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Gene expression biomarkers of the response to sleep loss with and without modafinil

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16. Abstract

Sleep disruption presents a substantial risk to health and safety, particularly due to the risks of performance degradation in safety-critical operations that can result in catastrophic injuries or mortality. Federal regulations exist to minimize the risks of fatigue with limitations on hours worked and requirements for fatigue risk management plans. Yet, even with workload controls and scheduled opportunities for rest, fatigue may be caused by factors such as personal and lifestyle choices, illness, and circadian disruption from travel across multiple time zones. Complicating risk mitigation is the challenge of identifying and measuring fatigue. Here, we report on gene expression biomarkers (biological indicators) for cognitive impairment during sleep loss. We observe hundreds of genes whose expression is associated with attention changes during one night of sleep loss. Several genes are identified that we previously associated with attention impairment in a separate study of sleep loss. The reproducibility of findings may indicate the robustness of these candidate fatigue impairment biomarkers. However, some biomarker genes only associate with certain tests of impairment (e.g., attention lapses but not self-reported fatigue), suggesting that different biomarker panels may be developed to assess the particular cognitive domains that need monitoring for a given safety critical operation. We also find that using a drug countermeasure (modafinil) not only helps mitigate impairment on tests of attention lapses, but also disrupts gene expression associations with attention lapses. Further research is needed to confirm whether this represents a unique effect of modafinil administration, or emphasizes the need to ensure biomarker validation occurs both in the presence and absence of countermeasures.

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Author Contributions

HAU conceived of the biomarker project in discussion with the NAMRU-D Fatigue Countermeasures Laboratory, oversaw project progress, conducted bioinformatics analyses, and drafted this report with input from coauthors. SJN oversaw laboratory work. VLW performed RNA extractions, and SKM performed RNA concentration and dilution. All authors have read and approved this manuscript.

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Conflicts of Interest

The authors declare that they have no competing interests.

Data Availability

To protect the privacy of subject participants and conform to the restrictions of the Institutional Review Board, raw and individual-level data will not be made available.

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Term	Definition
CAMI	Civil Aerospace Medical Institute
CFR	Code of Federal Regulations
F	Quasi-Likelihood F-statistic
FAA	Federal Aviation Administration
FDR	False Discovery Rate
GO	Gene Ontology
HGNC	HUGO Gene Nomenclature Committee
IPA	Ingenuity Pathway Analysis
logCPM	Log_2-Counts Per Million
logFC	Log_2-Fold Change
MTS	Match to Sample Test
NAMRU-D	Naval Medical Research Unit – Dayton
POMS-F	Profile of Mood States Questionnaire – Fatigue
NTSB	National Transportation Safety Board
PVT	Psychomotor Vigilance Test
RDM	Rapid Decision Making Test
RNA	Ribonucleic Acid
[R][D][X]	Model abbreviation nomenclature, where [R] is P (placebo), M (modafinil) or B (both) study runs; [D] is 5 or 8 for the number of data timepoints; [X] is P (PVT), R (RDM), S (MTS), F (POMS-F), or T (Timepoint)

Note: HUGO Gene Nomenclature Committee (HGNC) gene symbols are not included in the list of abbreviations, but when available, they appear along with a full gene name or description the first time a gene is introduced in the manuscript. Not all genes had a gene symbol assigned by the HGNC at the time of analysis.

Abstract

Sleep disruption presents a substantial risk to health and safety, particularly due to the risks of performance degradation in safety-critical operations that can result in catastrophic injuries or mortality. Federal regulations exist to minimize the risks of fatigue with limitations on hours worked and requirements for fatigue risk management plans. Yet, even with workload controls and scheduled opportunities for rest, fatigue may be caused by factors such as personal and lifestyle choices, illness, and circadian disruption from travel across multiple time zones. Complicating risk mitigation is the challenge in identifying and measuring fatigue. Here, we report on gene expression biomarkers (biological indicators) for cognitive impairment during sleep loss. We observe hundreds of genes whose expression is associated with attention changes during one night of sleep loss. Several genes are identified that we previously associated with attention impairment in a separate study of sleep loss. The reproducibility of findings may indicate the robustness of these candidate fatigue impairment biomarkers. However, some biomarker genes only associate with certain tests of impairment (e.g., attention lapses but not self-reported fatigue), suggesting that different biomarker panels may be developed to assess the particular cognitive domains that need monitoring for a given safety critical operation. We also find that using a drug countermeasure (modafinil) not only helps mitigate impairment on tests of attention lapses, but also disrupts gene expression associations with attention lapses. Further research is needed to confirm whether this represents a unique effect of modafinil administration, or emphasizes the need to ensure biomarker validation occurs both in the presence and absence of countermeasures.

Introduction

Despite increasing recognition of the importance of sleep, insufficient or mistimed sleep continues to present risks to health and safety. During the global severe acute respiratory syndrome-coronavirus 2 (SARS-COV-2) pandemic, fatigue received increased attention as both a symptom of infection and a consequence of the pandemic's impact on lifestyles and sleep habits. Sleep recently gained recognition as one of "Life's Essential 8" due to its importance for cardiovascular health (Lloyd-Jones et al., 2022). It is recommended that individuals 18 to 60 years old sleep at least seven hours each night (Consensus Conference Panel, 2015). In addition to well-recognized health effects, improper sleep poses serious risks in fields with safety-critical operations, such as transportation. It has been shown that 17 to 19 hours without sleep can result in performance impairment comparable to or worse than that associated with a blood alcohol concentration of 0.05% (Williamson & Feyer, 2000). Altogether, 20% of 182 major National Transportation Safety Board (NTSB) investigations from 2001 to 2012 implicated fatigue as a finding, contributing factor, or probable cause of the event (Marcus & Rosekind, 2016). More recently, a review of NTSB investigations from 2013 to 2019 decreased that percentage to approximately 12% for investigations across multiple transportation modes (Parenteau et al., 2023). However, in aviation fatigue was implicated in 28% of investigations, and overall, fatigued operators were more likely to be involved in accidents that led to fatalities or severe injuries (Parenteau et al., 2023).

A major challenge in fatigue management is the complex assortment of factors that can lead to fatigue. The U.S. Code of Federal Regulations (CFR) in 14 CFR Part 117.3 defines fatigue as "a physiological state of reduced mental or physical performance capability resulting from lack of sleep or increased physical activity that can reduce a flightcrew member's alertness and ability to safely operate an aircraft or perform safety-related duties." Multiple factors, including workload, sleep loss, and altered sleep timing or circadian disruption may lead to acute or chronic fatigue (Dijk & Swaen, 2003; Phillips, 2015; Shen et al., 2006). The likelihood of aircrew experiencing some of these fatigue-inducing factors can be reduced; for example, hours of service limitations and flight duty regulations can increase opportunities for rest and recovery. Another safety mechanism is individuals' subjective assessments of their own fatigue levels and fitness for duty, as explained in 14 CFR Part 117.5. However, self-reported fatigue does not always correspond with objective assessments (Ganesan et al., 2019; Lauderdale et al., 2008; McCauley et al., 2021), and operators may overestimate or underestimate fatigue risks.

In addition to the complex assortment of factors that can induce fatigue, individuals also vary in their responses to sleep disruption, further complicating fatigue risk management. Variation among individuals in the response to sleep loss has been recognized for decades (Wilkinson, 1961). Attention lapses, as measured by the Psychomotor Vigilance Test, or PVT, show a strong impact of sleep disruption in some individuals and little to no impact following experimental sleep deprivation in others (St. Hilaire et al., 2019; Tkachenko & Dinges, 2018; Van Dongen et al., 2004). A metric capturing changes in neurobehavioral performance rather

than rest opportunities could vastly improve the ability to tailor fatigue mitigation approaches to the unique needs of individual operators. Neurobehavioral performance tests like the PVT can be used to monitor aspects of cognitive impairment. However, time may not always be available for operators to conduct extended neurobehavioral test batteries, and their completion is impossible in some situations, such as postmortem accident investigations.

