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Undetermined implications of chronutrition: A missing curriculum in medicine

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Undetermined implications of chronutrition: A missing curriculum in medicine

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Editorial: Undetermined implications of chronutrition: a missing curriculum in medicine

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KEYWORDS

chronobiology, chrononutrition, sleep, chronic diseases, zeitgeber

Editorial on the Research Topic
Undetermined implications of chronutrition: a missing curriculum in medicine

Chronobiology (a.k.a. circadian biology) studies the rhythmicity of physiological processes throughout a 24-h cycle in any living organism. The body's timekeeping system is composed of a central (suprachiasmatic nucleus) clock and many peripheral (organ-specific) clocks, responsible for aligning body functions to timing photic (light/darkness) cues and non-photic (food intake) external cues called zeitgebers (1, 2). Even though chronobiology dates back to the Hippocratic era, its recognition as a scientific biomedical discipline began recently with the pioneering work conducted by Jeffrey Hall, Michael Rosbash, and Michael Young on the molecular mechanisms that govern circadian rhythmicity, research that earned them the Nobel Prize in 2017 (3). It is noteworthy that the circadian rhythm is controlled by transcriptional–translational feedback loops of clock genes and proteins that further interact with a well-orchestrated neuroendocrine system, connecting both central and peripheral clocks (4). However, the empirical observation of an "appetite-satiety" circadian rhythm that governs the body's energy balance, sleep, cardio-metabolic function, and many other nutrition-sensitive metabolic processes gave rise to chrononutrition as a scientific discipline. Chrono-disruptive eating behaviors have been implicated in many health disorders including sleep disturbances, cardiometabolic risk, unbalanced energy mobilization, body temperature deregulation, weight gain, psychosocial distress, and redox imbalances (3–5). Conversely, a substantial body of evidence indicates that meal timing, diet quality, and the regular intake of key dietary chronobiotics (e.g., phytomelatonin, tryptophane, short-chain fatty acids, and retinoic acid) help to synchronize internal clocks, reinforcing the idea that long-term dietary interventions with these food bioactives may be effective on restoring the body's circadian rhythmicity and metabolic homeostasis (5, 6).

In this special topic of *Frontiers in Nutrition*, new evidence has been gathered on the state of the art of chrononutrition and its implications. While remaining an emerging discipline in the biomedical curriculum, these studies offer valuable information in the form of narrative/systematic reviews, epidemiological studies, preclinical evidence, and clinical studies in this field. In their narrative review, Gangitano et al. documented the role of specific nutrient intake (chrono-disruptors or positive regulators) in the structure and

quality of sleeping patterns as a bidirectional phenomenon related to obesity and glycemic abnormalities. The authors particularly emphasize the role of the hormonal circuit that connects to this bidirectional axis (evidence from preclinical studies) and the beneficial effect of specific nutrients [e.g., tryptophan (Trp): large neutral amino acids (LNAA) ratio] and the negligible effectiveness of intermittent fasting (IF) protocols (clinical evidence). Regarding the latter, He et al. reported the short-term immunomodulatory, anti-obesogenic, and biochemical effects of IF protocols (mostly randomized-controlled trials), concluding that IF protocols induce modest immunomodulatory effects in healthy people and those with special physiological (e.g., pregnancy) and underlying pathophysiological (e.g., obese) conditions, but that such effects come from different mechanisms. Lastly, Qu documented, succinctly yet in-depth, the molecular crosstalk between the circadian clock and cancer development (e.g., oncogene primordial targeting)/progression (e.g., chromatin remodeling and angiogenesis) and its therapeutic implications, explicitly reviewing the effectiveness of IF and chrono-chemotherapy in cancer.

Epidemiological studies represent the first step when searching for scientific evidence linking the circadian rhythm with the health-disease continuum. In observational studies involving adults, it has been stated that night-shifting, evening chronotypes, and late sleep or meal timing are strong chrono disruptors that may lead to weight gain, hypertension/dyslipidemia, chronic inflammation, and ultimately cardiometabolic diseases and type-2-diabetes (7). However, little is known about the impact of chronodisruption in young or metabolically compromised populations. Juliana et al. studied the chrononutrition behavior of 409 young (21.5 $\pm$ 2.2 y old). Malayan participants during the COVID-19 pandemic, observing similar eating patterns to other young populations, variable sleep patterns, and a scarce yet significant chrono-disruptive eating pattern in participants with low body weight. Kuwahara et al. aimed to document the causal association of breakfast styles (Japanese, Western, and cereal consumers) with sleeping/eating patterns and lifestyle factors in Japanese children (aged 3–8 years), observing that those eating Japanese style breakfast > Western style breakfast and morning > evening type have better sleep and eating patterns. On the other hand, Teoh et al. followed 20 healthy primigravids during the second and third trimesters of pregnancy, documenting their eating patterns/style (3-day food records) and melatonin and cortisol levels (saliva, 5-time points in 24 h) during both periods; the authors' findings suggested that certain chrononutrition-related behaviors (e.g., eating window and breakfast skipping) have a significant influence on maternal melatonin and cortisol rhythmicity and so, targeting these intake behaviors may help to restore the circadian rhythm of melatonin and cortisol.

In the second and third stages of epidemiological research, the molecular/physiological mechanisms and benefits of chrononutrition interventions are commonly documented; this Research Topic includes two experimental studies using murine models and three clinical and community interventions that offer evidence in this regard. Zou et al. aimed to analyze the transcriptomic/metabolomic alterations in the rhythmic transcriptome and metabolism of meibomian glands (MGs; eyelid sebaceous glands) of C57BL/6J mice fed a balanced diet or a high-fat diet (HFD) and maintained in a 12/12 h dark/light cycle. The authors observed that HFD induces a chrono-disrupted state characterized by altered rhythmic oscillations of lipid components (enriched signaling pathways: glycerolipid/glycerophospholipid/ether metabolism, lipid storage deviations) but that HFD did not induce desynchrony of the light-regulated central clock pacemaker. Trebucq et al. induced a transient 12/12 h light/dark (LD) desynchronization state mimicking a chronic jet lag (CJL) condition to evaluate the ultimate effects on daily energetic homeostasis and weight gain, observing that this chronic misalignment causes glycemic abnormalities (nocturnal hyperglycemia, glucose intolerance, and hyperinsulinemia), high LDL, and weight gain. As for interventions with humans, Jacob et al. evaluated the eating behaviors/timing and certain psychological traits in 301 overweight/obese middle-aged participants (56% women) in weight loss programs, documenting that late eating is associated with a higher total energy intake and suboptimal eating behaviors, worsening factors associated with a higher risk of obesity. Marciniak et al. evaluated the plausible causal relationship between sex, chronotype, and age with saliva cortisol and dehydroepiandrosterone (DHEA) 64 h-rhythmicity after 1-day fasting in 49 obese individuals (50% women) observing mainly sex and chronotype-specific phasing alterations. Lastly, Albreiki et al. investigated the possible effect of melatonin supplementation on the transient fluctuation of plasma leptin and subjective appetite rating in nine young male eutrophic participants in a randomized three-way (two light exposures, one melatonin supplementation) cross-over overnight (6 PM-6 AM) study. The authors documented a positive impact of exogenous melatonin on subjective hunger and desire to eat and plasma leptin levels, despite a greater efficacy using MT1/MT2 receptor agonists to achieve appetite control.

Author contributions

RR: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing—review and editing. AG: Formal analysis, Supervision, Validation, Writing—review and editing. AW-M: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing—original draft, Writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acute impact of light at night and exogenous melatonin on subjective appetite and plasma leptin

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This study investigates the possible effect of exogenous melatonin on appetite control by investigating plasma leptin and subjective appetite parameters. Nine healthy male participants [26 ± 1.3 years, body mass index (BMI) 24.8 ± 0.8 kg/m^2] (mean ± SD) were recruited. The study was designed as a randomized three-way cross-over design; light (>500 lux) (LS), dark (<5 lux) + exogenous melatonin (DSC), and light (>500 lux) + exogenous melatonin (LSC), with an interval of at least 7 days between each session. Each session started at 18:00 h and ended at 06:00 h the following day. Participants were awake and in a semi-recumbent position during each clinical session. The meal times were individualized according to melatonin onset from 48 h sequential urine collection, whereas melatonin intake was given 90 min before the evening meal. Subjective appetite parameters were collected at 30 min intervals during each session. Plasma leptin was collected at specific time points to analyze pre-prandial and postprandial leptin. Subjective hunger and desire to eat were reported higher in LS than DSC and LSC (P = 0.03, and P = 0.001). Plasma leptin showed a significant increase in LSC and DSC (p = 0.007). This study suggested a positive impact of exogenous melatonin on subjective appetite and plasma leptin.

KEYWORDS

metabolism, melatonin, light at night, leptin, appetite

Introduction

Artificial light at night (ALAN) has grown exponentially over modern societies' natural nocturnal lighting levels. Although ALAN has provided substantial benefits to humankind, the adverse biological impacts of ALAN have been widely investigated (1–3). Extensive studies have been conducted to understand ALAN's mechanism and health hazards against human physiology. The link between ALAN and disruption of circadian rhythm has been well established (4, 5), which can contribute to alterations in metabolism (6, 7), and increased risk of chronic disorders such as obesity and type 2 diabetes (8). Furthermore, light can directly affect endocrine signaling from circadian dysregulation or impaired melatonin production (9).

Melatonin has been associated to various biological processes due to its widely distributed melatonin receptors. Various studies have linked melatonin to lipid and glucose metabolism (10, 11), vascular function (12), appetite (13), and behaviors (14). Therefore, melatonin suppression as a result of ALAN may potentially have an impact on metabolism. Melatonin acts as a hormonal mediator of photoperiodic information, regulating energy homeostasis by balancing energy intake and energy expenditure. Add to this, the diversity of melatonin binding sites in gastrointestinal tracts suggest various possible function of melatonin in appetite regulation.

Leptin is a hormone that is mainly released by adipose tissue to maintain energy balance and appetite regulation. Melatonin's role in the release of leptin has been highlighted in several animal studies. The administration of melatonin in middle-aged goldfish and rats results in a decrease in plasma leptin levels, and this impact was inverted in goldfish by the administration of a melatonin antagonist (15, 16). Other contradictory results revealed that consistent light exposure in rats could increase food intake consumption (17) or abolish day/light variation of leptin levels (18). Despite the controversial results in animal studies, the majority of studies suggested that melatonin administration may increase the circulating leptin levels (19). The role of melatonin in controlling appetite and weight has been discussed by Buonfiglio et al. (20) showing that deletion of melatonin receptor type 1 in the hypothalamus was associated with the malfunction of leptin signaling and leptin resistance (20).

There are few and controversial findings from human studies that discuss the impact of melatonin on appetite hormones such as leptin and ghrelin. Figueiro et al. (21) reported a boost in leptin levels and a drop in ghrelin in sleep-restricted individuals after morning light exposure (21). Similarly, an increased sense of hunger (22, 23), and reduced leptin (24, 25) were associated with sleep restriction or sleep debt. A recent study has shown a reduced body weight and body mass index (BMI) among overweight night shift workers after melatonin supplementation (26), whereas eating habits among female night shift workers with excessive weight have not changed after melatonin administration (27). In addition, a recent meta-analysis included seven clinical trials and 244 cases did not support the melatonin impact on body weight and appetite (28). We have previously shown that exogenous melatonin/ALAN was associated with reduced glucose tolerance, insulin insensitivity, and changes in lipid profile among healthy young males (10). We hypothesize that exogenous melatonin can alter subjective appetite and plasma leptin. Therefore, this study was conducted to assess the effects of exogenous melatonin administration on subjective appetite and plasma leptin among healthy young males.

Abbreviations: ALAN, artificial light at night; αMT6s, 6-sulfatoxymelatonin; CIU, clinical investigation unit; PSQI, Pittsburgh sleep quality index; HÖ, Horne-Östberg; RIA, radioimmunoassay; TAUC, total area under the curve; DLMO, dim light melatonin onset.

Methods

Ethic statement and recruitment

The ethics committee at the University of Surrey approved all parts of the study (UEC/2015/021/FHMS). The methodology and experimental design were discussed during an induction session for participants who met the inclusion and exclusion criteria (**Supplementary material A**). The chosen participants signed a written consent form before attending the clinical session, confirming they were aware of the potential hazards and discomforts. All included participants were a young male student from University of Surrey (Guildford, UK). All participant information was tagged and rigorously kept in accordance with the Data Protection Act (1998).

Screening procedures

Participants were required to complete multiple questionnaire such as general health questionnaire, Horne-Ostberg (HÖ) evaluation, Pittsburgh sleep quality index (PSQI), Munich chronotype questionnaire (MCTQ), and daily sleep diary. HÖ was completed to assess the chronotype of participants (29). PSQI evaluates the sleep quality assessment for all participants using PSQI was evaluated (30), whereas Munich chronotype was completed to evaluate sleep schedule during working/free days (31). Before the clinical session, participants wore Actiwatches to track their sleep-wake cycle. Furthermore, participants were not on night duties, or crossed more than 2 time zones in the month prior to the study session.

Caffeinated beverages, alcohol, strenuous activity, and analgesic medicine usage were prohibited for 24 h prior to the laboratory sessions. In addition, cosinor analysis

was used to determine acrophase of 6-sulfatoxymelatonin (aMT6s) (Stockgrand Ltd., University of Surrey, Guildford, UK), by analyzing a 48-h sequential urine collection from all participants. This will assist in determining the rising phase of participants' endogenous melatonin, thus allowing meal intakes (supper) to be individually assigned for each participants (**Supplementary materials B,C**). Participants who met the inclusion and exclusion criteria, plus passing the screening procedures were allowed to join the clinical sessions.

Meal and circadin timings

Table 1 shows the composition and macronutrients of meals served during the clinical session. Breakfast was served at 08:00 h, whereas lunch and the evening meal "supper" were tailored to the acrophase time of urinary aMT6s. All meals were served at the Clinical Investigation Unit, University of Surrey. The average fasting period between lunch and the super was 9–10 h. Circadin prolonged-release melatonin (Neurim Pharmaceuticals Ltd.) containing 2 mg melatonin was utilized to maintain increased melatonin levels for 8–10 h (32). Circadin was administered orally 90 min before the supper to ensure sufficient melatonin levels throughout the session (**Supplementary material B**).

Study session

Participants were randomized using a three-way cross-over design protocol: light session (LS) (>500 lux), light + Circadin session (LSC) (>500 lux) and dark + Circadin session (DSC) (<5 lux), with at least 7-day washout period. Each session began at 18:00 h and ended at 06:00 h the next day. Participants were assigned to one of two groups: A or B. Group A attended LS first, followed by DSC and then the LSC session, whereas Group B started with LSC first, followed by DSC and then the LS session (**Supplementary material D**). Sequencing effects were statistically evaluated in the data. **Figure 1** depicts the study protocol procedures. During the investigation, both body movement and posture were rigorously controlled. The participants were instructed to remain semi-recumbent (being at around 45°). They were also allowed to use the toilet following sample collection. Nonetheless, they had to rest in a semi-recumbent position for around 15 min before the next sample collection to ensure that all participants' bodily motions and energy consumption were consistent during the study sessions. The light intensity and irradiance were recorded and maintained during the three sessions. The **Supplementary Table E**. 1 shows total photon flux (photons/cm2/cm), light intensity (lux), and irradiance (w/m2).

At 08:00 h, each laboratory session began with breakfast. Participants were allowed to leave the CIU after breakfast, but to return at lunch time. After lunch, all participants were asked to stay at the CIU until the clinical session starts. The lunch and late evening test meals were tailored to the estimated melatonin onset from urinary aMT6 acrophase timing. Circadin was given 90 min before the late evening test meal in the DSC and LSC sessions to ensure that melatonin levels remained elevated throughout the session. Plasma leptin was collected hourly, beginning at 18:00 h and continuing until the late evening test meal. The samples were taken every 15 min for the first hour following the meal, then every 30 min until the session was over. The meal VAS questionnaire was recorded using a visual analogue scale (VAS PRO-Diary) at 30 min intervals throughout the session (**Figure 1**). VAS-Pro-Diary measured three types of subjective appetite: hunger, desire to eat, and fullness. Human leptin radioimmunoassay kit from Millipore Company (Billerica, MA, USA) was used to measure plasma leptin. The inter-assay and coefficient variation for plasma leptin was 4.1% for low-level quality control and 6.7% for high-level quality control. In contrast, Intra-assay for low and high-level quality controls were 5.5 and 4.2%, respectively.

