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RESEARCH PAPER

Exploring the impact of night shift work on methylation of circadian genes

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ABSTRACT

Night shift work is associated with increased breast cancer risk, but the molecular mechanisms are not well-understood. The objective of this study was to explore the relationship between night shift work parameters (current status, duration/years, and intensity) and methylation in circadian genes as a potential mechanism underlying the carcinogenic effects of night shift work. A cross-sectional study was conducted among 74 female healthcare employees (n = 38 day workers, n = 36 night shift workers). The Illumina Infinium MethylationEPIC beadchip was applied to DNA extracted from blood samples to measure methylation using a candidate gene approach at 1150 CpG loci across 22 circadian genes. Linear regression models were used to examine the association between night shift work parameters and continuous methylation measurements (β-values) for each CpG site. The false-discovery rate (q = 0.2) was used to account for multiple comparisons. Compared to day workers, current night shift workers demonstrated hypermethylation in the 5'UTR region of *CSNK1E* (q = 0.15). Individuals that worked night shifts for ≥10 years exhibited hypomethylation in the gene body of *NR1D1* (q = 0.08) compared to those that worked <10 years. Hypermethylation in the gene body of *ARNTL* was also apparent in those who worked ≥3 consecutive night shifts a week (q = 0.18). These findings suggest that night shift work is associated with differential methylation in core circadian genes, including *CSNK1E, NR1D1* and *ARNTL*. Future, larger-scale studies with long-term follow-up and detailed night shift work assessment are needed to confirm and expand on these findings.

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Introduction

Night shift work is defined as exposure to regular night-time work between the hours of 00:00 and 05:00 for at least three hours, and can include both rotating (i.e., alternating day and night shifts) and permanent night shift schedules [1]. An estimated 1.8 million Canadians work in a schedule with exposure to night shift work, the equivalent of 12% of the working population [2]. In European and North American countries, it is estimated that 13–15% of workers are night shift workers [3]. The proportion of female workers exposed to night shift work is increasing [2], and given that night shift work has been associated with a higher risk of breast cancer [4,5], there is a pressing need to understand the mechanisms by which night shift work may increase cancer risk so as to inform the development of effective intervention/prevention strategies.

It is hypothesized that exposure to artificial light at night and changes in sleep-wake cycles due to night shift work schedules could be responsible for an increase in the risk of cancer [6,7]. This may happen through a number of inter-related biological mechanisms, including suppression of melatonin production, adverse metabolic changes, changes in sex hormone levels, and alteration of the expression of circadian genes [6,7]. Disruption of DNA methylation, which is known to influence gene expression, is implicated in the aetiology of breast cancer, including changes to the methylation of circadian genes [8–11]. However, few

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studies have investigated the impact of night shift work on circadian gene methylation, and results among studies have differed with respects to specific genes impacted and direction of effects [12–16]. In fact, most studies examined very few circadian genes [12–14].

The objective of this study was to conduct an exploration of the relationship between night shift work and circadian gene methylation by examining well-defined night shift work parameters (current status, duration/years of night shift work, and night shift intensity) and DNA methylation levels at multiple CpG loci and gene regions across 22 circadian genes.

Materials and methods

Female hospital employees from Kingston Health Sciences Center (KHSC) enrolled in a previous study [17] were re-contacted to participate in a new cross-sectional study conducted from July 2019 to March 2020. Participants were eligible if they were 1) still employed at the hospital, 2) working the same schedule as previously recorded: either a shift schedule including a night component, or day-only schedule, and 3) not pregnant. In total, 74 female employees were included who work a fixed day schedule (n = 38) or a rotating schedule including nights (n = 36). Most participants were registered nurses from inpatient units (62%), but staff from laboratory, diagnostic, and support services were also included. This cross-sectional study was approved by the Health Sciences Research Ethics Board at Queen's University, and all participants provided written informed consent.

Night shift work

Information on history of night shift work was self-reported through a questionnaire. Night shift work parameters included: a) current night shift work status; b) years of night shift work exposure (duration); and c) average number of consecutive night shifts per week over the past two years. A typical night shift schedule consisted of a rotating shift schedule of two consecutive 12-hour day shifts, followed by two consecutive 12-hour night shifts with at least three working hours between midnight and 05:00, and five consecutive free days. This schedule was variable, with some participants working rotating weeks of only night shifts or only day shifts, or a mix of day and night shifts with no set schedule (for part-time workers). However, due to hospital policy, all night shifts were 12-hour shifts from 19:00 to 07:00. The typical day worker schedule included five consecutive 8-hour shifts starting at 08:00 or 09:00.