One aim of the Functional Genomics Research Team at the Federal Aviation Administration (FAA) Civil Aerospace Medical Institute (CAMI) is the development of novel fatigue impairment indicators to address the gap in metrics, specifically identifying ribonucleic acid (RNA) gene expression biomarkers of current impairment status. The definition proposed by the Biomarkers Definition Working Group (2001), as convened by the National Institutes of Health, is applied to describe a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." The FAA CAMI's Forensic Sciences Section conducts toxicological analyses on U.S. civilian pilot fatalities, testing for medications, drugs, glucose/glycated hemoglobin (HbA1c), ethanol/volatiles, and combustion gases to aid in aviation accident investigation (Cliburn et al., 2020). A genetic biomarker test on autopsy samples could augment biochemical toxicology analyses and provide further insights into possible impairment during an accident. Additionally, such biomarkers could provide an objective metric to aid accident prevention and fatigue risk management.

The present report is part of a series of FAA investigations into genetic changes associated with sleep disruption. This study was made possible by collaboration with the Naval Medical Research Unit – Dayton (NAMRU-D) Fatigue Assessment and Countermeasures team. The NAMRU-D team recruited volunteers to experience two separate instances of total sleep deprivation, one with the countermeasure drug modafinil and one with the administration of a placebo. Modafinil alleviates excessive sleepiness from sleep disorders and conditions, such as narcolepsy, and may have use as a potential fatigue countermeasure in military operations (Caldwell et al., 2020; Wingelaar-Jagt et al., 2022). Neurobehavioral performance was assessed throughout the study, as previously reported by NAMRU-D (Caldwell et al., 2020). The authors showed that modafinil administration helped mitigate decreases in performance following a night of sleep loss. The mitigating effect of modafinil was particularly noticeable for fatigue susceptible individuals who experienced greater impairment during sleep loss. Blood samples were collected from participants every four hours and provided to the FAA CAMI's Functional Genomics Research Team for gene expression analyses. Here, we report gene expression changes related to neurobehavioral performance with and without modafinil administration.

Materials and Methods

Sample collection and processing

Male subjects were exposed to one night of total sleep deprivation in two separate randomized study runs by NAMRU-D, differing by administration of 200 mg modafinil or a

placebo at midnight. Details of human recruitment and overall study design have been reported previously (Caldwell et al., 2020). Neurobehavioral tests comprised the number of attention lapses on the 10-minute Psychomotor Vigilance Test (PVT), the number correct in the Delayed Match to Sample (MTS), reaction time for correct responses in the Rapid Decision Making Test (RDM), and subjective self-report ratings of fatigue-inertia on a Profile of Mood States Questionnaire (POMS-F) (Caldwell et al., 2020). Although additional subjective and objective neurobehavioral measures were conducted, analyses focused on PVT, MTS, RDM, and POMS-F due to work by NAMRU-D indicating these test results changed with prolonged sleep deprivation. The current report supplements prior work with the addition of genetics analyses relative to these metrics, as described below.

During the study, eight consecutive blood draws were conducted at a frequency of one draw every four hours, beginning between approximately 12:00 and 13:00 hours on the first day and ending between ~16:00 and 17:00 hours on the second study day. A sample of 2.5 mL whole blood was collected into a PAXgene RNA blood tube (BD Biosciences, 762165) at each timepoint. Immediately after collection, PAXgene RNA blood tubes were inverted 10 times, then frozen at -80 ºC until RNA extraction at the FAA CAMI. Total RNA was extracted with the PAXgene Blood miRNA kit (QIAGEN, 763134) using a QIAGEN QIAcube robotic workstation, eluted in RNAse-free water, and manually purified with Agencourt® RNAclean XP beads (Beckman Coulter, A63987).

Purified total RNA was provided to the Baylor College of Medicine Human Genome Sequencing Center for library preparation and sequencing. Stranded total RNA-Sequencing libraries were made using the Illumina TruSeq Stranded Total RNA with Ribo-Zero Globin kit (20020612, Illumina Inc.). Preparation followed the manufacturer's instructions (Illumina Truseq® Stranded Total RNA Sample Preparation Guide; RS-122-9007DOC, Part # 15031048 Rev. E, October 2013). To improve ribosomal RNA/globin removal, the incubations of RNA with the ribosomal RNA/globin removal mix were increased to 68 ºC for 10 minutes followed by five minutes at room temperature, and the incubation with ribosomal RNA/globin removal beads was increased to five minutes at 50 ºC. During fragmentation, samples were incubated for three minutes at 94 ºC. Libraries were sequenced on an Illumina NovaSeq 6000 with the S4 reagent kit and 300 cycles to generate 2x150 nucleotide paired-end reads. To target 100 million forward plus reverse sequence reads each, 35 samples were pooled per lane.

Differential expression analyses

Demultiplexed fastq sequence files returned from Baylor College of Medicine Human Genome Sequencing Center to FAA CAMI were inspected for quality before and after trimming using FastQC version 0.11.9 (Andrews, 2010) and MultiQC version 1.10 (Ewels et al., 2016). Trimming of adapters and low-quality data was done with Trimmomatic version 0.39, specifically trimming the forward (R1) and reverse (R2) reads with the command java -jar : < Path> -threads 6 -phred33 -trimlog trimfile1 $R1 $R2 $R1paired $R1unpaired $R2paired $R2unpaired ILLUMINACLIP:< Path>/adapters/TruSeq3-PE-2.fa:2:30:10:8:TRUE

LEADING:20 TRAILING:5 SLIDINGWINDOW:5:20 MINLEN:50 (Bolger et al., 2014). Trimmed paired reads were mapped and counted using Rsubread version 2.4.2 set for reverse stranded paired reads, based on the GENCODE release 37 (GRCH38.p13) reference sequence for the primary assembly and the comprehensive annotation files (PRI file GRCh38.primary_assembly.genome.fa.gz and CHR GTF file gencode.v37.annotation.gtf.gz) (Frankish et al., 2020; Liao et al., 2019). Mapping was performed with the buildindex and align functions; settings primarily were left as defaults, except the align 'unique' argument was set to TRUE to remove multi-mapping reads. The Rsubread featureCounts function was used to produce count data for meta-features (genes), using the GENCODE GTF and primarily default settings, but modifying the argument countChimericFragments to FALSE in order to exclude chimeric fragments mapping to distinct chromosomes. Before differential expression analysis, subjects were removed from the dataset if they yielded results for fewer than nine blood draw timepoints. Also, samples that yielded low count data (sum of raw expression across all genes in the sample, more than two standard deviations below the average for all samples sequenced) were removed as potential outliers.

After subject and sample outlier removal, models were run to test for differential gene expression. First genes with low expression were removed from analysis, herein defined as genes with less than one count per million in at least 19 samples (selected due to having 19 replicate subjects). Then library size was normalized using the default trimmed mean of M-values (TMM) approach. Negative binomial generalized linear models were constructed with the R Bioconductor package edgeR version 3.38.1-3.38.4, with quasi-likelihood F-tests (Gentleman et al., 2004; Law et al., 2020; McCarthy et al., 2012; Robinson et al., 2009). All modeling was done with additive fixed effect terms.

To test for genes related to attention impairment, models were run on data across eight timepoints per study run, with terms to represent the individual subject participant effect, PVT lapses, and circadian rhythms specified as sin(2*pi*hour/24) + cos(2*pi*hour/24) (Table 1). Circadian rhythm modeling was based on the approach Law et al. (2020) recommended, with hour designating the hour of the day (0 for midnight, through 23 for 23:00 hours). Models with subject, PVT, and the circadian rhythm terms were tested separately, first on the study run with the administration of a placebo only, a second time with data from the study run with the administration of modafinil, and a third time combining the data from both placebo and modafinil study runs. In the models with data from both placebo and modafinil study runs, a binary factor term countermeasure was included to differentiate data at times after modafinil use (four to 16 hours after modafinil administration) from those without the drug (modafinil study run up to midnight, and all timepoints from the placebo run). Analyses of results focused on genes significantly related to the model term of PVT lapses with a threshold False Discovery Rate (FDR) of less than 0.05, based on Benjamini-Hochberg correction. In one case the genes related to the binary countermeasure term also were examined (Table 1). Although fold change can be used to further restrict gene lists, this is not always done (Koch et al., 2018). Here, we elected not to apply a fold change cutoff due to use of covariate model terms, and the desire to maximize the detected pool of candidate biomarker genes in this biomarker discovery research.