TABLE 1 Carbohydrate, protein, fat, fiber, and energy for each of the meals and overall composition of all three meals.

Meal/g	Energy Kcal	Protein (g)	CHO (g)	Fat (g)	Fiber (g)
Breakfast	627	15	98	16	14
Lunch	927	25	115	38	19
Test meal "Supper"	1,066	38	104	54	7
Total	2,620	78	317	105	40
% composition*		15%	59%	19%	7%

*Percentages were calculated proportionally from the total daily consumption.

Statistical analysis

Based on data from a prior investigation (7), a power calculation was performed using PS software (Vanderbilt University, Nashville, TN, US) with a power of 80% and a significance level of 0.05. This power estimate indicated that 12 or more participants were required; however, data sets were only retrieved from n = 9. The cosinor analysis to determine the peak of aMT6s was performed using a built-in calculation developed by Dr. D S Minors at University of Manchester (**Supplementary material F**).

Normality test using the D'Agostino-Pearson omnibus was assessed (GraphPad, San Diego, CA, USA). Mean, standard deviation, and standard error were calculated for all data. All hormonal and metabolic data were subjected to three-factor repeated measures (light, melatonin, and time) ANOVA, followed by Tukey's honest significance test using statistical analysis software (SAS) software SAS Institute Inc., Cary, NC, USA. The total area under the curve

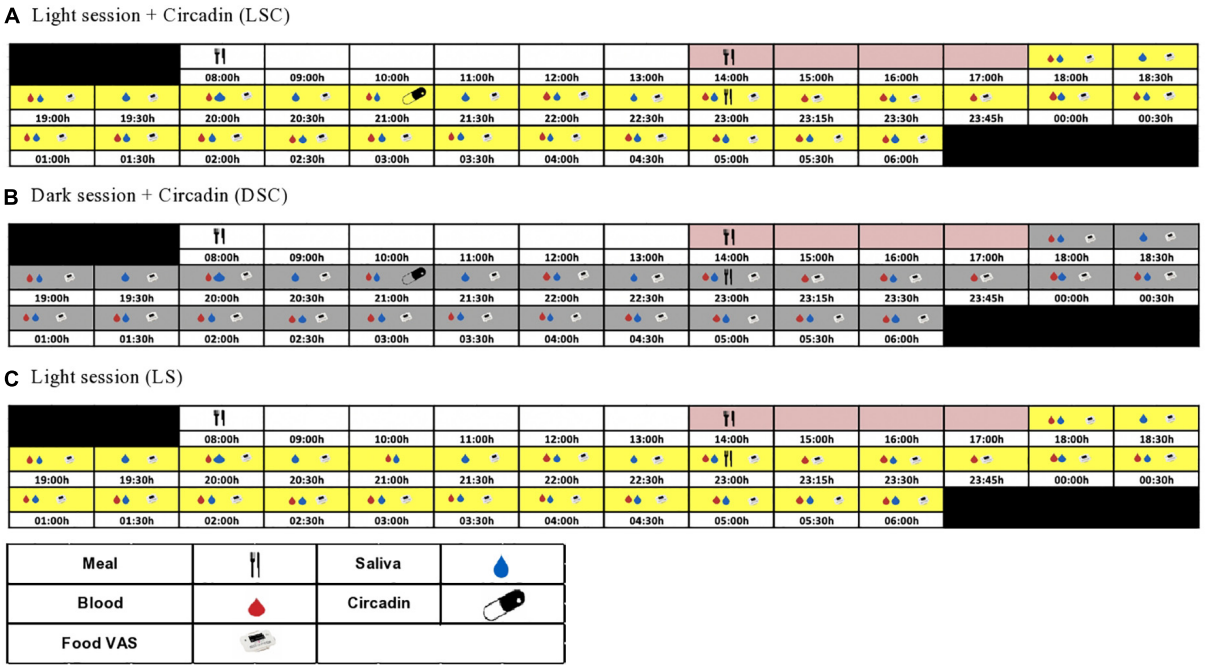

FIGURE 1
Study protocol. The schematic figure represents the study protocol for a participant with plasma melatonin onset at 20:30 h. All interventions (see key) were relative to each participants' melatonin onset.

(TAUC) and Incremental area under the curve (IAUC) were calculated using the trapezoidal and incremental rule (TAUC and IAUC). TAUC and IAUC were used to examine the hormonal and metabolic data, which was then followed by one-way ANOVA and Tukey's multiple comparison testing. The level of significance was fixed at $p \leq 0.05$.

Results

Twelve participants were recruited for this study, and only nine completed the study. All nine participants were males with an average age of 26 ± 1.3 and BMI of 24.8 ± 0.8 (Table 2). Participants' demographics, such as smoking and caffeine consumption, are shown in Table 2. The average score of PSQI was 3.5 ± 0.4, indicating good sleep quality, whereas HÖ scored 54.5 ± 2.6. In addition, three participants were classified as moderate morning type, whereas the remaining six were neither morning nor evening type. The mid-sleep time during the free days ranged between 03:30 to 06:30 h, and the average of all participants was 04:46 ± 00:22. Sleep parameters analyzed by Actiwatches showed no significant difference in sleep latency, efficiency and fragmentation index prior to each session (Supplementary material G). Participants have a sleep duration of approximately 6 h, with sleep efficiency above 70% and a fragmentation index over 40.

Subjective appetite scores

Figure 2 shows the repeated measure ANOVA of subjective hunger, desire to eat and fullness score in all three sessions (Figure 2). Participants felt more hunger and desire to eat in LS

TABLE 2 Participant demographics.

	All participants ($n = 9$) (mean ± SEM)
Age (year)	26 ± 1.3
Body weight (Kg)	75.3 ± 3.1
Height (m)	1.7 ± 0.02
BMI (kg/m^2)	24.8 ± 0.8
Caffeine/wk	6.2 ± 2.5
Alcohol/wk	0.2 ± 0.2
PSQI[a]	3.5 ± 0.4
HÖ[a]	54.5 ± 2.6
MCTQ[a] (h)	04:46 ± 00:22
RBC[a] (10^3/mm^3)	5.1 ± 0.1
WBC[a] (10^3/mm^3)	5.6 ± 0.4
PLT[a] (10^3/mm^3)	235 ± 11.7
HGB[a] (g/dl)	13.8 ± 0.3

Values are mean ± SEM. RBC, red blood cell; WBC, white blood cell; PLT, platelet; HGB, hemoglobin.
[a]Values given are those obtained during the screening session.

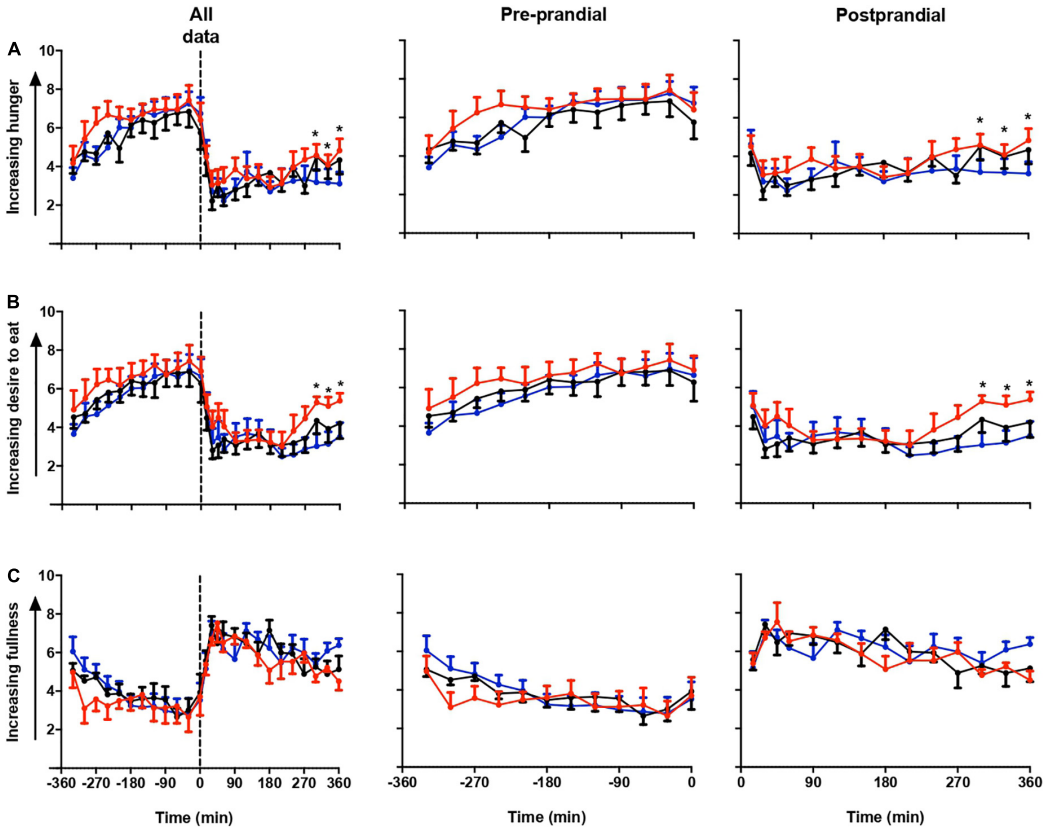

FIGURE 2
Subjective appetite parameters. Subjective hunger **(A)**, desire to eat **(B)**, and fullness **(C)** ratings (mean ± SEM) prior to and after a standard evening meal (time = 0 black dotted line) during LS (—●—), DSC (—●—), and LSC (—●—) in all participants (n = 9) (T = −330 to T = +360 min).

than in DSC and LSC (P = 0.03, and P = 0.001), respectively, whereas no significant difference was shown in the fullness score (P = 0.9). The significant differences were only reported at postprandial times (+270, +300, +330, and +360 min). The significant effects of times were reported in all three subjective parameters during all three sessions ($P < 0.001$).

Pre-prandial and postprandial of plasma leptin

Figure 3 shows the significant difference in plasma leptin during pre-prandial and postprandial times. There was a significant effect of melatonin (p = 0.007) and time ($p < 0.001$) between the three session. Plasma leptin showed significant increase in LSC and DSC compared to LS (p = 0.007). Significant differences were spotted at the postprandial time at +210 and +330 min (Figures 3A–C). Better visualization of leptin response can be seen when the data were plotted as a % leptin of T = 0 or T = −360 (Figures 4A–B). Further details about the plasma leptin for all participants are shown in Supplementary material I.

Total area under the curve and incremental area under the curve of plasma leptin

Total area under the curves and IAUCs of plasma leptin are presented in Figures 5A–B. Plasma leptin was considerably higher in DSC and LSC than LS (p = 0.01). The Tukey's multiple comparison testing showed the significant differences between LS and DSC (p = 0.04) and between LS and LSC (p = 0.01). TAUCs of plasma leptin in LS were 137.9 ± 21.1, 143.6 ± 22.2 in DSC, and 146.7 ± 27.5 ng/ml.min in LSC. No significant differences were reported in IAUC between all three session (p = 0.13). IAUC of plasma leptin in LS was 15.5 ± 2.5, 25.1 ± 7.1 in DSC, and 28.2 ± 8.1 ng/ml.min in LSC. Further details about the TAUC and IAUC of plasma leptin for all participants are shown in Supplementary materials J–K.

Discussion

This study investigated the effect of exogenous melatonin on plasma leptin as the primary appetite hormone was

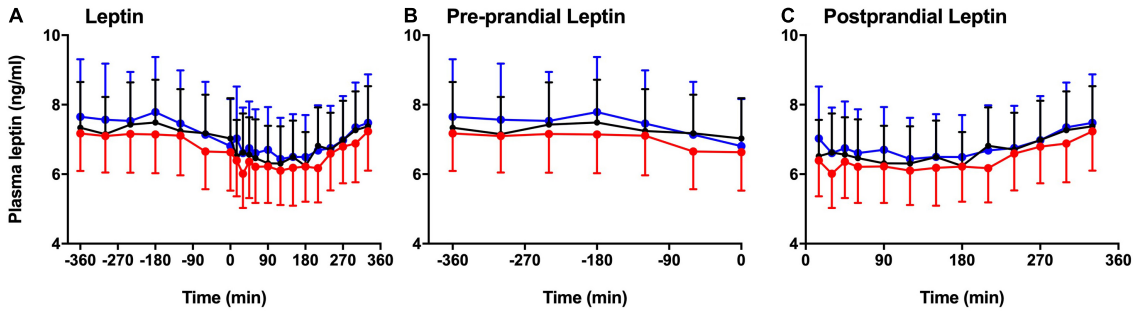

FIGURE 3
Pre-prandial and postprandial plasma leptin. Pre-prandial and postprandial plasma leptin (mean ± SEM) levels prior to and after a standard evening meal (time = 0 black dotted line) during LS (●), DSC (●), and LSC (●) in all participants (n = 9). **(A)** Plasma leptin between −360 to +330 min, **(B)** pre-prandial leptin between −360 to 0 min, and **(C)** postprandial leptin between +15 to +330 min [$F_{(1,496)}$ = 11.54, p = 0.007].

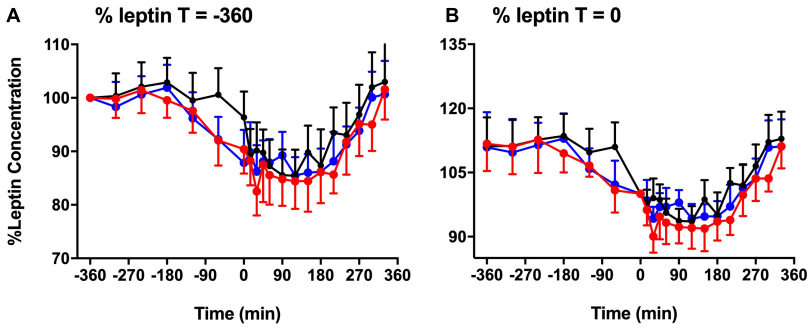

FIGURE 4
Basal leptin concentration. % basal leptin (mean ± SEM) levels directly prior to the test meal (T = 0) **(A)** and at time −360 **(B)** during LS (●), DSC (●), and LSC (●) (n = 9) (T = −360 to T = +330 min) [$F_{(1,496)}$ = 11.54, p = 0.007].

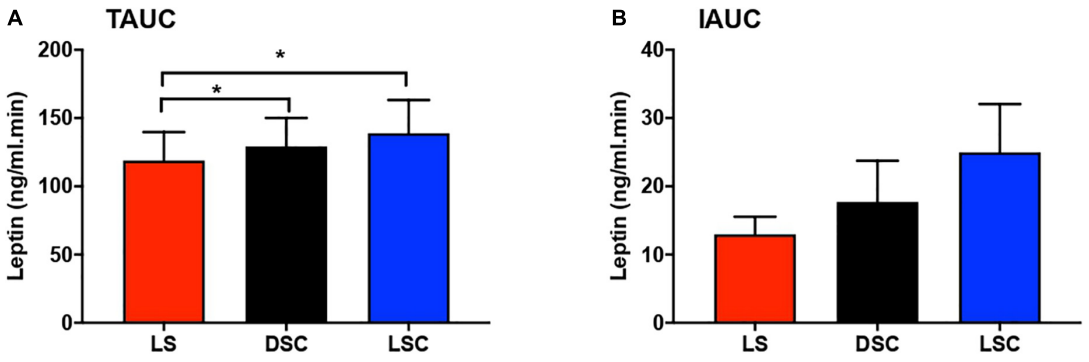

FIGURE 5
Total area under the curve (TAUC) and incremental area under the curve (IAUC) of plasma leptin. TAUC **(A)** and IAUC **(B)** for plasma leptin (mean ± SEM) during LS (■), DSC (■), and LSC (■) in all participants (n = 9) [TAUC: $F_{(1.5,12.1)}$ = 7.06, p = 0.01; IAUC: $F_{(1.9,15.3)}$ = 2.28, p = 0.13]. *Means $p < 0.05$.

investigated. This is the first study to assess the acute effect of exogenous melatonin on subjective appetite score and plasma leptin among healthy young males. This is part of a large clinical study that investigated the impact of exogenous melatonin/ALAN on hormones and metabolites (10). Data from recent studies revealed the presence of contradictory results on melatonin-leptin interactions. In some mammals, melatonin administration has increased leptin levels (33, 34), whereas leptin was decreased in middle-aged rats and goldfish (15, 16, 19). However, these factors influenced the above controversies

due to: (1) Different species; (2) Melatonin had adverse action on the adrenal glands, which triggered changes in glucocorticoids levels (35), or elevated the hypothalmo-pituitary-adrenal axis as result of pinealectomy (36); and (3) leptin suppression due to weight reduction (15, 37). Add to this, the possible differences in the physiological meaning of melatonin between nocturnal and diurnal animals could explain the discrepancy in the melatonin-leptin results reported in animal and human studies.

