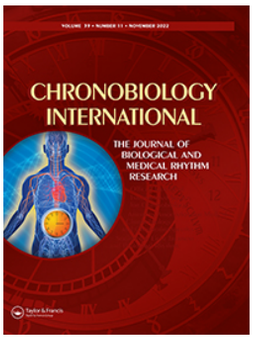

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Chronobiology International

The Journal of Biological and Medical Rhythm Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/icbi20

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To cite this article: Barbara N. Harding, Debra J. Skene, Ana Espinosa, Benita Middleton, Gemma Castaño-Vinyals, Kyriaki Papantoniou, José Maria Navarrete, Patricia Such, Antonio Torrejón, Manolis Kogevinas & Marissa G. Baker (2022) Metabolic profiling of night shift work – The HORMONIT study, Chronobiology International, 39:11, 1508-1516, DOI: 10.1080/07420528.2022.2131562

To link to this article: https://doi.org/10.1080/07420528.2022.2131562

Published online: 09 Oct 2022.

CHRONOBIOLOGY INTERNATIONAL
2022, VOL. 39, NO. 11, 1508–1516
https://doi.org/10.1080/07420528.2022.2131562

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ORIGINAL ARTICLE

Metabolic profiling of night shift work – The HORMONIT study

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ABSTRACT

Mechanistic studies are needed to understand how rotating shift work perturbs metabolic processing. We collected plasma samples (n = 196) from 49 males, rotating car factory shift workers at the beginning and end of a night-shift (22:00–06:00 h) and day-shift (06:00 h-14:00 h). Samples underwent targeted LC-MS/MS metabolomics and concentrations of 130 metabolites were log_2-transformed and pareto-scaled. An elastic net selected the most influential metabolites for linear mixed models examining within-person variation in metabolite levels at night-shift end (06:00 h) compared to day-shift start (06:00 h). Quantitative enrichment analysis explored differentially enriched biological pathways between sample time points. We included 20 metabolites (amino acids, biogenic amines, acylcarnitines, glycerophospholipids) in mixed models. Night-shift was associated with changes in concentrations of arginine (geometric mean ratio [GMR] 2.30, 95%CI 1.25, 4.23), glutamine (GMR 2.22, 95%CI 1.53, 3.24), kynurenine (GMR 3.22, 95%CI 1.05, 9.87), lysoPC18:2 (GMR 1.86, 95%CI 1.11, 3.11), lysoPC20:3 (GMR 2.48, 95%CI 1.05, 5.83), PCaa34:2 (GMR 2.27, 95%CI 1.16, 4.44), and PCae38:5 (GMR 1.66, 95%CI 1.02, 2.68). Tryptophan metabolism, glutathione metabolism, alanine metabolism, glycine and serine metabolism, and urea cycle were pathways differing between shifts. Night shift work was associated with changes in metabolites and the perturbation of metabolic and biochemical pathways related to a variety of health outcomes.

ARTICLE HISTORY
Received 24 June 2022
Revised 26 September 2022
Accepted 27 September 2022

KEYWORDS
Circadian; rotating shift work; shift worker; metabolomics; metabolism; occupational health

Introduction

Rotating night shift work is an increasingly common work scheduling arrangement. Night shift work results in circadian misalignment where the circadian timing system is misaligned to the behavioral sleep/wake and feeding/fasting cycles. Circadian rhythms drive a multitude of biological processes in the human body. These rhythms are regulated by a central clock in the suprachiasmatic nuclei (SCN) in the brain and peripheral clocks in virtually all tissues (Mohawk et al. 2012; Potter and Wood 2020). Circadian rhythms are influenced primarily by the 24-h light dark cycle (Papantoniou et al. 2014), but can also be influenced by timing of sleep, meals, and physical activity (Gabriel and Zierath 2019; Gangwisch 2009; Potter and Wood 2020; Wehrens et al. 2017).

Circadian clocks control changes in metabolism across the 24 h day (Davies et al. 2014; Mohawk et al. 2012), and there is an established link between night shift work, disrupted circadian rhythms and cardiometabolic disorders (Hulsegge et al. 2019; McAlpine and Swirski 2016; Scheer et al. 2009). However, the mechanisms underlying associations between night shift work and cardiometabolic disorders remain unclear. Therefore, mechanistic studies are needed to provide insight into how rotating night shift work may perturb metabolic processing.