Additional models were run to test for associations of gene expression with MTS, RDM, and POMS-F, for which data were only available across five timepoints. For comparison, tests with PVT were re-run on the corresponding five timepoints. For these model runs, data were available at the third through seventh timepoint (every four hours starting at approximately 20:00 hours on the first day of the study run). Models were constructed as noted above, either including a term for PVT lapses or replacing this term with the number correct for MTS, reaction time for correct responses for RDM, or subjective self-report ratings of fatigue-inertia for POMS-F (Table 1). However, circadian rhythm terms were not included. When preliminary tests included circadian rhythms, no differentially expressed genes were found at FDR<0.05 related to PVT, MTS, RDM, or POMS-F in tests of the placebo run, modafinil run, or the joint dataset combining information from both runs.

Table 1. Summary of models and terms[1] utilized to identify differential gene expression.

Model Abbreviation	Supplementary Table	Study Run	Measurement Times	Model Term Tested	Genes FDR<0.05 for Term Tested	Additional Model Terms
P8T	1	**P**lacebo	**8**	**T**imepoint	3719	Subject, Circadian
M8T	2	**M**odafinil	**8**	**T**imepoint	3455	Subject, Circadian
B8T	3	**B**oth	**8**	**T**imepoint	6049	Subject, Circadian, Countermeasure
P8P	4, 9	**P**lacebo	**8**	**P**VT	232	Subject, Circadian
M8P	none	**M**odafinil	**8**	**P**VT	0	Subject, Circadian
B8P	5, 9	**B**oth	**8**	**P**VT	248	Subject, Circadian, Countermeasure
B8P	6	**B**oth	**8**	Countermeasure	198	Subject, Circadian, **P**VT
P5P	7, 9, 10, 13	**P**lacebo	**5**	**P**VT	1169	Subject
M5P	11	**M**odafinil	**5**	**P**VT	0	Subject
B5P	8, 9, 12, 14	**B**oth	**5**	**P**VT	1406	Subject, Countermeasure
P5F	10	**P**lacebo	**5**	POMS-**F**	3	Subject
M5F	11, 15	**M**odafinil	**5**	POMS-**F**	418	Subject
B5F	12	**B**oth	**5**	POMS-**F**	110	Subject, Countermeasure
P5S	10	**P**lacebo	**5**	MT**S**	1	Subject
M5S	11, 16	**M**odafinil	**5**	MT**S**	596	Subject
B5S	12	**B**oth	**5**	MT**S**	108	Subject, Countermeasure
P5R	10	**P**lacebo	**5**	**R**DM	0	Subject
M5R	11	**M**odafinil	**5**	**R**DM	0	Subject
B5R	12	**B**oth	**5**	**R**DM	4	Subject, Countermeasure

Note: FDR = False Discovery Rate; PVT = Psychomotor Vigilance Test; POMS-F = Profile of Mood States Questionnaire – Fatigue, MTS = Match to Sample; RDM = Rapid Decision Making Test.

[1]The binary model term 'countermeasure' differentiated timepoints ≥4 hours after administration of modafinil, from all placebo times and from times up to midnight in the modafinil run. The circadian term consisted of sin(2*pi*hour/24) + cos(2*pi*hour/24), where hour designated the hour of the day (0 for midnight, through 23 for 23:00 hours).

Efforts were made to compare significantly differentially expressed genes in the current study at FDR<0.05, with a prior microarray study that had found 28 genes related to PVT lapses during total sleep deprivation at a less stringent cutoff of FDR<0.1 (Uyhelji et al., 2018). To improve compatibility with the current work, the microarray Affymetrix Transcript Cluster list

was submitted to biomaRt (http://useast.ensembl.org/biomart/martview) to check for annotation updates. Prior annotation of Transcript Cluster ID 8129428 as an uncharacterized locus or *FK506 binding protein* was updated to the annotation of *FKBP prolyl isomerase 1A (FKBP1A)*. Also, Transcript Clusters were updated from annotation as other members of the Speedy family to *Speedy/RINGO Cell Cycle Regulator Family Member E16 (SPDYE16)* and *Speedy/RINGO Cell Cycle Regulator Family Member E2B (SPDYE2B)*, and the previously unannotated 8180341, 8180342, and 8180343 Transcript Clusters were annotated as *Rac family small GTPase 1 (RAC1)*. Finally, Transcript Cluster 7977454 annotated as various members of the POTE family assumed an annotation of *POTE ankyrin domain family member G (POTEG)*. However, *SPDYE16, SPDYE2B,* and *POTEG* were not detected at levels passing the low-expression filtering and thus were not tested for differential expression in the current study, reducing the number of genes compared from 28 to 25 (Table 2).

Functional enrichment analyses

Functional enrichment analyses were conducted to improve understanding of the biological meaning of differentially expressed genes. Annotation information corresponding to the Ensembl v103 release was accessed using the R package biomaRt version 2.54.0 (Durinck et al., 2009). Unless otherwise indicated, gene annotations are based on the Ensembl gene identifier (e.g., ENSG00000127954.13), with HUGO Gene Nomenclature Committee or HGNC gene symbols included when available (e.g., *Six-Transmembrane Epithelial Antigen of the Prostate 4, STEAP4*). The R Bioconductor package goseq version 1.50.0 was used to test for enriched gene ontology categories (FDR<0.05) among genes related to variables of interest (PVT, MTS, RDM, POMS-F). Runs of goseq were conducted individually on the significantly differentially expressed genes from each of the separate model runs (Table 1) (Young et al., 2010). For this, annotations from biomaRt were used along with the gene lengths generated by featureCounts mapping with Rsubread. For the one gene that had two version identifiers, *Colony stimulating factor 2 receptor subunit alpha* (*CSF2RA*) with identifiers ENSG00000198223.17 and ENSG00000198223.17_PAR_Y), the version ENSG00000198223.17_PAR_Y was removed from the dataset before running goseq.

Additionally, the list of genes with FDR<0.05 for PVT lapses from the placebo run of the eight-timepoint model (P8P, Table 1) were input to QIAGEN Ingenuity Pathway Analysis (IPA) for a Core Analysis – Expression Analysis (Krämer et al., 2014). The $\log_2$-fold change values from the edgeR differential expression analysis were designated as IPA data type "Expr [expression] log ratio"; these values were used as the measurement type for the Core Analysis to allow calculations of directionality (z-scores). For the filter cutoff to identify which genes to include, genes with FDR<0.05 in relation to the PVT lapses term were selected. The "User Dataset" option was selected for the background reference set, representing all genes that passed the filtering for low expression in edgeR (18,248 minus 30 that did not generate 'mapped IDs' in IPA, leaving 18,218 analysis-ready reference genes). Species were limited to mammals (human, mouse, rat), and endogenous chemicals were excluded from interaction networks, but otherwise settings were left at default.