Consequently, our results revealed a notable increase in leptin level with melatonin administration because leptin was significantly higher in DSC and LSC than in LS. Eventually confirmed by TAUC and IAUC, this rise was quite evident when the data were split into "before and after meal periods" or "pre and post-prandial periods." Contrarily, our study conflicts with that of Figueiro et al. (21), who reported that leptin levels increased after morning light exposure (21). It likewise contradicts Cheung et al. (38), who reported no changes in leptin or ghrelin levels after exposure to blue-enriched light (38). Investigating the melatonin levels would have shed more light on their findings but both studies did not do that. Besides, their study designs did not cover the melatonin profile window. This opens a question whether changes reported above were due endogenous melatonin exerting metabolic effects.

The expression and release of the leptin gene were stimulated by glucocorticoid administration (39). We previously investigated cortisol in this study, yet cortisol's possible stimulatory effects were absent, as there were no apparent differences between the three sessions (10). Moreover, insulin-leptin interaction have been more controversial (40–42). Yet, Alonso-Vale et al. (43) showed that while insulin acted in synergy with melatonin to enhance the expression of the leptin gene, insulin or melatonin alone did not affect leptin gene expression. They further reported that through the Pertussis toxin (PTX)-sensitive Gi protein-coupled membrane receptor, melatonin could obstruct forskolin's inhibitory effect on both leptin synthesis and secretion in adipocytes (43). Insulin signaling is involved in leptin expression *via* melatonin receptor type I (MTI), and melatonin could increase its signaling effect (43). Although with melatonin administration, insulin levels were much lower (10), the melatonin-insulin action upon adipocytes cannot be unnoticed.

It is likewise essential to note that endocrine and metabolic status can be aggravated during major depression (44, 45). A recent meta-analysis have indicated metabolic and inflammatory dysregulation were strongly associated with atypical depression (46). One example is the report that those in a depressed mood tend to take more food (47). However, in this study, participants reported no difference in depressive mood between the three sessions. Meanwhile, this hypothetical mechanism is at odds with the notable increase in the subjective miserable score during LSC compared to LS (**Supplementary material H**). Nonetheless, analyzing short-term appetite control associated with other appetite biomarkers like cholecystokinin (CCK) and ghrelin could be more helpful in this condition. According to Spiegel et al. (23), the effect of insomnia or sleep deprivation on leptin levels is well documented (23). Nevertheless, during all three sessions in this study, participants were sleep deprived. This implies that leptin changes are not really influenced by sleep deprivation.

In this study, with the opposite in fullness score, compared to DSC and LSC, we saw significant increases in subjective hunger and desire to eat scores in LS. Our data revealed a notable effect of melatonin administration on subjective appetite scores. The significant differences shown in plasma leptin are explained by subjective hunger and desire to eat. These results contradicts to that of Cheung et al. (38), who reported that after exposure to blue-enriched light administration, both in the morning and evening, there were no changes in subjective hunger (38). This opens a question of whether melatonin intake can reduce body weight as reported in early type chronotype night shift workers (26). Our plasma leptin and subjective parameters support these findings, yet it is noteworthy to indicate that the evening test meal was served in the same room during the three sessions. Therefore, the smell of food by subjects or subjects with earlier dim light melatonin onset (DLMO) who had their meals could potentially influence our food VAS results.

There was an evident melatonin suppression increase in LS, shown in subjective appetite scores for hunger and desire to eat. Melatonin administration in this study, strengthened the possibility of a melatonin-leptin interaction. So, most likely because of the lower plasma leptin, participants reported higher hunger and desire to eat. So, according to Alonso-Vale et al. (43), forskolin's inhibitory effect on leptin synthesis and secretion in adipocytes is prevented by melatonin. Melatonin also increases the signaling power involved in insulin-induced leptin expression *via* MTI (43). Defined as the hormonal regulatory loop involving leptin-insulin interaction, never ignore the importance of the adipo-insular axis.

A key strength of the present study was the highly controlled in-laboratory and strict session monitoring of participants prior to attending the clinical session. For instance, melatonin intake and supper timing were individualized to ensure sufficient circulating melatonin when meal is given. A total of 9–10.5 h of fasting period was crucial to allow for sufficient collection for pre-prandial samples, and to ensure returning to basal fasting level at the beginning of the session. Light intensity and irradiance were well-controlled and monitored at the horizontal and vertical levels to ensure the same amount and correct intensity were delivered. Additionally, other non-photic factors capable of affecting melatonin, such as posture and calorie intake, were restricted. In contrast, there are multiple limitations that worth to be noted. This study was conducted only in males, with a low sample size. It is imperative to investigate the effect of melatonin in both genders and in a larger sample size to assess the significance level and minimize the limitations of

statistical power. Various eating patterns as a result of adopting eating patterns, such as in athletes or amongst mixed cultural backgrounds, were not considered, which may influence the appetite analysis (48–50). The meals were served in the same room, thus the smell of food affecting the food VAS results cannot be ignored. Short-term appetite hormones such as ghrelin and CCK would add significant value to this study.

According to the major findings of this study, our data suggested a positive impact of exogenous melatonin on subjective appetite and plasma leptin. Our recent findings along with previously reported evidences demonstrated could possibly influence leptin release by modulating insulin levels (10). These findings also indicate the short-term effect of ALAN, which may become aggravated in long-term exposure, such as in shift work. Furthermore, helping to minimize obesity, the ability of melatonin to alter subjective hunger and desire to eat, plus influencing plasma leptin, may regulate food intake. Finally, although melatonin is available "over the counter" in some countries, it is noteworthy mention that the greater efficacy of MT1/MT2 receptor agonist may be effective for improving appetite control.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the University of Surrey Ethics Committee (Study 1: EC/2013/93/FHMS and Study 2: UEC/2015/021/FHMS). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MA, BM, and SH designed the research. MA carried out the research and performed the data analysis. MA and GS wrote the manuscript. AB, NA, BM, and SH provided critical review. MA, GS, AB, BM, and SH have primary responsibility for the final content. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1079453/full#supplementary-material

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Chrononutrition is associated with melatonin and cortisol rhythm during pregnancy: Findings from MY-CARE cohort study

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Chrononutrition has been suggested to have an entrainment effect on circadian rhythm which is crucial for metabolic health. Investigating how chrononutrition affects maternal circadian rhythm can shed light on its role during pregnancy. This study aims to determine chrononutrition characteristics of healthy primigravida during pregnancy and its association with melatonin and cortisol rhythm across gestation. A total of 70 healthy primigravidas were recruited from ten randomly selected government maternal and child clinics in Kuala Lumpur, Malaysia. During the second and third trimesters, chrononutrition characteristics including meal timing, frequency, eating window, breakfast skipping, and late-night eating were determined using a 3-day food record. Pregnant women provided salivary samples at five time-points over a 24 h period for melatonin and cortisol assay. Consistently across the second and third trimesters, both melatonin and cortisol showed a rhythmic change over the day. Melatonin levels displayed an increment toward the night whilst cortisol levels declined over the day. Majority observed a shorter eating window (≤12 h) during the second and third trimesters (66 and 55%, respectively). Results showed 23 and 28% skipped breakfast whereas 45 and 37% ate within 2 h pre-bedtime. During the third trimester, a longer eating window was associated with lower melatonin mean ($\beta = -0.40$, $p = 0.006$), peak ($\beta = -0.42$, $p = 0.006$), and AUC_G ($\beta = -0.44$, $p = 0.003$). During both trimesters, a lower awakening cortisol level was observed in pregnant women who skipped breakfast ($\beta = -0.33$, $p = 0.029$; $\beta = -0.29$, $p = 0.044$). Only during the second trimester, breakfast-skipping was significantly associated with a greater cortisol amplitude ($\beta = 0.43$,

p = 0.003). Findings suggest that certain chrononutrition components, particularly eating window and breakfast skipping have a significant influence on maternal melatonin and cortisol rhythm. Dietary intervention targeting these characteristics may be useful in maintaining maternal circadian rhythm.

KEYWORDS

circadian rhythm, melatonin, cortisol, chrononutrition, dietary pattern, breakfast skipping, late-night eating, gestation

1. Introduction

There is a growing interest in chrononutrition, which studies the timing of food in relation to circadian rhythm alignment. Feeding cues have been found to predominantly entrain peripheral clocks in organs such as kidney and liver, which then interact with the master clock to keep the body's circadian rhythm synchronized (1). Food intake is naturally circadian, where energy stores are replenished through food intake during the active phase while fasting takes place during the sleep phase. Mistimed feeding in individuals at hours outside the normal sleep/wake cycle under a regular light/dark cycle can induce a disruption in metabolism (2, 3), which has been shown to be closely related to circadian rhythm (4). For instance, spontaneous food intake at the end of the resting phase (late afternoon) results in the most detrimental effects on energy homeostasis in obese rodents, whereas for humans, this critical period is at the early night or the beginning of the resting phase (5). Irregular feeding patterns such as delayed meal intake, breakfast skipping and late-night eating have been associated with circadian rhythm disruption, ranging from delayed glucose rhythms (6), phase delay in core body temperature (7), delayed peripheral clock genes (8) and blunted cortisol secretion (9).

During pregnancy, the food intake of women in terms of quality and quantity is a well-recognized determinant of maternal and infant outcomes, such as birth weight, gestation length and gestational weight gain (10). However, less is known about the relationship between maternal chrononutrition characteristics with pregnancy and infant outcomes. A recent review by Loy et al. (11) summarized that night eating during pregnancy was linked to metabolic implications, including the risk of gestational diabetes mellitus (GDM), undesired gestational weight gain pattern, postpartum weight retention and preterm birth. A more recent randomized controlled trial using a chrononutritional and sleep hygiene intervention showed improvement in maternal glycemic control among pregnant women with gestational diabetes mellitus (GDM) (12). This evidence linking chrononutrition with metabolic health and adverse birth outcomes suggests that the timing aspect of food intake during gestation may have a wider influence on the maternal physiological system, which is circadian in nature (13). Given the growing body of evidence demonstrating the association between disrupted circadian rhythm and poor pregnancy outcomes (14–16), unfavorable chrononutrition characteristics can be a potential modifiable approach to improve maternal and infant outcomes by intervening the temporal aspect of food intake.

To date, most studies involving the general adult population have shown the relationship between various chrononutrition aspects and adverse health outcomes, such as diabetes, cardiovascular diseases and obesity (17, 18). In the pregnant population, the impact of varying chrononutrition on maternal health has yet to be fully elucidated. Identifying the chrononutrition characteristics associated with maternal circadian rhythm during pregnancy can provide practical insights into the modifiable aspects of maternal nutrition through food intake, which can be targeted for maternal health promotion. Thus, this study aimed (1) to determine maternal circadian rhythm in terms of melatonin and cortisol levels during the second and third trimesters; (2) to identify maternal chrononutrition characteristics during the second and third trimesters; (3) to determine the association of maternal melatonin and cortisol levels with chrononutrition characteristics during the second and third trimesters. It is hypothesized that unfavorable chrononutrition characteristics including longer eating window, breakfast skipping and late-night eating are linked to differences in maternal melatonin and cortisol levels during the second and third trimesters of pregnancy.

2. Materials and methods

2.1. Study design and data collection

The results described in this paper were derived from a larger MY-CARE observational cohort study conducted to determine the effect of maternal circadian rhythm during gestation on birth and infant outcomes (19). Between June 2019 to October 2021, healthy primigravidas with singleton pregnancy, aged between 19 and 39 years, and in their first 20 weeks of gestation took part in the study. Subject recruitment

was carried out at ten randomly selected government maternal and child clinics (*Klinik Kesihatan Ibu dan Anak*) in Kuala Lumpur, Malaysia using purposive sampling. Pregnant women with the following conditions were excluded from the study: pre-existing health conditions such as diabetes mellitus, hypertension, and anemia, pregnancy-related complications, physical disabilities, consuming medicine or supplement containing melatonin or corticosteroids, using sleep medicine or recreational drugs, smoking, take part in shift work, or undertook transmeridian flight in the past 3 months upon recruitment.

The sample size needed for the study was calculated using G*POWER software, version 3.1.9.4[1]. Using a linear multiple regression model, effect size (f^2) of 0.338 (20), type I error rate (α) of 0.05 and power (1-β) of 90%, the minimum sample size required was 49. The required sample size was increased by 20% to 60 considering attrition and non-compliance. The final number of pregnant women after excluding dropout and non-compliance was 70. The flow of subject recruitment and participation was depicted in **Supplementary material**.

The socio-demographic characteristics of the pregnant women, such as age, race, educational level and household income level were collected using a face-to-face questionnaire at the clinic upon recruitment. Pre-pregnancy weight and height were obtained from the antenatal booklet to compute pre-pregnancy body mass index (BMI). Data collection was conducted only during the second and third trimesters with exclusion of the first trimester as pregnant women are normally in their late first trimester or early second trimester (week 9–12) at their first antenatal check-up.

2.2. Ethics approval and consent to participate

Pregnant women provided signed written informed consent before data collection commenced. The study was carried out in accordance with The Malaysian Code of Responsible Conduct in Research and the Helsinki Declaration. Ethical approval for the study was obtained from the Medical Research and Ethics Committee (KKM/NIHSEC/P19-125) on 29th April 2019.

2.3. Chrononutrition characteristics

The timing of food intake, types of food, beverage and supplement and the amount consumed were recorded using a 3-day food record. Pregnant women were instructed to record their food intake on three non-consecutive days (two weekdays and one weekend day) in order to capture differing eating patterns on weekdays and weekends. To reduce reporting bias among the pregnant women, a one-on-one briefing on the usage of the 3-day food record was conducted and visual aid of portion size using household measurements was provided along with the food record. To minimize recall bias, pregnant women were advised to record their food intake immediately after each eating event. A review of the food entries was conducted upon returning the 3-day food record. The ratio between reported total energy intake (EI) and basal metabolic rate (BMR) was calculated to identify underreporters. Underreporting was defined as having an EI:BMR ratio <1.2 (21) whereas an EI:BMR ratio of >2.4 was considered overreporting (22). BMR was calculated using the equation for Malaysian adults with the additional energy requirements during pregnancy (second trimester: 280 kcal/day, third trimester: 470 kcal/day) (23). In this study, 8.6% ($n = 6$) and 14.3% ($n = 10$) of pregnant women underreported their energy intake in the second and third trimesters, respectively. Hence, 64 and 60 pregnant women were included in the final analysis.

Chrononutrition characteristics that were assessed in this study include meal timing, meal frequency, eating window, largest meal, breakfast-skipping and late-night eating. Based on the food entries on the 3-day food record, mean meal timing for the main meals (breakfast, lunch, and dinner) and snacks was computed. Meal frequency is defined as the average number of meals and snacks consumed in a day. Eating window refers to the duration of time in minutes between the first eating event and the last eating event of the day (24). The largest meal of the day was identified based on the amount of calories consumed. In this study, breakfast is defined as the first meal of the day consumed no later than 10:00 h (25). Breakfast consisting of tea or water only was excluded (26). Pregnant women were categorized as breakfast skippers (ate breakfast $\leq$2 out of the 3 days of food record) or breakfast eaters (ate breakfast on all 3 days). Lunch, dinner and snacks were self-defined by the pregnant women. Pregnant women who ate within 2 h before sleep were categorized as late-night eaters (late-night eating $\geq$1/3 days) (24, 27).

2.4. Salivary melatonin and cortisol levels

Salivary samples were collected for a day during the second and third trimester, respectively. Pregnant women collected at least 3 ml of salivary samples using passive drool method to determine their melatonin and cortisol levels. Pregnant women were instructed to collect their first salivary sample of the day upon awakening, followed by 9:00 h and at every 6 h interval (15:00, 21:00, and 3:00 h) within a 24 h day. These timings were selected in reference to previous studies and to capture the changes in melatonin and cortisol secretion, particularly in the morning post-awakening, mid-day, before going to sleep and

1 http://www.gpower.hhu.de

mid-sleep (28, 29). They were briefed on the collection protocol at the clinics prior to home collection.

