of targeted therapies to improve symptomatology. Thus, there is a clear need to better understand the biological bases of unexplained hypersomnolence to advance clinical care through the development of advanced diagnostics and personalized therapeutics for these patients.

One of the great challenges that must be overcome in this area of research is the fact that hypersomnolence is a multifaceted symptom, for which no singular objective measure is able to fully capture the subjective complaint [2, 3]. It is widely appreciated that the multiple sleep latency test (MSLT), while highly useful in confirming type 1 narcolepsy, has limited test-retest reliability in disorders of unexplained hypersomnolence [4–6]. While the MSLT remains a key tool in the practice of sleep medicine, it quantifies sleep propensity during repeated nap opportunities and the presence of sleep onset REM periods, but does not capture other relevant aspects of hypersomnolence. Supplementary measures frequently capture other key facets of hypersomnolence, including the maintenance of wakefulness test, which quantifies the ability to remain awake under soporific conditions, infrared pupillometry, which quantifies drowsiness under resting conditions, the psychomotor vigilance task, which quantifies neurobehavioral alertness, and extended duration polysomnographic recordings, which measure excessive sleep duration. While these aspects of hypersomnolence correlate with subjective clinical complaints, they only marginally explain the variance of one another [7–9]. Thus, the measurable phenotype in patients with unexplained hypersomnolence can be quite complex, with variable combinations of abnormalities occurring among individual patients [7, 9].

Since there is heterogeneity among objective measurable deficits in persons with hypersomnolence, it is highly likely that there is also significant heterogeneity in the underlying biology associated with clinical complaints. Sleep medicine has traditionally focused on research that compares various disorders of excessive sleepiness defined by the International Classification of Sleep Disorders (ICSD) both against one another and healthy persons. The diagnostic criteria for disorders such as idiopathic hypersomnia have shifted over time, often with limited clinical or biological data to support alterations in nosology. To disentangle the undoubtedly complex causes of unexplained central nervous system hypersomnolence requires that research efforts focus on the biology of specific and measurable phenotypes that may cut across traditional diagnostic boundaries, rather than nosological distinctions defined largely by expert opinion [10].

The importance of using refined and objective phenotypes is particularly salient for the study of the genetics that may be related to hypersomnolence complaints. Effort has been made to utilize genome-wide association studies (GWAS) to identify candidate single nucleotide polymorphisms (SNPs) that are associated with various sleep phenotypes [11]. Unfortunately, the SNPs that are statistically related to sleep measures in these large-scale and hypothesis-free investigations often have modest effect sizes on the overall phenotype, suggesting that genetic variation alone is unlikely to account for extremes of daytime sleepiness and/or sleep duration observed in clinical practice [12–16]. Additionally, the sleep phenotype used in GWAS studies is almost universally based on self-report, which may be both inaccurate and have limited connection to relevant underlying genetic variability. Thus, while GWAS can help identify potential genes linked to broad phenotypic traits, alternative approaches are needed to connect findings from large-scale population-based GWAS investigations to smaller populations of heterogeneous and disordered patients.

In this context, epigenetics, the study of molecular modifications not affecting the genetic code itself, may be highly applicable to the study of unexplained hypersomnolence. Employing epigenetic methodologies is a crucial step in clarifying how candidate genes are related to specific observable and measurable phenotypes in sleep disorders. In particular, combining epigenetics with a detailed and objective phenotyping approach is likely to advance our understanding of how specific gene regulatory elements are related to the pathophysiology of human disease. Therefore, this investigation examined alterations in DNA methylation, a key component of epigenetic regulation, in a well-characterized sample of patients with unexplained hypersomnolence, to determine if epigenetic modification of candidate genes were related to specific aspects of the hypersomnolence phenotype.

Methods

Participants

All participants were clinical patients referred by their treating clinician for sleep testing to evaluate complaints of hypersomnolence at Wisconsin Sleep, the sleep clinic and laboratory affiliated with the University of Wisconsin-Madison. For inclusion, participants had to be (1) free of psychotropic medications at the time of and preceding sleep procedures, (2) not have any identifiable cause of hypersomnolence [e.g. sleep apnea defined as apnea hypopnea index (AHI) > 5/h), evidence of sleep deprivation prior to sleep testing (using either sleep logs and/or actigraphy), complaints of cataplexy suggesting type 1 narcolepsy, etc.], and (3) have no sleep onset REM periods on either overnight polysomnography (PSG) or MSLT. Participants provided informed consent for all study procedures, including both sleep phenotyping measures not part of routine clinical care, as well as collection of biospecimens for subsequent genetic analysis. The University of Wisconsin-Madison Health Sciences Institutional Review Board approved the study.