Results

Differential expression related to time awake

Based on analyses of genes changing in response to timepoint (i.e., continued wakefulness), this study found a substantial impact of sleep loss on gene expression. In the eight-timepoint models (Table 1), there were 3,719 significantly differentially expressed genes with FDR<0.05 related to timepoint in the placebo model run P8T (Supplementary Table 1), 3,455 in the modafinil run M8T (Supplementary Table 2), and 6,049 in the analysis of both placebo and modafinil study runs with model B8T (Supplementary Table 3). There was substantial overlap between the list of significantly differentially expressed genes relative to timepoint and those relative to PVT lapses. Of the 232 genes significantly differentially expressed relative to PVT lapses in model run P8P (Table 1), only seven were unique to PVT lapses and not found in the P8T model run. These were *Family with Sequence Similarity 177 Member B (FAM177B); Novel Transcript, Antisense to FAM38A*; *Novel transcript*; *Tudor Domain Containing 9 (TDRD9)*; *Long Intergenic Non-Protein Coding RNA 2432 (LINC02432); Novel Transcript, Sense Intronic to TNFSF13B*; and *CD177 molecule (CD177)*. Conversely many genes only were related to timepoint (3,494) as found in the P8T model, and not related to PVT in the P8P model. However, this study aimed to interrogate biomarkers for impairment from sleep loss rather than molecular changes from extended wakefulness. Thus, these timepoint-related candidate biomarkers of the response to continued wakefulness were not analyzed in detail.

The correlation between timepoint as an indication of continuous wakefulness and PVT lapses was tested in R with the Hmisc version 4.7-0 package (Harrell Jr & Dupont, 2006) using the rcorr function. Spearman rank (rho= 0.66) and Pearson (r= 0.55) correlations between PVT lapses and the eight timepoints were significant (P<0.001). Thus, timepoint was not included as a model term in tests of the relationship of gene expression to PVT, and similarly, it was excluded from tests of MTS, RDM, and POMS-F (Table 1).

Differential expression related to PVT

Most subjects showed elevated PVT lapses in response to total sleep deprivation. Two of the 19 subjects exhibited seven or fewer PVT lapses, possibly indicating a tendency toward fatigue resistance. Although two persons is too few for a specific analysis of fatigue resistance, for further consideration of resistance, see Caldwell et al. (2020). For the remaining 17 subjects, the highest number of lapses observed was 24 ± 8 (mean ± 1 standard deviation) across all data points, ranging from 10 to 34 lapses. Generalized linear modeling was used to test for significantly differentially expressed genes related to PVT lapses in the full eight-timepoint model. Tests yielded 232 significantly differentially expressed genes for the placebo model run P8P (185 up-regulated, 47 down-regulated; Supplementary Table 4), but zero genes for the modafinil run M8P (Table 1). Modeling data from both placebo and modafinil study runs (B8P; Table 1) yielded 248 significantly differentially expressed genes related to PVT lapses (107 up-regulated, 141 down-regulated; Supplementary Table 5), and 198 related to the binary drug

countermeasure usage term (84 up-regulated, 114 down-regulated; Supplementary Table 6). Of the 248 genes related to PVT lapses in the B8P model, 99 (approximately 40%) were among the 232 significantly differentially expressed genes in the placebo run. For tests of expression related to PVT (models P8P and B8P testing for the PVT model term, Table 1), down-regulated genes (i.e., those with a negative $\log_2$-fold change, as reported in Supplementary Table 4-5) are inversely related to PVT lapses (i.e., lower gene expression is observed as lapses increase). For tests related to the drug usage countermeasure term in the B8P model (Table 1), down-regulated genes (negative $\log_2$-fold change in Supplementary Table 6) are more highly expressed after modafinil use (i.e., higher gene expression four to 16 hours after modafinil administration as compared to expression at times prior to or without the drug).

For the five-timepoint models, there were 1,406 significantly differentially expressed genes relative to PVT lapses in the B5P analysis of both placebo and modafinil study runs, and 1,169 in the placebo-only P5P run (including 806 also significant across the two tests; Supplementary Table 7, Supplementary Table 8, Table 1). Yet, similar to the eight-timepoint modafinil model, the five-timepoint modafinil-only model M5P yielded zero significant genes for PVT. There was overlap in genes with FDR<0.05 for PVT between five and eight-timepoint models (Supplementary Table 9). The analysis of data from both placebo and modafinil runs yielded 105 genes significantly differentially expressed in both the five and eight-timepoint models (FDR<0.05 for PVT lapses in B8P and in B5P). In contrast, analysis of just the placebo run resulted in 136 genes differentially expressed that were common to both the five and eight-timepoint models (P8P and P5P). For example, the gene *Six-Transmembrane Epithelial Antigen of the Prostate 4 (STEAP4)* was differentially expressed in both five and eight-timepoint placebo runs (but not analysis of both placebo and modafinil runs). The gene *Syndecan Binding Protein (SDCBP)* was differentially expressed in both five and eight-timepoint placebo runs (P8P and P5P), as well as the five-timepoint B5P (but not eight-timepoint B8P) analysis of both placebo and modafinil runs. Altogether 47 significantly differentially expressed genes were noted across all four tests (analysis of both runs in the five and eight-timepoint models B5P and B8P, plus the placebo-only run in the five and eight-timepoint models P5P and P8P). Of the 28 genes significantly related to PVT in a prior microarray study of total sleep deprivation (Uyhelji et al., 2018), the current study identified 11 genes at FDR<0.05 for PVT lapses in P8P, B8P, P5P, and/or B5P models (Table 2).

Table 2. List of genes associated with PVT lapses during total sleep loss in Uyhelji et al. 2018, and their Benjamini-Hochberg corrected P-value (FDR) in the current study models[1].

Ensembl Gene ID	HGNC Sybmol	P8P	B8P	P5P	B5P	M5F	M5S
ENSG00000142634.13	EFHD2	0.767	0.834	0.670	0.639	0.163	0.347
ENSG00000122417.15	ODF2L	0.560	0.550	0.994	0.938	0.909	0.605
ENSG00000151151.6	IPMK	0.158	0.129	0.090	0.129	0.145	0.222
ENSG00000136167.15	LCP1	0.128	0.076	**0.044**	**0.018**	0.229	**0.046**
ENSG00000100644.17	HIF1A	0.289	0.385	**0.032**	**0.037**	**0.041**	0.058
ENSG00000103569.10	AQP9	0.065	0.066	**0.025**	**0.014**	0.097	**0.046**
ENSG00000087253.13	LPCAT2	0.072	0.086	**0.020**	**0.031**	0.230	0.158
ENSG00000189067.14	LITAF	0.152	0.136	0.078	0.061	0.111	0.053
ENSG00000166747.13	AP1G1	0.436	0.558	0.173	0.053	0.264	0.077
ENSG00000188895.12	MSL1	0.237	0.183	0.133	**0.038**	0.145	**0.045**
ENSG00000177885.15	GRB2	0.199	0.095	0.096	**0.036**	0.149	**0.045**
ENSG00000180871.8	CXCR2	0.519	0.473	0.336	0.182	0.460	**0.045**
ENSG00000163464.8	CXCR1	0.338	0.185	0.221	0.072	0.331	**0.047**
ENSG00000157551.19	KCNJ15	0.067	0.200	0.062	0.095	0.190	0.069
ENSG00000157557.13	ETS2	0.078	**0.034**	**0.030**	**0.011**	0.053	**0.046**
ENSG00000075785.14	RAB7A	0.654	0.428	0.741	0.607	0.883	0.212
ENSG00000113742.14	CPEB4	0.137	0.082	**0.020**	**0.003**	0.064	**0.044**
ENSG00000113369.9	ARRDC3	0.480	0.884	0.163	0.241	0.265	0.218
ENSG00000088832.18	FKBP1A	0.997	0.572	0.546	0.475	0.750	0.521
ENSG00000137312.15	FLOT1	0.672	0.176	0.247	0.056	0.145	**0.046**
ENSG00000012660.14	ELOVL5	0.053	0.078	**0.043**	0.064	0.144	**0.046**
ENSG00000127954.13	STEAP4	**0.029**	0.060	**0.042**	0.069	0.244	0.106
ENSG00000137575.12	SDCBP	**0.049**	0.056	**0.023**	**0.017**	0.065	**0.046**
ENSG00000047644.19	WWC3	0.845	0.476	0.717	0.616	0.132	0.218
ENSG00000136238.18	RAC1	0.629	0.635	0.131	0.056	0.289	0.053

Note: PVT = Psychomotor Vigilance Test; FDR = False Discovery Rate; HGNC = HUGO Gene Nomenclature Committee.