Metabolic profiling (metabolomics) has the ability to characterize metabolic phenotypes that may be associated with night shift work and disease pathology (Davies et al. 2014; Hancox et al. 2021). Leveraging metabolomic profiling allows for the investigation of genotype and environmental effects, integrating information derived from changes at the gene transcript level, the protein level and the posttranslational modification level together (Raamsdonk et al. 2001). The few studies that have incorporated metabolomics into analyses of night shift work have found that the normal rhythmicity of metabolite concentrations is absent or shifted

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following night shift work, indicating these changes may be due to the shifted behavioral cycles rather than the phase of the central SCN circadian clock (Kervezee et al. 2019, 2018; Skene et al. 2018). Moreover, these analyses only included data from simulated night shift studies, and data from few participants (n = 14 in Skene et al. 2018, n = 9 in Kervezee et al. 2019 and n = 8 in, 2018).

We undertook the present study to examine how shift work is related to changes in the plasma metabolomic profile within a population of male rotating shift workers who worked both day and night shifts in a slow backward rotation. We hypothesized that night shift work would up or down regulate metabolites relative to day shift work and be associated with biological pathway perturbations.

Methods

Study population

This study included 49 adult male backward rotating (counterclockwise) shift workers from the HORMONIT study who were working at a car factory in Barcelona, Spain (HORMONIT n.d.). Participants rotated through 3 weeks of night shifts (22:00–06:00 h), followed by 3 weeks of evening shifts (14:00–22:00 h) and 3 weeks of early morning shifts (06:00–14:00 h). All participants worked five-day work weeks from Monday-Friday followed by two days off on Saturday and Sunday. Participants were sampled on two days. One day of sample collection occurred during an early morning shift on the 2nd or 3rd week of the early morning shift rotation. The other day of sample collection occurred during a night shift, again on the 2nd or 3rd week of the night shift rotation. A venipuncture blood sample was taken at the beginning and end of each work shift on both sample collection days, resulting in a total of up to four blood samples collected for each study participant.

The study protocol was reviewed and approved by The Parc de Salut Mar Clinical Research Ethics committee (#2015/6351). All participants were given a leaflet with study information and signed an informed consent form. A total of 71 men volunteered for the study, of whom 7 were found to be ineligible and 8 withdrew prior to study start due. Ultimately, 56 participants were enrolled in the study, of whom 49 had complete metabolomic data and are included in the following analyses.

Covariates

At each of the two sample collection points, participants responded to questionnaires collecting data on demographics, work-related aspects, lifestyle factors such as smoking, alcohol use, caffeine use and dietary habits, medical history and medication use and sleep-related information. We also collected information on daylight length (hours) on the days that blood samples were collected (using values available from the National Oceanic and Atmospheric Association calculator and inputting the latitude 41° 230 N and longitude 2° 100 E for Barcelona) (US Department of Commerce et al., 2005). Furthermore, participants wore an actigraphy device (Actigraph 2 GT3X+, USA) capable of measuring triaxial acceleration during the 24 h of the early morning shift collection period and during the 24 h of the night shift collection period to collect information on daily step counts and sleep (Madrid-Navarro et al. 2019). Finally, information on the urinary 6-sulfatoxymelatonin (aMT6s, the major melatonin metabolite) was collected from 24-h urine voids. Using the 24-h urine voids, we applied a cosinor analysis to plot the aMT6s level throughout the 24-h period of data collection and then extracted data on the aMT6s level corresponding to the clock hour of blood collection (Harding et al. 2022).

Outcomes

Participants provided blood samples at both the start (06:00 h) and end (14:00 h) of the early morning shift and the start (22:00 h) and end (06:00 h) of the night shift. Targeted metabolomic analysis was performed on a total of 196 plasma samples using the AbsoluteIDQ® p180 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria), and a Waters Xevo TQ-S triple-quadrupole mass spectrometer coupled to an Acquity UPLC system (Waters Corporation, Milford, MA, USA). Methods for these analyses have been described in detail elsewhere (Honma et al. 2020; Skene et al. 2017). Briefly, plasma samples were prepared according to the manufacturer's instructions adding several stable isotope–labelled standards to the samples prior to derivatization and extraction. Using reverse-phase UPLC-MS/MS (ultra-performance liquid chromatography/mass spectrometry) or FIA-MS/MS (flow injection analysis-MS/MS), 185 metabolites from 5 different compound classes (namely acylcarnitines, amino acids, biogenic amines, glycerophospholipids, and sphingolipids) could be quantified (Davies et al. 2014). Sample order was randomized and three levels of quality controls (QC) were run on each plate.

