ORIGINAL ARTICLE

Common Genetic Variation Near Melatonin Receptor 1A Gene Linked to Job-Related Exhaustion in Shift Workers

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Study Objectives: Tolerance to shift work varies; only some shift workers suffer from disturbed sleep, fatigue, and job-related exhaustion. Our aim was to explore molecular genetic risk factors for intolerance to shift work.

Methods: We assessed intolerance to shift work with job-related exhaustion symptoms in shift workers using the emotional exhaustion subscale of the Maslach Burnout Inventory-General Survey, and carried out a genome-wide association study (GWAS) using Illumina's Human610-Quad BeadChip (n = 176). The most significant findings were further studied in three groups of Finnish shift workers (n = 577). We assessed methylation in blood cells with the Illumina HumanMethylation450K BeadChip, and examined gene expression levels in the publicly available eGWAS Mayo data.

Results: The second strongest signal identified in the GWAS (p = 2.3 × 10E-6) was replicated in two of the replication studies with $p < .05$ (p = 2.0 × 10E-4 when combining the replication studies) and indicated an association of job-related exhaustion in shift workers with rs12506228, located downstream of the melatonin receptor 1A gene (*MTNR1A*). The risk allele was also associated with reduced *in silico* gene expression levels of *MTNR1A* in brain tissue and suggestively associated with changes in DNA methylation in the 5' regulatory region of *MTNR1A*.

Conclusions: These findings suggest that a variant near *MTNR1A* may be associated with job-related exhaustion in shift workers. The risk variant may exert its effect via epigenetic mechanisms, potentially leading to reduced melatonin signaling in the brain. These results could indicate a link between melatonin signaling, a key circadian regulatory mechanism, and tolerance to shift work.

Keywords: genome-wide association study, job-related exhaustion, shift work, *MTNR1A*, DNA methylation.

Statement of Significance

This study represents the first systematic search for molecular genetic risk factors for intolerance to shift work, assessed with job-related exhaustion symptoms in shift workers. The study suggests a potential genetic risk variant for intolerance to shift work located near melatonin receptor 1A gene (*MTNR1A*). This variant is linked to lower expression levels of *MTNR1A* and suggestively to changes in DNA methylation in the regulatory region of *MTNR1A*. We propose that the reduced melatonin signaling through decreased *MTNR1A* expression may increase the rhythm-changing effects of nocturnal light in shift workers and worsen circadian disruption. Given the wide use of melatonergic drugs, defining the role that *MTNR1A* plays in tolerance to shift work warrants further investigation.

INTRODUCTION

In the Western world, about one in five employees is engaged in shift work.[1] Performing shift work long-term has been linked to a number of adverse health effects, such as an increased risk of type 2 diabetes,[2] coronary heart disease,[3] and breast cancer.[4] In daily life, the most common health-related effects of shift work are disturbed sleep–wake cycle and the associated sleep loss, which promote fatigue, and at worst can lead to shift work sleep disorder.[5] Shift workers are also at higher risk for work-related stress syndrome burnout.[6,7] Clearly, individual differences do exist regarding tolerance to shift work since not all shift workers suffer from fatigue or other adverse consequences.[8]

The definition of intolerance to shift work and methods to assess it vary.[9] The traditional definition offered by Andlauer et al.,[10] and a later definition by Reinberg et al,[11] both include persistent fatigue as one of the key symptoms, along with digestive troubles, persistent sleep alterations,[10,11] regular use of sleep medication, and changes in behavior.[11] Persistent fatigue, which is long-lasting and not recovered by days-off,[12] closely resembles emotional exhaustion (job-related exhaustion), the core symptom of burnout also linked to ineffective rest,[13] which can be measured by structured tools such as the Maslach Burnout Inventory-General Survey (MBI-GS).

Individual risk factors for intolerance to shift work include female gender, neuroticism and its related personality traits, and the morningness chronotype, especially with night work.[9,14] Genetic variants may also affect tolerance to shift work.[8,9] Interactions between circadian genes and a person's sleep strategy (the timing and amount of sleep when switching from one shift to another) have been detected in adaptation to work schedules,[15] and the frequencies of serotonin transporter gene variants and cryptochrome circadian clock 1 gene (*CRY1*) variants vary between day workers and rotating night workers, potentially reflecting genotype-based selection.[16,17] In a study of tolerance to sleep deprivation, a common consequence of shift

work, the major part of the inter-individual variability in neurobehavioral deficits during sleep deprivation was due to trait-like differential vulnerability,[18] such that could be explained by genetic factors, for example. To our knowledge, no systematic molecular genetic search on the risk factors for intolerance to shift work has been carried out previously.

To identify molecular genetic risk factors for intolerance to shift work, we performed a genome-wide association study (GWAS) of job-related exhaustion, as measured by the MBI-GS, in shift workers of the Finnish general population and sought to replicate the strongest findings in subjects from the general population and subjects from two independent Finnish occupational groups, airline workers and nurses engaged in shift work. To investigate if an interaction of genetic risk factors and the shift work environment is mediated through molecular mechanisms affecting gene expression, we also studied *in silico* gene-expression levels, and investigated blood-cell DNA methylation levels.

METHODS

Population-Based Study Subjects

Carried out from 2000 to 2001 by the National Public Health Institute of Finland, the Health 2000 survey is an epidemiological cohort representative of the Finnish population over the age of 30 (n = 8028).[19] Subjects in our studies comprised individuals under 65 years old who perform three-shift work, two-shift work, or regular night work (Table 1). These subjects were defined based on the following questions asked of those indicating that they had been working in the last 12 months: "What sort of hours do/did you work in your main occupation: regular day-job (between 6 am and 6 pm), regular evening job, regular night job, two-shift work, three-shift work, periodical work, only weekend work, other sort of working time?" We included those reporting two-shift work, three-shift work, or regular night work. In Finland, three-shift work usually refers to 8-hour shift work schedules with morning/day, evening, and night shifts; while, two-shift work refers to shift work with only two different shifts which are normally the morning/day and the evening shift.