[1]See Table 1 for model abbreviations. Data are not shown for P5F, P5S, P5R, B5F, B5S, B5R, M5P, or M5R, where all 25 genes from the Uyhelji et al. 2018 paper yielded FDR≥0.05.

Differential expression related to MTS, RDM, and POMS-F

In addition to the number of PVT attention lapses, models were run to test for other cognitive (RDM and MTS) and subjective (POMS-F) impacts (Table 1). Tests of RDM yielded four differentially expressed genes in the B5R model of data from both placebo and modafinil study runs (Supplementary Table 12), but zero genes in the separate placebo P5R (Supplementary Table 10) or modafinil M5R models (Supplementary Table 11). Differentially expressed genes from the B5R model included *DNA damage inducible transcript 4 (DDIT4)*,

FKBP prolyl isomerase 5 (FKBP5), *RNA, U1 small nuclear 28, (RNU1-28P)*, and a novel transcript (ENSG00000223561) lacking an HGNC symbol at the time of analysis (Supplementary Table 12). Testing for genes related to either MTS or POMS-F yielded a somewhat opposing trend to PVT (Table 1), with a mere three and one significantly differentially expressed genes in the placebo run (P5F, P5S, Supplementary Table 10), >400 genes each differentially expressed in the modafinil run (M5F, M5S, Supplementary Table 11), and 108 to 110 genes differentially expressed in analysis of both placebo and modafinil study runs (B5F, B5S, Supplementary Table 12). In analysis of both runs in B5F, B5R, B5S, and B5P models, the two genes *DDIT4* and *FKBP5* were differentially expressed with respect to all four tested terms (POMS-F, MTS, PVT, and RDM), and another seven genes had FDR<0.05 for three of the four terms (Table 1, Supplementary Table 12). In the placebo P5F, P5R, P5S, and P5P models, three genes were differentially expressed for two terms: *C-X-C motif chemokine receptor 4 (CXCR4)*, *DDIT4*, and *KLF transcription factor 9 (KLF9)*. Genes *CXCR4* and *DDIT4* were differentially expressed relative to POMS-F and PVT, while *KLF9* was differentially expressed relative to MTS and PVT (Supplementary Table 10). For the modafinil models M5F, M5R, M5S, and M5P, no genes were significant for three or all four terms of POMS-F, MTS, PVT, and RDM (Supplementary Table 11). There were 87 genes differentially expressed relative to both POMS-F and MTS terms; all other genes were only differentially expressed relative to a single term (including *DDIT4* and *FKBP5*, with FDR<0.05 only for POMS-F) or not differentially expressed. The gene *CXCR4* was differentially expressed relative to POMS-F and MTS, while *KLF9* was not differentially expressed relative to any of the four tested terms in the modafinil models.

Of the genes previously identified as PVT biomarker candidates in (Uyhelji et al., 2018), none were significant at FDR<0.05 for POMS-F, MTS, or RDM in the five-timepoint placebo-only run, or the analysis of both placebo and modafinil runs. However, in the five-timepoint modafinil-only M5F model, *HIF1A* was significantly associated with POMS-F, and 11 genes were significantly associated with MTS in M5S models (Table 2).

Functional enrichment

In the eight-timepoint model, goseq functional enrichment tests of the 232 genes significant for PVT lapses in the placebo model P8P yielded five over-represented gene ontology (GO) functional terms at FDR<0.05. These included one biological process term related to the immune system (GO:0043312, neutrophil degranulation) and four cellular components (GO:0070062, extracellular exosome; GO:0070821, tertiary granule membrane; GO:0005576, extracellular region; GO:0005615, extracellular space). No genes were differentially expressed relative to PVT lapses in the modafinil M8P or M5P models, and hence, there was no functional enrichment. While there was differential gene expression for the analysis of both placebo and modafinil study runs in the B8P model, there was no GO functional enrichment relative to PVT lapses at FDR<0.05. However, in that same B8P model, there was functional enrichment of the 198 genes (Table 1) differentially expressed relative to the countermeasure term reflecting

modafinil usage. At an FDR<0.05, significant GO terms included biological processes GO:0060337 for type I interferon signaling pathway, GO:0045071 for negative regulation of viral genome replication, and GO:0043312 for neutrophil degranulation. The terms related to cellular functions were GO:0009986 for cell surface, GO:0005886 for plasma membrane, and GO:0070062 for extracellular exosome. The only enriched molecular function was GO:0005102 for signaling receptor binding.

In the five-timepoint model of the placebo run, there was no significant GO enrichment for POMS-F, MTS, or RDM based on separate goseq tests of the significantly differentially expressed genes from P5F, P5S, and P5R models. However, there were 25 over-represented terms based on the significantly differentially expressed genes for PVT lapses from the P5P model (Supplementary Table 13). These included functions related to the immune system (e.g., GO:0043312, neutrophil degranulation) and GO:0007165 signal transduction. In tests of genes differentially expressed in B5F and B5R models, there was no functional enrichment relative to POMS-F or RDM (FDR>0.4 for all GO terms). However, for the MTS model B5S, there were three over-represented cellular components at FDR<0.05: GO:0030863, cortical cytoskeleton; GO:0005829, cytosol; and GO:0005737, cytoplasm. There also was one over-represented biological process (GO:0043066, negative regulation of apoptotic process) but zero enriched molecular functions. There were 23 over-represented gene ontology functional terms for genes related to PVT lapses in the five-timepoint model of both runs (Supplementary Table 14), again including GO:0043312, neutrophil degranulation and GO:0007165, signal transduction. In the five-timepoint model of the modafinil run, there were 13 over-represented terms for POMS-F and 36 for MTS (Supplementary Table 15, Supplementary Table 16). There was no differential gene expression and, therefore, no functional enrichment for PVT or RDM based on the five-timepoint modafinil models M5P and M5R.

Pathway analysis

Ingenuity pathway analysis predicted many activated or inhibited pathways (positive or negative z-score, respectively). Pathway prediction was run on the dataset of 232 genes differentially expressed relative to PVT in the eight-timepoint placebo model P8P. The top canonical pathway with the lowest P-value (3.43E-04) was Toll like receptor signaling, based on six significantly differentially expressed genes: *Interleukin 18 (IL18), Interleukin 1 Receptor Associated Kinase 3 (IRAK3), Toll like Receptor 2 (TLR2), Toll Like Receptor 4 (TLR4), Toll Like Receptor 8 (TLR8),* and *TNF Alpha Induced Protein 3 (TNFAIP3)*. Among the upstream regulator genes with pathways predicted by upstream analysis, the lowest P-value for overlap (7.96E-07) between genes known to be regulated by a transcriptional regulator and dataset genes was associated with the upstream regulator *Ras Related 2* (*RRAS2*). This was based on 10 dataset genes: *ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS5), Nicotinamide phosphoribosyltransferase (NAMPT), Versican (VCAN), CD36 molecule (CD36), Interleukin 18 (IL18), Myelin protein zero like 2 (MPZL2), Solute carrier family 2 member 3 (SLC2A3), Sphingomyelin synthase 2 (SGMS2), Serpin family B member 8 (SERPINB8),* and *TNF alpha induced protein 3 (TNFAIP3)*. Another upstream regulator was *HIF1a*, based on a network of 13 dataset genes (Figure 1). There were 248 causal networks generated by the IPA upstream analysis with a P-value<0.05. This included a network with SDCBP as the master regulator in a network supported by 19 differentially expressed dataset genes (Figure 2) and an overlap P-value of 3.51E-06.