DNA methylation

Fasting blood samples (4–5 mL) were collected by the study nurse. In order to minimize diurnal variation in lymphocyte cell counts, all blood samples were collected within the same three-hour time window (06:30 to 09:30). Blood was collected in PAXGene DNA tubes and stored in a − 20°C freezer. To isolate buffy coats, genomic DNA was directly extracted from thawed buffy coats using the QIASymphony SP (Qiagen, Crawley, UK) instrument and the QIAsymphony DNA Midi Kit (Qiagen, Crawley, UK). DNA was quantified using the Quant-iTTM PicoGreen dsDNA kit (Invitrogen), and was bisulphite converted according to manufacturer specifications. DNA was whole-genome amplified, enzymatically fragmented, purified, and applied to the Illumina Infinium MethylationEPIC beadchip (Illumina, San Diego, CA). The EPIC beadchips were analysed using the Illumina iScan system (Illumina, San Diego, CA). Quality control measures included verifying the integrity of the DNA using agarose gel electrophoresis and randomization of samples across chips and plates.

The *minfi* R package was used to perform quantile normalization of the methylation data. Multidimensional scaling plots and principal component analyses were performed to identify sources of variation among samples; no strong sources of variation were identified, and no probes were removed (see Supplementary Figure S1). Filtering was done using the methods described by Maksimovic et al [18]. We excluded probes that had detection p-values greater than or equal to 0.05 in at least one sample (n = 6,831), SNP-related probes (n = 28,767), and cross-reactive probes (n = 40,775) [19]. Since all participants were female, no probes located on the sex

chromosomes were removed. β-values were also calculated using the *minfi* package.

The array included 1150 CpG loci distributed across the 22 circadian genes, as sourced from the UCSC Genome Browser, hg19 assembly [20] (padding of ± 5000 base pairs): *CLOCK, ARNTL, ARNTL2, NPAS2, PER1, PER2, PER3, CRY1, CRY2, NR1D1, NR1D2, RORA, DEC1, BHLE41, TIMELESS, CSNK1A1, CSNK1D, CSNK1E, CSNK2A1, CSNK2A2, MTNR1A* and *MTNR1B*. These genes were chosen *a priori* because many are considered core clock genes whose protein products are essential for the generation and regulation of circadian rhythms [21].

Estimation of white blood cell type distribution

Proportions of six white blood cell types (neutrophils, monocytes, B-cells, Natural Killer (NK) cells, CD4 + T-cells, and CD8 + T-cells) were estimated from methylation data using Houseman's reference-based approach implemented in the 'estimatecellcounts2' function from the *FlowSorted.Blood.EPIC* package in R[22,] [22].

Statistical analysis

We applied multiple linear regression techniques to examine the association between night shift work parameters and continuous β-values for each CpG site. Confounders in the relationship between night shift work and circadian gene methylation were chosen *a priori* using a directed acyclic graph (DAG) (see Supplementary Figure S2). All analyses were adjusted for age (<40 years/40-54 years/55 + years), household income (<$75,000/$75,000–99,999/$100,000+), part/full-time status, occupational exposure to radiation (very often/often vs. not often), and occupational exposure to disinfectants (very often/often vs. not often). Proportion of leukocytes cell types (excluding neutrophils to avoid multicollinearity) were also included in all models to adjust for leukocyte cell profile. To account for multiple testing, q-values were calculated based on the false discovery rate (FDR) [23]. In this exploratory study, associations with a q-value less than or equal to 0.20 were deemed noteworthy.

Since core circadian genes are known to regulate downstream expression of genes related to inflammation, it is possible that inflammation could be a mediator in the relationship [24,25]. A sensitivity analysis was also conducted that did not adjust for white blood cell composition (a potential proxy for inflammation). In addition, smoking status and alcohol use are strong predictors of DNA methylation [26,27]. A sensitivity analysis was also conducted that additionally adjusted for smoking status (current or recently quit/past smoker/never smoker) and alcohol use (never or <1 drink a month/1-4 drinks a month/2 + drinks a week). We also examined the association using continuous M-values, rather than β-values. However, results were similar to our original findings and are not presented. All data analyses were conducted using R (version 4.0.4).

