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Original Article

Sleep interruption associated with house staff work schedules alters circadian gene expression

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ABSTRACT

Background: Epidemiological studies indicate that disruption of circadian rhythm by shift work increases the risk of breast and prostate cancer. Our studies demonstrated that carcinogens disrupt the circadian expression of circadian genes (CGs) and circadian-controlled genes (CCGs) during the early stages of rat mammary carcinogenesis. A chemopreventive regimen of methylselenocysteine (MSC) restored the circadian expression of CGs and CCGs, including *PERIOD 2* (*PER2*) and estrogen receptor β (*ERS2*), to normal. The present study evaluated whether changes in CG and CCG expression in whole blood can serve as indicators of circadian disruption in shift workers.

Methods: Fifteen shift workers were recruited to a crossover study. Blood samples were drawn before (6 PM) and after (8 AM) completing a night shift after at least seven days on floating night-shift rotation, and before (8 AM), during (1 PM), and after (6 PM) completing seven days on day shift. The plasma melatonin level and messenger RNA (mRNA) expression of *PER2*, nuclear receptor subfamily 1, group d, member 1 (*NR1D1*), and *ERS2* were measured, and the changes in levels of melatonin and gene expression were evaluated with statistical analyses.

Results: The mRNA expression of *PER2* was affected by shift ($p = 0.0079$); the levels were higher in the evening for the night shift, but higher in the morning for the day shift. Increased *PER2* expression ($p = 0.034$) was observed in the evening on the night versus day shifts. The melatonin level was higher in the morning for both day shifts ($p = 0.013$) and night shifts ($p < 0.0001$).

Conclusion: Changes in the level of *PER2* gene expression can serve as a biomarker of disrupted circadian rhythm in blood cells. Therefore, they can be a useful intermediate indicator of efficacy in future MSC-mediated chemoprevention studies.

1. Introduction

Epidemiological studies indicate that shift workers, including nurses, pilots, and flight attendants, are at an increased risk of breast, prostate, and colon cancers. By contrast, blind people, whose biological rhythms are not regulated by light and dark cycles and whose melatonin levels are diminished, show reduced risk of these cancers [1–3]. Recent meta-analysis results further provided evidence for the positive dose–response relationship between breast cancer risk and increasing years of employment and cumulative shift work [4]. These findings are corroborated by animal studies indicating that disruption of the biological clock by exposure to constant light, light at night, pinealectomy, or jet-lag protocols (repeated disruption of circadian rhythm) increase the incidence of spontaneous and carcinogen-induced tumors and accelerates tumor growth in rat mammary tumor models [5,6]. Based on these combined human and animal data, the International Agency for Research on Cancer classified disruption of circadian rhythm by shift work as a probable human carcinogen (Type 2A) [7].

The circadian rhythm regulates biological processes ranging from gene expression to behavior in a precise and sustained rhythm controlled by a molecular oscillator that functions with a periodicity of ~24 h. The biological clock functions not only in the central pacemaker,

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the suprachiasmatic nucleus (SCN), but also in most peripheral organs and cells, including mammary glands [8] and white blood cells [9]. The SCN synchronizes peripheral clocks through humoral (eg, melatonin and glucocorticoids) and neuronal pathways upon exposure to external cues, particularly light and nutrients. In mammalian cells, the molecular oscillator is regulated by the coordinated function of interlocked transcriptional/translational feedback loops. Heterodimers of Bmal1 and Clock activate transcription by binding to E-box elements in the promoters of circadian genes (CGs), including *Per*, *Cry*, and *Rev-ErbAα* (nuclear receptor subfamily 1, group d, member 1 (*Nr1d1*)), and in numerous circadian-controlled genes (CCGs) that include growth regulatory genes, DNA damage response and repair genes, and tumor suppressor genes [2]. Previous clinical studies indicated that shift work influences the regulation of peripheral CG and CCG expression in human blood cells; however, these findings remain controversial and inconclusive [10,11].