The metabolite levels in each QC were compared to the expected values and the percent coefficient of variation (CV%) was calculated. Metabolites where >25% of concentrations were below the limit of detection, out of range, or the QC2 coefficient of variance was >30%, were

excluded (n = 50). Values < 1E −10 were replaced by 1E-10 for computational purposes. The remaining 130 quantified metabolites comprised 5 acylcarnitines, 21 amino acids, 8 biogenic amines, 13 lysophosphatidylcholines, 69 glycerophospholipids, and 14 sphingolipids.

Statistical analysis

Prior to undertaking statistical analyses, we applied log transformation (base 2) to all 130 metabolite values due to the log-normality of the data values. Metabolomics data are commonly normalized or scaled, which is done to reduce systematic variation and better ensure true biological variation can be revealed (Fan et al. 2019). We applied a pareto-scaling procedure utilizing Metaboanalyst 5.0 (Pang et al. 2021). Pareto-scaling was done to give each metabolite a similar distribution and reduce the influence of the larger metabolite values, as pareto-scaling will decrease larger fold changes more than smaller fold changes (Eriksson et al. 1999; van den Berg et al. 2006; Worley and Powers 2013). We examined basic differences for all 130 metabolites between the four time points using a one-way repeated measures ANOVA. We also examined differences in metabolite concentrations between the two time points that coincided in clock time (start of the day shift at 06:00 h and end of the night shift at 06:00 h) using paired t-tests. All p-values from the ANOVA and paired t-tests were corrected using a Benjamini Hochberg correction to control for the false discovery rate (FDR) due to multiple comparisons. To allow more potential metabolite differences to be found in these exploratory analyses, an FDR < 0.1 was considered significant. For all comparisons, we summarized the top metabolite differences between the time points using tables and box plots.

To pick the best subset of the 130 metabolites to include in subsequent mixed models investigating differences in metabolite levels between shifts, we fit an elastic net model including the two collection points that coincided in clock time (06:00 h at the beginning of the early morning shift, and at the end of the night shift). We used the R package *glmnet* to fit the elastic net, using the binomial family option (Friedman et al. 2010). For the elastic net, an elastic net mixing parameter, α, equal to 0.85, and a λ value equal to 0.05 were chosen using leave one out cross validation (LOOCV) and included in the model. Twenty metabolites were selected by the elastic net model, including amino acids (alanine, arginine, aspartate, citrulline, glutamine, phenylalanine), the biogenic amine kynurenine, acylcarnitines (carnitine and tetradecenoylcarnitine), and glycerophospholipids (lysoPCaC18:2, lysoPCaC20:3, lysoPCaC26:0, lysoPCaC28:0, PCaaC32:0, PCaaC34:2, PCaaC40:3, PCaeC30:0, PCaeC36:4, PCaeC38:1, and PCaeC38:5).

Using the 20 metabolites selected from the elastic net, we fit a linear mixed model to examine within-person differences in metabolite levels during the night shift compared to the day shift as the reference. The mixed model was adjusted for hours of daylight on the day of sample collection, aMT6s levels at the time of sample collection, 24-h step count, smoking, and duration of sleep in the 24 hours prior to sample collection. Beta coefficients were exponentiated and results are presented as Geometric mean ratio (GMR).

Quantitative enrichment analysis

To identify biological pathways that may be differentially enriched between time points, we utilized the MetaboAnalyst 5.0 Quantitative Enrichment Analysis module. With this module, we used enrichment analysis to look for groupings of metabolites in the same biological pathway that differed significantly between samples collected at the beginning of a day shift compared to the end of a night shift (both 06:00 h in clock time). To undertake the enrichment analysis, the $\log_2$-transformed concentrations for all 130 metabolites in our set were matched to their Human Metabolome Database identifier, uploaded to MetaboAnalyst 5.0, pareto scaling was applied, and their concentrations by shift schedule were compared to metabolite sets found in 99 Small Molecule Pathway Database (SMPDB) human metabolic pathways. Differences in pathways between shifts were quantified with Benjamini Hochberg FDRs, as an indication of how likely the pathway perturbation is related to true biologic variation, and not chance alone. The top five pathways found to be most different between day shift and night shift that included at least three metabolites from our set were further investigated to determine if the pathway may have biological relevance to shift work.