Hypersomnolence phenotyping procedures

The hypersomnolence phenotype was determined using four parameters: (1) mean sleep latency on MSLT, (2) total sleep time on ad libitum polysomnography, (3) pupillary unrest index measured by infrared pupillometry, and (4) lapses on the psychomotor vigilance task. All measures were collected within a single sleep laboratory visit.

Participants arrived at the sleep laboratory for testing at approximately 19:00–20:00. After polysomnographic set-up,

participants determined their bedtime and were only disturbed after sleep onset if technical issues arose with the recording that would inhibit sleep scoring/staging. The end of the PSG recording was determined by the patient informing the technician that they were ready to get up for the day, rather than a universally applied standard wake time. An MSLT was subsequently performed, with sleep latency defined as the time from lights out to the first 30-s epoch scored as any stage of sleep. The nap was terminated after 20 minutes (if no sleep was achieved) or 15 min after the first epoch of scored sleep [17]. Both PSG and MSLT were collected using Alice Sleepware (Phillips Respironics, Murrysville, Pennsylvania, United States) and scored following standard criteria [18].

Following MSLT naps 1 and 3, the pupillographic sleepiness test (PST) and the psychomotor vigilance task were collected with values at each timepoint averaged. The PST is an assessment of drowsiness under constant darkness that records oscillations of the pupil diameter via a computer-based infrared video technique [19]. These undulations result from progressive reduction of noradrenergic central activation from the locus coeruleus, resulting in disinhibition of the parasympathetic Edinger-Westphal nucleus [19]. The fluctuations in pupil diameter are utilized to calculate the pupillary unrest index (PUI), defined by absolute values of cumulative changes in pupil size based on the mean values of consecutive data sequences [20], with higher values suggestive of increased drowsiness. Reproducibility, reliability, and normative values of the PUI have been established for adults [19, 21–23]. The PSTEco system (AMTech, Germany) was used for ascertainment of PUI in this investigation and was applied following established protocols [19].

The 10-minute psychomotor vigilance task (PVT) was collected after PST to minimize the potential impact of time-on-task effects from this measure of neurobehavioral alertness on PUI. The PVT is a well-validated measure used in sleep research that quantifies the ability to sustain attention and respond in a timely manner to salient signals [24]. The PVT requires responses to a stimulus (digital counter) by pressing a button as soon as the stimulus appears, which stops the stimulus counter and displays the reaction time in milliseconds for a 1-s period. The number of lapses (failure to respond within 500 ms of stimulus; Tukey transformed) was considered the primary PVT measure of interest in this investigation.

DNA extraction and methylation detection

Saliva samples were collected from participants approximately two hours after awakening using Oragene kits for DNA methylation analysis (DNA Genotek, Canada). DNA was extracted following the manufacturers protocol. Genomic DNA samples were resolved on a 1% agarose gel, to verify the DNA was of high molecular weight, and quantified using Qubit (Qiagen, USA). Genome-wide DNA methylation levels were determined using the HumanMethylationEPIC array and raw intensity data files were imported into the R package *minfi* to assess sample quality, calculate the detection *p*-value of each tested probe, filter probes, and determine beta values [25]. Probes were background- and control-corrected, followed by subset-quantile within array normalization to correct for probe-type bias [25–27]. Probes were removed from further analysis if: one sample or more exhibited a detection *p*-value >.01; the probe contained a known single nucleotide polymorphism (SNP); the probe was derived from a sex chromosome; the probe measured methylation at a cytosine followed by a nucleotide other than guanine; or the probe was a cross-reactive probe. With these filtration criteria, 97,964 probes were discarded and 768,127 probes were available for further analysis.