Pregnant women were requested to carry out their salivary sampling on a work-free day when they can freely choose their sleep and wake timings and follow their usual routine without the constraint of work commitment. Additionally, they were advised to avoid the intake of chocolate, banana, alcohol, caffeine or drinks containing artificial colorants on the sampling day. The consumption of prescribed over-the-counter medications within 12 h prior to sampling was required to be reported. Pregnant women were asked to avoid consuming a major meal or brushing teeth with toothpaste within 30 min prior to sampling. After collection, pregnant women stored their samples in their home freezer until collection by the researcher.

The samples were analyzed using direct salivary melatonin enzyme-linked immunosorbent assay (ELISA) kits from IBL International (Hamburg, Germany) and direct saliva cortisol ELISA kit from LDN Labor Diagnostika Nord GmbH & Co. KG (Nordhorn, Germany) according to the manufacturer's instructions. Each sample was measured in duplicate to generate an average concentration value. Samples with concentrations that exceeded the standard range of the ELISA kit were diluted 2-fold and re-analyzed. The intra-assay and inter-assay coefficient of variation were 2.4 and 13.0% for melatonin measurements and 3.4 and 13.9% for cortisol measurements.

2.5. Chronotype

Chronotype of the pregnant women was assessed using the Morningness-Eveningness Questionnaire (MEQ). The MEQ was administered once upon recruitment and was not assessed repeatedly considering that chronotype is a stable trait with minor advances over years (30). The chronotypes of pregnant women were categorized by the following cut-offs: definite to moderate morning (score = 59–86), intermediate (score = 42–58), and definite to moderate evening (score = 16–41) (31).

2.6. Statistical analysis

All statistical analyses were conducted using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). Continuous data with a normal distribution were reported as mean and standard deviation (SD) whereas median and interquartile range (IQR) were reported for data with a skewed distribution. Categorical variables were reported in percentage. Outliers and skewness of data distribution were determined using the Shapiro Wilk test. Outliers were excluded from analyses. A p-value of <0.05 was considered statistically significant. To check for the differences in melatonin and cortisol measurements between the second and third trimesters, paired sample t-test (for parametric variables) and Wilcoxon's sign rank test (for non-parametric variables) were conducted.

Based on the four melatonin levels across the day (9:00, 15:00, 21:00, and 3:00 h), mean, maximal level, amplitude, area under the curve with respect to ground (AUC_G), and area under the curve with respect to increase (AUC_I) were computed. The maximal level is determined based on the highest melatonin level among the four collection timings. Amplitude is calculated as the ratio of the maximal melatonin level to the lowest melatonin level. It is commonly examined as a marker that indicates the robustness of melatonin secretion across the day. Both AUC_G and AUC_I were tabulated using the trapezoid method (32). The AUC_G represents the total melatonin production across the day, whereas AUC_I indicates the increment of melatonin secretion over the 24 h day. A more positive value of melatonin AUC_I indicates a steeper increment in melatonin level across the day. Melatonin level at z-awakening was not analysed in this study as the aim of collecting salivary sampling at awakening was to determine awakening cortisol level, which is a commonly examined feature of cortisol rhythm but not for melatonin rhythm.

On the other hand, mean, awakening level, amplitude, diurnal slope, morning slope, evening slope, and AUC_G were calculated based on the five salivary samples analysed for cortisol levels. Amplitude and AUC_G of cortisol were calculated based on the same method for melatonin. Diurnal cortisol slope was calculated by dividing the difference in the cortisol levels at 9:00 and 21:00 h by the difference in hours, representing the diurnal cortisol decline. To further examine the variation in the rate of cortisol decline in the morning and from afternoon to the evening, morning slope (difference in the cortisol levels at 9:00 and 15:00 h over difference in hours) and evening slope (difference in the cortisol levels at 15:00 and 21:00 h over difference in hours) were calculated. A more negative slope represents a steeper rate or slope of cortisol decline over the period, whereas a more positive slope represents a flatter decline in cortisol level.

In this study, potential covariates that may be associated with salivary melatonin and cortisol levels include study design-related factors (gestation week at sampling), maternal socio-demographic characteristics [maternal age, race, household income level, educational level, and pre-pregnancy body mass index (BMI)], situational factors (wake and sleep time on sampling day and sleep duration), and fetal sex. Preliminary analyses were conducted using univariate tests to examine the univariate associations between the abovementioned variables and salivary melatonin and cortisol parameters. Variables that showed significant association were included as controls in the subsequent analyses.

Hierarchical linear regression analyses were performed to examine the association of maternal melatonin and cortisol variables with chrononutrition characteristics while adjusting for covariates. The potential covariates identified from the preliminary analyses were entered in the first step, whereas chrononutrition variables were entered in the

second step of the hierarchical regression model. Entering the independent variables and covariates by the enter method allows the examination of the incremental predictive effect of chrononutrition variables on outcome variables. Collinearity between variables entered in the model was tested with variance inflation factors (VIFs), where VIF greater than five indicates collinearity.

3. Results

3.1. Subject characteristics

Mean age of pregnant women in this study was 28.7 years old. The majority was Malay ($n = 39$, 55.7%) and attained tertiary education ($n = 59$, 84.3%). Around 47.1% ($n = 33$) came from households with middle income. In terms of pre-pregnancy BMI, 10.0% ($n = 7$) were underweight, whereas 22.8% ($n = 16$) were overweight or obese before pregnancy. The majority are of intermediate chronotype (71.4%), with only 4.3% are evening chronotype. The subject characteristics were summarized in Table 1.

TABLE 1 Characteristics of the study sample ($n = 70$).

	Mean	SD	Range	*n*	%
Age (years)	28.7	3.7	20–37	—	–
Gestational week (weeks)	–	–	–	–	–
T2	20.0	3.7	13–27	–	–
T3	32.9	2.1	28–37	–	–
Pre-pregnancy BMI (kg/m^2)	23.3	4.9	15.8–42.3	–	–
Underweight	–	–	–	7	10.0
Normal	–	–	–	47	67.1
Overweight	–	–	–	12	17.1
Obese	–	–	–	4	5.7
Ethnicity	–	–	–	–	–
Malay	–	–	–	39	55.7
Chinese	–	–	–	26	37.1
Indian	–	–	–	2	2.9
Others	–	–	–	3	4.3
Educational level	–	–	–		
Secondary	–	–	–	11	15.7
Tertiary	–	–	–	59	84.3
Household income (RM)*	–	–	–	–	–
Low (<2300)	–	–	–	7	10.0
Middle (2300–5599)	–	–	–	33	47.1
High (≥5600)	–	–	–	30	42.9
MEQ score	53.9	6.8	–	–	–
Morningness	–	–	–	17	24.3
Intermediate	–	–	–	50	71.4
Eveningness	–	–	–	3	4.3

BMI, body mass index; RM, ringgit Malaysia; T2, second trimester; T3, third trimester.
*Based on the definition of 10th Malaysia Plan.

3.2. Circadian variation in salivary melatonin level

Melatonin levels and variables were listed in Supplementary material. A distinct change in melatonin levels across the days was observed (See Figure 1). Melatonin secretion showed a significant increment from 21:00 to 3:00 h, with relatively low levels at 9:00 and 15:00 h in both the second and third trimesters. No significant difference in melatonin levels across all collection timings (all $p > 0.05$). The melatonin rhythm variables including mean, maximal level, AUC_G, and AUC_I were maintained across trimesters (all $p > 0.05$). Melatonin amplitude was significantly lower in the third trimester as compared to the second trimester ($p = 0.020$), indicating an attenuated melatonin response over the day.

3.3. Circadian variation in salivary cortisol levels

Pregnant women showed a distinct decline in cortisol levels over the day during the second and third trimesters of pregnancy, as depicted in Supplementary material. An overall increase in cortisol levels in the third trimester compared to the second trimester was observed (See Figure 2), with significant differences in cortisol levels across all collection timings (all $p < 0.05$). Mean and AUC_G of cortisol were also significantly higher in the third trimester (both $p < 0.001$), indicating a greater melatonin output. However, there was no significant variation in the amplitude and patterns of cortisol decline, namely diurnal slope, morning slope, evening slope, and AUC_I as pregnancy advanced.

3.4. Chrononutrition characteristics

Chrononutrition characteristics of pregnant women were listed in Table 2. Meal timing, frequency and eating window did not significantly vary by trimester. The majority of the pregnant women followed an eating window of 12 h or below both in the second (65.6%, $n = 42$) and third (55.0%, $n = 33$) trimesters. A higher proportion of pregnant women in this study reported having lunch or dinner as their largest meal of the day. In the third trimester, the prevalence of breakfast skipping was significantly higher ($p = 0.005$), whereas the prevalence

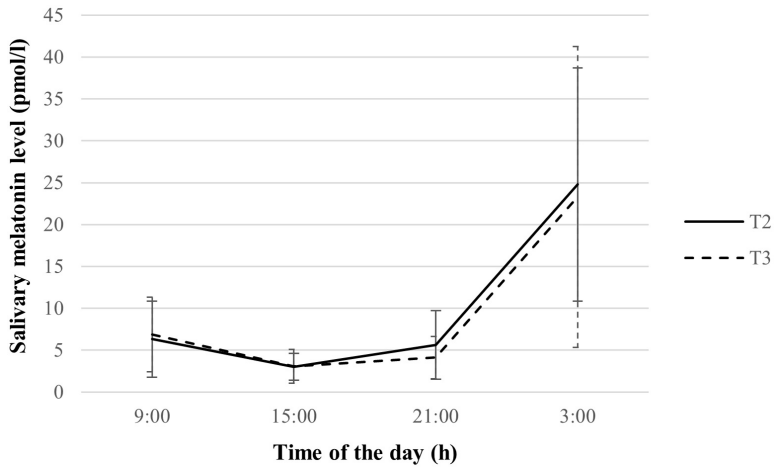

FIGURE 1
Salivary melatonin levels of pregnant women during the second and third trimesters (n = 70). Values are expressed in median ± median absolute deviation. T2, second trimester; T3, third trimester.

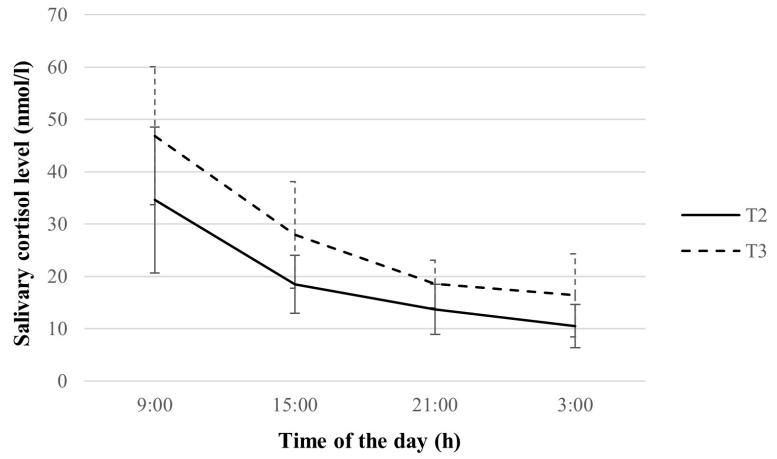

FIGURE 2
Salivary cortisol rhythm of study subsample during the second and third trimesters (n = 70). Values are expressed in median ± median absolute deviation. T2, second trimester; T3, third trimester.

of late-night eaters was significantly lower than in the second trimester (p = 0.002).

3.5. Association between maternal melatonin levels and chrononutrition characteristics

As shown in Table 3, significant associations were found between longer eating window and lower mean (β = –0.40, p = 0.006), peak (β = –0.42, p = 0.006), and AUC_G (β = –0.44, p = 0.003) of melatonin rhythm in the third trimester. Each of them explains an additional 14.4, 15.2, and 17.3% of the variance. No significant association was observed in the second trimester. Results for the non-significant models were listed in Supplementary material.

3.6. Association between maternal cortisol levels and chrononutrition characteristics

In both the second and third trimesters, a lower awakening cortisol level was observed in pregnant women who skipped breakfast (T2: β = –0.33, p = 0.029; T3: β = –0.29, p = 0.044), explaining an additional variance of 8.0 and 14.7% of the variance (See Table 4). Only during the second trimester, breakfast-skipping was significantly associated with a greater

TABLE 2 Chrononutrition characteristics of the pregnant women during second (n = 64) and third trimester (n = 60).

Variable	T2 (n = 64)	T3 (n = 60)	P-value
n (%)/Mean ± SD			
Meal timing			
Breakfast	8:41 ± 0:47	8:32 ± 0:43	0.510
Morning snack	10:37 ± 0:54	10:20 ± 0:39	0.427
Lunch	13:18 ± 0:55	13:11 ± 0:58	0.320
Afternoon snack	16:22 ± 1:07	16:25 ± 1:02	0.301
Dinner	19:54 ± 0:55	19:57 ± 0:52	0.876
Supper	22:41 ± 1:18	22:25 ± 1:07	0.827
Meal frequency	4.45 ± 0.79	4.28 ± 0.78	0.079
Eating window	11.95 ± 1.43	11.91 ± 1.63	0.708
≤12 h	42 (65.6)	33 (55.0)	–
12–14 h	18 (28.1)	20 (33.3)	–
>14 h	4 (6.3)	7 (11.7)	–
Largest meal[a]	–	–	0.104
Breakfast	9 (14.1)	7 (11.7)	–
Morning snack	1 (1.6)	3 (5.0)	–
Lunch	25 (39.1)	26 (43.4)	–
Afternoon snack	1 (1.6)	–	–
Dinner	28 (43.8)	24 (40.0)	–
Supper	–	–	–
Breakfast-skipping	–	–	0.005**
Breakfast eater	49 (76.6)	43 (71.7)	–
Breakfast skipper	15 (23.4)	17 (28.3)	–
Late night eating	–	–	0.002**
Non-late-night eater	35 (54.7)	38 (63.3)	–
Late-night eater	29 (45.3)	22 (36.7)	–

Independent t-test was carried out for continuous variables while McNemar's test was used for categorical variables. Paired sample t-test was conducted to determined differences between second and third trimesters based on sample's data. [a]Fisher's exact test was performed as expected count less than five was more than 20%. SD, standard deviation; T2, second trimester; T3, third trimester. **$p < 0.01$.

cortisol amplitude (β = 0.43, p = 0.003). It explains an additional 13.5% of the variance. Results for the non-significant models were listed in **Supplementary material**.

4. Discussion

The results from this study provided an overall understanding of maternal chrononutrition characteristics and circadian rhythm in terms of melatonin and cortisol levels during the second and third trimesters of pregnancy. Overall, the mean salivary melatonin and cortisol level in this sample was approximately eight times lower than one study that measured salivary melatonin [Shimada et al. (33)] over two time points among healthy pregnant women. As compared to a previous study that reported a significant rise in serum melatonin levels as pregnancy advanced, with levels in the third trimester being around 1.4 to 3-fold higher, the overall mean melatonin levels in the present study did not differ significantly between the second and the third trimester (34–37). Increment of cortisol levels in both trimesters were approximately 2–3 times higher than the observations in previous studies (38–41).

Findings of the present study suggested that certain chrononutrition characteristics, particularly breakfast skipping and eating window can influence maternal melatonin and cortisol rhythm during pregnancy after controlling for covariates. Pregnant women who skipped breakfast were associated with a lower awakening cortisol level both during the second and third trimesters. Trimester-specific associations were also detected. Only during the second trimester, breakfast skippers showed a significantly higher cortisol amplitude. Additionally, a longer eating window during the third trimester was associated with an overall lower melatonin output in comparison to several studies [Nakamura et al. (35)], as measured by mean, peak and AUC_G. Lowered melatonin levels during pregnancy have been shown to have negative effects by increasing risks of pre-eclampsia and intrauterine growth retardation (35).

The timing of the major meals (breakfast, lunch, and dinner), snacks, and supper overlaps with the typical meal patterns reported by other studies (42–44), which indicates that the intake of major meals are typically within a certain time frame across geography. A higher proportion of the pregnant women in this study had their lunch or dinner as the largest meal of the day, which is consistent with previous studies that reported a higher proportion of energy consumed at lunch and dinner, i.e., during the later times of the day (45, 46). The prevalence of breakfast-skipping in this study was at the upper end as compared to the previously reported prevalence among pregnant women (6–31%) (47–49). Pregnant women tend to be vulnerable to meal skipping due to factors such as morning sickness, sensitivity to smell, and reduced appetite (50–52). Furthermore, in this study, pregnant women who skipped breakfast had a significantly later wake and sleep time on both workdays and free days as well as a lower mean MEQ score or greater eveningness than breakfast eaters, implying that this eating behavior can be related to their chronotype preference. Taken together, the patterns of delayed food intake and breakfast skipping may also be related to the characteristics of the study sample, which largely comprised of working pregnant women living in the urban city. In an urban environment, work and social commitments, eating out culture and around-the-clock dining are factors that likely promote higher energy intake toward the later times of the day and breakfast-skipping (53, 54). This phenomenon may not be unique to the current study sample but is prevalent in urban population.