Results

Participants in this study were males with a mean age of 38 (SD ± 9) years. More than half of the population had a BMI ≥ 25 kg/m^2 (53%). A large portion of participants reported smoking during their day (63%) or their night (59%) shift. Participants exercised more during their day shift than their night shift (Table 1).

When examining differences in metabolite concentrations between the two time points that coincided in clock time (start of the day shift, 06:00 h and end of the night shift, 06:00 h), we found significant differences (FDR < 0.1) for several glycerophospholipids (lysoPCaC18:2, lysoPCaC20:4, PCaaC32:0, PCaaC34:2, and

Table 1. Characteristics of HORMONIT participants (N = 49).

Variable	Mean (SD) or %
Age, years	38 (9)
BMI kg/m^2	
< 25	47%
25–30	35%
≥ 30	18%
Education	
Primary	29%
Professional	71%
Smoking	
Smoking during day shift	63%
Smoking during night shift	59%
Minutes of daylight	
Minutes of daylight day	719 (103)
Minutes of daylight night	766 (114)
6-Sulphatoxymelatonin level	
6-Sulphatoxymelatonin mesor day shift	7.7 (3.4)
6-Sulphatoxymelatonin mesor night shift	7.1 (4.0)
Daily step count	
Step count day shift	20014 (3382)
Step count night shift	18998 (3225)
Sleep duration	
Sleep duration day shift (h)	6.0 (1.6)
Sleep duration night shift (h)	6.1 (1.9)

lysoPCaC20:3) and amino acids (alanine, citrulline, tryptophan, and phenylalanine) (Table 2). When visually examining the difference in concentration levels from the five metabolites with the most pronounced differences at the 06:00 h time points, the majority of metabolites appeared to be lower at the start of the day shift than they were at the end of the night shift (lysoPCaC18:2, lysoPCaC20:4, PCaaC32:0, and citrulline) (Figure 1).

Results from the linear mixed models showed several metabolites with intra-individual differences in their concentrations at the end of the night shift (06:00 h) compared to the reference day shift sample (06:00 h) (Table 3). This includes the amino acids arginine (GMR 2.30, 95%CI 1.25, 4.23) and glutamine (GMR 2.22, 95% CI 1.53, 3.24), the biogenic amine kynurenine (GMR 3.22, 95%CI 1.05, 9.87), and the glycerophospholipids lysoPCaC18:2 (GMR 1.86, 95%CI 1.11, 3.11), lysoPCaC20:3 (GMR 2.48, 95%CI 1.05, 5.83), PCaaC34:2 (GMR 2.27, 95%CI 1.16, 4.44), PCaeC36:4 (GMR 2.04, 95%CI 1.00, 4.15), and PCaeC38:5 (GMR 1.66, 95%CI 1.02, 2.68). In all instances, these metabolites were higher at the end of the night shift period compared to the start of the day shift period at the same clock hour.

We included the top five pathways that contained at least three of the metabolites in our metabolite set. These included: tryptophan metabolism, glutathione metabolism, alanine metabolism, glycine and serine metabolism, and the urea cycle. Table 4 outlines the pathways that were found to differ between day shift and night shift, including information on the biological relevance of the pathway.

Discussion

We used a repeated design to compare metabolomic profiles among individuals working a night shift rotation compared to a day shift rotation in a real-world setting, and found that night shift work perturbed metabolism. In particular, we found that the levels of several amino acids, several glycerophospholipids and one biogenic

Table 2. Top 20 metabolites with the greatest differences in metabolite concentrations (μM) between the start of the day shift and end of the night shift (06:00 h).