Results

Characteristics of study population

Current night shift workers were more likely to be younger and postmenopausal than day workers and were more likely to report working 10 or more years of night shifts (Table 1). Day and night workers were similar in terms smoking status, part-time status, and white blood cell composition. Current night shift workers were also more likely to report being regularly exposed to radiation and disinfectants, compared to day workers, more likely to have a higher household income over $100,000 CDN, and slightly more likely to report a family history of cancer (Table 1).

Night shift work and circadian gene methylation

Results for the 10 circadian CpG sites with the lowest q-values in association with current night shift status, night shift duration, and night shift intensity are summarized in Tables 2–4, respectively. Results for all 1150 CpG loci are provided in Supplementary Tables S1–3. These results are also summarized as a volcano plot in Figure 1, where loci with the strongest associations (q ≤ 0.20) are highlighted for each analysis.

Current shift workers had 1.7% higher mean methylation levels at cg14718583, compared

Table 1. Demographic data among current day and night shift study participants.

	Current day worker (n = 38)	Current night shift worker (n = 36)
Age (years) *(n, %)*		
<40	7 (18.4)	16 (44.4)
40–54	24 (63.2)	11 (30.6)
≥55	7 (18.4)	9 (25.0)
Menopausal status *(n, %)*		
Postmenopausal	12 (31.6)	14 (38.9)
Premenopausal	26 (68.4)	22 (61.1)
Education *(n, %)*		
High school	2 (5.3)	0 (0.0)
Post-secondary diploma	19 (50.0)	19 (52.8)
University undergraduate degree or higher	17 (44.8)	17 (47.2)
Household Income (in Canadian $) *(n, %)*		
<$75,000	10 (26.3)	3 (8.3)
$75,000-$99,999	9 (23.7)	8 (22.2)
≥$100,000	19 (50.0)	25 (69.4)
Smoking status *(n, %)*		
Never smoked	24 (63.2)	25 (69.4)
Past smoker	10 (26.3)	9 (25.0)
Currently smoke/recently quit	4 (10.5)	2 (5.6)
Family history of cancer *(n, %)*		
Yes	17 (44.7)	19 (52.7)
No	21 (55.3)	16 (44.4)
Missing	0 (0.0)	1 (2.8)
Alcohol consumption *(n, %)*		
≥2+ drinks/week	10 (26.3)	10 (27.7)
1–4 drinks/month	18 (47.4)	15 (41.7)
Never or <1 drink/month	10 (26.3)	11 (30.6)
Occupational exposure to radiation *(n, %)*		
Not often	32 (84.2)	19 (52.8)
Very often/often	6 (15.8)	17 (47.2)
Occupational exposure to disinfectants *(n, %)*		
Not often	15 (39.5)	1 (2.8)
Very often/often	23 (60.5)	35 (97.2)
White blood cell-type composition *(mean, SD)*		
B cells (%)	2.73 (1.40)	3.59 (1.98)
CD8 T cells (%)	6.91 (2.95)	6.95 (3.92)
CD4 T cells (%)	18.47 (4.95)	19.51 (6.40)
Natural killer cells (%)	3.14 (2.24)	2.59 (2.41)
Monocytes (%)	6.92 (2.50)	6.63 (2.16)
Neutrophils (%)	61.31 (7.40)	59.96 (7.56)
Characteristics of work schedule		
Status *(n, %)*		
Part-time	5 (13.2)	5 (13.9)
Full-time	33 (86.8)	31 (86.1)
Night shift work duration *(n, %)*		
<10 years	26 (68.4)	10 (27.8)
≥10 years	12 (31.6)	26 (72.2)
Night shift work intensity *(n, %)*		
<3 consecutive night shifts/week	-	25 (69.4)
≥3 consecutive night shifts/week	-	11 (30.6)

to day workers (95% CI: 0.87, 2.52; q-value: 0.18). Cg14718583 is located in the 5′UTR of the *CSNK1E* gene (Table 2). Individuals that worked night shifts for ≥10 years had 1.6% lower mean methylation levels at cg20667664 (95% CI: −2.33, −0.87; q-value: 0.08) compared to those that worked night shifts <10 years. Cg20667664 is located in the gene body of *NR1D1* (Table 3). Those who worked ≥3 consecutive night shifts per week had 2.2% higher mean methylation levels at cg21078679 (95%: 1.15, 3.27; q-value: 0.15) compared to those who worked <3 consecutive night shifts per week. Cg21078679 is located in the gene body of *ARNTL*.