TABLE 3 Hierarchical linear regression models predicting maternal melatonin parameters from chrononutrition characteristics (T2: *n* = 64; T3: *n* = 60).

	Melatonin during T3					
	Mean		Maximal level		AUC_G	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value	β (95% CI)	*P*-value
Step 1:						
Maternal age	0.07 (−0.55, 0.92)	0.613	−0.04 (−2.09, 1.53)	0.756	0.12 (−0.97, 2.43)	0.388
Pre-pregnancy BMI	0.24 (−1.36, 9.30)	0.140	0.19 (−5.32, 20.50)	0.242	0.23 (−3.58, 20.73)	0.162
Gestational week	−0.01 (−1.39, 1.30)	0.950	−0.12 (−4.59, 1.86)	0.397	0.10 (−1.95, 4.13)	0.473
Fetal sex	0.07 (−4.23, 7.07)	0.615	0.03 (−12.24, 14.99)	0.839	−0.09 (−16.93, 8.89)	0.533
Sleep time	−0.30 (−5.43, 0.19)	0.067	−0.29 (−12.84, 0.81)	0.082	−0.27 (−11.89, 0.99)	0.096
ΔR^2 for step 1	0.126	0.306	0.136	0.302	0.087	0.541
Step 2:						
Eating window	−0.40 (−4.07, 0.72)	0.006**	−0.42 (−9.76, 1.72)	0.006**	−0.44 (−9.61, −2.09)	0.003**
ΔR^2 for step 2	0.144	0.006**	0.152	0.006**	0.173	0.003**
F_{model}	2.592	0.032*	2.625	0.031*	2.474	0.039*

AUC_G, area under the curve with respect to ground; β, standard coefficients; BMI, body mass index; T3, third trimester. $^{**}p < 0.01$; $^{*}p < 0.05$.

TABLE 4 Hierarchical linear regression models predicting maternal cortisol parameters from chrononutrition characteristics (T2: *n* = 64; T3: *n* = 60).

	Cortisol during T2				Cortisol during T3	
	Awakening		Amplitude		Awakening	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value	β (95% CI)	*P*-value
Step 1:						
Maternal age	−0.11 (−1.95, 0.92)	0.474	−0.01 (−0.15, 0.16)	0.967	0.09 (−1.28, 2.44)	0.534
Pre-pregnancy BMI	0.18 (−0.48, 2.31)	0.195	−0.22 (−0.30, 0.02)	0.085	0.01 (−1.73, 1.82)	0.960
Household income level	0.32 (0.65, 16.68)	0.035*	0.18 (−0.33, 1.51)	0.201	0.14 (−5.44, 15.26)	0.345
Gestational age	0.18 (−0.48, 2.35)	0.192	0.23 (−0.02, 0.29)	0.090	−0.14 (−4.84, 1.64)	0.324
Fetal sex	−0.19 (−16.91, 2.66)	0.150	0.05 (−0.86, 1.32)	0.679	−0.18 (−21.53, 4.13)	0.179
Wake time	0.02 (−3.61, 4.26)	0.868	−0.29 (−0.96, −0.07)	0.025*	0.41 (2.60, 13.26)	0.004**
ΔR^2 for step 1	0.170	0.147	0.156	0.158	0.088	0.110
Step 2:						
Breakfast-skipping						
No	1.00		1.00		1.00	
Yes	−0.33 (−25.37, −1.47)	0.029*	0.43 (0.78, 3.55)	0.003**	−0.29 (−30.77, −0.41)	0.044*
ΔR^2 for step 2	0.080	0.029*	0.135	0.003**	0.147	0.044*
F_{model}	2.285	0.043	3.045	0.009**	2.306	0.042*

β, standard coefficients; BMI, body mass index; T2, second trimester; T3, third trimester. $^{**}p < 0.01$; $^{*}p < 0.05$.

On the other hand, around 33–46% of the pregnant women in this study are late-night eaters or ate within 2 h before sleep. Late-night eating and breakfast-skipping are typically seen in individuals with an evening tendency due to a collective delay in behaviors, including food intake (55). Further analysis showed no significant correlation between breakfast eating or chronotype with night eating in this study. This may be because the majority of pregnant women reporting night eating in this study were due to the consumption of maternal milk at night before sleep instead of eating a major meal. In addition, with the majority of pregnant women consuming their largest meal either in the afternoon or at night, it is possible that they are more likely to consume food closer to bedtime due to an overall delayed food intake pattern.

In this study, a longer eating window, which implies a shorter fasting cycle relative to the feeding cycle, was associated with an overall reduced output of melatonin rhythm, namely mean, peak and AUC_G. The association between a longer eating window and lower melatonin secretion may be a result of a circadian mismatch between the active-feeding/resting-fasting cycle and the circadian regulation of metabolic physiological processes. The study of eating windows

is based on time-restricted eating (TRE) which involves limiting daily food intake to 8–12 h to prolong a fasting period (56). Aligning the feeding/fasting cycle to hormonal regulation across the 24 h day means restricting energy intake to the active phase of the day whereas stored energy is used during the resting phase (57). This result can be explained by the role of melatonin in regulating the daily distribution of metabolic processes and hormones to synchronize the active/feeding phase to the high insulin sensitivity phase and the resting/fasting phase to the insulin-resistant phase of the day (57, 58). Previous studies examining the effect of fasting on melatonin found reduced nocturnal melatonin levels (59, 60) and phase advance of melatonin rhythm in response to fasting (61). As the results from the aforementioned studies were participants who were subjected to 48–72 h of fasting, they may not be directly comparable with the results in this study. However, taken together, these findings support the hypothesis that altering the length of the feeding/fasting cycle can have an impact on circadian melatonin secretion.

The association between eating window and melatonin rhythm in this study appeared to be trimester specific. As the median and patterns of eating window did not significantly vary across trimester, the trimester-specific association may be related to the physiological changes that occur as pregnancy advances. An example of physiological changes as demonstrated in this study is the elevation of cortisol level in the third trimester. Melatonin and cortisol are physiologically linked, and both play various key roles in circadian system and energy homeostasis along with other hormones, such as insulin, leptin, and ghrelin. Although melatonin levels remain relatively stable across trimester, the larger, collective physiological changes may potentially confound the trimester-specific association. Nonetheless, the mechanistic pathway underlying this trimester-specific association requires further study. Based on the results, it remains unclear what is the desirable range of eating window to maintain melatonin secretion. Majority of the pregnant women in this study had an eating window of 8–12 h. Further study is recommended to examine the different range of eating window and its influence on circadian melatonin secretion.

Additionally, it was found that pregnant women who skipped breakfast had a significantly higher cortisol amplitude during the second trimester and a lower awakening cortisol level during both the second and third trimesters. These associations between breakfast-skipping and cortisol level or amplitude have been reported previously. A study conducted by Witbracht et al. (62) reported that both non-stimulated and meal-stimulated cortisol levels over the day in women who regularly skip breakfast were elevated, particularly at midday. Furthermore, a smaller morning to evening cortisol amplitude or a blunter diurnal cortisol response was observed. These results were contrary to the findings in the present study where an increased amplitude and reduced awakening level were found. It is plausible that the lower cortisol awakening level and higher cortisol amplitude observed among breakfast skippers is due to delayed cortisol rhythm, where the increment in diurnal cortisol level occurs later post-awakening as compared to non-breakfast skippers. Breakfast-skipping is linked to evening chronotype and later sleep/wake timing (63, 64), which are associated with a delayed circadian phase (65–67). This explanation is supported by the observation that breakfast-skippers in this study had a significantly later wake and sleep time as well as a greater eveningness during the second and third trimesters.

Contrary to previous studies that demonstrated an association between night eating syndrome (NES) or late eating (lunch at 16:30) and differences in circadian melatonin and cortisol measurements such as lower amplitude and delayed circadian phase (9, 68), this study did not find such association. Delayed melatonin and cortisol rhythm along with blunted melatonin and cortisol secretion are prominent characteristics of circadian misalignment (69). The inconsistency in findings between the previous studies and the current study may be due to the difference in the definition of late-night eating, which in this study is defined as eating within 2 h before sleep. Examining the pattern and caloric intake of late-night eating may help to provide more comprehensive insights into its influence on circadian rhythm.

The current study findings should be interpreted in light of its limitations. Although the use of a 3-day food record comprising two weekdays and one weekend day is representative of the individual's usual food intake, it may not be sufficient to capture daily variability in eating behaviors. Furthermore, the 3-day food record was self-reported by the participants. Hence there was a possibility of reporting bias. Salivary sampling was conducted at specific time-points in a 24 h day, which may not be adequate to capture individual variability in circadian melatonin and cortisol secretion, particularly with respect to the circadian peak and nadir. Furthermore, as both the salivary samplings and 3-day food record were not conducted on the same day, the associations found in this study may be confounded by factors contributing to day-to-day variability that were not studied in this research. The findings may not be generalized to other populations as the study was conducted among the urban pregnant women population. However, the study has several important strengths. Measuring both melatonin and cortisol levels as markers of circadian rhythm provides a more comprehensive understanding of the patterns of maternal circadian rhythm during pregnancy. It also allows the examination of the differential association of chrononutrition with melatonin and cortisol. Potential covariates of melatonin and cortisol secretion were also taken into account in the regression models. This study collected data during both the second and third trimesters which allow the detection of trimester-specific associations among the same pregnant women. Besides, this study focused on healthy primigravida, which would reduce the confounding effect of pregnancy-related complications on dietary intake and circadian rhythm.

5. Conclusion

Findings of this study suggested that certain chrononutrition characteristics, specifically breakfast skipping and longer eating window can contribute to significant lowered melatonin secretion and awakening cortisol levels among pregnant women. This indicates the potential influence of meal timing and pattern in altering maternal circadian rhythm, which is linked with disparities in pregnancy outcomes. Dietary intervention may need to consider incorporating chrononutrition characteristics on top of food quantity and quality to promote optimal pregnancy outcomes through maintaining circadian rhythm.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Research and Ethics Committee (KKM/NIHSEC/P19-125). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ANT was responsible for data collection, data analyses, and writing the manuscript. SK, NHMS, and SRS were responsible for the conception of the study, provided input to data analysis, and reviewed the manuscript. NAB, MT, and SS guided in lab analysis results and reviewed the manuscript with substantial contribution to the interpretation of the results. All authors approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chrononutrition behavior during the COVID-19 pandemic and its relationship with body weight among college students

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Introduction: Students in colleges are exposed to unhealthy lifestyles and poor dietary choices. They are at risk of being overweight, skipping meals, and developing eating disorders. However, there is a paucity of information on their chrononutrition behavior, which is very important, especially concerning the timing of food consumption across the day. Therefore, the present study aimed to investigate chrononutrition behavior and its potential association with body weight status among college students in Malaysia.

Methods: This cross-sectional study was conducted on 409 college students aged above 18 in Malaysia. The chrononutrition behavior was assessed using the validated Chrononutrition Profile Questionnaire (CP-Q). The questionnaire was distributed using an online platform. Participants self-reported their body weight and height, and the Body Mass Index (BMI) was computed. Data were analyzed using the SPSS software.

Results: A total of 409 participants were recruited, with a mean age of 21.5 ± 2.2 years. The prevalence of underweight, normal, and overweight was 24.7, 49.4, and 25.9%, respectively. The chrononutrition behavior revealed that participants ate breakfast about four times/week (mean 4.27 ± 2.43 days), and only 135 (33.0%) consumed breakfast daily. The largest meal consumed was during lunch (75.8%), and the mean of snacking after the last meal was 3.23 ± 2.01 days. The prevalence of night eating was low, and most participants (70.9) did not wake up at night to eat. The frequency, however, was significantly higher in the underweight group compared to the normal weight group ($p < 0.05$). We observed a significant association between BMI and eating window, evening latency, evening eating, and night eating. It was found that the underweight had a poor eating window ($p < 0.01$), poor evening latency ($p < 0.01$), poor evening eating ($p < 0.01$), and poor night eating ($p < 0.05$) compared to those with normal and overweight BMI groups. In contrast to predictions, poor chrononutrition behavior was more likely to predict being underweight compared to normal ($p < 0.05$).

Conclusion: Underweight young adults are more likely to have poor chrononutrition behavior. The results of the present study suggest that future nutrition education should also focus on the chrononutrition behavior of college students.

KEYWORDS

chrononutrition, chronotype, college students, young adults, overweight

1. Introduction

Eating habits have been a major concern among college students. They are at risk of developing unhealthy eating habits such as breakfast skipping, fast food consumption, and high sugar intake (1) that may hinder their health and psychological wellbeing (2, 3). Skipping breakfast affects the hypothalamic-pituitary function and the reproductive cycle by disturbing the central clock system, which leads to ovarian and uterine malfunction (4). The post-adolescent students' food habits may deteriorate early in college life due to the large number of students who move in alone. The transition to college life, together with academic and lifestyle challenges, was postulated with these significant changes in their nutrition intake. Unhealthy lifestyles, including poor sleep habits, skipping food, poor eating patterns, increased alcohol intake and reduced physical activity, were also reported among college students (5). Consequently, the incidence of weight gain and increased adiposity during college life was identified in previous meta-analyses (6, 7). Nevertheless, the prevalence of being underweight among young adults and college students remains a major concern (8, 9).

While many factors related to overweight and underweight among college students have been reported, there is a paucity of information on their chrononutrition behavior. The emerging field of chrononutrition provides valuable information on managing food intake across the day. Chrononutrition is the interplay between nutrition and circadian rhythm (10). The two crucial elements are dietary components that regulate the circadian system and meal timings that synchronize misaligned molecular clocks (11). In a healthy human, the circadian rhythms play major physiological functions, including the 24-h biological cycle, behavioral, physical, and mental changes. The timing of eating is tied to the internal 24-h biological timing system (the circadian clock) and influence the metabolic process of the body due to the complex interaction between circadian biology, nutrition, and human metabolism (12). Chrononutrition behavior refers to the behavioral patterns that are likely to influence one's chrononutrition profile, and these include (i) eating at night, (ii) time-restricted feeding, (iii) breakfast eating, (iv) the timing of the largest meal, (v) the time of evening eating, and (vi) the time between eating and sleep time (13). The chrononutrition behavior may affect an individual's adiposity, which may be related to the physiological adaptation to sleeping and irregularities in eating following circadian times (14). Despite using less energy, individuals with more irregular eating patterns, were more likely to develop obesity and the metabolic syndrome (14).

Studies have shown that eating at different times of the day may affect one's Body Mass Index (BMI). Inappropriate eating habits such as skipping breakfast and night eating were associated with a high BMI (15, 16). Eventually, high-calorie intake during breakfast was significantly associated with higher weight loss compared to large consumption of calories during dinner (17). It was also reported that individuals who had a high-calorie intake at later time of the day, were exposed to the risk of developing obesity. This is due to the process of lipogenesis and accumulation of adipose tissues, which tend to occur during the period of the last meal (18). Late eating may increase hunger and altered appetite-regulating hormones and altered adipose tissue gene expression favoring increased lipid storage (19).

However, the habit of skipping breakfast was also reported as a means of reducing total daily energy intake (20, 21). Indeed, it raises questions about meal timing studies such as intermittent fasting and time-restricted feeding, which indicate that skipping or delaying breakfast may reduce body weight (22, 23). The relationship between body weight and the meal timing pattern remains unclear, and it is an important research area to explore. Thus, the present study aimed to investigate the potential association of chrononutrition behavior with BMI among young adults, especially college students. To the best of our knowledge, no regional (Malaysia and Southeast Asia) studies on chrononutrition among college students has been published yet. Eating behavior of college students may carry over to later life, hence early identification related to the behavior and its effect on nutritional status (BMI) should be identified and intervene earlier. Consistent with previous studies, we hypothesized to observe significant differences concerning chrononutrition patterns and body weight status.

2. Materials and methods

2.1. Subjects

A cross-sectional study of chrononutrition behavior and weight status was conducted on college students in Malaysia's Klang Valley. The recruitment was conducted *via* social media, and electronic forms of questionnaires were distributed. Inclusion criteria were college students aged 18−35 years, literate in the English language, and having access to the internet. Those individuals with a known diagnosis of sleep disorder were excluded. The sample size was derived using OpenEpi calculated software, with 95% confidents interval and 80% power of the study, and yielded at least 385 participants.