Comprised of equal numbers of cases and controls for metabolic syndrome, the GenMets subcohort from the Health 2000 survey was selected for genome-wide genotyping (n = 2130).[20] Our initial GWAS analyses comprised GenMets shift workers (Health 2000 GWAS study, n = 176 with successful genotyping and answered to MBI-GS) and the analyses were adjusted for the case-control status of GenMets (73 cases and 103 controls). A description of the selection of cases and controls is provided in the Supplementary Methods and basic characteristics of the cases and controls are provided in Supplementary Table S1. The two variants with the strongest association in GWAS were genotyped in the rest of the Health 2000 cohort, which was not part of GenMets, and those shift workers were used in our first replication analysis (Health 2000 replication study, n = 241 with successful genotyping and answered to MBI-GS) (Table 1). The interaction analysis of the shift-work status included the shift working subjects in both the Health 2000 GWAS and Health 2000 replication studies and, in addition, non-shift-working subjects of the Health 2000 (n = 2484 with successful genotyping and answered to MBI-GS).

The genetic fine mapping investigation was supplemented with two additional Finnish population cohorts, Vantaa 85+ (n = 532)[21] and Kuopio 75+ (n = 601),[22] which contained no information on shift work or job-related exhaustion. For a total of 14 subjects from Health 2000 and Vantaa 85+, we performed capillary sequencing, and thereafter Vantaa 85+ Kuopio 75+

Table 1—Study Characteristics.

Study	Health 2000 GWAS study[a]	Health 2000 replication study[b]	Nurse study[c]	In-flight workers of the airline study[d]	Non-flight workers of the airline study[d]
Occupation	All	All	Nurses	Pilots and flight attendants	Workers at airport, eg, in catering or in cargo
Shift work type	Three-shift, two-shift or regular night work	Three-shift, two-shift or regular night work	Three-shift work	night shifts ≥3/mo or/and early morning shifts ≥1/wk	night shifts ≥3/mo or/and early morning shifts ≥1/wk
Number of shift workers	176	241	73	263	343
MBI-GS exhaustion, mean (*SD*)	1.17 (1.23)	1.10 (1.18)	1.25 (1.08)	1.77 (0.87)	2.26 (1.22)
Age mean (*SD*, range)	46.7 (7.6, 30–61)	42.7, (8.4, 30–60)	47.7 (6.8, 31–59)	44.3 (8.2, 24–64)	43.8 (8.4, 24–60)
Males (%)	42.0	38.6	0	21.7	65.3

GWAS = genome-wide association study; MBI-GS = Maslach Burnout Inventory-General Survey.

[a]Genmets subcohort of the Health 2000 Survey designed originally for case-control study of metabolic syndrome.

[b]Respondents of the Health 2000 survey who participate in the genetic study and were not part of Genmets.

[c]Subset of nurses from the Finnish Public Sector Study.[25]

[d]Employees of the airline company in the follow-up phase of the study for diabetes screening and prevention implementation program in the occupational health care of the Finnish airline.[24]

were used in the fragment analysis of the microsatellite marker found by sequencing (Supplementary Methods).

Occupational Study Subjects

Used as additional replication material and in methylation analyses, occupational subjects comprised employees from two sectors. The baseline phase of the airline study for prevention of type 2 diabetes was collected during the volunteer employee health checks of a Finnish airline operating primarily along Asia–Europe traffic routes.[23] Of the 2312 study subjects, those with full baseline data and no diabetes ($n = 40$ with diabetes based on their baseline glucose levels) were invited to the follow-up study 2.5 years later, and 1347 participated.[24] The participants of the follow-up study who had information from MBI-GS and from work schedules, gave permission for DNA sampling, and had successful genotyping, numbered 821. The shift-working subjects were defined by the questions "How many night shifts do you have per month (at least 3 hours between 23.00–06.00)?" and "How often in your shift work schedule you have following working times: Early morning shifts starting before 6 am?" The answers were further verified from the working schedule system. As shift workers, we classified subjects with night shifts ≥3/mo, and/or early morning shifts ≥1/wk. Of the included study subjects, 606 were classified as shift workers and comprised our airline study, which included in-flight workers ($n = 263$), who served as pilots or flight attendants, and non-flight workers ($n = 343$), who held various occupations at the airport. The non-flight workers constituted a separate group, analyzed post hoc, because they had much higher exhaustion scores compared to the other occupational and population-based groups (Table 1). In addition to shift-working non-flight workers, non-flight workers who did not perform shift work ($n = 193$ with successful genotyping and answered to MBI-GS) were included in the interaction testing of shift work.

The second occupational group included nurses from the Finnish Public Sector Study ($n = 73$ with successful genotyping and answered to MBI-GS)[25] (Table 1). All the nurses had a shift work schedule with three different shifts: morning, evening, and night.

For all the contributing studies, the local institutional review boards on human research approved the study protocols, and the participants gave their informed consent.

Measure for Job-Related Exhaustion

Job-related exhaustion was assessed in all studied data sets using the emotional exhaustion subscale from the MBI-GS.[26,27] The MBI-GS questionnaire is suitable for any profession, and its reliability and validity have been confirmed.[28,29] The emotional exhaustion subscale comprises five symptom statements with a frequency scale ranging from 0 (never) to 6 (daily). One missing value was allowed. After logarithmic transformation to achieve a normal distribution, the sum score for emotional exhaustion was treated as a quantitative trait.