Statistical analyses

Methylation levels (i.e. beta-values) were calculated in *minfi* as the ratio of methylated to total signal (i.e. beta-value = methylated signal/[methylated signal + unmethylated signal + 100]), where beta-values range from 0 (unmethylated) to 1 (methylated). Beta values were further converted to *M*-values (i.e. logit-transformed beta-values) for differential analysis, as *M*-values are more appropriate for statistical testing. Individual models for each independent objective variable of interest [i.e. average PUI on infrared pupillometry, total sleep time (TST) on PSG, mean sleep latency (MSL) on MSLT, and average PVT lapses] were generated, while also accounting for the dependent variables of age, sex, body mass index (BMI), and HumanMethylationEPIC Beadchip identification number. Score on the Epworth Sleepiness Scale (ESS) was similarly evaluated as a subjective measure of daytime sleepiness for comparison purposes [28]. Since saliva tissues may be confounded due to latent factors such as heterogeneous cell populations, the R package *sva* was employed to identify any surrogate variables not accounted for in the model [29]. In each model, one surrogate variable was identified and adjusted for in the model using *sva*. Linear regression for each tested CpG using a multivariate model was employed using the R package *limma* [30].

To assess systematic bias of the linear regression model, the genomic inflation factor was calculated for the obtained *P*-values for each model, yielding a genomic inflation factor of: PUI (λ = 0.99), TST (λ = 1.05), MSL (λ = 1.05), PVT (λ = 1.37), and ESS (λ = 1.19). In the latter two cases (PVT and ESS) the slightly elevated λ score suggests modest inflation of the generated *P*-values, indicative of potential bias in the model. As such, these two models were reanalyzed without adjusting for surrogate variables and the genomic inflation factor was reassessed. Without surrogate variable adjustment, the genomic inflation factors were: PVT (λ = 0.956) and ESS (λ = 0.76), indicating a more unbiased approach without adjusting for surrogate variables in the cases of the independent variables PVT and ESS. In addition, the R package NHMMfdr used here reports a BIC score based on the *p*-values generated from model fitting for both the adjusted and unadjusted models. Model fitting in NHMMfdr resulted in a lower BIC score using the unadjusted models for PVT and ESS. Together, these data supported that differential methylation modeling should not adjust for the identified surrogate variable for the PVT and ESS variables. Notably, the deflated lambdas for these two cases would result in type II error (i.e. false negative), and may be caused by population stratification in our sample, potentially stemming from the higher proportion of female to male participants.

The methylation levels of immediately flanking probes tested by array-based platforms often exhibit dependence upon one another, and because corrections for multiple testing such as the Benjamini-Hochberg false discovery rate may be inefficient under a varying dependence structure, the R package *NHMMfdr* was used to detect the adjusted local index of significance (aLIS), an extension of adjusted *p*-values, for each probe by first converting all *p*-values to *z*-scores, followed by employing a Hidden Markov Model to determine the "aLIS *p*-value" [31]. An aLIS

p-value threshold of <.05 was used to identify significant differentially methylated loci.

Results

Participant demographics and phenotype

A summary of the 28 patients included in these analyses is provided in Table 1. The sample was predominantly (75%) female. While the mean age was 31.7 ± 11.9 years, the overall range was relatively broad (20–66 years). The mean Epworth Sleepiness Scale score was 12.8 ± 3.6 for the sample. Overall, there also was a wide range of values for each hypersomnolence phenotypic trait of interest (e.g. TST, MSL, PUI, and PVT) across the sample, with minimal correlation between values for any objective measure (Supplementary Figure 1).

PAX8/PAX8-AS1 DNA methylation levels are associated with sleep duration

We first took a hypothesis-driven approach by examining the DNA methylation levels of eleven genes previously associated with the hypersomnolence phenotype (Table 2). Genomic DNAs from saliva were examined using the HumanMethylationEPIC beadchip array, which provides a quantitative measure of DNA methylation levels at 866,091 CpG/CpH dinucleotides across the genome at single-nucleotide resolution, including enhancers and all coding regions. Initial differential methylation analyses centered only on the 832 CpG sites annotated to these eleven genes and utilized independent regression models of the five hypersomnolence scores as the explanatory variable (Methods). These analyses revealed that only nine CpG sites exhibited significant associations between DNA methylation levels and any of the 5 hypersomnolence measures. All nine differentially methylated CpG sites were annotated to the paired box 8 (*PAX8*) gene and its related antisense gene (*PAX8-AS1*) and these methylation levels were correlated to total sleep time measured using ad libitum PSG (aLIS *p*-value < .05). Among these nine differentially methylated positions (DMPs) was a notable cluster of five CpG sites within a small (~400 base pair) region located in the body of the *PAX8* gene and promoter of *PAX8-AS1* (Figure 1). All five DMPs within this cluster had a positive correlation (mean 0.31 ± 0. 09) with sleep duration (i.e. increasing methylation was associated with longer sleep time). To quantify the magnitude of the effect of methylation of the 5 DMPs, the sample was also stratified into those with TST <9 and ≥9 h [32, 33]. Participants with hypersomnia had mean methylation of these 5 DMPs that was 8.6% greater than those without excessive sleep duration (77.4% vs. 68.6%; *p*-value = 0.026; Supplementary Figure 2).