This study was approved by the UiTM Ethics Research Committee [REC/06/2021 (UG/MR/589)] and digital informed consent was obtained from all participants. Data on body weight and height were self-reported, and guidelines on how to measure weight and height correctly were included in the instructions. Data collection was performed in the third quarter of 2021, when Malaysia was still in the COVID-19 pandemic but moving to the endemic transition phase. The lockdown is over, and there was no restriction on going out. During this period, college students still adapted to online learning and physical attendance at the institution was not compulsory.

TABLE 1 Background of participants.

Variables	Mean ± SD/frequency (*n*)
Sex	
Male	43 (10.5)
Female	366 (89.5)
Age (years)	21.45 ± 2.216
Height (cm)	157.95 ± 7.151
Weight (kg)	56.12 ± 13.999
Body Mass Index (kg/m^2)	22.4 ± 5.13
Underweight (<18.5)	101 (24.7)
Normal (18.5−24.9)	202 (49.4)
Overweight (>25)	106 (25.9)

Data were presented as *n* (%)/mean ± standard deviation (SD).

TABLE 2 Mean intake (days per week) of breakfast, snacking after last meal, and eating at night.

BMI/meal timing	Underweight (n = 101)	Normal weight (n = 202)	Overweight (n = 106)	P-value
Breakfast	4.30 ± 2.49	4.38 ± 2.41	4.03 ± 2.40	0.477
Snacking after the last meal	3.53 ± 2.11	3.11 ± 2.01	3.19 ± 1.86	0.253
Night eating	1.15 ± 1.81[a]	0.67 ± 1.56[b]	0.73 ± 1.48	0.044*

Data were presented as mean ± standard deviation (SD) with significant values of *$p < 0.05$. Significant difference between [a] and [b] by Tukey *post hoc* analysis.

TABLE 3 Association between largest meal of the day and timing of sleep with BMI.

BMI/largest meal and sleep	Underweight (n = 101)	Normal weight (n = 202)	Overweight (n = 106)	P-value
Largest meal of the day				
Breakfast	5 (5.0)	7 (3.5)	4 (3.8)	0.235
Lunch	64 (63.4)	154 (76.2)	76 (71.7)	
Dinner	32 (31.6)	41 (20.3)	26 (24.5)	
Time of sleep				
Workdays				
9 p.m.−12 a.m.	50 (49.5)	103 (51.0)	53 (50.0)	0.967
12−6 a.m.	51 (50.5)	99 (49.0)	53 (50.0)	
Freedays				
9 p.m.−12 a.m.	37 (36.6)	73 (36.1)	40 (37.7)	0.369
12−6 a.m.	64 (63.4)	129 (63.9)	96 (62.3)	

Data were presented as n (%). Analyzed using Chi-Square Test.

2.2. Instruments

The Chrononutrition Profile Questionnaire (CP-Q) was used to determine the chrononutrition behavior (13). Data from CP-Q measures the chrononutrition pattern on typical work/school as well as free days and can compute eating misalignment. Regarding the present study, we measured six chrononutrition behaviors: Breakfast skipping, largest meal, evening eating, evening latency, night eating, and eating window. Breakfast skipping refers to days per week during which individuals skip breakfast. The largest meal refers to the meal in which the largest number of calories are consumed. Evening eating refers to the last eating event, while evening latency refers to the duration of time between an individual's last eating event and sleep onset. Night eating refers to one or more days per week during which individuals wake up in the night to eat. Lastly, the term "eating window" refers to the duration of time between one's first and last eating events of the day. Results were further scored into three; good, fair, and poor behavior, a scoring cut-off from a study by Engwall (24).

Body Mass Index was computed by dividing weight (kg) by height squared (m^2). It was then further classified as: <18.5 kg/m2 underweight, 18.5−24.9 kg/m2 normal, and >25 overweight (25).

2.3. Statistical analysis

All analyses were performed using SPSS statistical software version 22.0 (SPSS Inc., Chicago, IL, USA). The participant's background including sex, age, height, weight, and BMI were reported descriptively in mean (standard deviation) or frequency. Normality tests were conducted before parametric analyses. One-way ANOVA was used to investigate differences in mean intake (days) of breakfast, snacking after last meals, and night eating between the different BMI groups. Pearson chi-squared analyses were used to determine the differences between BMI and chrononutrition behaviors. All significant variables ($p < 0.05$) were then analyzed in a logistic regression model to determine the predictor of BMI.

3. Results

A total of 409 participants with a mean age of 21.45 ± 2.2 years completed the questionnaire. Most of the participants were females (89.5%), and almost half of them were of normal weight (49.4%). The mean BMI was 22.4 ± 5.13 kg/m^2. A quarter of our participants were either underweight (24.7%) or overweight (25.9%) (Table 1).

As presented in Table 2, mean intake (days per week) of breakfast and snacking after the last meal showed no significant differences between BMI categories. However, we found that the underweight group significantly had a higher intake (days per week) of night eating compared to normal weight group ($p < 0.05$). The largest meal consumed by all BMI categories was during lunch (Table 3). Timing of sleep during both free days and workdays, did not show any different between BMI. However, observing the sleeping patterns of the participants revealed that half of them sleep after midnight even on workdays. During free days, the participants were more likely to sleep after midnight.

Chi-square analyses revealed a significant different between BMI categories and eating window, evening latency, evening eating, and night eating ($p < 0.05$) (Table 4). It was found that being underweight was associated with poor eating habits compared to being normal and overweight. Further analyses using logistic regression identified that significant predictors of being underweight included poor eating window, poor evening latency, poor evening eating, and poor night

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TABLE 4 Association between the scoring of chrononutrition behavior with BMI.

Chrononutrition behavior	Underweight (*n* = 101)	Normal weight (*n* = 202)	Overweight (*n* = 106)	Total (*n* = 409)	*P*-value
[a]Eating window					
Good (≤12:00)	54 (53.5)	132 (65.3)	83 (78.3)	269 (65.8)	0.001*
Fair (12:01−14:00)	31 (30.7)	55 (27.2)	19 (17.9)	105 (25.7)	
Poor (>14:00)	16 (15.8)	15 (7.4)	4 (3.8)	35 (8.6)	
[b]Breakfast skipping					
Good (1 day/week or less)	40 (39.6)	79 (39.1)	34 (32.1)	153 (37.4)	0.768
Fair (2−3 days/week)	21 (20.8)	45 (22.3)	26 (24.5)	92 (22.5)	
Poor (≥4 days/week)	40 (39.6)	78 (38.6)	46 (43.4)	164 (40.1)	
[c]Evening latency					
Good (>6:00)	6 (5.9)	23 (11.4)	8 (7.5)	37 (9)	0.001*
Fair (2:01−6:00)	67 (66.3)	143 (70.8)	92 (86.8)	302 (73.8)	
Poor (≤2:00)	28 (27.7)	36 (17.8)	6 (5.7)	70 (17.1)	
[d]Evening eating					
Good (<20:00)	6 (5.9)	41 (20.3)	19 (17.9)	65 (16.1)	0.002*
Fair (20:00−22:59)	66 (65.3)	129 (63.9)	72 (67.9)	267 (65.3)	
Poor (≥23:00)	29 (28.7)	32 (15.8)	15 (14.2)	76 (18.6)	
[e]Night eating					
Good (1 day/week or less)	72 (71.3)	171 (84.7)	88 (83.0)	331 (80.9)	0.042*
Fair (2−3 days/week)	14 (13.9)	17 (8.4)	12 (11.3)	43 (10.5)	
Poor (≥4 days/week)	15 (14.9)	14 (6.9)	6 (5.7)	35 (8.6)	
[f]Largest meal					
Breakfast	5 (5.0)	7 (3.5)	4 (3.8)	16 (3.9)	0.235
Lunch	64 (63.4)	154 (76.2)	76 (71.7)	294 (71.9)	
Dinner/supper	32 (31.7)	41 (20.3)	26 (24.5)	99 (24.2)	

Data were presented as *n* (%).
Analyzed using the Chi Square Test with significant values at $*p < 0.05$.
[a]Eating window describes the duration between first and last eating events, presented in HH:MM format (hours per day).
[b]Breakfast skipping describes the frequency of breakfast skipping, presents in days/week.
[c]Evening latency describes the duration between last eating event and sleep onset, presents in HH:MM format (hours per day).
[d]Evening eating describes the risk of eating late in the waking day, presented in HH:MM format (time).
[e]Night eating describes frequency of night eating, presented in days/week.
[f]Largest meal describes the meal in which largest among of food is eaten. HH:MM, hour:minute. The percentages may not total 100 due to rounding.

TABLE 5 Predictors of being underweight or overweight.

Variables	Underweight			Overweight		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Poor eating window	2.607	1.205−5.644	0.015*	0.424	0.136−1.322	0.139
Poor evening latency	2.981	1.069−8.312	0.037*	0.479	0.147−1.561	0.222
Poor evening eating	6.193	2.294−16.719	<0.001*	1.012	0.446−2.296	0.978
Poor night eating	2.545	1.168−5.544	0.019*	0.833	0.309−2.242	0.717

Analyzed using Logistic regression with significant values at $*p < 0.05$. Reference category: Normal weight.

eating (Table 5). All this poor chrononutrition behavior was strongly related to being underweight.

4. Discussion

Chrononutrition is closely related to the individual body's circadian rhythm; the biological clock that regulates the sleep and wake cycle. Its response is highly influenced by changes in the environment, primarily to light and darkness. It may not be forgotten that the light/dark cycle and food intake are important circadian rhythm regulators controlled by the central clock system. These oscillations that occur in the body influence physical, mental, and behavioral changes following a 24-h cycle (18). On the other hand, the peripheral clocks in each body system that controls localized physiological processes which include glucose and lipid

homeostasis, hormonal secretion, the immune responses, and the digestive system is highly influenced by the nutritional intake and physical activity patterns (26). Current studies suggest that eating time highly influences body weight; specifically, eating meals late at night may impact the desynchronization of the internal biological clock. Charlot et al. (27) suggested to prioritize in matching daily application of eating time to individual's circadian rhythms for optimal metabolic health (27). Important circadian hormones affecting body weight, namely cortisol, serotonin, melatonin, insulin, and insulin growth factor 1 (IGF-1) are highly synchronized with the biological clock. Hence, desynchronization of meal intake will further affect these hormones and is phenotypically reflected in body weight (28).

The results of the present study found that more than 24% of participants had their largest meal at night during dinner or supper. The prevalence is in accordance with the current trend worldwide seen among young adults. Swiss adults aged 18−26 years (29), US college students (30), and Turkish university students (31) were all found to have a similar trend of night eating. Despite the fact that night eating is associated with increased body weight (15), the young adults who were underweight in our sampling frame, showed a significantly higher prevalence of night eating. Zooming at other related research that found the association between obesity and night eating, the sampling frame focused on adults at the age of more than 30 years (15, 32, 33). Guentcheva et al. (34) also found that young adults with night eating problems have lower BMI compared to those without the problem. However, the difference was not statistically significant (34). Another population of young adults that was similar to the present study was the finding among Pakistan college students who were underweight and had poorer eating habits than those with normal BMI and overweight (35). Another important aspect that must be considered in a self-reporting questionnaire to assess dietary habits is the bias that may exist in the accuracy of habitual nutritional behaviors (36). Thus, this may suggest that the effect may arise later in life. To establish the cause and effect, another important factor that must be included in future studies is the duration of individual eating habits being established.

It is important to highlight that altered food intake as shown among these young adults may affect their hormonal patterns in the long run. Since the human body has the capability of adapting and compensating for changes, the changes may not be apparent at an early age. However, recent studies have started to discuss the potential ripple effects of poor chrononutrition on health at a later age. Evidence shows that insulin sensitivity is highly associated with circadian regulation; hence, the thermic effect of food is reduced in the evening. Therefore, it was suggested that blood sugar and insulin responses to carbohydrates are more exaggerated at night than during the day (37). Recent studies suggest that endometriosis, commonly manifested by dysmenorrhea, arises as a result of the modern dietary lifestyle, thus showing the evidence of gynecological disorders being closely linked to dietary practices (38, 39). It has been reported that female students who skipped breakfast had a higher incidence of dysmenorrhea (40).

Theoretically, the time frame of intake would be higher among individuals with short sleep duration because the total caloric intake is directly associated with the time spent awake (41). The regulation will be in synchrony with normal physiological adaptation to the environmental influence (42). However, no significant differences were observed in participants, eating behaviors with their sleep duration. The finding is consistent with the findings obtained among American (43) and Brazilian adults (44).

The present study found that there was a vast heterogeneity in sleeping patterns among each participant. Benham (45) reported that the COVID-19 pandemic had a significant effect on students' sleeping patterns. The study found that students went to bed significantly later during the pandemic and there were pronounced delays in waketimes (45). Since the present study was conducted during the pandemic after the lockdown, the wide range of waking time and sleeping time shown in the study population may have been affected by the asynchronous format of their classes' format. Additionally, during the sampling, some of the undergraduates were still having online classes, hence, eliminating the need for early waketime.

Based on Abraham et al. (1), young adults usually establish their eating habits during their years in college and the behavior often continues through adulthood. Furthermore, Das and Evans (46) highlighted that body weight is part of the barriers and promoting factors for lifestyle choices to maintain health. The best eating habits and ideal weight can only be achieved with the help of proper knowledge about nutrition, but this knowledge needs to be combined with favorable environmental conditions such as access to nutritious food and physical activity activities (47, 48). In earlier studies, it has been reported that the absence of healthy food in educational institutions was a barrier to healthy eating (49). Self-efficacy, dietary preferences, body image, conformity to friends and parents, socioeconomic position, and the accessibility of food in the community are a few of the variables that may directly or indirectly influence adolescent eating behaviors (50).

Interestingly, earlier studies have also reported the fact that adolescents and young adults also had the habit of not consuming a proper lunch until they returned home at 03.00−04.00 p.m. (49). It has been reported that individuals who receive parental encouragement and support, tend to eat healthier foods and have better eating habits (51, 52). The underweight participants are less particular in their eating time frame (46). Good eating habits may not be the main priority being emphasized by those underweight. However, they may suffer from health consequences at a later age if their eating habits continue. Result from this study opens a new gap of study that warrant serious attention. Despite plethora of publications on eating habits of university students, most studies focused on eating disorders among those who are overweight and obese (7, 53). However, cohort studies are needed to elucidate the impact of eating disorders among those who are underweight and its association with their later health impact.

We admit a few limitations in our study. The cross-sectional design employed may be insufficient to arrive at any definite conclusion. Our study had a small sample size, hence unable to generalize to all college students in Malaysia. Besides that, participation in this study is voluntary basis, hence, resulted in marked volume of women participants as compared to the men. It was earlier reported that there are large variety in eating behavior among college students with conflicting results on the differences reflected between gender (54, 55). As weight and height data were reported, it may introduce bias, which is the limitation of all self-reported surveys. However, we provided an insight into the situation happening in the younger population. It is paramount to include the

underweight in any nutrition intervention as well, as they are prone to poor eating habits.

5. Conclusion

This study found that underweight college students have poor chrononutrition behavior as compared to the rest. The general lack of knowledge regarding healthy and timely eating among college students is a cause of concern. These population of interest tend to skip meals and develop various eating disorders. These young adults are constantly exposed to unhealthy lifestyles and poor dietary choices without knowing the consequences of such exposure. The dietary habits of this population can be duly addressed with proper education and health screening programs. There is also a need to build strong social support and a framework to promote healthy eating among college-going young adults. Future long-term research studies should be conducted to arrive at a definite conclusion.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee, Universiti Teknologi MARA. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NT designed the research, supervised the project, and wrote the manuscript. NJ contributed to the research design and wrote the manuscript. KH conducted the experiment and drafted the manuscript. WW conducted the experiment. SD edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of the one-day fasting on cortisol and DHEA daily rhythm regarding sex, chronotype, and age among obese adults

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Introduction: Physiological and biochemical processes in the human body occur in a specific order and show rhythmic variability. Time dependence characterizes the secretion of cortisol and dehydroepiandrosterone (DHEA). One-day fasting implies alternating fasting days and eating days. The study aimed to determine how 24-h fasting affects the daily rhythm of cortisol and DHEA levels in obese people while taking into account gender and chronotype.