Accumulating evidence indicates that the circadian clock prevents carcinogenesis and that frequent disruption of circadian rhythm is an important tumor-promoting factor [1,12,13]. Long-term shift work is associated with repeated phase shifts over the course of several days, leading to delayed resetting of the circadian clock. Chronic jet-lag protocols, which model long-term shift work, promote tumor development in mice by disrupting the circadian expression of major CGs (eg, period 2 [*Per2*]), activating oncogene expression, and inhibiting DNA repair signaling pathways [13]. Models of chronic jet lag also accelerate liver carcinogenesis by eliminating the rhythmic expression of *Per2* and other tumor suppressor genes [14,15]. Moreover, *in vivo* and *in vitro* studies have demonstrated that knocking out or mutating the *Per2* gene increases the incidence and accelerates the growth of spontaneous and chemical-induced tumors [1,2], suggesting that chronic disruption of normal circadian rhythm also promotes carcinogenesis. Our previous studies demonstrated that disruption of CG expression (eg, *Per2*) by a single carcinogenic dose of mammary tumor-specific carcinogen, *N*-nitroso-*N*-methylurea (NMU), ablated the rhythmic expression of several key DNA damage responsive and repair genes in rat mammary glands. The rhythmic expression of *Per2* and most DNA response and repair genes was also disrupted in the mammary glands of rats exposed to a weeklong, 12-h-advanced jet-lag protocol (unpublished data). A chemopreventive regimen of dietary *L*-methylselenocysteine (MSC), which reset the rhythms and restored the expression levels of CGs and CCGs, especially *Per2* and estrogen receptor β (*ERβ*, *Ers2*), inhibited rat mammary carcinogenesis [8,16]. These results suggested that the circadian expression of *Per2* plays a pivotal role in mammary carcinogenesis and its prevention.

Light exposure at night and sleep deprivation influence circulating levels of several hormones including melatonin (a hormone with direct oncostatic properties), cortisols, and reproductive hormones to increase the risk of breast cancer [2,17,18]. Among shift workers, melatonin secretion was strongly influenced by the number of night shifts, specific work schedule, and light intensity exposure during the study period [19–22]. Chronic reduction in melatonin levels among night-shift workers may be an important carcinogenic mechanism.

Given that up to 30% of the workforce is employed in occupations that require chronic rotation into work shifts resulting in exposure to light at night (hospital staff, flight crews, janitorial staff, firefighters, police, soldiers, factory workers, etc.), the health implications of circadian disruption may be significant [23,24]. In addition, exposure to increasing amount of light due to light pollution, television and computer monitors, smartphones, and other electronics continue to extend the daily period of light exposure [25]. Therefore, strategies that mitigate the effects of light at night on sleep patterns and disruption of circadian rhythm have the potential to significantly improve public health. Given the individual variation in phase and susceptibility to circadian disruption, finding molecular biomarkers for circadian disruption is essential to developing intervention strategies for preventing the adverse effect of chronic work schedule changes. The aim of the present study was to determine whether expression changes in CGs and CCGs in peripheral blood samples can serve as biomarkers for assessing the peripheral circadian rhythm in shift workers.

2. Materials and methods

2.1. Study design and subject recruitment

The study design was approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey (now Rutgers, the State University of New Jersey). Briefly, the goal was to recruit 15 hospital interns and residents to a crossover biomarker study. Blood samples were drawn before (6 PM) and after (8 AM) completing a night shift after seven days on a floating night-shift rotation, and before (8 AM), during (1 PM), and after (6 PM) completing seven days on the day shift. The plasma melatonin level and mRNA expression levels of *PER2*, *NR1D1*, and *ERS2* were measured in the peripheral whole blood samples.

Study volunteers were recruited among the residents and interns of the Departments of Family Medicine and Pediatrics at the Robert Wood Johnson University Hospital after consultation with the residency program director and the chief resident. Volunteers were recruited primarily by using flyers and communicating with the chief resident.

2.1.1. Subject selection criteria

When a potential subject contacted the recruiting office, the recruiter briefly screened the subject over the phone using a questionnaire to verify that they were healthy and eligible to participate in the research study. If no exclusions were present, an initial appointment was scheduled in the morning at the end of the night shift. All appointments in the Clinical Research Center (CRC) of Robert Wood Johnson Medical School were held in the Robert Wood Johnson University Hospital. Informed consent was obtained from each subject at this time. The first round of study blood samples was collected, with a fasting ChemScreen and complete blood count (CBC), to verify the absence of significant abnormalities.

2.1.2. Inclusion criteria

Healthy men and women, aged 21–34 years, who regularly work the night shift and who were not taking selenium-containing supplements were eligible for inclusion in the study.

2.1.3. Exclusion criteria

Exclusions beyond the current use of selenium supplements included pregnancy or breast-feeding, major cardiovascular conditions, major chronic lung disease, and current or past cancer therapy. Common medical conditions and their treatments such as diabetes, asthma, obesity, and use of medications, including hormonal contraceptives, were recorded but not noted for exclusion in this working population. Subjects with significant anemia or liver function test abnormalities were excluded. Subjects received the results of the laboratory tests to share with their personal physician.