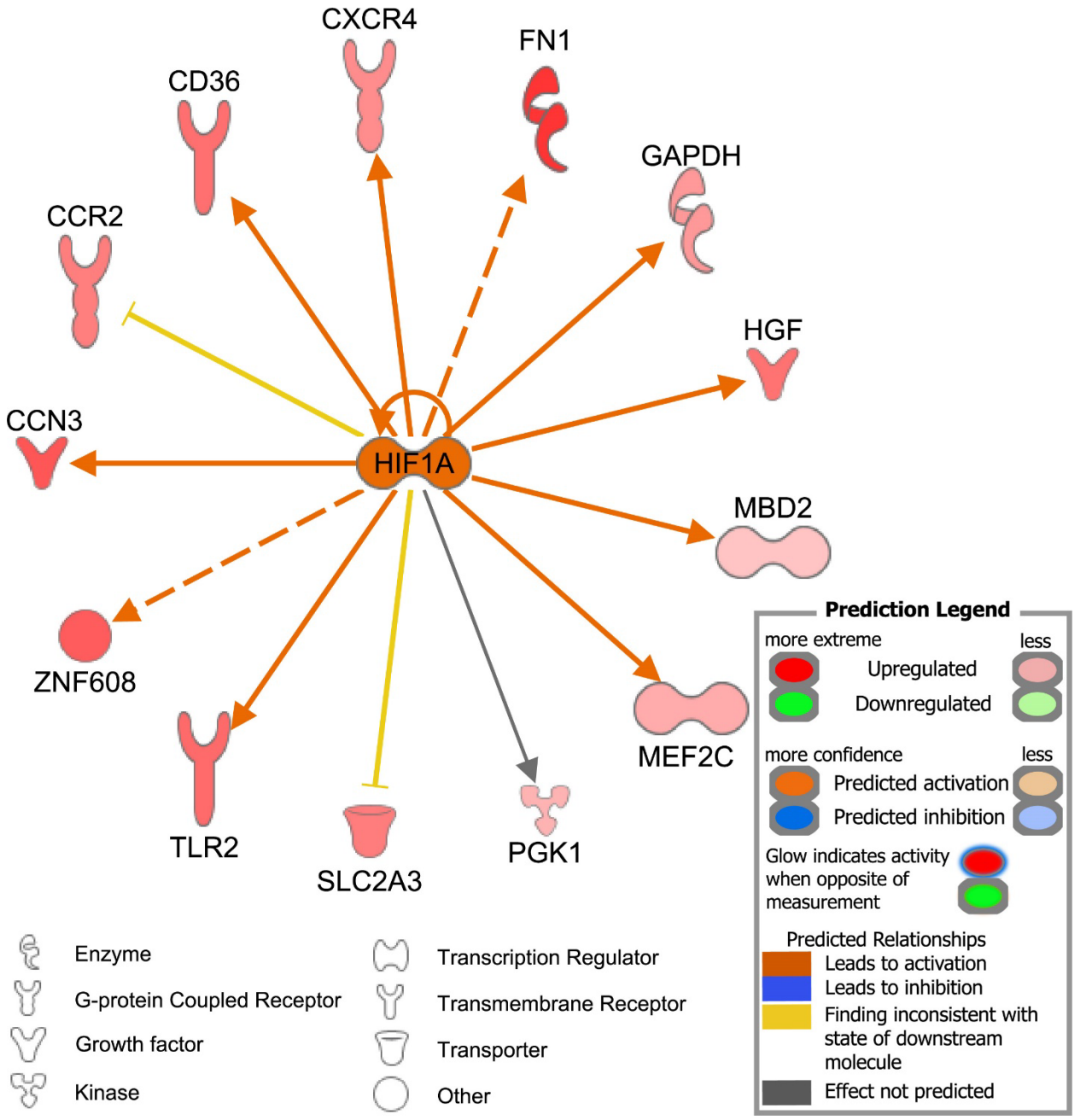

Figure 1. Ingenuity Pathway Analysis network pathway with *HIF1a* as an upstream regulator, based on genes differentially expressed relative to PVT lapses in the eight-timepoint placebo-only run. Solid lines indicate direct interactions; dashed lines reflect indirect or inferred relationships between molecules.

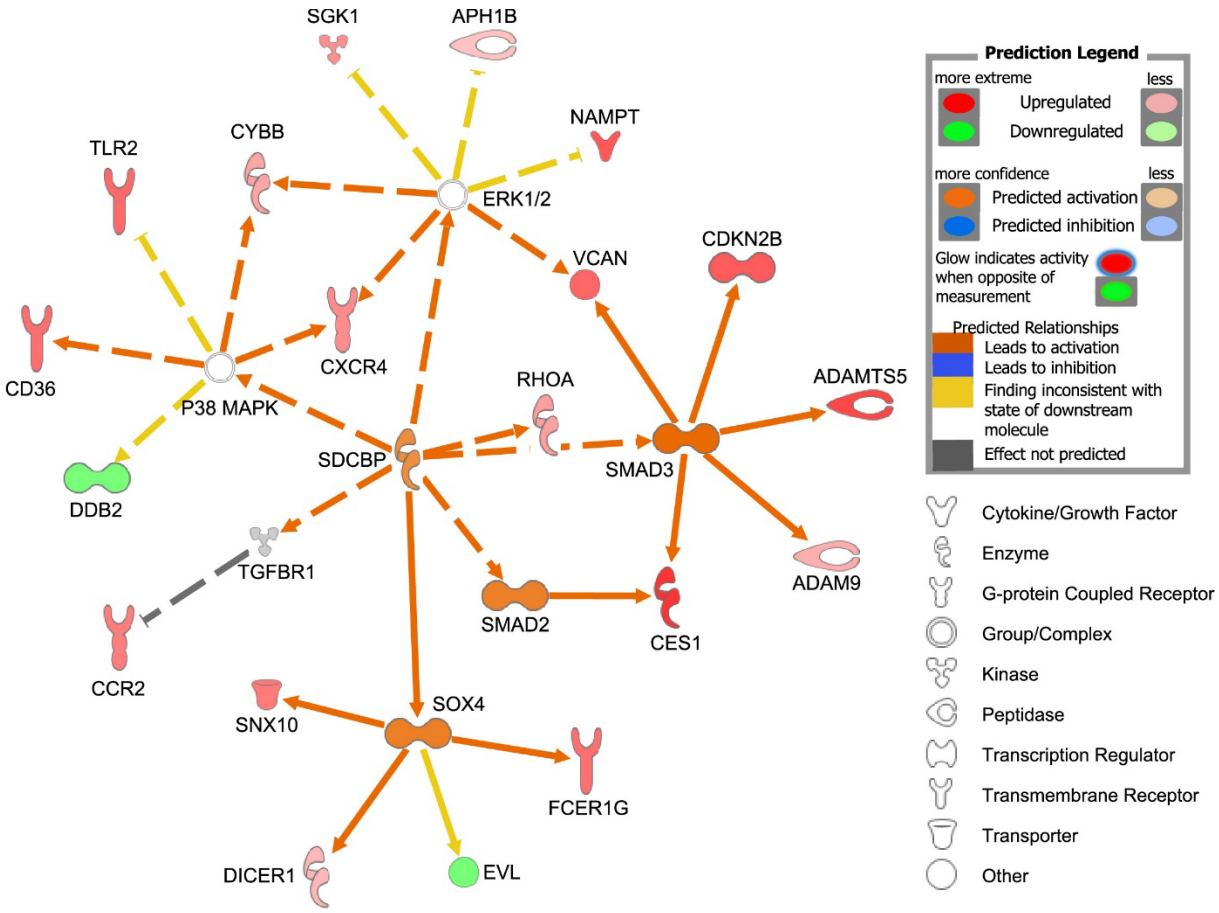

Figure 2. Ingenuity Pathway Analysis causal network pathway with *SDCBP* as a master regulator, based on genes differentially expressed relative to PVT lapses in the eight-timepoint placebo-only run. Solid lines indicate direct interactions; dashed lines reflect indirect or inferred relationships between molecules.