Methods: Forty-nine obese patients (BMI 32.2–67.1kg/m^2; 25 women and 24 men) underwent a 3-week hospital-controlled calorie restriction diet to reduce body weight. During hospitalization, patients fasted for 1day, during which only water could be consumed. Samples of whole mixed unstimulated saliva were collected at 2–3-h intervals over a 64-h period and analyzed for cortisol and DHEA by immunoassays. The individual chronotypes were assessed by the morning and evening questionnaire, according to Horne and Östberg. Three components of daily rhythm were evaluated: amplitude, acrophase, and the so-called MESOR.

Results: Cortisol rhythm showed differences in amplitude (p=0.0127) and acrophase (p=0.0005). The amplitude on the fasting day was 11% higher (p=0.224) than the day after. The acrophase advanced on the day of fasting, 48min earlier than the day before (p=0.0064), and by 39min to the day after fasting (p=0.0005). In the rhythm of DHEA, differences were found in the MESOR (p=0.0381). The MESOR on the fasting day increased.

Discussion: Our results obtained during 64 consecutive hours of saliva sampling suggest that one-day fasting may affect three components of cortisol and DHEA daily rhythm. Additionally, no differences were found in the daily rhythm between the morning and evening chronotypes and between females and males. Although aging did not influence daily cortisol rhythm, DHEA amplitude, MESOR, and acrophase changed with age. To the best of our knowledge, this is the first presentation of changes in DHEA rhythm during one-day fasting.

KEYWORDS

cortisol, DHEA, fasting, obesity, rhythm

Introduction

Rhythmicity seen in many processes, including metabolism, reveals both individual habits (e.g., sleep and mealtimes) and the effect of internal body clocks (1). The central pacemaker controls behavioral, metabolic, and physiological rhythms and can synchronize the peripheral oscillators (2). The lack of synchronization between the central clock and peripheral clocks, e.g., by changing the timing of food intake and the diet composition, may lead to the desynchronization of rhythms and the development of metabolic disorders, including obesity or type 2 diabetes (3, 4). Synchronization mechanisms imply various humoral signals, e.g., circulating components such as glucocorticoids (5). Cortisol and DHEA belong to the group of steroid hormones (6, 7). The circadian rhythm of cortisol and DHEA secretion is regulated by the central pacemaker, the so-called biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and is dependent on the time of sleep and wakefulness (8, 9). Circadian variation in DHEA is also documented, with peaks occurring in the early morning hours (10–12). DHEA is secreted synchronously with cortisol in response to corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (13). Cortisol plays a major role in maintaining the body's homeostasis, it is involved in the regulation of carbohydrate, lipid, and protein metabolism. It stimulates gluconeogenesis in the liver and inhibits glucose consumption in peripheral tissues. Cortisol increases lipolysis through its catabolic effect, thus altering adipose tissue (14, 15). It has also been reported that a high and long-term rise in cortisol concentration (as opposed to a short-term increase during a one-day fast) results in increased consumption of high-fat snacks and sweets (16). Overstimulation of the hypothalamic–pituitary–adrenal (HPA) axis may play a role in the pathogenesis of diseases coexisting with obesity (17), e.g., it correlates with excessive food intake and causes emotional eating (18). Moreover, excessive levels of glucocorticoids reduce the activity of the satiety hormone – leptin (19). It is a common belief that visceral adipose tissue has the most significant impact on elevated cortisol levels (20, 21). The amount of visceral fat correlates with the increased HPA axis reactivity, especially in the morning and in response to acute stress (21). DHEA has anti-obesity (22), anti-diabetic (23), and anti-atherosclerotic properties (24). The ratio of cortisol to DHEA seems to be of particular importance, including indicators of metabolic and cardiovascular outcomes in obese patients after bariatric surgery (25). DHEA and dehydroepiandrosterone sulfate (DHEA-S) are defined as large reservoirs that easily converting into more potent androgens in peripheral tissues. A decrease in the levels of these reservoirs can lead to health problems such as obesity and insulin resistance by reducing the inhibitory effect of glucocorticoids (26). This is further exacerbated by increased cortisol levels upon aging (27). On the other hand, since DHEA concentration gradually decreases with age, the implementation of replacement therapies of DHEA might be an effective anti-aging therapy (28, 29).

Food intake is one of the synchronizers of the circadian rhythm (30). One-day fasting is one type of intermittent fasting (IF), comprising a fasting day (no foods and drinks of any energy value, or reduction of food consumption to 25% of usual intake, approximately 500 kcal) and an eating day during which you can consume food without restrictions (31). The participants of the one-day fasting study showed a 3–7% higher body weight loss after 2–3 months of intermittent fasting compared to the control group, and improvement in lipid profile, blood pressure, and insulin sensitivity were also observed (32). Fasting increases the serum cortisol concentration by activating the HPA axis (33). Furthermore, this method of feeding improves metabolism, which in turn prolongs the life of animals, as well as slows down the aging process (34). It is believed that a hypocaloric diet can control metabolism by influencing the biological rhythms of metabolic processes (35). It has also been reported that during fasting, cortisol affects the pathways of metabolic processes in the liver, adipose tissue, and skeletal muscles, and also the regulation of clock gene expression, which in turn influences the biological rhythms of metabolic processes (36). DHEA is a weak androgen and an important precursor to androgens in males and oestrogens in females (37). In addition, DHEA counteracts the effect of cortisol on the immune system and cognitive functions (38–40) improvement of cognitive functions, enhancement.

In our study, we aimed to determine the influence of 1-day fasting on the daily rhythm of cortisol and DHEA in patients with obesity. It is hypothesized that 1-day fasting modifies the daily rhythm of cortisol and DHEA in obese individuals.

Materials and methods

Study population and study design

A total of 49 patients with obesity, including 25 women and 24 men, were registered. The mean age (±SD) of participants was 48.6 ± 12.8, and the median (min-max) was 47 (26–70). This study enrolled patients with BMI ≥ 30 kg/m^2, age > 18 years old, who were referred to initiate a weight loss regimen on doctor's advice. Exclusion criteria were a history of or current disease (central nervous system, psychiatric, active liver disease, immune, gastrointestinal, endocrine, and hematologic), a history of eating disorders (anorexia, bulimia), alcohol/drug abuse, and ongoing antibiotic therapy and steroid therapy, and shift work, night work. Eligible participants must maintain a regular sleep–wake cycle, residing in bed between 22:00 and 24:00 habitually.

Patients were treated for obesity at the Department of Gastroenterology, Dietetics and Internal Diseases of the Poznan University of Medical Sciences. Participants who qualified for the study were subjected to a three-week dietary treatment in a hospital setting. They used an individually selected diet with a 25–30% reduction in the daily caloric supply (reduction of 500–1,000 kcal) in relation to the total energy requirement, calculated according to the Harris and Benedict formula and the physical activity index (41). All patients received the same type of diet provided by a hospital, with the same proportion of nutrients: 20% protein, 25–30% fat, and 50–55% carbohydrates. In addition, on the second day of hospitalization (day 2), patients underwent a 24-h fast with only water intake *ad libitum*. Simultaneously, multiple saliva samplings were collected over a 64-h period at 2–3-h intervals starting at 08:00 (Figure 1).Bodyweight measurement was conducted, including body composition analysis using the BIA electrical bioimpedance method, which allowed the estimation of both the percentage and weight of adipose tissue and muscle tissue in patients (BIA; Tanita/Acern Body Composition Analyzer, Japan).

All patients gave written informed consent and entered the study. Patient baseline characteristics are given in Table 1. The study was approved by the Ethics Committee of the Poznan University of Medical Sciences (No. 249/19) and followed the principles of the Declaration of Helsinki.

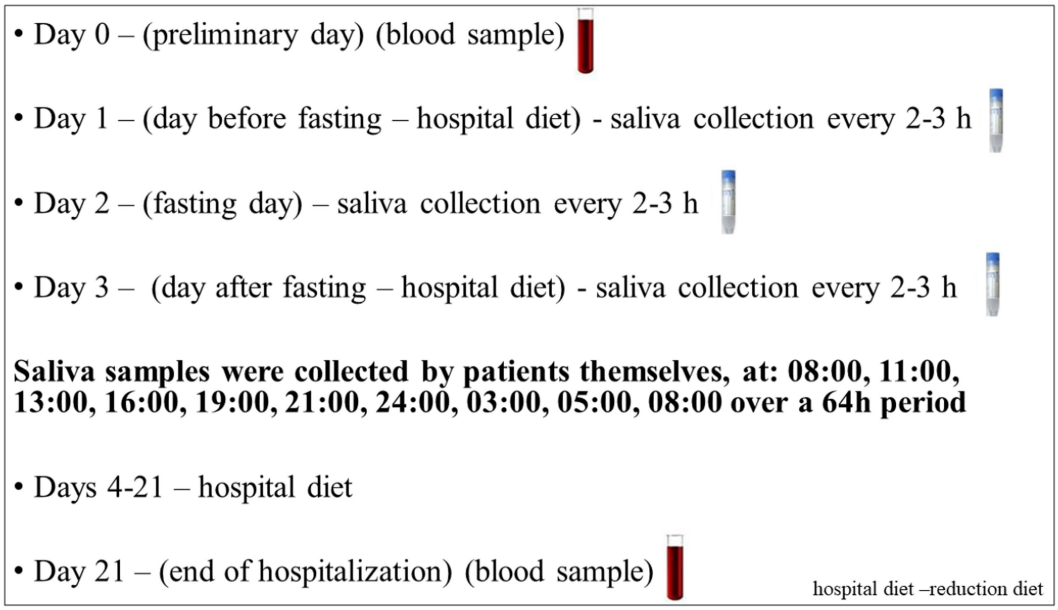

FIGURE 1
Study protocol. Each subject underwent a protocol schedule with 3-week controlled calorie restriction diet in the hospital. Unstimulated saliva were collected over a 64-h period at 2–3-h intervals starting at 08:00 during the course of three consecutive days for the daily rhythm of cortisol and DHEA. In addition, an initial and final sample of blood on day 0 and day 21 was obtained for biochemical parameters.

Saliva collection

Unstimulated whole mixed saliva was collected using Salivette swabs (Sarstedt, Nümbrecht, Germany), per the manufacturer's instructions. All patients were provided with written instructions for the saliva sampling. Samples were collected over a 64-h period at 2-3-h intervals starting at 08:00 (Figure 1). Following centrifugation, the samples of clear saliva were stored at minus 80°C until assayed in batch (9).

Biochemical measurements

The concentrations of cortisol and DHEA were measured with specific immunoassays from Demeditec Diagnostics GmbH (Kiel, Germany). The sensitivity of the assays was 0.019 ng/ml for cortisol and 6.4 pg/ml for DHEA. Intra- and inter-assay precision for cortisol was (CV%) 7.0 and 7.4%, and for DHEA (CV%) 7.4 and 7.0%, respectively. The assays were performed according to the manufacturer's instructions. All other measurements were performed by the central laboratory of the university hospital. Insulin resistance was calculated from the fasting levels of blood glucose and insulin using the homeostasis model assessment (HOMA) index (42).

Questionnaire

An individual's chronotype was determined using the morningness-eveningness questionnaire (MEQ), as described by Horne and Ostberg (43). This tool could decide on their chronotype, morningness, or eveningness. It may lead to understanding the peak hours of physical and psychological performance and sleep and awakening preferences of these respondents. The subjects were categorized into the morning types, evening types and intermediate types (43).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism TM 8 software (Software Inc., United States). The normality of the distribution was tested using Shapiro–Wilk's test. The data that did not follow a Gaussian distribution or ordinal data were analyzed with the Wilcoxon test, Mann–Whitney test or the Kruskal–Wallis test with Dunn's *post-hoc* test. The relationship between the variables was analyzed with Spearman's rank correlation coefficient, and categorical data were analyzed with the X^2 test. The results are presented as individual data with the medians and interquartile ranges (min-max). A value of $p < 0.05$ was considered significant. In order to analyze changes over time, for related samples, the ANOVA, two-way ANOVA and appropriate *post-hoc* tests were used. The cortisol and DHEA results were assessed in terms of outliers' values in the Grubbs' test. Therefore, in the entire further study, the cortisol and DHEA results were presented without outliers.

The cosine analysis

The daily rhythm was assessed by a single cosine test using MemCalc/Win (GMS, Tokyo, Japan). The daily rhythm is described by the midline estimating statistic of rhythm (MESOR), amplitude, and acrophase. The MESOR is the mean of all values across the circadian rhythm. The amplitude is half the difference between the highest and the lowest points of the cosine function, best fitting the data. The acrophase represents the time point when the circadian cycle reaches the peak value.

TABLE 1 Baseline characteristics of the participants.

Parameters	All participants $n=49$	Female (F) $n=25$	Male (M) $n=24$	p-Value F vs. M
Age, years	47 (26–70)	45 (26–68)	49.5 (29–70)	0.3734
Morbid obesity BMI > 40, *n*	29	13	16	0.2952
Glucose intolerance, *n*	20	11	15	0.1931
Diabetes type 2, *n*	6	4	2	0.4087
Dyslipidaemia, *n*	14	7	7	0.9280
Weight, kg	130.2 (89.2–189.4)	112.5 (89.2–189.4)	142.0 (103.3–189.2)	**0.0013**
BMI, kg/m^2	41.5 (32.2–67.1)	41.6 (32.2–67.1)	41.5 (32.7–57.5)	0.7336
Fat tissue, %	41.6 (32.6–53.2)	43.4 (33.7–53.2)	40.0 (32.6–48.8)	**0.0076**
Fat tissue, kg	51.7 (31.9–100.2)	48.9 (31.9–100.2)	55.2 (34.4–88.7)	0.8729
Visceral fat tissue	18.5 (6–40)	13.5 (6–25)	24.0 (17–40)	**<0.0001**
Muscle tissue, kg	70.3 (31.7–95.6)	59.4 (48.9–84.7)	80.3 (31.7–95.6)	**<0.0001**
Body water (TBW), %	41.3 (33.4–48.6)	43.5 (33.7–53.2)	40.0 (32.6–48.8)	**<0.0001**
Glucose, mg/dl	103.0 (84–313)	103.0 (86–313)	107.5 (84–247)	**0.0473**
Insulin, mU/ml	18.0 (5.8–88.8)	16.9 (5.8–58.3)	23.3 (7.3–88.8)	0.1696
HOMA-IR	5.4 (1.5–49.3)	4.1 (1.5–26.5)	5.8 (1.5–49.3)	**0.0192**
HbA1c, %	6.4 (5–27.4)	5.7 (5–13)	6.1 (5.2–27.4)	0.1504
Cholesterol, mg/dl	180.5 (106–274)	178.5 (112–267)	182.0 (106–274)	0.7984
LDL, mg/dl	106.8 (37.6–204)	103.5 (56.2–142.3)	114.9 (37.6–204)	0.2343
HDL, mg/dl	45.0 (31–100)	49.0 (32–100)	44.0 (31–65)	**0.0146**
Triglycerides, mg/dl	135.0 (50–454)	134.0 (50–454)	137.0 (77–259)	0.5039
CRP, mg/L	6.3 (0.4–16.2)	7.9 (0.4–16.2)	4.0 (0.6–11.5)	**0.0152**
Morning chronotype, *n*	36	20	16	0.5343
Intermediate chronotype, *n*	11	4	7	0.2891
Evening chronotype, *n*	2	1	1	0.9764

The data are given as medians and range (min-max), $n=49$, day 0. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; CRP, C-reactive protein; HbA1c, glycated hemoglobin. The data were analyzed with the Mann–Whitney test and X^2 test. A value of $p<0.05$ was considered significant. Bolded values are statistically significant.

Results

Description and distribution of the obese subjects

Almost 60% (59%) of participants ($n=29$) enrolled in the study had morbid obesity with BMI above 40 kg/m^2. Among them, 40.8% ($n=20$) had glucose intolerance, 12% ($n=6$) diabetes type 2, and 29% ($n=14$) dyslipidemia. The females were predominant (51%) among the study group. As expected, female patients were found to have a lower 26% body weight, 35% muscle tissue, and 3.5% body water with 3.4% higher fat tissue % as compared with male participants. Female patients had lower glucose, insulin resistance (HOMA) index, and higher HDL and CRP concentrations than men (Table 1).

Patients' chronotypes

Patients could be classified as morning chronotypes 73%, ($n=36$), evening chronotypes 5%, ($n=2$) and 22%, ($n=11$) and intermediate chronotypes (Table 1). Due to the small number of participants with the pure evening chronotype, for this analysis, the patients with intermediate and evening chronotypes were grouped together and designated further as evening chronotypes. These patients were compared to those with the morning chronotype. Such an approach was used previously in other studies (34, 35).