2.2. Sleep logs

To enhance compliance with completing the daily sleep log, subjects received a weekly phone call or e-mail reminder, with their permission. Each sleep log documents the daily sleep and wake time, and nap and medications taken. The subjects were asked to sleep a total of at least 8 h in bed, that is, 10 PM to 6 AM for the day shift and 10 AM to 6 PM for the night shift. Fig. 1 is a representative example of a completed standard sleep log.

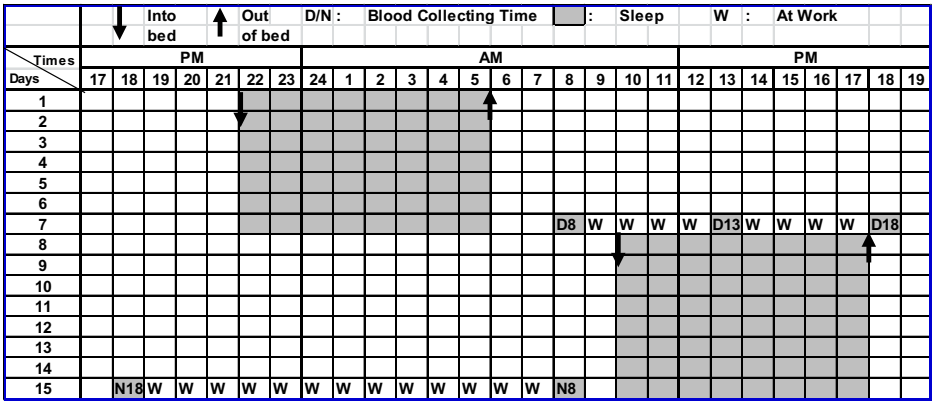

Fig. 1. Sleep log and sample collection protocol diagram. At the end of seven days on day shift, three blood samples were drawn for plasma melatonin determination and gene expression assessment at three time points, respectively. The first sample was obtained right after coming to work (~8 AM, D8), the second was obtained after lunch (~1 PM, D13), and the third was collected before leaving for home (~6 PM, D18). Following at least one week of night shift, two blood samples were obtained before (~6 PM, N18) and after (~8 AM, N8) working the night shift.

2.3. Biosample collection

Blood was sampled from 15 subjects for a crossover biomarker study. Blood samples were drawn before (6 PM) and after (8 AM) completing a night shift after at least seven days on a floating night-shift rotation, and before (8 AM), during (1 PM), and after (6 PM) completing seven days on the day shift. Samples on the night shift at midnight corresponding to noontime (1 PM) on the day shift were not collected. At each time point, 3 ml of blood from each subject was collected in a PAXgene tube (BD Biosciences, San Jose, CA, USA) for RNA extraction. Another 10 ml of blood was collected in a BD sodium heparin tube (BD Biosciences) for determining the plasma melatonin level. The project staff recovered and transported the samples to the Bionomics Research Technology Center (BRTC) at our university for registration, processing, and analysis.

2.4. Determination of blood melatonin levels

Plasma was separated from peripheral blood by centrifugation. The melatonin levels were then determined using a Melatonin ELISA Kit™ (GenWay Biotech, San Diego, CA, USA). Melatonin was extracted from the plasma samples with C18 extraction columns; then, this was incubated overnight with melatonin biotin and melatonin antiserum in a 96-well plate coated with goat polyclonal anti-rabbit immunoglobulin G (IgG) at 4 °C. After incubation with anti-biotin antibody conjugated to alkaline phosphatase, an enzymatic color reaction was observed following addition of p-nitrophenyl phosphate as a substrate. Finally, the absorption density was determined in a SpectraMax V5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm with 600–650-nm reference wavelengths.

2.5. Quantitative real-time polymerase chain reaction analysis of messenger RNA expression

RNA was extracted with a PAXgene Blood RNA MDx Kit (BD Biosciences) using a BioRobot Universal System (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed to complementary DNA (cDNA) and amplified using an Applause WT-Amp Plus ST RNA Amplification system (NuGEN, San Carlos, CA, USA). The cDNA was subjected to real-time polymerase chain reaction (PCR) amplification using a Universal Probe Library (Roche Applied Science, Madison, WI, USA) and TaqMan Universal Master Mix (Life Technology, Grand Island, NY, USA) in an ABI Prism 7900 Sequencing Detector (Life Technology), according to the manufacturer's instruction. The primers used in PCR were 3′-attggcaatgagcggttc-5′ (sense) and 3′-ggatgccacaggactccat-5′ (antisense) for *β-ACTIN*, 3′-tgtggtcccag tgtatgacct-5′ (sense) and 3′-ccgtgatggaggacttgc-5′ (antisense) for *ERS2*, 3′-gtcccacgagcaccttatg-5′ (sense) and 3′-gagtcctcatggccgttg-5′ (antisense) for *PER2*, and 3′-caactccctggcgcttac-5′ (sense) and 3′-tctgcagagacaagcaccac-5′ (antisense) for *NR1D1*. A no-template control and normal human RNA were included in each assay as negative and positive controls, respectively. *β-ACTIN* was used as an endogenous control for normalizing the data. Each assay was run in triplicate. The comparative Ct method was used to analyze the relative messenger RNA (mRNA) expression levels.