Metabolite	Class	P value	FDR	Concentration (μM) at start of day shift (06:00 h), median (IQR)	Concentration (μM) at end of night shift (06:00 h), median (IQR)
alanine†	amino acid	0.00	0.00	406.0 (346.0, 465.0)	292.0 (255.0, 360.0)
lysoPCaC18:2†	glycerophospholipids	0.00	0.00	60.2 (49.5, 76.4)	82.4 (65.0, 102.0)
lysoPCaC20:4†	glycerophospholipids	0.00	0.00	9.5 (7.5, 11.7)	12.0 (10.2, 13.9)
PCaaC32:0	glycerophospholipids	0.00	0.06	9.7 (8.7, 10.8)	10.9 (9.3, 13.0)
citrulline†	amino acid	0.00	0.06	34.1 (28.7, 40.6)	41.6 (33.8, 51.0)
PCaaC34:2	glycerophospholipids	0.00	0.06	149.0 (136.0, 160.0)	159.0 (145.0, 168.0)
tryptophan†	amino acid	0.00	0.06	72.0 (65.1, 86.2)	85.1 (70.3,93.9)
lysoPCaC20:3	glycerophospholipids	0.00	0.06	3.7 (3.3, 4.5)	4.5 (3.4, 5.9)
phenylalanine†	amino acid	0.01	0.08	66.7 (57.0, 73.0)	72.3 (63.4, 85.8)
PCaaC36:2	glycerophospholipids	0.01	0.12	127.0 (118.0, 135.0)	134.0 (124.0, 143.0)
ornithine	amino acid	0.01	0.12	60.5 (52.3, 69.7)	66.3 (56.3, 83.5)
butyrylcarnitine	acylcarnitines	0.01	0.12	0.20 (0.15, 0.23)	0.22 (0.18, 0.27)
lysoPCaC28:1	glycerophospholipids	0.01	0.12	0.26 (0.21, 0.29)	0.27 (0.23, 0.33)
lysoPCaC18:1	glycerophospholipids	0.02	0.16	31.3 (25.1, 38.0)	34.3 (28.9, 42.5)
arginine†	amino acid	0.02	0.16	90.7 (83.9, 107.0)	104.0 (83.4, 124.0)
PCaaC34:1	glycerophospholipids	0.03	0.21	121.0 (113.0, 134.0)	128.0 (119.0, 140.0)
SM(OH)C16:1	sphingolipids	0.03	0.21	3.7 (3.1, 4.5)	4.0 (3.7, 4.6)
lysoPCaC26:1	glycerophospholipids	0.03	0.21	0.10 (0.08, 0.13)	0.11 (0.10, 0.12)
SMC16:0	sphingolipids	0.03	0.21	135.0 (114.0, 151.0)	149.0 (125.0, 165.0)
PCaeC42:5	glycerophospholipids	0.03	0.22	2.0 (1.7, 2.3)	2.1 (1.7, 2.4)

† indicates that these metabolites were also included in the list of 20 top metabolite differences from the one-way repeated measures ANOVA analysis

Table 3. Mixed model results from the start of the day shift and end of the night shift sampling time points that coincide in clock time (06:00 h).

		Minimally adjusted[a]				Fully adjusted[b]			
Metabolite	Class	GMR	95%CI		P value	GMR	95%CI		P value
alanine	amino acid	0.98	0.54	1.79	0.95	0.70	0.25	1.98	0.51
arginine	amino acid	1.71	1.15	2.54	0.01	2.30	1.25	4.23	0.01
aspartate	amino acid	0.99	0.93	1.06	0.79	0.97	0.88	1.07	0.56
citrulline	amino acid	2.08	1.30	3.33	0.00	1.94	0.92	4.08	0.08
glutamine	amino acid	1.64	0.85	3.18	0.14	2.22	1.53	3.24	0.00
phenylalanine	amino acid	1.26	0.64	2.47	0.50	1.66	0.63	4.37	0.30
kynurenine	biogenic amine	1.69	0.75	3.83	0.21	3.22	1.05	9.87	0.04
carnitine	acylcarnitines	1.16	0.57	2.39	0.68	1.06	0.38	2.95	0.91
tetradecenoylcarnitine	acylcarnitines	0.60	0.34	1.08	0.09	0.84	0.33	2.12	0.71
lysoPCaC18:2	glycerophospholipids	1.70	1.11	2.59	0.02	1.86	1.11	3.11	0.02
lysoPCaC20:3	glycerophospholipids	1.77	1.06	2.94	0.03	2.48	1.05	5.83	0.04
lysoPCaC26:0	glycerophospholipids	1.03	0.59	1.80	0.92	0.65	0.28	1.53	0.33
lysoPCaC28:0	glycerophospholipids	1.90	1.15	3.14	0.01	0.74	0.30	1.80	0.51
PCaaC32:0	glycerophospholipids	1.45	0.88	2.40	0.15	0.96	0.43	2.13	0.91
PCaaC34:2	glycerophospholipids	1.54	0.54	4.44	0.42	2.27	1.16	4.44	0.02
PCaaC 40:3	glycerophospholipids	0.80	0.59	1.08	0.15	0.93	0.48	1.81	0.82
PCaeC30:0	glycerophospholipids	2.18	1.33	3.55	0.00	1.06	0.34	3.26	0.92
PCaeC36:4	glycerophospholipids	1.00	0.64	1.57	0.99	2.04	1.00	4.15	0.05
PCaeC38:1	glycerophospholipids	1.19	0.77	1.83	0.43	1.38	0.67	2.86	0.38
PCaeC38:5	glycerophospholipids	1.33	0.92	1.92	0.12	1.66	1.02	2.68	0.04

[a]Adjusted for daylight, [b] adjusted for daylight, aMT6s level, step count in last 24 hours, smoking in last 24 hours and sleep duration during last 24 hours

amine were higher during the night shift than the day shift after at least one week of adaptation.