Analyses that did not include adjustment for white blood cell composition and analyses that additionally adjusted for smoking and alcohol intake did not produce materially different results (results not shown).

Discussion

In this exploratory study, three differentially methylated loci in core circadian genes were identified among women working night shifts. We found evidence of *CSNK1E* hypermethylation in current shift workers, hypomethylation of *NR1D1* in workers with ≥10 years of night shift work duration, and hypermethylation of *ARNTL* in those who worked ≥3 consecutive night shifts per week. Our exploratory analysis suggests that different parameters of night shift work are associated with differential methylation in core circadian genes, including *CSNK1E, NR1D1* and *ARNTL*, and highlights the need for further evaluation of these genes (and others) in future studies.

CSNK1E is a member of the casein kinase I protein family, and is known to encode the kinase CK1ε, which phosphorylates a number of genes including the *PERIOD* genes *PER1* and *PER2* [28]. Phosphorylation of PER2 is one of the key circadian pacemakers of the mammalian circadian clock, and is known to be involved in controlling the timing and structure of sleep patterns [29,30]. It is unclear how hypermethylation at the 5′UTR region is related to gene expression, as it is likely to be gene dependent [31]. Both *CSNK1E* and *PER2* have been implicated in cancer, including breast cancer. Studies have found that *CSNK1E* is overexpressed in cancer tissues, while *PER2* is under-expressed, which supports the notion that *CSNK1E* promotes cancer development by downregulation of the *PERIOD* genes [10,32–35].

Table 2. Circadian gene methylation differences between current night shift and day shift workers.

Illumina ID	Chromosome	Position	Gene	Gene Group[a]	Relation to CpG Island[b]	Mean[c] methylation in day workers (SD)	Mean[c] methylation in night shift workers (SD)	Adjusted[d] mean difference (95% CI)	q-value[e]
cg14718583	22	38,725,060	CSNK1E	5′UTR	Open sea	87.72 (1.84)	88.57 (1.57)	1.69 (0.87, 2.52)	0.18
cg20667664	17	38,254,448	NR1D1	Body	North Shore	78.97 (1.65)	78.29 (1.51)	−1.53 (−2.38, −0.67)	0.52
cg12596843	11	45,864,825	CRY2	-	North Shelf	12.73 (1.28)	12.41 (0.84)	−0.89 (−1.43, −0.35)	0.67
cg23929615	22	38,794,688	CSNK1E	TSS200	Island	52.41 (2.28)	51.06 (2.09)	−2.07 (−3.34, −0.79)	0.67
cg10472395	15	61,070,548	RORA	Body	Open Sea	82.50 (1.38)	81.96 (1.45)	−1.09 (−1.80, −0.39)	0.69
cg10976861	2	239,149,937	PER2	TSS1500	South Shore	33.86 (2.11)	32.64 (1.61)	−1.58 (−2.60, −0.55)	0.69
cg26113056	15	61,521,643	RORA	TSS200	Island	8.97 (1.56)	8.57 (1.21)	−1.03 (−1.71, −0.35)	0.69
cg23965982	15	61,055,848	RORA	Body	Open Sea	83.96 (1.91)	83.44 (1.41)	−1.23 (−2.07, −0.38)	0.78
cg16774421	12	27,481,875	ARNTL2	-	North Shelf	92.20 (1.33)	91.85 (1.14)	−0.91 (−1.54, −0.27)	0.78
cg13184823	4	187,476,599	MTNR1A	TSS200	Island	38.96 (8.07)	41.24 (6.26)	5.42 (1.59, 9.25)	0.78

a. Functional region of gene as indicated in Illumina annotation.
b. Position relative to CpG Island as indicated in Illumina annotation.
c. Unadjusted mean.
d. All analyses adjusted for age, income, part/full-time status, occupational exposures to radiation and disinfectants, and leukocyte cell profile. Day workers are the referent category.
e. Q-values represent p-values adjusted for multiple testing using the FDR method.

Table 3. Circadian gene methylation differences between workers with ≥10 years of night shift work duration and workers with <10 years.