2.6. Statistical analyses

Descriptive statistics, including means, standard deviations, minimums, medians, and maximums, summarized the distribution of the data. In addition, visual inspection of histograms (not shown) confirmed approximate normality of the data and minimal outliers. Mixed linear models assessed whether the change in outcomes from the sampling time period (morning to evening) differed depending on the shift (day or night). The data from the noontime samples were excluded from this analysis, as there was no sampling corresponding to this time period during the night shift. Therefore, no shift comparisons of interest were possible with this time point. This linear model included indicator variables to represent sampling time and shift, with an interaction between the two to assess the hypothesis of central interest. A random effect for shift nested within individuals accounted for correlation between measurements. When indicated by a lower Akaike information criterion (AIC) relative to compound symmetry, the variance–covariance structure within shift was allowed to be unstructured such that the variance of the outcome varied by sampling time (morning vs. evening). This was appropriate for melatonin, but for none of the other outcomes (data not shown). Post hoc contrasts of evening and morning sampling times within shift and then shifts within sampling times were conducted. Note that, for each endpoint, data for subjects without complete data were excluded from the analyses.

3. Results

3.1. Participant characteristics

Fifteen subjects met the inclusion/exclusion criteria, including 11 women and four men ranging in age from 21 to 34 years

Table 1
Summary of sleep logs and sample collection.

Sample #	Subject ID	Age	Gender	Day Shift				Night Shift		
				Sleep time (hours) between 10 pm to 9 am	Blood Drawing Time			Sleep time (hours) between 10 pm to 9 am	Blodd Drawing Time	
					Morning	Noon	Evening		Morning	Evening
1	N01	34	F	5.5	6:45:00 AM	1:00:00 PM	6:00:00 PM	0.4	9:10 AM	7:10 PM
2	N02	27	F	7.2	8:52:00 AM	1:05:00 PM	7:15:00 PM	6.5	8:10 AM	7:45 PM
3	N03	29	M	5.8	7:30:00 AM	12:15:00 PM	7:20:00 PM	6.1	8:00 AM	7:45 PM
4	N04	28	F	Not Availabe	9:05:00 AM	12:45:00 PM	6:05:00 PM	Not Availabe	8:15 AM	7:55 PM
5	N06	26	F	7.0	8:14:00 AM	1:15:00 PM	6:50:00 PM	1.0	8:55 AM	7:50 PM
6	NO7	24	M	7.4	8:05:00 AM	1:05:00 PM	6:20:00 PM	Not Availabe	8:50 AM	7:40 AM
7	N08	27	F	4.5	8:15:00 AM	12:25:00 PM	7:30:00 PM	5.1	7:40 AM	7:35 PM
8	N10	25	F	6.9	8:05:00 AM	1:00:00 PM	7:35:00 PM	6.0	7:55 AM	7:55 PM
9	N11	28	F	7.0	8:20:00 AM	12:41:00 PM	7:32:00 PM	Not Availabe	7:55 AM	7:51 PM
10	N12	28	M	Not Availabe	8:03:00 AM	1:25:00 PM	7:59:00 PM	0.0	7:45 AM	7:24 PM
11	N14	25	F	6.3	8:05:00 AM	1:20:00 PM	7:40:00 PM	6.3	8:25 AM	7:40 PM
12	N15	29	F	8.0	6:45:00 AM	12:30:00 PM	7:00:00 PM	Not Availabe	8:40 AM	6:52 PM
13	N18	21	F	Not Availabe	7:15:00 AM	12:30:00 PM	4:50:00 PM	Not Availabe	7:35 AM	6:10 PM
14	N19	29	F	Not Availabe	7:58:00 AM	12:08:00 PM	5:35:00 PM	Not Availabe	6:44 AM	5:55 PM
15	N20	25	M	6.9	6:45:00 AM	1:30:00 PM	5:45:00 PM	3.1	8:43 AM	5:00 PM
Mean (hours)				6.6				3.8		
Standard Deviation				1.0				2.7		
Standard Error				0.3				0.8		