Table 1. Demographics and sleep phenotypic data for the sample (N = 28)

Characteristic	Value
Female sex, *n* (%)	21 (75)
Age in years, mean (*SD*)	31.7 (11.9)
BMI in kg/m^2, mean (*SD*)	26.2 (5.3)
MSLT MSL in minutes (*SD*)	12.8 (4.2)
PSG TST in minutes (*SD*)	521.7 (112.4)
PUI (*SD*)	5.9 (2.5)
Lapses, mean (*SD*)	2.5 (1.4)

Genome-wide DNA methylation levels are associated with other aspects of the hypersomnolence phenotype

We additionally conducted genome-wide exploratory comparisons of the five independent continuous variables of hypersomnolence scores as the explanatory variable (Methods), which each yielded a unique set of DMPs (Supplementary Dataset). Notably, DMPs were found in two genes that have been associated with hypersomnolence in other investigations, but were not included in the initial a priori candidate genes either because their genetic association to hypersomnolence was published after the study was conducted or the relationship between the gene/protein was not previously established by genetic/epigenetic studies. First, *LHX6* contained 11 DMPs associated with mean sleep latency on the MSLT (Supplementary Dataset). Among these 11 DMPs was a notable cluster of 6 CpG sites within a small (~500 base pair) region that were located in the body of the *LHX6* gene. All six DMPs within this cluster had a negative correlation with MSLT MSL (i.e. increasing methylation was associated with reduced sleep latency). Second, *GABRA2* contained seven DMPs associated with PUI using infrared pupillometry (Supplementary Dataset). These seven DMPs were within a small (~300 base pair) region located in the promoter of the *GABRA2* gene. The majority (6/7) of these DMPs had a negative correlation with sleep duration (i.e. decreasing methylation was associated with increasing PUI). Significant findings were not found using either ESS or PVT as the independent variable in either the hypothesis-driven or genome-wide analyses.

Discussion

This investigation demonstrates that *PAX8/PAX8-AS1* DNA methylation levels are associated with sleep duration quantified using in-laboratory ad libitum polysomnography in a well-characterized, unmedicated group of clinical patients with unexplained hypersomnolence. Specifically, nine differentially methylated sites within *PAX8/PAX8-AS1* were associated with sleep duration, of which five formed a distinct cluster of CpG sites within a small (~400 base pair) region in the body of the *PAX8* gene and promoter of *PAX8-AS1*. Since antisense genes frequently play key roles in reducing expression of overlapping sense genes and form self-influencing circuits with regulatory advantages over transcription factor proteins [45], the location and clustering of these significantly differentially methylated CpG sites increase the likelihood they may directly impact both *PAX8* and *PAX8-AS1* expression. In fact, >70% of the transcripts in humans have antisense transcripts that are generated by independent promoters [46]. Antisense transcript can function by the act of its own transcription in *cis* (controlling genes locally on the DNA strand involved in its origination), which regulates sense gene expression by interfering/blocking sense transcriptional machinery [47]. A recent report has examined the relationships between *PAX8-AS1* methylation and gene expression levels, demonstrating that increased methylation results in reduced gene expression of *PAX8-AS1* [48]. Notably, the DMR examined in this prior investigation contained six out of the nine DMPs in *PAX8/PAX8-AS1* identified in this study, including