Genotyping and Quality Control

Genome-wide genotyping in the Health 2000 GWAS study was performed using Illumina's Human610-Quad DNA Analysis BeadChip (Illumina, Inc., San Diego, CA). Markers with MAF <1% or Hardy–Weinberg equilibrium (HWE) $p < 1 \times 10^{-6}$ were excluded from the analyses. For individuals and markers, the call rate was >95%. Data selection and genotyping in GenMets have been described previously in detail.[27]

Genotyping of the Health 2000 replication study and of the occupational studies (HWE $p > 1 \times 10^{-6}$) was performed by MassARRAY genotyping using iPLEX Gold chemistry (Sequenom, San Diego, CA). In the Health 2000 replication study of 270 shift workers who answered the MBI-GS, the genotyping success rate was 97.4% for rs12506228 and 96.6% for rs3821986. Call rate for individuals was 95% and altogether 28/270 subjects were excluded. The success rates for genotyping of rs12506228 were 100% in the nurse study and 99.9% in the in the airline study. The call rate for individuals was 95% in the airline study, and two subjects from the non-flight workers of the airline were removed.

Capillary sequencing was employed to sequence of the haplotype region of rs12506228, whilst fragment analysis was used to further investigate the related microsatellite marker (Supplementary Methods and Table S9).

Association Analyses

For genetic association analyses we utilized linear regression analysis of PLINK, version 1.07,[30] with an additive model and covariates of age and sex. In the GWAS, case-control status for metabolic syndrome and 20 EIGENSTRAT principal components were used as covariates to control for enrichment of the cases of metabolic syndrome in the Health 2000 GWAS study and potential population stratification, respectively. PLINK was also used for the haplotype analyses (Supplementary Methods) and interaction testing, which was calculated using PLINK's interaction command which adds the interaction term on the linear regression model. Beta and p values for the interaction term were reported in the model including covariates of age and sex. The main effect of the shift work environment on job-related exhaustion was calculated with a linear regression model in SPSS (IBM, SPSS statistics, version 23), as this was unavailable in PLINK. Haplotype structure was determined with the confidence interval method of the Haploview software, version 4.2 (Haploview, version 4.2, Daly lab at the Broad Institute, MA).[31] In the meta-analysis, we assumed a similar true genetic risk effect in all the study groups which all included shift workers with a Finnish background, and Health 2000 GWAS and Health 2000 replication studies were part of the same sampling procedure. All the studies included in the meta-analysis (the Health 2000 studies, in-flight workers of airline study, and the nurse study) also had female prominence, mean ages, and exhaustion scores close to each other (Table 1). Meta-analyses were performed using the fixed-effect model in GWAMA.[32] Heterogeneity between studies was tested with GWAMA using measures of Cochran's Q-test and I^2 index. In the case of heterogeneity, the source of heterogeneity was searched and analysis excluding those studies was also performed. The random-effect model was omitted due to the low number of studies in the meta-analysis (2–4), which renders it unreliable.[33]

In the GWAS, correction for multiple testing was handled by considering the level of significance as $p < 5 \times 10^{-8}$, which is the classical threshold used in GWAS studies. In other instances,

Bonferroni corrections were performed with the formula $p_{Bonferroni} = 1-(1-p)^k$, where p is the uncorrected p value and k number of tests.

Gene Expression in eGWAS Mayo Data

To examine association of rs12506228 with melatonin receptor 1A gene (*MTNR1A*) expression, we used publicly available eGWAS Mayo data through the National Institute of Aging Genetics Data Storage Site (NIAGADS).[34] The data set included gene expression profiles of cerebellum and temporal cortex, and genome-wide single nucleotide polymorphism (SNP) genotyping data for patients with neuropathologically verified Alzheimer's disease (AD) pathology and non-AD individuals, many of whom had other brain pathologies (55% with progressive supranuclear palsy, 13% with Lewy body disease, 12% with corticobasal degeneration).To avoid the effect of widespread neurodegeneration related to AD, only the non-AD subjects (n = 177, cerebellum) were included in this study. Gene expression levels were measured with the Whole Genome DASL assay (Illumina, Inc., San Diego, CA). Probes with detection in less than 75% of the samples were excluded. A probe for *MTNR1A* was excluded in the temporal lobe samples and therefore only results for cerebellum were reported. The SNP genotyping was based on Illumina's HumanHap300-Duo Genotyping BeadChips. Available statistics from the eGWAS Mayo data were based on linear regression analysis of PLINK using an additive model and covariates of age at death, gender, PCR plate, and RNA integrity number (RINmean).[2]

DNA Methylation Analyses

For the methylation study, to minimize variation in methylation levels due other reasons, subjects were excluded from all groups due to several circumstances, including: smokers; those using hormonal medication or medication affecting cognitive functions; and alcohol users exceeding the risk-use guidelines in Finland. The analyses of DNA methylation included 20 pilots (all males), 20 flight attendants (all females) from the airline study, as well as 20 nurses from the Finnish Public Sector Study (all females). In pilots and flight attendants, both individuals engaged and not regularly engaged in flights that cross time-zones (a minimum of four time-zones for over 3 years prior to measurement) were included (10 + 10 in both occupational groups). Individuals in the nurse study[35] had worked for at least 3 years in the same ward either collectively evaluated to be a high-stress (n = 10) or low-stress (n = 10) environment.[35] The groups were matched pairwise for age as well as possible with the limited number of subjects.