Discussion

Healthy sleep is vital to overall health, and sleep disruption can reduce safety-critical operator performance in fields such as transportation. Individuals respond differently to extended periods of wakefulness (St. Hilaire et al., 2019; Tkachenko & Dinges, 2018; Van Dongen et al., 2004), and thus, a tailored data-driven approach based on performance monitoring may improve current approaches to safety risk management. Ultimately, it may be more informative to detect performance impairment as the outcome of sleep disruption rather than relying on the identification of potential causes of fatigue, such as sleep loss or altered timing of sleep. This study advances the aim of developing metrics for fatigue safety risks from sleep loss by reporting on gene expression biomarkers associated with impairment in the presence and absence of the countermeasure drug modafinil.

Modafinil and treatment effects

Modafinil administration seemed to diminish the ability to detect differential expression related to some neurobehavioral performance metrics. Analyses of the placebo run alone or joint analysis of data from both runs yielded differential gene expression related to neurobehavioral

performance impairment. For instance, hundreds of genes were differentially expressed relative to PVT lapses in the P8P and B8P models (Table 1). However, analyses of the modafinil run by itself (M8P, Table 1) yielded no differentially expressed genes relative to PVT attention lapses. For the five-timepoint modafinil models, tests of self-reported fatigue (POMS-F assayed in M5F) yielded 418 differentially expressed genes, and tests of the number correct in MTS (M5S model) yielded 596 differentially expressed genes (Table 1, Supplementary Table 11). There was no differential expression for either PVT lapses (M5P) or reaction time in the RDM test (M5R).

Differences in separate models of the modafinil and placebo study runs suggest the potential for chemical countermeasures, such as modafinil, to alter the relationship between gene expression and neurobehavioral performance changes during sleep loss. Future work is needed to explore molecular mechanisms. Modafinil has been shown to attenuate performance impairment (Caldwell et al., 2020). Modafinil may disrupt associations of gene expression with performance seen in placebo models (e.g., P8P vs. M8P; Table 1) due to possible effects of the drug on performance, on gene expression, or simultaneous effects on both. This highlights a potential need to validate fatigue impairment biomarker candidates in the presence of drugs or countermeasures that may be administered. Notably, modafinil is a countermeasure that may have additional considerations in a military setting, but at the time of writing, would be considered a Do Not Issue or defer exam matter for civilian aviation pilot medical certification as a category IV controlled substance (Federal Aviation Administration, 2023). The Fatigue Countermeasures Laboratory at NAMRU-D demonstrated that modafinil use more strongly mitigated declines in performance among subjects that are more vulnerable to impairment from sleep loss, at least for the MTS and PVT assays (Caldwell et al., 2020). Variation in the response to the countermeasure among individuals more or less susceptible to fatiguing conditions, could perhaps add noise that inhibits the ability to find an overall relationship between performance and gene expression in the presence of modafinil. Whether this finding is unique to modafinil or could be shared by other fatigue countermeasures, such as caffeine, warrants further research.

Biomarkers for time awake and attention impairment

Total sleep deprivation resulted in thousands of differentially expressed genes relative to time awake. This finding was expected, as it is well-known that sleep deprivation and circadian disruption influences gene expression (Arnardottir et al., 2014; da Costa Souza & Ribeiro, 2015; Laing et al., 2018; Möller-Levet et al., 2013). Due to the correlation between PVT lapses and hours of wakefulness (timepoint), tests for the primary variables of interest (PVT, POMS-F, MTS, and RDM) did not include a model term for timepoint. Indeed, of the 232 genes differentially expressed relative to PVT lapses in the eight-timepoint placebo run P8P, a separate model run testing for timepoint revealed all but seven of the 232 genes also were significantly associated with study timepoint in the P8T model. Again, this inter-relationship was anticipated, as prior work has indicated continued wakefulness leads to increased attention lapses in most individuals (Uyhelji et al., 2018). Furthermore, studies have indicated relationships between gene expression and circadian rhythms, including disruption of rhythmic gene expression from

insufficient sleep (Möller-Levet et al., 2013). Thus, initial models with the full eight-timepoint dataset included terms to address the circadian component. However, with only five timepoints of data, analyses of MTS, RDM, and POMS-F were limited to excluding the circadian component (Table 1).

Among the genes differentially expressed with relation to PVT lapses, both analyses of individual genes and functional enrichment implicate changes in the immune system. Well-known associations of sleep, circadian rhythms, and the immune system support the present study's findings (Besedovsky et al., 2012; Foo et al., 2019; Irwin, 2019). Gene ontology analyses revealed functional enrichment of categories related to neutrophil degranulation, the cellular response to diacyl bacterial lipopeptide, and the Toll like receptor TLR6:TLR2 signaling pathway. Partial sleep deprivation has previously been shown to result in TLR4 stimulating an increase in monocyte production of Interleukin 6 and Tumor Necrosis Factor (Irwin, 2019). Here individual genes with known roles in the immune response, including chemokine receptors and interleukins, were among those differentially expressed relative to PVT lapses. Indeed *C-X-C motif chemokine receptor 4 (CXCR4)* was significantly related to PVT lapses in the placebo run and in the analysis of both placebo and modafinil runs, for both five and eight-timepoint models (P8P, B8P, P5P, B5P). *Interleukin 18 (IL18)* was related to PVT lapses in the placebo run and in the analysis of both placebo and modafinil runs for the eight-timepoint model (P8P and B8P), and in the placebo run for the five-timepoint model (P5P). These findings are congruent with previous reviews reporting a relationship between cytokines and sleep (Haspel et al., 2020; Krueger, 2008; Liu et al., 2021; Opp, 2005).

Additionally, there is an implication of the hypoxic response, with current and prior results (Uyhelji et al., 2018) indicating that *HIF1a* is differentially expressed following sleep loss in association with attention impairment. In the present study, the transcription factor *HIF1a* was significantly related to PVT lapses in the five-timepoint models of the placebo run (P5P) and the analysis of both placebo and modafinil runs (B5P; Table 2). Although it was not significantly differentially expressed in the eight-timepoint models that included terms for circadian rhythmicity, *HIF1a* was an upstream regulator predicted by IPA Core Analysis of the 232 genes related to PVT in the eight-timepoint placebo run (Figure 1). Other authors have suggested that sleep loss may influence the response to environmental hypoxia, and even increase the risk of experiencing acute mountain sickness (Fabries et al., 2022). Review articles also have proposed bidirectional relationships between the circadian clock and responses to hypoxia (Gabryelska et al., 2022; Peek, 2020). If sleep loss and/or circadian disruption (e.g., jet lag) interacts with physiological and molecular responses to hypoxic exposure at altitude, sleep disruption may have nuanced effects on frequent flyers and aircrew. Aircrews in the civilian or military sector may experience mild hypoxia (Nicholson et al., 2021; Shaw et al., 2021). Further research is needed to explore the relationship between sleep disruption and hypoxia.

Substantial overlap between genes related to PVT lapses in the current and prior research (Uyhelji et al., 2018), presents strong evidence of reproducible molecular changes that may be

used as a fatigue impairment metric. Indeed, some genes significantly related to PVT lapses in the current study were found not only in the prior microarray study by Uyhelji et al. with linear modeling (Table 2), but also in a re-analysis of data from that prior study employing a hierarchical clustering approach (Satterfield et al., 2019). Genes detected in the re-analysis include the following five genes: *Lysophosphatidylcholine acyltransferase 2* (*LPCAT2), ETS proto-oncogene 2, transcription factor (ETS2), ELOVL fatty acid elongase 5 (ELOVL5), STEAP4, and SDCBP.* Although this reproduction of results appears to indicate biomarker robustness, little research exists relating molecular changes such as gene expression levels to neurobehavioral performance decrements during sleep disruption. Some studies have been conducted of genetic variants such as single nucleotide polymorphisms that may indicate an inherited greater resistance to the impairing effects of sleep loss. However, such possible biomarkers of predisposition may not indicate current impairment status as studied here. For example, it has been suggested that variants of the gene *Adenosine A2a receptor (ADORA2a)* may be associated with resistance to attention lapses during total sleep loss (Erblang et al., 2021). Yet this gene was not differentially expressed relative to PVT lapses in any of the eight timepoint models, and was not significantly related to PVT, POMS-F, RDM, or MTS in the five timepoint models of placebo, modafinil, or both runs.