Table 2. A priori list of genes contributing to hypersomnolence

Gene	Name	aLIS *p*-value	Ref.
PDE4D	Phosphodiesterase 4D	N.S.	[34, 35]
NPR2	Natriuretic peptide receptor 2	N.S.	[36]
SP140	Speckled protein 140	N.S.	[36]
PAX8	**Thyroid-specific transcription factor paired box gene 8**	**0.021**	[12, 13]
IL1RN	Interleukin-1 receptor antagonist	N.S.	[12, 37]
ADORA2A	Adenosine A(2) receptor	N.S.	[38, 39]
AR	Androgen receptor	N.S.	[13]
OPHN1	Oligophrenin-1	N.S.	[13]
PER3	Period 3	N.S.	[39, 40]
CACNA1C	Calcium voltage-gated channel subunit Alpha1 C	N.S.	[41–43]
ZFYVE28	Zinc finger FYVE-type containing 28	N.S.	[44]

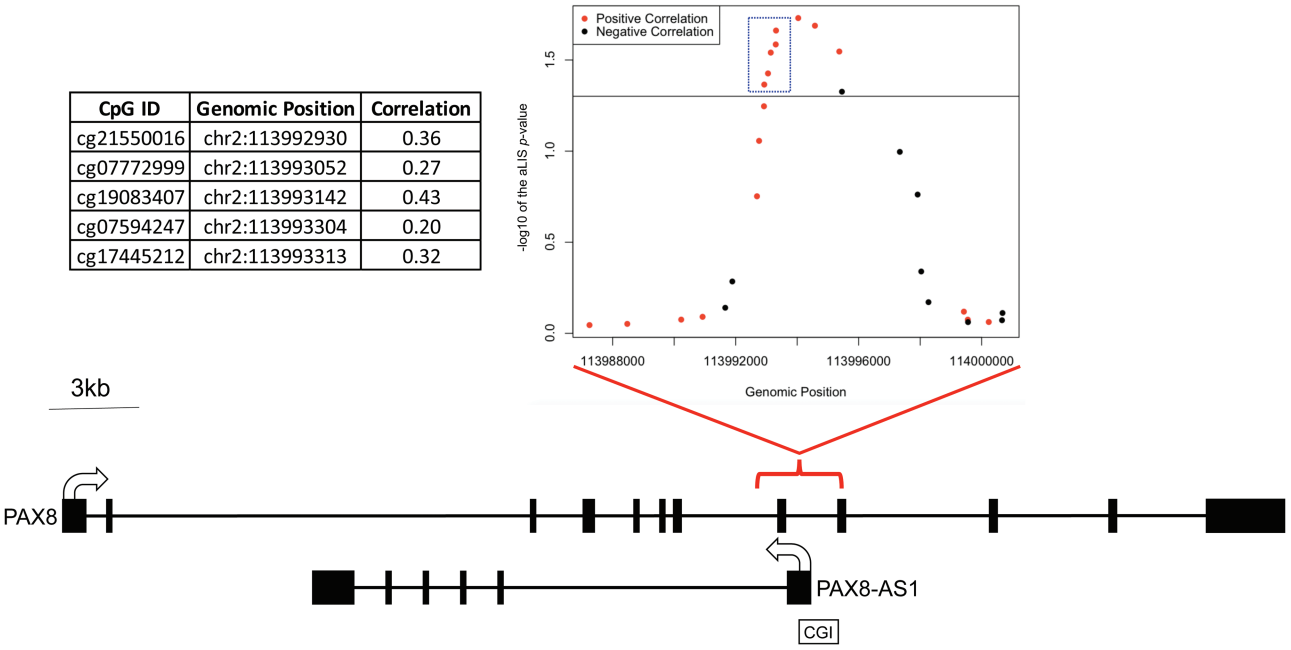

Figure 1. Relative positions of PSG-associated differentially methylated positions at the *PAX8* and *PAX-AS1* loci. Schematic of *PAX8* and its antisense gene *PAX8-AS1*. The relative positions of probes measuring methylation levels of CpG sites annotated to *PAX8* and *PAX8-AS1* with their genomic 5'-3'positions are provided (inset panel; x-axis) vs. the $-\log_{10}$ of the aLIS adjusted *p*-value (y-axis). All probes were tested for positive correlations with PSG (red dots) and negative PSG (black dots) sleep duration. The level of aLIS *p*-values <0.05 (black line) is indicated. Dotted blue box denotes cluster of five CpG sites near *PAX8-AS1* promoter. The inset table provides the Illumina provided CpG ID number (CpG ID), the CpG chromosome (chr) number and genomic position, and the correlation R matrix between DNA methylation levels and total sleep duration for these five CpG sites.

all five DMPs located within the promotor of *PAX8-AS1*. Thus, despite the lack of gene expression data in this investigation, based on prior literature, increased methylation in this region would be predicted to decrease *PAX8-AS1* expression, and theoretically increase overall expression of the sense (i.e. *PAX8*) gene. However, the implications of the methylation patterns found on the *PAX8* gene and *PAX8-AS1* promoter require further formal investigation and may also represent a biomarker for sleep duration, not directly related to *PAX8* gene expression.