(mean ± standard deviation [SD] = 27 ± 3.0), who consented to participate in the study. Blood samples were collected from all 15 subjects. The requested sleep log was completed by eight of 15 study subjects, with seven subjects providing incomplete sleep logs. Among the eight participants who provided both night- and day-shift data for nighttime sleep, there was no statistically significant difference in the duration of nighttime sleep (average sleep hours between 10 PM and 6 AM in a 24-h period) across shifts ($p = 0.073$). However, three of the subjects reported substantially less sleep during the night shift (5.1, 6, and 3.8 h less), while the remaining reported similar amounts of sleep between shifts (ranging from 0.9 h less and 0.6 h more) (Table 1). Our ability to recruit and retain subjects for this study was limited by the required timing of the final blood draw (day shift: 6 PM; night shift: 8 AM), which required that the participants return to the hospital a few hours after completing the night shift.

3.2. Biomarker analyses

The plasma samples from all 15 subjects were analyzed for melatonin levels. The total RNA samples from all 15 subjects were used to determine the mRNA expression levels of the *PER2*, *NR1D1*, and *ERS2* genes. The raw means and standard deviations of expression levels of these genes are given in Table 2. The means and standard deviations of differences for day versus night shifts and morning versus evening sampling times are presented in Table 3. The latter table also presents *p*-values for these comparisons as well as for the

Table 2
Summary statistics for outcome measures at each sampling time and shift.

Shift	Sampling Time	*PER2* ($N = 14$)		*NR1D1* ($N = 14$)		*ERS2* ($N = 13$)		Melatonin ($N = 15$)	
		Mean (Std)	Min, Med, Max	Mean (Std)	Min, Med, Max	Mean (Std)	Min, Med, Max	Mean (Std)	Min, Med, Max
Day	Morning	0.87 (0.21)	0.59, 0.82, 1.29	1.12 (0.34)	0.66, 1.04, 2.12	0.79 (0.28)	0.33, 0.75, 1.33	18.6 (16.6)	0.3, 10.8, 57.2
	Noontime*	0.78 (0.23)	0.43, 0.79, 1.21	1.18 (0.51)	0.59, 1.01, 2.66	1.00 (0.54)	0.42, 0.77, 1.91	2.8 (2.9)	0, 1.7, 9.5
	Evening	0.74 (0.22)	0.39, 0.70, 1.25	1.17 (0.31)	0.67, 1.20, 1.94	0.80 (0.20)	0.44, 0.81, 1.11	5.7 (7.5)	0, 2.6, 28.7
Night	Morning	0.70 (0.16)	0.48, 0.68, 0.98	1.09 (0.49)	0.47, 0.90, 2.11	0.88 (0.41)	0.40, 0.76, 1.76	26.5 (16.7)	3.0, 26.4, 58.1
	Evening	0.98 (0.45)	0.41, 0.86, 2.23	1.16 (0.45)	0.58, 1.08, 1.88	0.88 (0.37)	0.37, 0.78, 1.51	2.7 (3.5)	0, 1.8, 10.8

* Results of samples collected at noontime were not used in statistical analysis in Table 3.

Table 3
Means and standard deviations of differences within person combined with *p*-values based on mixed linear models for comparisons of interest.

Comparison	Within	*PER2* ($N = 14$)		*NR1D1* ($N = 14$)		*ERS2* ($N = 13$)		Melatonin ($N = 15$)	
		Mean (Std)	*p*-value*	Mean (Std)	*p*-value	Mean (Std)	*p*-value	Mean (Std)	*p*-value*
Shift: day versus night	Sampling time: morning	–0.17 (0.24)	0.1269	–0.03 (0.53)	0.8472	0.08 (0.37)	0.4932	7.9 (18.7)	0.2056
	Sampling time: evening	0.24 (0.48)	0.0337	–0.01 (0.47)	0.9636	0.09 (0.49)	0.5157	–3.0 (7.3)	0.1641
Sampling time: morning versus evening	Shift: day	–0.13 (0.28)	0.2005	0.05 (0.35)	0.6349	0.00 (0.21)	0.9361	–12.8 (19.4)	0.0127
	Shift: night	0.28 (0.45)	0.0105	0.08 (0.50)	0.5025	0.00 (0.22)	0.9962	–23.8 (17.9)	<0.0001

PER2: Overall *p*-value for interaction between shift and sampling time, equivalent to testing whether difference between –0.17 and 0.24 (or –0.13 and 0.28) is different than one might expect by chance, is given by $p = 0.0079$ ($F_{1,26} = 8.29$).
NR1D1: Overall *p*-value for shift × sampling time interaction is 0.8889 ($F_{1,26} = 0.02$).
ERS2: Overall *p*-value for shift × sampling time interaction is 0.9574 ($F_{1,24} = 0.00$).
Melatonin: Overall *p*-value for shift × sampling time interaction is 0.1207 ($F_{1,28} = 2.56$).
* Gives *p*-value for specified comparison within strata (similar to a paired *t*-test).