In our mixed model, which included the confounders of daylight, aMT6s levels, daily step count, smoking, and sleep duration, two non-essential amino acids, arginine and glutamine, were found to be significantly different between the day and night shift. The former is important in immune function, has a putative role in preventing or treating circulatory diseases, is produced and used by the body when fatigued, and is important in the formation of urea (PubChem n.d.-a); the latter is important for the regulation of ammonia levels in the body, and is necessary for a range of vital functions, including muscle growth, immune system support, and synthesizing neurotransmitters, nucleotides, and nucleic acid (PubChem n.d.-b). Five glycerophospholipids, a class of glycerol-based phospholipids that are the key component of biological membranes, and important for signal processing and induction (PubChem n.d.-c), also differed significantly between day and night shift. In a previous simulation study by Skene et al. (2018) (3-day simulated day shift sleeping period 22:00 h-06:00 h, 3-night simulated night shift sleeping period 10:00 h-18:00 h), similar differences in the levels of several amino acids and glycerophospholipids after simulated night shift work were observed, providing evidence that the circadian rhythm of many metabolites shifted as a result of night shift work. In the same study, Skene et al. (2018) also found an alteration in kynurenine levels associated with night-shift work, a biogenic amine found to be significantly different in our mixed model.

Several pathways that were identified as being perturbed in our dataset from the quantitative enrichment analysis have also been found to be perturbed in other shift work and sleep studies, adding to the evidence of their potential disruption due to working at night. Bhat et al. (2020) and Davies et al. (2014) reported on tryptophan metabolism being altered in both rats and humans experiencing sleep deprivation, with sleep deprivation also increasing production and accumulation of kynurenic acid, which is the byproduct of tryptophan metabolism and is related to adverse neurological outcomes (Bhat et al. 2020; Davies et al. 2014). In a simulated shift work study by Skene et al., serine, glycine, alanine, and glutathione pathways were found to be enriched in day workers compared to night workers, similar to our finding in rotating shift workers (2018).

A strength of this study was the sampling strategy employed. By design, we sampled the same participants both when they were in their 2nd or 3rd week of day shift and again when they were in their 2nd or 3rd week of night shift work (repeated design). While some individuals are capable of faster and more complete adaptation to night shift work than others, we expected that during the 2nd or 3rd week of the night shift, the majority of participants would exhibit metabolite rhythms that were nearly opposite to the "normal" daytime conditions due to the shifting of their central SCN clock and their peripheral clocks (Pickel and Sung 2020; Skene et al. 2018). Although we are unable to comment on the rhythmicity of metabolites in our study (having only collected samples at two time points on any given day) we did observe that the concentrations of