While the specific determinants of habitual sleep time remain unknown, recent genome wide association studies (GWAS) have highlighted the role of *PAX8/PAX8-AS1* in human sleep duration. *PAX8* likely serves several roles as a master transcription factor crucial to embryonic development and maintenance functions after birth [49]. Using both self-report and accelerometer data in population-based cohorts, SNPs in *PAX8/PAX8-AS1* have repeatedly demonstrated significant effects on sleep duration [12–16]. However, the SNPs themselves impact sleep duration only marginally (i.e. only a few minutes longer sleep per day). Thus, while hypothesis-free GWAS investigations have pointed to a role of *PAX8/PAX8-AS1* in habitual sleep duration, specific sequence variability in *PAX8/PAX8-AS1* is unlikely to be a major contributing factor in pathological hypersomnia. In this investigation, we have further extended these well-replicated associations between *PAX8/PAX8-AS1* and sleep duration by finding a significant association between *PAX8/PAX8-AS1* DNA methylation levels and nocturnal sleep duration in symptomatic patients. The magnitude of the effect between sleep duration and *PAX8/PAX8-AS1* methylation levels (e.g. ~10%) observed in this investigation is on par with methylation level changes that are the epigenetic hallmark of complex disease phenotypes [50]. Unique to our approach was the application of

deep phenotyping [10], which carefully and objectively quantified several different facets of hypersomnolence, including ad libitum polysomnography to measure total sleep time.

There are several lines of evidence that further support the role of *PAX8/PAX8-AS1*, and particularly its epigenetic modification, on sleep duration and hypersomnia. First, one of the few compounds that has demonstrated a measurable impact on excessive sleep duration in idiopathic hypersomnia is levothyroxine, evaluated in a small open-label study in euthyroid patients [51]. While *PAX8* is expressed in many tissues, it is predominantly expressed in fetal and adult thyroid tissue, and mutations in *PAX8* have been associated with thyroid dysgenesis and thyroid cancers. Second, fluctuations in cerebral *PAX8* methylation levels mirror patterns of total sleep quantity across the lifespan, with a high rate of change early in the first few years of life, followed by a general plateau beginning in the second decade [52]. Thus, it is probable given the large number of GWAS studies implicating *PAX8/PAX8-AS1* in sleep duration, relevant supportive data in the literature, and our findings linking *PAX8/PAX8-AS1* methylation to sleep duration, that DNA methylation changes in *PAX8/PAX8-AS1* may be a biomarker for, or mechanistically related to hypersomnia.

The relevance of these findings to disorders associated with excessive sleep duration requires further clarification as well as replication of findings. First, all DNA was obtained from saliva samples collected approximately 2 h after awakening in the morning, making it unclear whether these changes in DNA methylation levels represent a state or trait marker for hypersomnia. Since epigenetic modifications can be impacted by a night of sleep itself [53, 54], a key step in advancing this line of research will be clarifying whether *PAX8/PAX8-AS1* DNA methylation levels are influenced by sleep loss and/or extension in healthy individuals. In addition, there may have been unmeasured circadian factors that influenced results, that should be considered in future studies. Evaluation of the potential mechanistic role of *PAX8/PAX8-AS1* DNA methylation levels on sleep duration in healthy persons through gene expression analyses (e.g. transcriptomic, proteomics, etc.) will also help clarify and strengthen the relationship between these genes and total sleep time. In addition, since the measurement of total sleep time using extended sleep duration recordings is not yet standardized across the few sleep laboratories conducting such procedures [9, 55–57], replication of these findings in other available clinical samples is warranted. Future replication in other centers would further increase the generalizability of these results and the likelihood that *PAX8/PAX8-AS1* DNA methylation levels can be developed as a diagnostic tool and treatment target in pathological hypersomnia for more widespread application in clinical care.