Illumina ID	Chromosome	Position	Gene	Gene Group[a]	Relation to CpG Island[b]	Mean[c] methylation in <10 years workers (SD)	Mean[c] methylation in ≥10 years workers (SD)	Adjusted[d] mean difference (95% CI)	q-value[e]
cg20667664	17	38,254,448	NR1D1	Body	North Shore	79.13 (1.73)	78.15 (1.33)	−1.60 (−2.33 – 0.87)	0.08
cg22405816	9	118,135,937	DEC1	5′UTR	Open Sea	84.68 (2.47)	83.25 (2.65)	−1.84 (−2.87, 0.81)	0.32
cg00436663	22	38,793,933	CSNK1E	Body	Island	7.54 (0.93)	7.07 (0.85)	−0.74 (−1.18, – 0.31)	0.32
cg24219929	15	60,884,748	RORA	Body	Island	9.83 (7.03)	6.37 (3.38)	−4.81 (−7.63, – 1.98)	0.32
cg18204040	12	26,279,190	BHLHE41	TSS1500	Island	10.69 (0.72)	10.20 (0.69)	−0.56 (−0.90, −0.22)	0.32
cg06233947	16	58,230,251	CSNK2A2	Body	North Shore	92.20 (1.17)	91.32 (1.49)	−1.13 (−1.81, – 0.44)	0.32
cg26724232	15	60,885,912	RORA	Body	South Shore	81.46 (3.30)	83.61 (2.65)	2.24 (0.85, 3.63)	0.32
cg09473510	4	187,476,573	MTNR1A	TSS200	Island	19.92 (8.21)	23.99 (7.45)	5.19 (1.97, 8.41)	0.32
cg13154331	22	38,795,868	CSNK1E	TSS1500	South Shore	70.52 (2.44)	71.55 (2.17)	1.52 (0.57, 2.48)	0.32
cg07250429	15	61,051,202	RORA	Body	Open Sea	84.76 (1.22)	83.98 (1.57)	−1.03 (−1.68, −0.38)	0.32

a. Functional region of gene as indicated in Illumina annotation.
b. Position relative to CpG Island as indicated in Illumina annotation.
c. Unadjusted mean.
d. All analyses adjusted for age, income, part/full-time status, occupational exposures to radiation and disinfectants, and leukocyte cell profile. Workers with <10 years of night shift work duration are the referent category.
e. Q-values represent p-values adjusted for multiple testing using the FDR method.

We found evidence of hypomethylation in the body of *NR1D1* (also known as REV-ERBα) in workers with longer duration of night shift work. *NR1D1* is involved in circadian feedback loops as a transcriptional repressor and is thought to play an essential role in circadian clock regulation [36]. Once activated, *NR1D1* controls rhythmic oscillations of *ARNTL* by suppressing its transcription

Table 4. Circadian gene methylation differences between workers with ≥3 consecutive night shifts/week and <3 consecutive night shifts/week.

Illumina ID	Chromosome	Position	Gene	Gene Group[a]	Relation to CpG Island[b]	Mean[c] methylation in <3 shifts/week workers (SD)	Mean[c] methylation in ≥3 shifts/week workers (SD)	Adjusted[d] mean difference (95% CI)	q-value[e]
cg21078679	11	13,377,829	ARNTL	Body	Open sea	85.79 (1.36)	87.71 (2.14)	2.21 (1.15, 3.27)	0.15
cg26449680	22	38,714,272	CSNK1E	TSS200	South Shore	37.84 (2.92)	40.81 (2.69)	1.71 (0.65, 2.77)	0.97
cg15603424	11	13,300,592	ARNTL	5′UTR	Island	8.10 (4.32)	12.34 (5.28)	4.72 (1.65, 7.79)	0.97
cg14718583	22	38,725,060	CSNK1E	5′UTR	Open Sea	87.97 (1.76)	89.07 (1.48)	1.65 (0.56, 2.75)	0.97
cg27004243	17	8,055,360	PER1	5′UTR	Island	6.00 (0.64)	5.45 (0.87)	−0.75 (−1.24, −0.25)	0.97
cg03092603	22	38,712,210	CSNK1E	5′UTR	North Shore	7.10 (0.72)	6.52 (0.60)	−0.72 (−1.22, −0.22)	0.97
cg23633210	2	101,600,602	NPAS2	Body	Open Sea	86.66 (1.58)	87.94 (1.36)	1.47 (0.43, 2.51)	0.97
cg17367616	12	27,485,428	ARNTL2	TSS1500	North Shore	56.72 (2.93)	55.81 (3.87)	−2.56 (−4.48, −0.65)	0.97
cg04324336	15	60,941,269	RORA	Body	Open Sea	86.00 (1.45)	84.89 (1.48)	−1.14 (−2.11, −0.18)	0.97
cg08924113	12	27,497,913	ARNTL2	Body	Open Sea	79.10 (4.68)	76.00 (7.06)	−4.30 (−7.94, −0.66)	0.97