DNA methylation was assessed using Illumina Infinium HumanMethylation 450k BeadChips (Illumina, Inc., San Diego, CA). First, 500 ng of genomic DNA was treated with sodium bisulfite using the EZ96 DNA Methylation Kit protocol (Zymo Research). DNA methylation was assessed using Illumina Infinium HumanMethylation 450k BeadChips (Illumina, Inc.). A duplicate sample was included in each chip to control for the batch effect and methylation controls were included for 0% and 100% methylation (Epitech). DNA samples were processed according to the manufacturer's protocol. Preprocessing, correction, and normalization steps were implemented in the R-studio environment (version 0.97.318) using the Bioconductor software packages (version 2.12). Methylation data was extracted as raw signals (IDAT files) and loaded into the R environment using the minfi software package (version 1.18.2).[36] A subset quantile normalization approach (SWAN[37]) was used to adjust the intensities in each array and to correct for technical differences between type-I and type-II assays. At this stage, probes with a detection of $p > .01$ for any DNA sample or missing measurements, known cross-reactive probes, probes on X and Y chromosomes, and probes that contain either a SNP at the CpG interrogation or at the single nucleotide extension were excluded from subsequent analysis. All methylation values were converted to M values (log2 ratio of the measured intensities of the methylated probe vs. the unmethylated probe) in order to provide a more accurate interpretation of the methylation levels at the extreme ends (high and low methylation) compared to the beta values.[38] The lymphocute cell counts (CD8T, CD4T, natural killer cells, B cells, monocytes, and granulocytes) were derived from the methylation data as implemented in the minfi package.

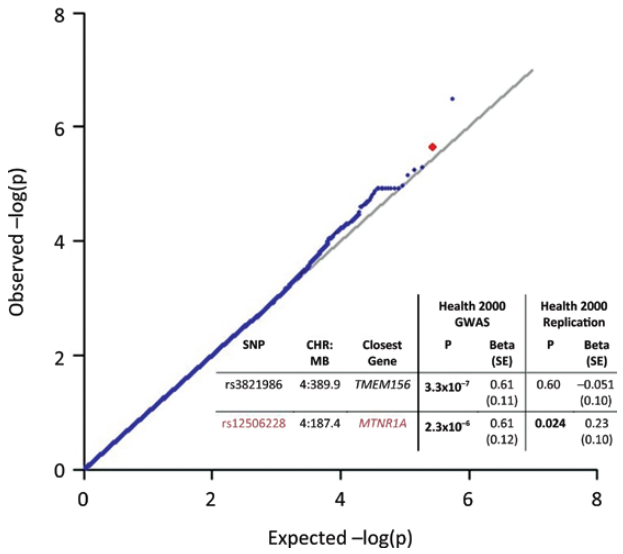
The association of the SNP with the CpG sites was calculated separately for each occupational group using a linear regression analysis with an additive model in SPSS (IBM, SPSS Statistics, version 23) to avoid a variance due to group, and then combined using the fixed-effect model in GWAMA. Lymphocyte cell counts for the six cell lines and methylation chip (slide) were added on the regression model as covariates. One individual from the nurses was excluded due to incomplete bisulfite conversion, and one individual from the pilot group due to outlying methylation value for cg04188238 (>3.0 *SD* from the mean *M*-value). Age was a non-significant covariate, and it was not included in the model.

RESULTS

GWAS and the First Replication Analysis Revealed an Association of a Variant Close to *MTNR1A* With Job-Related Exhaustion Among Shift Workers

To identify genetic risk variants for intolerance to shift work, we first performed a GWAS for job-related exhaustion among shift workers in the Health 2000 GWAS study (n = 176) (Figure 1, Supplementary Figure S1, Supplementary Table S2). No genome-wide significant associations ($p < 5 \times 10E\text{-}8$) emerged, but the two SNPs with the strongest association with the trait (suggestive association, $p < 5.0 \times 10^{-6}$), rs3821986 and rs12506228, were selected for genotyping in the Health 2000 replication study (n = 241). This replication study revealed no association of rs3821986 with job-related exhaustion, but an association with rs12506228 was replicated ($\beta = 0.23$, $p_{Bonferroni} = .048$) (Figure 1). rs12506228 is located 70 kb downstream from the gene encoding for the melatonin receptor type 1A (*MTNR1A*).

To determine whether the effect of the risk variant rs12506228 on job-related exhaustion was specific to shift workers, all workers from Health 2000 (417 shift workers and 2484 non-shift workers) were analyzed for gene-environment interactions. Among shift workers the association with job-related exhaustion was detected (Supplementary Table S10), and we saw a significant interaction effect with rs12506228 and shift-work status ($\beta = 0.33$, $p = 6.1 \times 10^{-5}$). No association with job-related exhaustion was seen among non-shift workers ($\beta = 0.0024$, $p =$



SNP	CHR: MB	Closest Gene	Health 2000 GWAS P	Health 2000 GWAS Beta (SE)	Health 2000 Replication P	Health 2000 Replication Beta (SE)
rs3821986	4:389.9	*TMEM156*	$3.3x10^{-7}$	0.61 (0.11)	0.60	–0.051 (0.10)
rs12506228	4:187.4	*MTNR1A*	$2.3x10^{-6}$	0.61 (0.12)	**0.024**	0.23 (0.10)

Figure 1—Genome-wide association study (GWAS) for job-related exhaustion in shift workers and the first replication study. A quantile–quantile plot was calculated for expected and observed test statistics for the GWAS in the Health 2000 GWAS study (n = 176). Replication analyses were performed in the Health 2000 replication study (n = 241) for the highest ranking two variants (table embedded). CHR:MB, chromosome:locus in megapairs.