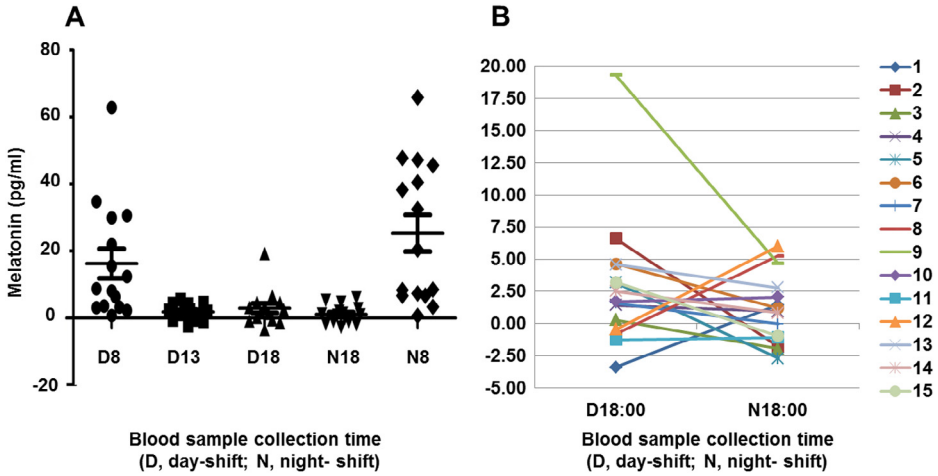

Fig. 2. Plasma melatonin levels of subjects studied. Peripheral blood samples were collected from 15 subjects at indicated time points. Plasma melatonin levels were determined with a Melatonin ELISA Kit. Each dot or line represents a result from an individual subject in the scatter (A) or line (B) chart.

interaction effect assessing the effect of shift on the difference between day and night shifts.

3.2.1. Plasma melatonin levels

Overall, the melatonin levels are significantly higher at 8 AM (D8: 18.6 ± 16.6 pg/ml; N8: 26.5 ± 16.7 pg/ml) than that at 1 PM (2.8 ± 2.9 pg/ml) and 6 PM (D18: 5.7 ± 7.5 pg/ml; N18: 2.7 ± 3.5 pg/ml), regardless of whether participants were working the day shift or night shift, with one exception. Subject No. 9 had an unusually high melatonin level (19.5 pg/ml) compared with the average level at 6 PM (N18) (Fig. 2 and Tables 2 and 3). This subject was a statistical outlier and hence excluded from the gene expression study.

The average plasma melatonin level was significantly affected by sampling time (day shift: $p = 0.013$; night shift: $p < 0.0001$), but this was not significantly related to working time (day versus night shift) (Table 3). Further, the change from morning to evening did not depend significantly on work shift ($p = 0.1207$).

3.2.2. Gene expression profiling

Steady-state mRNA levels for the CGs (*PER2* and *NR1D1*) and CCGs (eg, *ERS2*) were determined in whole peripheral blood samples before and after a floating night-shift rotation of 1 week. The results from 14 subjects were used in statistical analyses for *PER2* and *NR1D1*, and the results from 13 subjects for *ERS2* after exclusion of subject No. 9. In the day shift, the average of *PER2* mRNA levels was lower in the evening (0.74 ± 0.22) than in the morning (0.87 ± 0.21); however, in the night shift, the *PER2* mRNA level was significantly higher at night (0.98 ± 0.45) than in the morning (0.70 ± 0.16) ($p = 0.011$). This change in *PER2* mRNA expression differed significantly depending on work shift (day or night, $p = 0.0079$). A change in *PER2* mRNA expression ($p = 0.034$) during night shift (0.98 ± 0.45) versus day shift (0.74 ± 0.22) was observed based on the levels in the samples collected in the evening. However, the expression of *NR1D1* and *ERS2* mRNA was not significantly affected by sampling time and schedule changes (Fig. 3 and Tables 2 and 3). Sensitivity analyses, repeating the above linear modeling while including the incomplete data, provided similar results to those presented here.