a. Functional region of gene as indicated in Illumina annotation.
b. Position relative to CpG Island as indicated in Illumina annotation.
c. Unadjusted mean.
d. All analyses adjusted for age, income, part/full-time status, occupational exposures to radiation and disinfectants, and leukocyte cell profile. Workers with <3 consecutive night shifts/week are the referent category.
e. Q-values represent p-values adjusted for multiple testing using the FDR method.

[36]. Although the role of DNA methylation within the gene body is poorly understood, there is evidence that hypomethylation in the gene body affects gene splicing, transcription factors, and chromatin formation [31,37]. It has also been observed that many gene bodies become hypomethylated in cancer [38]. The role of *NR1D1* in cancer is less clear, but has been implicated in the development and progression of gastric cancer [39], and activation of *NR1D1* suppress proliferation of breast cancer cells [40]. *NR1D1* is more commonly implicated in glucose regulation, lipid metabolism, and regulation of inflammatory functions [36].

We found evidence of hypermethylation in the body of *ARNTL* (also known as *BMAL1*) among night shift workers working three or more consecutive night shifts a week. *ARNTL* is a known transcriptional activator which forms a core component of the circadian clock [41]. *ARNTL* forms a heterodimer with the *CLOCK* gene that initiates transcription of the *PERIOD* genes, *CRY* genes, and *NR1D1*[42]. *ARNTL* is also shown to have cancer promoting effects in breast cancer, although the specific molecular mechanism is not well understood [42]. Overexpression of *ARNTL* is shown to promote the invasion and metastasis of breast cancer cells, and hypomethylation at the promoter region has been observed in breast cancer tissue [10,42,43]. Conversely, hypermethylation at the promoter region (associated with gene silencing) is linked with the development of neoplasia, such as lymphocytic leukaemia, and ovarian cancer [44,45].

Studies that have examined the relationship between night shift work and methylation of circadian genes have produced mixed results with regards to specific circadian genes identified and direction of effects. Similar to our study, Bhatti et al. (2015) found differential methylation in the 5'UTR gene of *CSNK1E* gene [15]. However, contrary to our findings, they found hypomethylation in the *CSNK1E* gene, hypomethylation in *ARNTL* gene near the transcription start site, and found no differences in *NR1D1*[15]. Samulin Erdem et al. (2017) examined promoter methylation in female nurses with breast cancer (n = 278 cases) or without breast cancer (n = 280 controls) [12]. Similar to our study, among cases they found hypermethylation