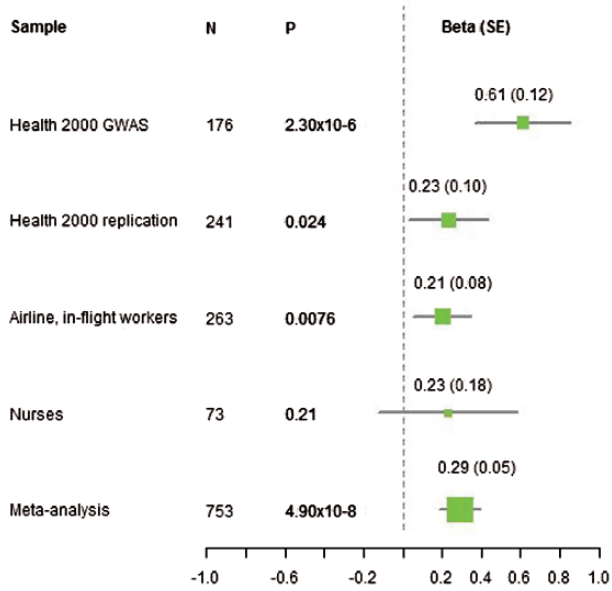


Figure 2—Meta-analysis for the association of rs12506228 with job-related exhaustion in shift workers. The Forrest plot presents the effect sizes (β) and standard errors (*SE*) for the linear regression analysis of each study.

.94). Shift work environment in itself presented no significant main effect on job-related exhaustion (β = 0.010, p = .84). Thus, in the Finnish population-based GWAS and replication studies, we were able to show a suggestive association of rs12506228, a variant close to *MTNR1A*, with job-related exhaustion, specifically in shift workers.

Association With *MTNR1A* Variant was Replicated in an Occupational Cohort

We next attempted to find further evidence for the association of rs12506228 with job-related exhaustion in shift workers by replicating the analysis in two occupational groups with shift-work schedules, in-flight workers of a Finnish airline (n = 263) and three-shift-working nurses (n = 73), using the same measure as the population-based studies—MBI-GS. We found a significant replication in the in-flight workers (β = 0.21, p = .0076). The smaller nurse study, while not significant, showed a similar effect size and direction (β = 0.23, p = .21). There was an insufficient number of in-flight workers and nurses who lacked shift work, rendering the calculation of interaction with shift work impossible.

Shift-working non-flight workers from the same Finnish airline (n = 343) differed greatly compared to other groups in their level of job-related exhaustion (t test, $p = 8.91 \times 10^{-9}$ compared to the in-flight workers; Levene's test of equality, p < .05), and gender distribution (Table 1). Post hoc analysis in this group showed a nonsignificant trend of association of rs12506228 with job-related exhaustion, but an opposite direction of effect, and a weaker size of effect, in comparison to other groups (β = −0.15, p = .08). There was also no interaction with shift work (n = 343 + 193, β = −0.21, p = .13), and no association with job-related exhaustion among non-shift-working non-flight workers (β = 0.073, p = .51).

Meta-analysis Showed Association With the *MTNR1A* Variant and Heterogeneity Between Studies

Combining the results from the population-based Health 2000 cohort, the in-flight airline workers and the nurses, the fixed-effect meta-analysis for the association of rs12506228 with job-related exhaustion in shift workers reached genome-wide significance (β = 0.29, $p = 4.90 \times 10^{-8}$) (Figure 2). The meta-analysis showed, however, moderate inter-study heterogeneity (i^2 = 0.64, Q statistics p = .040). The source of heterogeneity was the Health 2000 GWAS study, which showed a bigger effect size compared to other studies (Figure 2). The three replication studies showed no heterogeneity (i^2 = 0, Q statistics p =.97) and their fixed-effect meta-analysis showed significant replication signal (n = 577, β = 0.22, p = 1.95 × 10E-4). Thus, the original association of rs12506228 with job-related exhaustion in shift workers was replicated highlighting a genetic region near *MTNR1A*, a receptor for melatonin.

Fine Mapping of the rs12506228 Genomic Region Revealed Linked SNPs and a Novel Microsatellite Marker

To investigate if the marker rs12506228 was in linkage disequilibrium (LD) with other, potentially causative variants, we carried out a haplotype analysis of rs12506228 and the surrounding region, and examined the haplotype area by capillary sequencing. By performing the haplotype analysis, we were able to narrow down the genomic region of interest to an 11-kb area centromeric to *MTNR1A* (Figure 3, Supplementary Results, Supplementary Figure S2, Supplementary Table S3). The