4. Discussion

The aim of this study was to determine whether changes in the expression of CGs and CCGs in whole blood samples can serve as indicators of circadian disruption in shift workers. The available circadian biomarkers could provide a facile indication of the responses of a person's circadian regulation to work schedules, light at night, and other stressors that disrupt normal circadian patterns of gene expression. In addition, circadian biomarkers could be used to monitor individual responses to pharmaceutical, dietary, and lifestyle interventions designed to restore the circadian rhythm of the affected individual toward normal. To evaluate the potential of using CG and CCGs as circadian indicators, samples were drawn from study participants before (6 PM) and after (8 AM) completing a night shift after at least seven days on a floating night-shift rotation, and before (8 AM), during (1 PM), and after (6 PM) completing seven days on the day shift. Descriptive statistics and mixed linear models were then used to evaluate changes in the levels of melatonin and gene expression. The results showed that the *PER2* mRNA expression in blood cells was significantly affected by shift, such that the levels were higher in the evening for the night shift but higher in the morning for the day shift. Increased *PER2* expression was observed in evening samples on the night shift versus the day shift. This finding is similar to those of previous studies on CG expression in simulated or real shift workers, albeit with different study designs [10,11]. Other studies indicated that the expression of *PER2* in peripheral blood cells is influenced by the polymorphism of genes important in regulating circadian rhythm [26] and by the antioxidant status of blood [27]. Genetic polymorphisms were not examined in the present study, but they could explain the unusually high *PER2* expression levels observed in one study subject. The observation that *PER2* levels are also dependent on the antioxidant status of the blood support the present study hypothesis that antioxidants such as MSC could restore CG expression in peripheral cells of shift workers, independently of melatonin levels.

Substantial changes were not found for *NR1D1* or *ERS2*, suggesting that the expression of the latter genes was very low in blood cells at the tested time points. However, our animal studies indicated that basal *Ers2* expression levels are very low in the absence of MSC. Thus, although the changes in *ERS2* expression levels were not statistically significant between the day and night shift, a significant increase in shift workers is expected after MSC supplementation.

The melatonin level was significantly higher in the morning samples for both day and night shifts, respectively, but it was not significantly affected by shift in the current study. This finding suggested that melatonin was not significantly suppressed during night

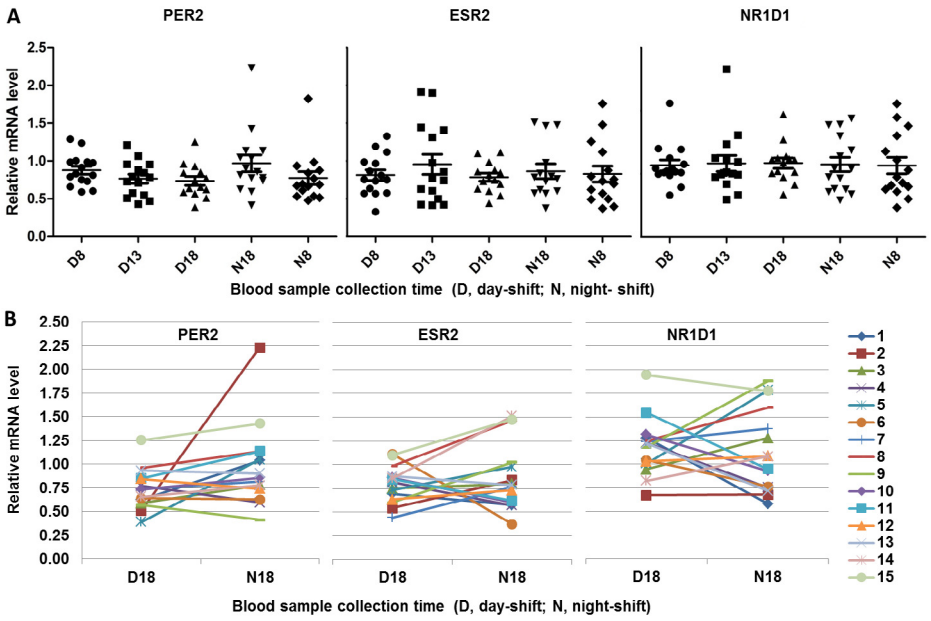

Fig. 3. Messenger RNA expression levels of CGs (*PER2* and *NR1D1*) and estrogen receptor 2 (*ERS2*) gene at different times during day shift and night shift. Total RNA was extracted from frozen peripheral blood sample and then subject to quantitative real time RT-PCR to determine the mRNA expression level. A, scatter chart showing distribution of mRNA levels of each gene at each time point in all subjects; B, line chart presenting a change of mRNA expression level of each gene after night shift compared with that during day shift in individual subject. *Y*-axis, relative mRNA expression level; *X*-axis, sample collection time.