In the secondary genome-wide analysis, DMPs also were found in two genes that have been associated with hypersomnolence in other investigations, *LHX6* and *GABRA2*. LHX6-positive GABA-releasing neurons in the zona incerta promote NREM sleep, likely by inhibiting hypocretin positive neurons, consistent with a plausible connection between these findings and sleep propensity quantified by the MSLT [58]. A SNP in *GABRA2* was recently associated with self-reported daytime sleepiness in a large-scale GWAS study, published after the study methods were executed [59]. Together, these data suggest that genes other than *PAX8* may exhibit disruptions in DNA methylation levels correlated to other aspects of hypersomnolence. Thus, future studies that both replicate the primary finding of an association between PAX8/PAX8-AS1 methylation and sleep duration, as well as associations between LHX6 and MSLT sleep latency and GABRA2 and infrared pupillometry, are indicated.

There are limitations of this investigation that are meritorious of discussion. First, our relatively small sample size may have impacted findings. However, our deep phenotyping approach likely increased power to identify relevant associations between DNA methylation levels and hypersomnolence measures. Accordingly, the sample size utilized here is similar to other prior investigations in sleep and neuropsychiatric disorders that have applied similar deep phenotyping and epigenetic methods [53, 60–64]. Second, while we utilized four objectively measured hypersomnolence characteristics (ad libitum PSG, MSLT, PVT, and infrared pupillometry), there may be other relevant procedures that quantify other aspects of hypersomnolence not captured here (e.g. maintenance of wakefulness test) that could also be associated with altered DNA methylation levels. In a related vein, the use of an alternative measure derived from the PVT (e.g. reaction time) may have yielded different results than the use of lapses. However, lapses were utilized here as the PVT variable most relevant to hypersomnolence given their association with daytime sleepiness, the absence of an effect of sex on this measure, and because response times greater than 500 ms fall well outside the normal range for reaction times across all adult age groups, consistent with a pathological aspect of hypersomnolence, rather than a state or trait measure of motor function [65]. It is noteworthy that we found no significant associations between subjective daytime sleepiness quantified by the ESS and DNA methylation of any gene, using both targeted and genome-wide approaches, suggesting objective phenotyping is a key factor in linking DNA methylation levels to hypersomnolence traits. Given the exploratory nature of this study and the need to balance type I and type II error in a relatively small (but well characterized) dataset, Bonferroni correction for the number of hypersomnolence phenotypes was not applied, instead utilizing a limited number of a priori candidate genes for initial hypothesis testing with subsequent aLIS correction (an extension of a family-wise correction) for multiple comparisons. This approach is consistent with other investigations that have examined epigenetic changes with different sleep parameters using hypothesis-driven approaches [66]. Nonetheless, these results should be considered preliminary and require replication in other cohorts. Third, the sample was predominantly female, consistent with other studies of persons with unexplained hypersomnolence [67]. While sex was controlled for as a covariate, we were not able to further clarify specific sex-related effects of *PAX8/PAX8-AS1* methylation on sleep duration. Fourth, actigraphy preceding in-laboratory procedures was available on approximately half of participants, with others completing sleep logs, consistent with the clinical nature of the sample. Thus, we are unable to examine relationships between habitual sleep estimates using accelerometry and *PAX8/PAX8-AS1* DNA methylation levels, and rely instead on the gold-standard, but cross-sectional, EEG-based measurements of total sleep time. Future investigations in both healthy and disordered persons that apply actigraphy, and measure longitudinal stability of the association between *PAX8/PAX8-AS1* methylation and sleep duration outside of the laboratory environment are likely to further extend this line of inquiry.

In summary, our investigation demonstrates a significant association between *PAX8/PAX8-AS1* DNA methylation levels and objectively quantified sleep duration in persons with

unexplained hypersomnolence. These results represent a key step forward in understanding the underlying biology that may be related to both sleep duration and pathological hypersomnia, bridging key findings in prior GWAS studies to disordered populations in sleep medicine. This work will serve as a nidus for further investigation that clarifies the role of *PAX8/PAX8-AS1* regulation and expression in both sleep need and duration for healthy persons and patients with pathological hypersomnia.

Data Access

De-identified data underlying this article will be shared on reasonable request to the corresponding authors.

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Disclosure Statement

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