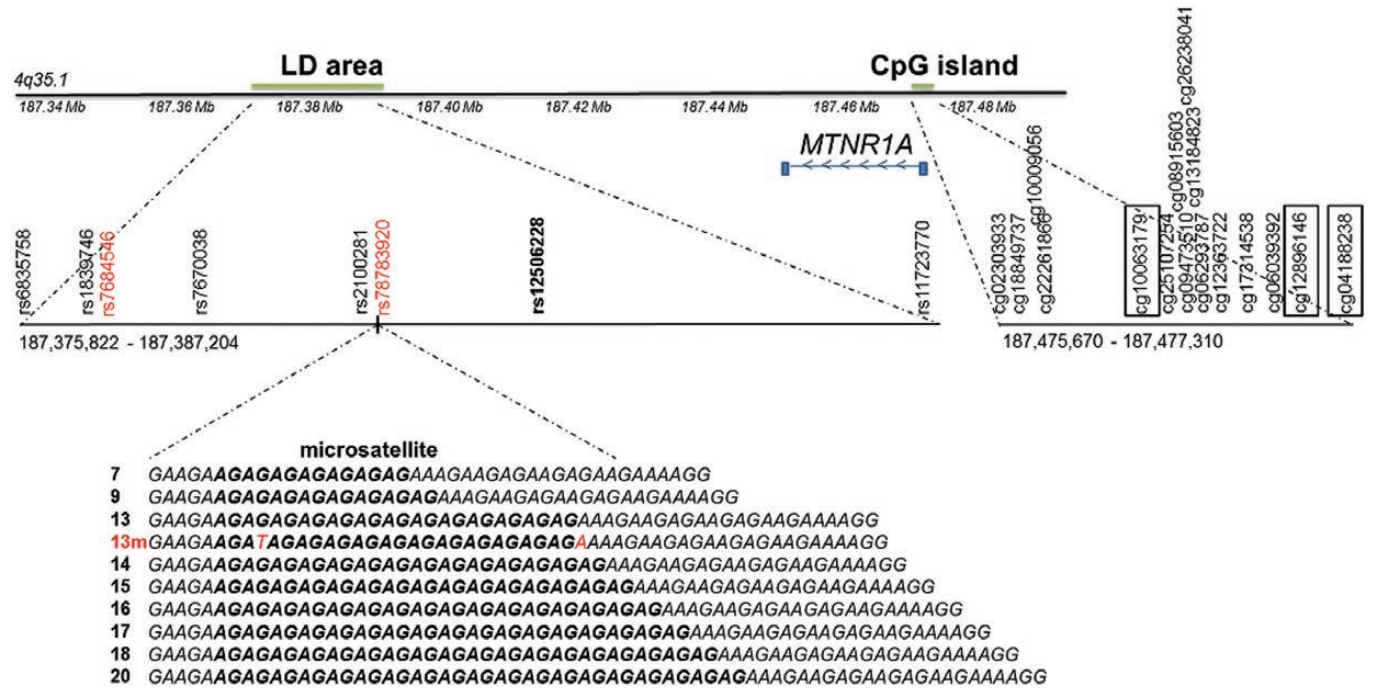


Figure 3—Genetic map of the *MTNR1A* area. Rs12506228 was part of a 7-SNP haplotype (rs6835758-rs1839746-rs7670038-rs2100281-rs12506228-rs6846710-rs11723770). Capillary sequencing of that haplotype area in 14 individuals revealed two SNPs and an allele (13m) of a novel microsatellite marker perfectly linked to rs12506228 (marked with red). In the CpG island and shores of the 5' regulatory region of *MTNR1A*, three of the 16 methylation sites (marked with box) showed nominally significant association with rs12506228. LD, Linkage disequilibrium.

sequencing of that region (bp 187 375 836–187 388 519) led to the identification of three variants which segregated fully with rs12506228: two SNPs (rs7684546 and rs78783920) and an intermediate-length allele (named here 13m) of a novel microsatellite marker with an unknown function (Supplementary Table S4). In addition, in the Genmets Illumina chip data rs12506228 showed strong LD (R^2 = 0.936) with one of the haplotype markers, rs11723770, but in the sequencing that variant was at the unsuccessfully genotyped self-chain region. The microsatellite was further characterized with fragment analysis in Vantaa 85+ and Kuopio 75+ which revealed 10 different repeat polymorphisms (Figure 3, Supplementary Table S5). Since the SNPs and the 13m allele of the microsatellite were virtually in complete LD with the tagging variant rs12506228, their effect could not be distinguished from that of rs12506228 with our study size (Supplementary Results).

rs12506228 was Associated With *MTNR1A* Gene Expression Levels

To explore if the association of rs12506228 with job-related exhaustion in shift workers is mediated through changes in gene expression, we performed an *in silico* search of the publicly available eGWAS Mayo data[34] for allele-specific expression differences in *MTNR1A* in the post-mortem human cerebellum (n = 176). The *MTNR1A* expression levels in the carriers of the risk allele A of rs12506228 were significantly reduced ($\beta = -0.26$, p = .0046). The association of rs12506228 with *MTNR1A* was the most significant of the 38 available SNPs that were situated less than 100 kb from *MTNR1A*. These findings suggest that the reduced *MTNR1A* expression linked to the risk allele is a mediating mechanism for the association of rs12506228 with job-related exhaustion in shift workers.

rs12506228 Linked to Methylation at the 5' Regulatory Region of *MTNR1A*

The interaction of rs125056228 with the shift work environment and the association of that variant with gene expression led us to search for epigenetic changes as a mediating mechanism for the detected associations. We explored the association of rs12506228 with methylation of the CpG sites available in the Illumina HumanMethylation450K BeadChip in the 5' regulatory region of *MTNR1A* (n = 16). In the meta-analysis of pilots, flight attendants, and nurses (n = 59), three of these CpG sites presented nominally significant association ($p < .05$) so that cg04188238 and cg12896146 showed higher methylation levels in association with the A allele of rs12506228 (cg04188238 $\beta = 0.148$, p = .0075, $p_{\text{Bonferroni}}$ = .114; cg12896146 $\beta = 0.15$,p = .019, $p_{\text{Bonferroni}}$ = .264) and cg10063179 lower methylation levels ($\beta = -0.141$, p = .0048, $p_{\text{Bonferroni}}$ = .074) (Figure 4, Supplementary Table S6). Thus, 3/16 CpG sites at the regulatory region of *MTNR1A* showed nominally significant associations of $p < .05$ with rs12506228, but these did not remain significant after Bonferroni correction. Many of the 16 sites are highly intercorrelated, however, thus do not represent independent tests, (eg, Spearman's $\rho = 0.632$ between cg04188238 and cg12896146, Supplementary Table S7), making Bonferroni correction a conservative approach for correction of multiple testing.