work, which is consistent with the recent observation in Canadian [28] and Scandinavian [10] rotating-shift nurses by other researchers. However, given the small sample size, insignificant difference in actual nighttime sleep, and the failure to control confounding parameters, such as lighting conditions on the daytime versus nighttime, eating times across shifts, and individual sleeping habits (delayed vs. advanced sleep), clear-cut conclusions cannot be drawn on the association between melatonin level and shift work. Future studies will have to factor in these limitations.

Although the sample size was relatively small, we were able to demonstrate that changes in steady-state levels of *PER2* (and possibly other CGs) in whole blood cells can serve as biomarkers of circadian disruption by shift work. Accumulating evidence indicates that, in shift workers, circadian disruption is associated with increased production of oxidative stress, decreased DNA repair and immune function, gastrointestinal and metabolic disturbances, as well as neuroendocrine perturbations [29–31]. These physiological and biological changes are related to an increased risk of developing various chronic diseases, including cardiovascular diseases, metabolic syndrome (obesity and diabetes), cancer, neurodegenerative diseases, and psychological disorders [32]. Therefore, biomarkers that reflect the changes in the circadian rhythm of shift work may be early indicators of an increased risk of multiple diseases and disorders, including cancer.

Data from our preclinical studies in rats indicated that, in addition to light at night, exposure to a single dose of a chemical carcinogen also ablates circadian expression of genes involved in DNA repair and tumor suppression in the target organ, the mammary gland. Moreover, a chemopreventive dose of dietary methylselenocysteine (MSC) reset the circadian expression of genes whose expression was disrupted by the carcinogen, providing a mechanistic link between chemoprevention and circadian rhythm [16].

Translating these findings to shift workers requires developing biomarkers to monitor circadian disruption by shift work, and to assess the efficacy of chemopreventive agents in restoring a more normal peripheral rhythm. The aim of this study was to evaluate the use of CG and CCG expression in peripheral white blood cells as biomarkers for the effects of shift work on circadian rhythm. Our study showed that the differences in *PER2* expression in blood cells during morning and evening sampling times were significantly affected by shift work. Further, this indicated that working time indeed disrupted the peripheral circadian rhythm, followed by entrainment such that the peak times for *PER2* expression were moved to the morning while working the night shift. These findings suggest that *PER2* can be a reliable biomarker for the change in circadian rhythm, and it can reflect the impact of shift work on peripheral circadian rhythm, serving as an intermediate end point in longitudinal intervention studies in shift workers.

Although the best form of cancer treatment is obviously prevention, the vast majority of human chemoprevention trials directed at the population as a whole have demonstrated little or no benefit [33]. A recent example is the Selenium and Vitamin E Cancer Prevention Trial (SELECT) on prostate [34], which yielded negative–neutral results for prostate cancer [35]. Follow-up studies further suggested that, while there was no significant effect in the population as a whole, men with polymorphisms in the SEP15 selenoprotein genes or with low level of selenium may benefit [36]. These findings support the hypothesis that the use of mechanistic data to match preventive agents to specific environmental or occupational exposures may increase the success of intervention trials [33,35,37]. The present findings also provide a mechanistic basis for future studies comparing the effects of shift work on the epigenetic regulation of CG expression in shift-worker cohorts.

5. Conclusions

The differences in *PER2* during morning and evening sampling times were significantly affected by shift, such that levels were higher in the evening for the night shift but higher in the morning for the day shift, with a significant difference between night and day shift in the evening sample. The melatonin level was significantly higher in the morning samples for both shifts without a significant difference between night and day shift. These results indicate that night versus day shift has a significant impact on *PER2* gene expression,

suggesting that *PER2* expression can be used as an indicator of disrupted circadian rhythm in the blood samples of shift workers. Lack of significance in melatonin levels could be caused by the lack of significant difference in nighttime sleep and in lighting conditions across shifts. Increasing sample size, measuring lighting during night work, and recording accurate sleep logs may be helpful. From these studies, we conclude that changes in the level of *PER2* gene expression can be used as a biomarker of disrupted circadian rhythm in human blood cells, and they can be useful in longitudinal intervention studies with dietary chemopreventive agents (eg, MSC) to restore the peripheral circadian rhythm in shift workers.

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Conflict of interest

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: http://dx.doi.org/10.1016/j.sleep.2015.06.011.

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