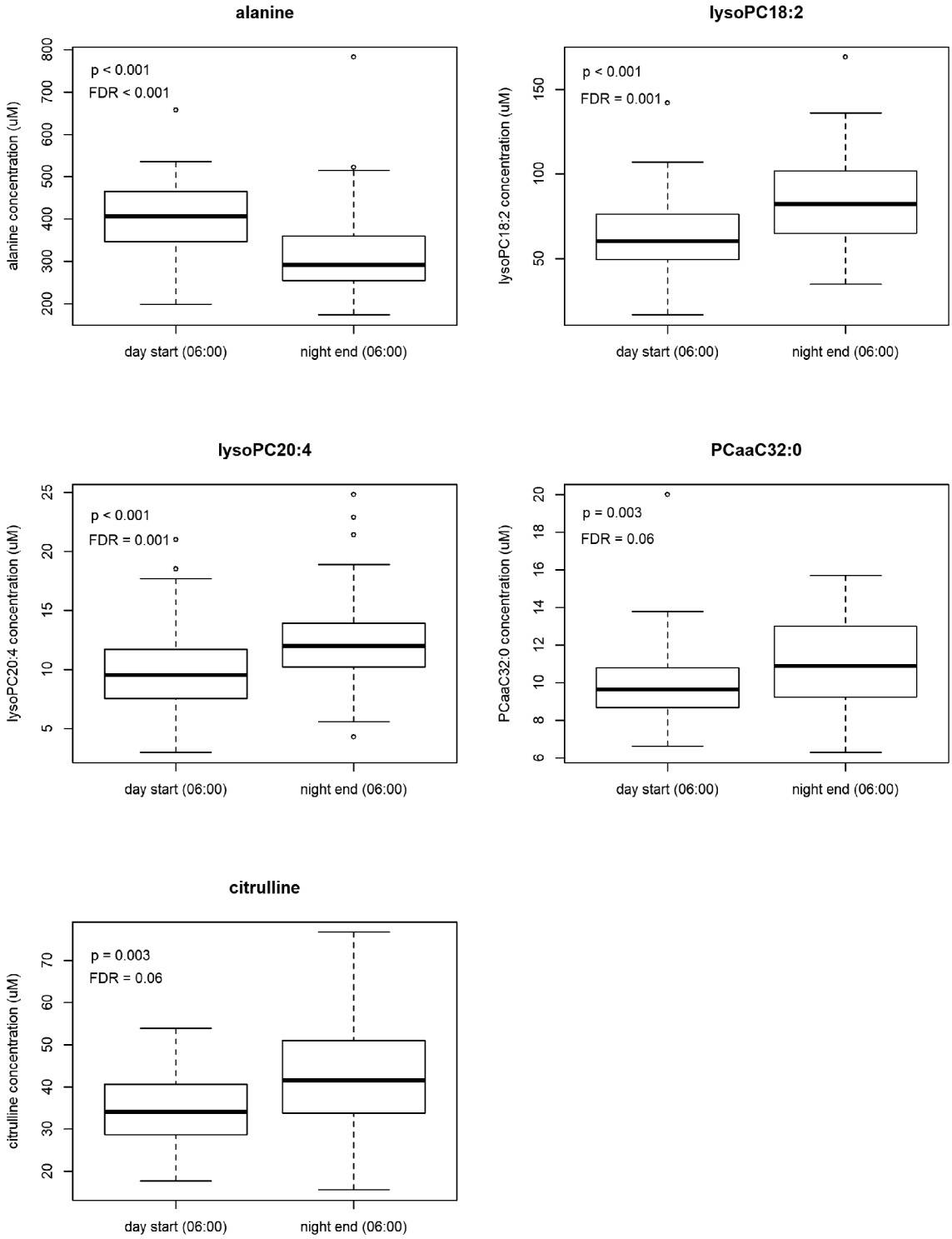

Figure 1. Boxplots showing raw metabolite concentrations (μM) at the start of the day shift and end of the night shift sampling time points that coincide in clock time (06:00 h) for the top 5 metabolite differences using t tests.

several metabolites were significantly altered in the night shift period compared to the same clock hour in a day shift period, even with presumed biological adaptation to the night shift.

When we examined metabolite levels at each of the four sample time points, we noted that for the majority of metabolites, the concentration decreased over the working day during the day shift and similarly during the night shift. Interestingly, for the majority of metabolites, the metabolite concentrations at the start of the night shift were higher than at the start of the day shift (Supplemental Figure 1). To hypothesize the reason for

Table 4. Findings from enrichment analysis. Using normalized and scaled data from the start of the day shift and end of the night shift sampling time points that coincide in clock time (06:00 h).

Pathway name	Total Compound	Hits	Raw p	FDR	Biologic relevance
Tryptophan Metabolism	60	5	0.00	0.03	TRP regulates immunity, neuronal function, metabolic stress, intestinal homeostasis
Glutathione Metabolism	21	3	0.00	0.03	GSH provides antioxidant defense, nutrient metabolism, regulates cellular events
Alanine Metabolism	17	3	0.00	0.03	ALA is used for protein biosynthesis, provides energy under fasting conditions
Glycine and Serine Metabolism	59	7	0.01	0.08	GLY/SER provide essential precursors for proteins, nucleic acids, lipids
Urea Cycle	29	7	0.02	0.15	Converts excess ammonia (from amino acid catabolism) into urea in the liver cells