DISCUSSION

We present here a GWAS followed by replication studies to explore genetic differences in tolerance to shift work, assessed

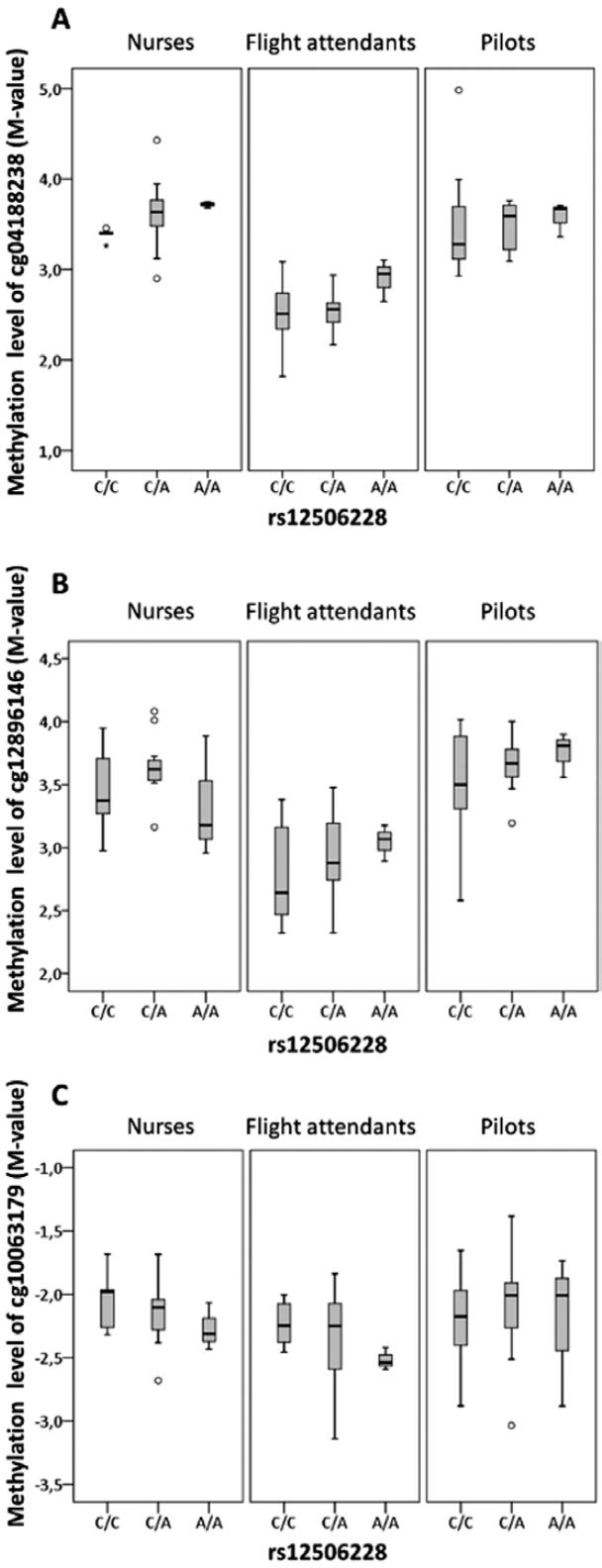


Figure 4—Rs12506228 and methylation levels of CpG sites cg04188238, cg12896146, and cg10063179 in the 5' regulatory region of *MTNR1A*. Boxplots for the methylation M-values in the groups of nurses (n = 5 (C/C) + 11 (C/A) + 3 (A/A), flight attendants (n = 10 (C/C) + 7 (C/A) + 3 (A/A), and pilots (n = 9–10 (C/C) + 7 (C/A) + 3 (A/A)) for (A) cg04188238, (B) cg12896146, and (C) cg10063179.

by measuring job-related exhaustion in shift workers. The second strongest signal identified in the GWAS, observed at rs12506228, showed two independent replications and the meta-analysis for replication studies was highly significant ($p = 2.0 \times 10\text{E-}4$). Even though no candidate genes were selected prior to investigation, that SNP finding is situated close to the gene coding for the melatonin receptor type 1A, one of two high-affinity receptors for melatonin, a hormone that serves as an indicator of our biological night and circadian rhythm. The genetic association analyses were complemented with a database search on gene expression using the eGWAS Mayo data, which showed decreased brain expression of *MTNR1A* in carriers of the rs12506228 risk allele. In addition, rs12506228 showed tendency for association with blood DNA methylation in the 5' regulatory region of *MTNR1A,* which suggests that rs12506228 may regulate gene expression through differential methylation.

We sought to find differences in job-related exhaustion symptoms specifically in shift workers, in order to estimate the ability to cope with the circadian challenge that shift work presents. The second strongest signal identified in the GWAS, rs12506228, showed significant replication in two of the replication data sets. Among the replication data sets, the general population of the Health 2000 replication cohort, in-flight airline workers, and nurses, the effect size was similar, and smaller to that in the initial GWAS study. This is likely due to the initial study's tendency to overestimate the genetic effect, as reported previously.[39] Nonetheless, among the individuals in the post-hoc study comprising non-flight workers of the airline with remarkably higher job-related exhaustion scores (Table 1), the same variant showed a nonsignificant trend of association with an opposite direction of effect. We assume that the effect of rs12506228 in most of the occupational groups is close to that in the replication studies, β (standardized) ~ 0.2, but not all special occupational groups show similar results. The background for this heterogeneity, perhaps related to the level and background of job-related exhaustion symptoms, must be studied further. Attempts to replicate this genetic association in different populations and occupational groups are encouraged.