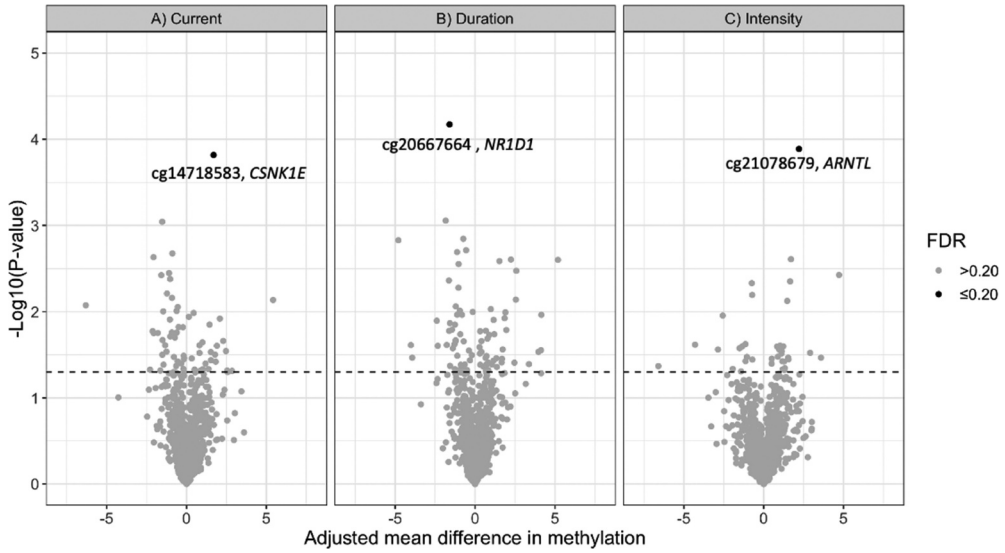

Figure 1. Volcano plot of results from the analysis of 1150 CpG loci across 22 circadian genes in association with A) current night shift work vs. day work, B) night shift work duration (≥10 years vs. <10 years) and C) night shift intensity (≥3 consecutive night shifts/week vs. <3 night shifts/week). The figure plots p-values versus the effect size (adjusted mean difference in methylation β-values). Dotted line represents p = 0.05. Loci with FDR ≤0.20 are highlighted.

in *ARNTL* in shift workers with 3 or more consecutive night shifts and <5 years of duration [12].Reszka et al. (2018) also examined promoter CpG methylation among female workers in healthcare (n = 347 rotating shift workers, n = 363 day workers) and observed that women with a longer lifetime duration of shift work (>10 years) had lower methylation in *ARNTL*, compared to those with 10 or less years of shift work duration [14]. Other studies have found that night shift work is associated with differential in methylation in other core circadian genes, including *CLOCK, PER1, PER2, CRY1*, and *CRY2*$^{-}$ [14,16].

Differences across studies may be attributable to multiple factors, including lack of adjustment for white blood cell composition, differences in confounder adjustment, and timing of blood collection. In addition, the majority of studies have compared associations among long-term night shift workers and day workers. It remains unclear how various parameters of night shift work such as cumulative night shift work (combining intensity and duration of shift work exposure) may influence circadian gene methylation. Epidemiologic studies examining night shift work and breast cancer risk suggest that female workers with a long duration of shift work (over 20 years) and a higher intensity of night shifts have the highest risk of breast cancer [4,5]. In our sample, 9 of 11 current night shift workers who reported working ≥3 night shifts a week also reported ≥10 years of night shift work duration. However, night shift workers in our sample were typically scheduled to work a rotating schedule that included two night shifts per week, meaning Individuals who opted-in to working ≥3 consecutive nights could be systematically different than those who do not. Well-powered studies with detailed assessment of night shift work and longitudinal follow-up are therefore needed to fully assess the relationship between night shift work and circadian gene methylation.

The evaluation of methylation for 22 circadian genes using microarrays is a major strength of this study. We were able to assess methylation at 1150 CpG sites within 22 circadian genes at single-nucleotide resolution. This includes extensive coverage of CpG Islands, genes, and enhancers. The use of several night shift work parameters is also a strength allowing for an assessment of how different aspects of night shift work, including years of night shift work and night shift intensity, may relate to methylation in circadian genes [1]. In general, self-reported exposure to night shift work is shown to be highly valid, limiting the potential for exposure misclassification and bias [46]. Furthermore, we carefully selected confounders using a causal model that was constructed based on hypothesized and tested relationships in the literature.

Limitations include a modest sample size, however, this is an exploratory analysis designed to explore the relationship between shift work and DNA methylation and inform future larger-scale studies. DNA methylation was only measured at one point in time, which may not reflect long-term impacts [47]. Considering DNA methylation is known to change over a person's life course and is susceptible to environmental influence, future studies should examine the relationship longitudinally at multiple time points [48]. Although differential gene expression in the identified genes has been linked to cancer, it remains unclear if the magnitude of methylation changes observed at each loci would impact gene expression and/or have downstream carcinogenic effects. Due to the cross-sectional design, selection bias (i.e., the healthy shift worker effect) is also possible [49]. Older workers who have remained in night shift work for many years may represent a cohort of healthier individuals that are tolerant to shift work, and workers more susceptible to circadian misalignment (and potentially more susceptible to disease risk) may select out of working night shifts [49]. If present, this may have attenuated our effect estimates. This also means we cannot rule out that our observed associations could be explained by reverse causation; it is possible that methylation patterns influencing adaptability to shift work (e.g., chronotype) may have affected a participant's willingness to work night shifts long-term.

In conclusion, this exploratory study suggests that various night shift work parameters are associated with differential methylation in core circadian genes, including *CSNK1E, ARNTL* and *NR1D1*. In order to better understand how night shift work may impact circadian gene methylation, large, well-powered studies with detailed assessments of night shift work and long-term follow-up are needed.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability

The data that support the findings of this study are available from the corresponding author, JA Ritonja, upon reasonable request.

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