The five genes currently and previously related to PVT lapses have immune regulation and signaling roles. The product of gene *LPCAT2* interacts with Toll like receptors to mediate an immune inflammatory gene expression response to bacterial antigens such as lipopolysaccharides (Abate et al., 2020). In a study of patients with depression, those who responded to a therapeutic exposure to sleep deprivation showed differential expression of *LPCAT2* as compared to non-responders (Foo et al., 2019). The gene *ETS2* is a member of the E26 transformation-specific family of transcription factors, which have roles in processes such as normal cell development, but whose dysregulation is associated with cell proliferation in cancer (Fry & Inoue, 2018; Gutierrez-Hartmann et al., 2007). The *ELOVL5* gene product is an enzyme involved in the regulation of lipogenesis and synthesis of polyunsaturated fatty acids (Shikama et al., 2015). Another fatty acid elongase gene, *ELOVL2*, has been associated with self-reported sleep duration in a genome-wide association study (Scheinfeldt et al., 2015). The gene *Six-transmembrane epithelial antigen of the prostate 4 (STEAP4)* encodes a metalloreductase with proposed roles in responding to inflammatory stress (Scarl et al., 2017). More recent work has indicated a possible association with restless leg syndrome (Tilch et al., 2020). The gene *SDCBP*, also known by gene synonyms *Melanoma differentiation associated gene-9 (MDA9)* or *Syntenin*, encodes a scaffolding protein with roles in cell signaling and cancer progression (Boukerche et al., 2008). It further appeared as a master regulator of a causal network predicted by IPA Core Analysis in the current study (Figure 2).

Biomarkers across measures of fatigue impairment

Of the possible biomarker genes related to PVT lapses, there was partial overlap with lists of genes related to other cognitive and subjective variables in the five-timepoint model tests.

Those genes that respond to tests of PVT as well as either RDM and/or MTS may be associated with overall neurobehavioral performance inhibition. Yet the fact that many genes respond uniquely to one or the other variable suggests the potential to develop distinct panels of genes to target the specific types of performance most relevant to a given operational setting. The POMS-F is a subjective self-report of fatigue, and it is interesting to find minimal overlap between genes responding to PVT and POMS-F. In the placebo run, of the 1,169 genes related to PVT lapses, only two were related to POMS-F (*CXCR4* and *DDIT4*) and one to MTS (*KLF9*). Of course, with zero genes related to PVT lapses in the modafinil models (M8P and M5P), there was no overlap to investigate. For analysis of data from both placebo and modafinil study runs, of the 1,406 genes significant for PVT lapses, 69 were significant for POMS-F, 68 for MTS, and four for RDM. This included *KLF9*, again significant for PVT and MTS, as well as *CXCR4* and DDIT4, again significant for PVT and POMS-F. The gene *FKBP5* was not significantly related to PVT lapses in the eight-timepoint models (P8P, M8P, B8P). In the five-timepoint models, it was related to POMS-F, MTS, PVT, and RDM in the analysis of both runs, only to PVT in the placebo run, and only to POMS-F in the modafinil run. Low levels of overlap between genes related to POMS-F with the more objective performance metrics PVT, MTS, and RDM is consistent with self-reported fatigue not always corresponding to objective metrics of neurobehavioral performance.

For those genes differentially expressed across multiple model terms, known functions often were related to the immune system, corticosteroid signaling, and circadian rhythms. Glucocorticoids are hormones that suppress inflammation (Irwin, 2019). The gene *DDIT4* has previously been identified as a potential biomarker of melatonin phase and is associated with human circadian rhythms (Foo et al., 2019; Laing et al., 2018). The *DDIT4* gene (also known as *Regulated in development and DNA damage response 1* or *REDD1*) is involved in the response to stressors such as hypoxia, with a role in regulating the activity of the mammalian target of rapamycin (mTOR) (Tirado-Hurtado et al., 2018; Zhidkova et al., 2022). The gene *KLF transcription factor 9* (*KLF9*, also known as *Basic transcription element-binding protein 1* or *BTEB1*) belongs to the Krüppel-like family of transcription factors. The *KLF9* gene encodes a transcription factor regulated by electrical activity, corticosterone, and thyroid hormone T3 (Moore et al., 2011). It has been suggested that KLF9 in the human epidermis may influence circadian expression of target genes (Spörl et al., 2012), and that it is a key regulator of glucocorticoid signaling (Gans & Coffman, 2021). Also important to glucocorticoid responses is the gene *FKBP5*, itself regulated by environmental stressors (Gans & Coffman, 2021; Zannas et al., 2016) and a possible circadian phase biomarker (Laing et al., 2017). Chemokine receptor *CXCR4* is part of a superfamily of G protein-coupled receptors and regulates biological processes ranging from the immune response to organogenesis (Busillo & Benovic, 2007). Roughly a decade ago, research supported a role for cortisol inducing *CXCR4* upregulation and mediating immune system rhythmicity, particularly a morning decrease in circulating T cells (Besedovsky et al., 2014). More recently, work in mice demonstrated increased levels of CXCR4 on B cells within blood and brain associated with sleep deprivation (Korin et al., 2019).

Of genes previously related to PVT lapses by Uyhelji et al. (2018), one gene (*HIF1a*) was related to POMS-F in the modafinil-only run M5F (Table 2). Another 13 genes were related to MTS in the modafinil only run M5S: *Lymphocyte cytosolic protein 1* (*LCP1)*, *Aquaporin 9 (AQP9)*, *MSL complex subunit 1 (MSL1)*, *Growth factor receptor bound protein 2 (GRB2)*, *C-X-C motif chemokine receptor 2 (CXCR2), C-X-C motif chemokine receptor 1 (CXCR1)*, *ETS proto-oncogene 2, transcription factor (ETS2)*, *Cytoplasmic polyadenylation element binding protein 4 (CPEB4), ELOVL fatty acid elongase 5 (ELOVL5), Syndecan binding protein (SDCBP)*, and *flotillin 1 (FLOT1)*. None were significantly related to POMS-F, MTS, or RDM in the placebo run or in tests of both runs. Different results with modafinil vs. placebo administration again may be a feature unique to modafinil or underlie the importance of considering countermeasure use in developing biomarker panels for fatigue impairment. However, the limited number of timepoints for MTS, POMS-F, and RDM hindered the ability to use models that explicitly incorporated terms for circadian rhythms. Thus, some of the biomarkers related to variables tested in the five-timepoint models possibly could represent genes affected by circadian rhythms, including genes related to PVT in the five but not eight-timepoint models, such as *FKBP5*.

Conclusion

Several lists of candidate biomarker genes for fatigue impairment were generated in this study. These included biomarkers for subjective self-reported fatigue as measured by the POMS-F assay, and those for objective cognitive performance tests with the PVT, RDM, and MTS assays. For the civilian aviation sector, genes found in placebo runs without a drug countermeasure may be the most applicable candidates. Nonetheless genes significant in tests of both placebo and modafinil study runs also may be informative. The subset of genes related to PVT lapses in this study, that also were found in prior investigation of gene expression changes related to PVT during sleep loss, may represent robust biomarker candidates for fatigue-related attention impairment. Future work is needed to validate these candidate genes outside sleep clinics and in operational settings. Considering that variation in biomarker detection may depend on which cognitive or subjective test is used (PVT, RDM, MTS, POMS-F), another critical need is to identify aspects of cognition and physiology most important to operational safety. This will facilitate the development of biomarker panels for application to fatigue risk mitigation.

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