In addition to the environment, genomic variants also affect DNA methylation.[40] The risk allele rs12506228A was suggestively linked to methylation of the CpG sites in the 5' regulatory region of *MTNR1A*. This finding might explain lower expression levels of *MTNR1A* detected in association with rs12506228A, since DNA methylation is considered to participate in the control of gene expression. In our study, however, methylation was measured from blood cells, whereas SNP-expression associations were detected in cerebellar brain tissue. The inter-individual variation in the DNA methylation patterns of the peripheral blood cells is reported to reflect that in the cerebellum of the same individual (correlation = 0.76, $p < .001$, $n = 2$).[41] When examining similarities in methylation quantitative loci (meQTL) between peripheral blood and five brain areas, significant overlap occurred which varied from 6.6% to 35.1% depending on brain area. This result was evident despite comparing different types of tissue samples from different individuals, and the donors for blood or postmortem brain samples also derived from different ancestral backgrounds.[42] Thus, we suggest that the peripheral blood serves as a surrogate for methylation differences in brain, and that rs12506228 may regulate *MTNR1A* expression in brain

via methylation differences. However, the mechanism underlying the suggestive association of rs12506228 with methylation in the 5' region of *MTNR1A* remains to be clarified, and replication for brain specimens assessed for methylation and expression data is needed to confirm this hypothesis.

Proposed Mechanisms

Association of the risk allele of rs126506228 and reduced *MTNR1A* expression was visible in cerebellum, but because of the similarities in SNP-expression associations in the cerebellum and other brain areas,[34] the association may be similar in the suprachiasmatic nucleus (SCN). This could lead to reduced melatonin signaling to SCN through type 1A melatonin receptors in the carriers of the risk allele. According to previous studies,[43–46] and discussed in[47,48], physiological melatonin signaling may stabilize the circadian rhythm against light-induced phase shifts, although some controversy remains.[49] This effect of melatonin is likely conveyed through the inhibitory effect of melatonin receptors type 1A in the SCN.[48] The relative shortage of melatonin receptors type 1A in the SCN in carriers of the risk allele of rs12506228 may, thus, lead to a higher sensitivity to nocturnal light. In night shift workers, despite nocturnal light, there is melatonin secretion when they are working their shift.[50] If the stabilizing melatonin signal is, however, reduced due to relative lack of type 1A melatonin receptors, it would lead to an increased phase-shifting effect of nocturnal light. Because most night shift workers[51] or rotational shift workers lack time to adjust their biological rhythms to the activity rhythm of their working shift, they are unlikely to benefit from a pronounced phase-shifting effect of light but it rather increases circadian disruption,[52] which could lead to symptoms of intolerance to shift work.[9,53]

Limitations and Strengths

The biggest limitation for our study is the small study size and limited power, especially in the initial GWAS study (power calculations, Supplementary Methods). Given the modest study size, our study showed a surprisingly consistent genetic association with significant replications for rs12506228 in two separate studies. One reason may have been our relatively genetically homogenous group of individuals (only Finnish subjects) which was further limited to a specific group of workers, shift workers.[54] The phenotype was also defined similarly across all studies using the MBI-GS questionnaire, a measure that was able to reveal a replicable molecular genetic risk factor among the whole worker population.[27] For example, in the search for the genetic background of depression, a phenotype that presents substantial etiological heterogeneity, the CONVERGE consortium showed that the use of a homogenous subjects, in terms of ethnicity and phenotype, resulted in greater success at finding genetic risk variants.[55] We attribute the appearance of this seemingly true positive finding, based on significant replications, to the homogeneity of our subjects. Still, false negative findings could exist but cannot be examined further in this study due to a lack of genome-wide data in the replication studies.

The initial GWAS study was originally selected for the case-control study of metabolic syndrome, which somewhat enriched that group for metabolic syndrome and did not keep it representative of Finnish population. However, covariation for the metabolic syndrome status was performed in the GWAS, and the first replication study represented the rest of the same population-based group from which the initial selection for GWAS was made. No significant differences were detected in the job-related exhaustion scores or the key covariates among cases and controls (Supplementary Table S1), and nearly similar sized effects of rs12506228 were detected when the cases and controls for metabolic syndrome in the initial GWAS study were analyzed separately (Supplementary Table S8).

A limitation of our study is the use of only subjective measures. There is no consensus on good objective measures for intolerance to shift work, even though some have measured circadian rhythms, for example, with actigraphy or cognitive performance tasks administered at different times during the shift.[9] We assessed job-related exhaustion, which is a subjective experience by nature and cannot be measured objectively, but with questionnaires like MBI-GS, a commonly used measure for burnout validated across occupations.[29] Registrations of sleep or circadian rhythm at the magnitude needed for molecular genetic studies are very resource intensive. Hypotheses created in association studies with subjective measures and more subjects may be tested in studies with experimental settings and fewer subjects.

CONCLUSIONS

Overall, we show here that genetic risk variants associating with the level of job-related exhaustion in shift workers may be identified using a GWAS-based approach. One of the most significant findings of our GWAS replicated independently in two of our replication studies, and we were also able to suggest an epigenetic regulatory mechanism for how the risk variant could reduce *MTNR1A* expression. Reduced internal melatonin signaling through decreased *MTNR1A* expression may lead to an increased sensitivity of the circadian regulatory system to nocturnal light and, therefore, diminish an individual's capacity to cope with changing schedules. To further examine the proposed model, experimental settings are necessary. Coping with irregular working hours is an important issue for our modern society and we are only beginning to shed light on the biological differences which contribute to the phenomenon.

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at *SLEEP* online.

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DISCLOSURE STATEMENT

None declared.