this finding, both internal and external factors must be considered. Internal biological cues, related to circadian rhythms or diurnal variation could drive differences in the metabolites that are tested at differing clock times (06:00 h versus 22:00 h). However, external factors must also be considered. Prior to starting a 06:00 h early morning shift, participants likely wake up and go straight to work. In contrast, participants may have eaten, engaged in physical activity, or a variety of other social activities in the hours leading up to the night shift start (22:00 h). Prior research has shown that feeding is an external cue that exerts one of the strongest influences on the synchronization of peripheral clocks both in rodents and humans (Damiola et al., 2000; Pickel and Sung 2020; Wehrens et al. 2017) and that feeding and meal timing results in acute changes of some metabolite levels. In addition, time of sleep onset, sleep duration and other lifestyle habits influence metabolite levels (Dollet and Zierath 2019). With our study design, we are unable to separate out how these varying external cues may impact metabolite levels. While measured perturbations to the metabolomic profile may be due to lifestyle habits, timing/quality of meals, or physical activity, these are downstream effects of night shift work. Therefore, our findings provide further evidence that night shift work, either through changing dietary, lifestyle, sleeping, and other habits, and/or through disruption to internal biological rhythms, perturbs the metabolomic profile.

This study has several strengths including its inclusion of a larger cohort of real-world rotating shift workers. Prior metabolomic studies included a smaller sample size and shift work was simulated in controlled laboratory conditions. We also have repeated measures that controls for individual differences, with data on metabolite levels available at four different time points per participant, combined with data on many covariates and potential external cues, allowing us to further account for factors that may influence acute changes in plasma metabolite levels. This study also has limitations including the fact that we had only four time points where we were able to determine metabolite concentrations (two samples per shift). Therefore, we are unable to say if our findings truly reflect a shift in the timing of the metabolite rhythms, or if our results can be explained fully or in part by acute effects of the feeding/fasting state, sleep deprivation or other lifestyle behaviors on the day of sampling (Davies et al. 2014). Despite our careful efforts in taking two samples during each shift, discrete sampling cannot track shifted peripheral clock rhythms. While we asked participants to report eating times during the day and night shifts when urine samples were collected, unfortunately these data were not able to be included due to high degree of missingness and lack of clarity on whether the times reported were AM or PM. Recognizing that feeding/fasting state and timing of meals can be related to metabolite rhythms, collecting more complete meal data will be an important area to improve for future studies.

Additionally, this study only included men. It should also be noted that the targeted metabolomics kit and the reverse-phase UPLC-MS/MS platform used is restricted to analyzing five metabolite classes, being enriched for glycerophospholipids. Considering the established link between night shift work, obesity and cardiometabolic dysfunction, this metabolomics platform was appropriate. We found several lipids were altered, but caution is advised in not over-interpreting the influence of night shift on lipid levels because of the features of the metabolomics kit chosen. Although reverse-phase UPLC-MS/MS is considered the gold standard in metabolomics due its reproducibility and ease of use, polar metabolites, for example, sugars, organic acids, and nucleotides/nucleosides cannot be measured. To measure polar metabolites a hydrophilic interaction liquid chromatography (HILIC) system comprised of UPLC coupled to a triple-quadrupole mass spectrometer operating in both positive and negative electrospray ionization modes would be needed.

In summary, we found that night shift work was associated with changes in the levels of several amino acids, glycerophospholipids and one biogenic amine, and the perturbation of primary metabolic and biochemical pathways. These changes are related to a variety of health outcomes ranging from

cardiometabolic and cancer-related, to neurologic and kidney or liver function. Although additional research is needed, particularly among larger diverse populations and with biological samples collected during several time points throughout a shift, these findings provide insight into additional mechanistic pathways that may explain the associations between night shift work and many common non-communicable diseases.

Acknowledgements

The authors thank Emilia Molinero, Mayte Martín Bustamante and Elena Juanola Pages from the Generalitat de Catalunya for all of their support in planning this study. The authors also thank Ms. Holly-May Lewis and the Metabolomics Core Facility at the University of Surrey. In addition, the authors thank the study participants for their important contributions.

Disclosure statement

JMN, PS and AT work at the Occupational Health service of the car factory, which was the setting of the present study. Within the HORMONIT study working group they express their own views and do not represent the company. All other authors have no disclosures.

Funding

The study was partially supported by the Instituto de Salud Carlos III-FEDER [PI14/00444]. ISGlobal acknowledges support from the Spanish Ministry of Science and Innovation through the "Centro de Excelencia Severo Ochoa 2019-2023" Program [CEX2018-000806-S], and support from the Generalitat de Catalunya through the CERCA Program.

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Data availability statement

The data underlying this analysis is available at: https://github.com/bakermarissa/HORMONIT

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