Saliva samples

Cortisol

Saliva samples were collected during the 64-h study (Figure 2). The cosinor method was used to assess the biological rhythm of cortisol. Salivary concentrations of cortisol measured over a 24-h period displayed significant fluctuations. The cosine analysis revealed that these changes exhibited a daily rhythm, and the characteristics of these rhythms are given in Table 2. Differences in amplitude ($p=0.0127$) and acrophase ($p=0.0005$) were noticed. The amplitude on day 2 (fasting day) was 11% ($p=0.224$) higher than the amplitude on day 3 (the day after fasting). On the other hand, acrophase was shifted on the fasting day, 48 min earlier compared to day 1 ($p=0.0064$) and by 39 min from day 3 ($p=0.0005$; Table 2). After dividing the patients by gender, differences in amplitude ($p=0.03$) and acrophase ($p=0.0006$) were observed in the group of women. The amplitude on the fasting day was 12% higher compared to day 1 and 13% higher on day 3 (the day after fasting). Furthermore, the acrophase was shifted on the fasting day by 66 min compared to day 1

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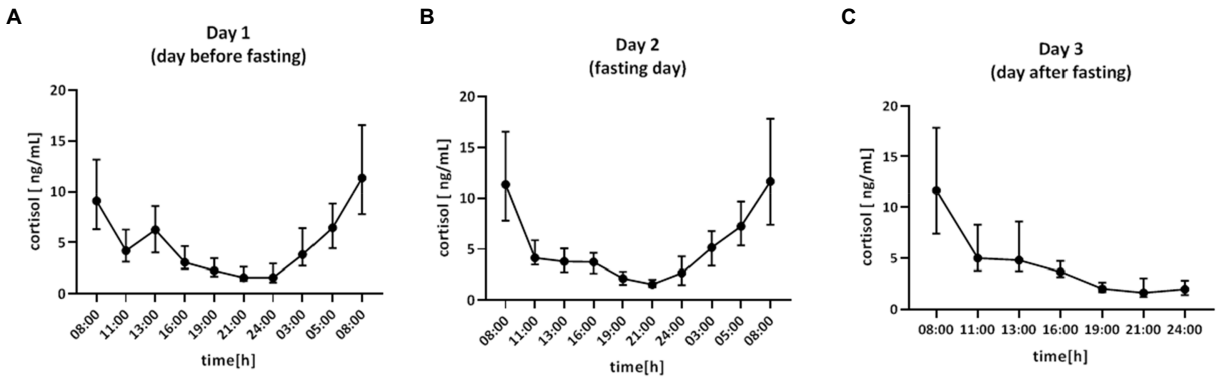

FIGURE 2
Time course of cortisol concentration (ng/ml) for 64h in: **(A)** day 1 (day before fasting), **(B)** day 2 (fasting day), **(C)** day 3 (day after fasting), *n*=49. Each value is the median (min-max).

TABLE 2 The characteristics of daily cortisol and DHEA rhythm during 64h.

Parameters	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p
Cortisol *n*=49							
MESOR, ng/ml	4.973 (2.632–7.83)	5.025 (2.543–7.05)	5.175 (1.208–10.84)	0.3288	ns	ns	ns
Amplitude, ng/ml	3.724 (0.1374–10.23)	4.289 (0.1839–10.96)	3.801 (0.275–8.808)	**0.0127**	ns	ns	**0.0224**
Acrophase, clock time	08:24 (04:42–10:48)	07:36 (05:00–10:24)	08:15 (06:18–11:54)	**0.0005**	**0.0064**	ns	**0.0005**
DHEA, *n*=15							
MESOR, pg/ml	55.25 (11.4–393.4)	60.52 (7.34–435.4)	45.0 (10.35–277.2)	**0.0381**	ns	ns	ns
Amplitude, pg/ml	32.12 (2.963–235.6)	42.85 (4.924–176.5)	30.2 (1.75–128.9)	0.2818	ns	ns	ns
Acrophase, clock time	5:14 (1:58–20:44)	5:13 (00:04–10:05)	7:00 (2:08–20:26)	0.0569	ns	ns	ns
Cortisol/DHEA ratio, *n*=15							
05:00	56.5 (7.7–949.6)	194.4 (9.7–1891)	–	–	**0.043**	–	–
08:00	87 (7.7–373)	101.3 (11.2–2,284)	255.4 (5.7–3,038)	0.3441	ns	ns	ns
24:00	48.3 (4.8–1842)	24.5 (3.8–2,939)	30.5 (5.2–6,122)	0.7659	ns	ns	ns

The data are given as medians and ranges (min-max). The daily rhythm is described by the midline estimating statistic of rhythm (MESOR), amplitude, and acrophase. The MESOR is the mean of all values across the circadian rhythm. The amplitude is half the difference between the highest and the lowest points of the cosine function, best fitting the data. The acrophase represents the time point when the circadian cycle reaches the peak value. DHEA, dehydroepiandrosterone; ns, non-significant. The data were analyzed with a one-way ANOVA test. A value of $p < 0.05$ was considered significant. Bolded values are statistically significant.

($p = 0.0008$) and by 48 min to day 3 ($p = 0.0374$). However, no differences were found in the cortisol rhythm between men and women (Table 3). The study group was divided according to chronotype. Differences were noticed for acrophase ($p = 0.025$) in people with morning chronotype. Acrophase was shifted on the fasting day by 57 min earlier than day 1 ($p = 0.0202$) and by 42 min compared to day 3 ($p = 0.0034$). No differences were found in the cortisol rhythm between the morning and evening chronotypes (Table 4). The study group was also divided according to age. No differences were found in the daily rhythm of cortisol between younger (<50 years old) and older (>50 years old) participants (Table 5).

The area under the curve (AUC) for cortisol concentration was calculated to compare the total concentration of cortisol on day 1 and day 2. There were no differences in AUC ($p = 0.8766$; Figure 3A).

DHEA

Saliva samples for DHEA were collected during the 64-h study (Figure 4). The cosine analysis revealed that these changes exhibited a daily rhythm, and the characteristics of these rhythms are given in Table 2. Differences in MESOR ($p = 0.0381$) were noticed. The MESOR on day 2 (fasting day) was 11% higher compared to the MESOR on day 1 (the day before fasting) and 13.4% higher than the MESOR on day 3 (the day after fasting). No differences were found in the DHEA rhythm in the group of women, men and between these two groups (Table 3). After dividing the patients by chronotype differences in acrophase, were observed ($p = 0.456$) in the morning chronotype group and amplitude ($p = 0.0394$) evening chronotype group (Table 4). The acrophase was shifted on the fasting day by 66 min compared to day 1 and by 266 min to day 3 ($p = 0.0417$). The amplitude on day 2 (fasting day) was 57.2% lower than the amplitude on day 1 and 12.7% lower than the amplitude on day 3. No differences were found in the DHEA rhythm between the morning and evening chronotypes (Table 4). The study group was divided according to age. The differences were found in the parameters of DHEA rhythm between younger (<50 years old) and older (>50 years old) participants ($p < 0.01$, 2-way ANOVA). The MESOR and amplitude in older patients were reduced, and the acrophase was delayed (Table 5).

The area under the curve (AUC) for DHEA concentration was calculated to compare the total concentration of DHEA on day 1 and day 2. There were no differences in AUC ($p = 0.6355$; Figure 3B).

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TABLE 3 The characteristics of daily cortisol and DHEA rhythm during 64h according to sex.

Parameters	Female (F)							Male (M)							F vs. Mp
	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1vs. D3p	D2 vs. D3p	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p	
Cortisol															
	n = 25							*n* = 24							
MESOR, ng/ml	4.816 (2.632–7.83)	4.842 (2.543–7.04)	4.885 (2.841–7.2)	0.5806	ns	ns	ns	5.185 (3.201–7.38)	5.08 (2.719–7.04)	5.453 (2.557–10.48)	0.4059	ns	ns	ns	ns
Amplitude, ng/ml	3.812 (0.137–8.747)	4.511 (0.1839–10.96)	3.6 (0.275–9.808)	**0.03**	ns	ns	ns	3.585 (1.543–6.614)	3.876 (0.6909–7.004)	4.298 (0.6294–8.407)	0.0912	ns	ns	ns	ns
Acrophase, clock time	08:18 (04:42–10:48)	07:12 (05:18–09:24)	08:00 (07:06–09:48)	**0.0006**	**0.0008**	ns	**0.0374**	08:33 (05:36–10:36)	07:54 (05:36–10:18)	08:30 (06:18–11:54)	0.0865	ns	ns	ns	ns
DHEA															
	n = 8							*n* = 7							
MESOR, pg/ml	51.46 (14.18–323.9)	67.31 (20.63–269.8)	49.41 (11.7–211.9)	0.1495	ns	ns	ns	55.25 (11.4)	51.29 (7.34–435.4)	42.32 (10.35–277.2)	0.1916	ns	ns	ns	ns
Amplitude, pg/ml	27.92 (2.963–122.5)	35.62 (6.135–130.6)	30.99 (3.271–84.82)	0.7943	ns	ns	ns	44.79 (6.105–235.6)	44.57 (4.924–176.5)	30.2 (1.75–128.9)	0.4861	ns	ns	ns	ns
Acrophase, clock time	6:06 (02:35–20:44)	5:06 (00:35–8:30)	8:10 (3:22–20:26)	0.0789	ns	ns	ns	5:14 (1:58–13:22-)	6:56 (00:04–10:05)	4:51 (02:08–11:08)	0.6197	ns	ns	ns	ns

The data are given as medians and ranges (min-max). DHEA, dehydroepiandrosterone; ns, non-significant. The data were analyzed with a one-way ANOVA test and with two-way ANOVA for female vs. men. A value of $p < 0.05$ was considered significant. Bolded values are statistically significant.

Cortisol/DHEA ratio

The cortisol to DHEA ratio for all time points of saliva sampling was calculated.

Only in the morning at 05:00 did the ratio differ between pre-fasting and fasting day, whereas no significant differences could be found between any other time points of the experimental days (Table 2).

Calorie restriction

The 3-week calorie restriction resulted in a modest but significant reduction in body weight and – consistently – in BMI, %, kg of adipose tissue, visceral fat, body water, and muscle tissue (Table 6). In addition, a more significant decrease in glycated hemoglobin and total cholesterol, and triglyceride levels was observed (Table 6). Spearman correlations between cortisol and DHEA rhythm and changes in anthropological and biochemical parameters after dietary restriction were calculated. There were no correlations between those parameters.

Discussion

The present study assessed the daily rhythm of cortisol and DHEA in obese subjects following a one-day fasting diet. There are only very few scientific reports on the daily rhythm of DHEA. To the best of our knowledge, this is the first study to assess the relationship between fasting and DHEA rhythm. On the fasting day, we found differences in the amplitude and acrophase of the daily cortisol rhythm and the MESOR of the daily DHEA rhythm. We have shown that one-day fasting causes significant changes between the minimum and maximum values of the cortisol rhythm curve. The amplitude on the fasting day was higher than on the other sampling days. Moreover, a shift in the phase of the cortisol rhythm has been observed. Acrophase on the fasting day occurred earlier. The MESOR of the cortisol rhythm did not differ between days.

In a partially contradictory study by Bergendahl et al. (44), the authors also noticed that fasting caused an increase in amplitude and MESOR of the circadian rhythm of serum cortisol; however, it did not affect the acrophase. The duration of fasting was identical to our study and amounted to 24 h, though the study group consisted of slim men. In another study involving lean people and overweight patients, an 84-h fast was implemented, and an increase of the MESOR and the amplitude of the cortisol rhythm was observed (44). A plausible explanation for the discrepancies in the results of MESOR and acrophase with our study could be due to the differences in the study protocol, i.e., duration of fasting (5 days vs. 1 day fast) and different characteristics of the subjects (lean vs. obese). However, in all studies, the amplitude of the cortisol rhythm was increased. The suprachiasmatic nucleus of the hypothalamus drives the 24-h pattern in cortisol (45). The animal study by Girotti et al. (46) showed that the feeding cue is a strong synchronizer of the HPA axis. In their review, Nakamura et al. (33) emphasize that fasting has a

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TABLE 4 The characteristics of daily cortisol and DHEA rhythm during 64h according to chronotype.

Parametrs	Morning chronotype (M)							Evening chronotype (E)							M vs. Ep
	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p	
Cortisol															
	n = 36							*n* = 13							
MESOR, ng/ml	4.932 (2.632–7.83)	4.738 (2.543–7.04)	5.061 (2.557–7.1)	0.931	ns	ns	ns	5.18 (3.94–7.25)	5.55 (4.134–6.42)	6.054 (3.603–7.7)	0.783	ns	ns	ns	ns
Amplitude, ng/ml	3.243 (0.1374–9.06)	4.083 (0.1839–10.96)	3.579 (0.275–8.407)	0.065	ns	ns	ns	4.756 (1.961–7.187)	4.489 (1.439–9.794)	4.344 (0.6796–8.808)	0.2108	ns	ns	ns	ns
Acrophase, clock time	08:33 (04:42–10:48)	07:36 (05:00–10:24)	08:18 (06:18–11:54)	**0.025**	**0.0202**	ns	**0.0034**	08:18 (05:36–10:12)	07:42 (05:30–08:42)	08:06 (07:06–10:12)	0.1803	ns	ns	ns	ns
DHEA															
	n = 10							*n* = 5							
MESOR, pg/ml	72,15 (14.18–393.4)	67.31 (19.51–435.5)	49.41 (26.84–277.7)	0.3675	ns	ns	ns	31.81 (11.4–121.0)	38.07 (7.34–176.8)	15.52 (10.35–107.3)	0.0934	ns	ns	ns	ns
Amplitude, pg/ml	47.97 (2.963–235-6)	43.71 (14.82–176.5)	38.78 (20.42–128.9)	0.8302	ns	ns	ns	23.59 (6.105–129.5)	10.1 (4.924–123.3)	11.57 (1.75–84.82)	**0.0394**	ns	**0.0342**	ns	ns
Acrophase, clock time	4:50 (1:47–20:44)	3:44 (00:04–8:30)	8:10 (2:08–15:41)	**0.0456**	ns	ns	**0.0417**	6:48 (5:08–13:22)	6:11 (4:48–10:05)	6:44 (4:51–20:26)	0.9537	ns	ns	ns	ns

The data are given as medians and ranges (min-max). DHEA, dehydroepiandrosterone; ns, non-significant. The data were analyzed with a one-way ANOVA test and with two-way ANOVA for morning chronotype vs. evening chronotype. A value of $p < 0.05$ was considered significant. Bolded values are statistically significant.

powerful effect on increasing cortisol secretion. In this respect, our results obtained during several consecutive days of saliva sampling might confirm that fasting may elicit changes in the cortisol rhythm.

Obesity is associated with increased inactivation of cortisol and impaired hepatic regeneration of cortisol from cortisone (47). Moreover, obesity is accompanied by an altered circadian rhythm of cortisol (48, 49). On the other hand, weight loss associated with a very low-calorie diet (VLCD) in obese people normalizes the production of cortisol (48, 50, 51). In the study by van Rossum et al. (52), a moderate calorie restriction diet for 6 months did not affect the circadian cortisol rhythm.

According to Al Safi et al. (48), the cortisol concentration in obese patients was significantly higher compared to lean individuals. Furthermore, obese subjects showed no decrease in cortisol concentration in the evening. In contrast, the study by Parra et al. (51) showed that in obese men, the secretion of cortisol in the fasting state and after a meal was lower than in lean subjects. Our study showed that the total cortisol concentration measured by AUC did not change during fasting (Figure 3). The current research indicates a relationship between fasting and the circadian cortisol rhythm. The observed alteration in cortisol rhythm might indicate disturbances in regulating the HPA axis under the influence of fasting.

When analyzing the study group according to gender, we found that female participants presented alterations in the amplitude and acrophase of the cortisol rhythm. The amplitude of the rhythm on the fasting day was higher, and the acrophase occurred earlier. There were no differences in the MESOR parameter. In contrast, none of the parameters characterizing the cortisol rhythm differed in the male subjects.

A study by Vance et al. (53) examined the effect of 5-day fasting on cortisol secretion in lean men. The authors noted changes in the amount of cortisol, and the amplitude of the circadian rhythm decreased (53). Multiple studies have been inconclusive, with some reports showing a higher cortisol concentration in women and others in men.

One of the hypotheses explaining these differences may be the use of different biological materials in the studies. In the research that assessed sera or blood plasma, the cortisol concentration was higher in men (54, 55), while an inverse relationship was observed in studies involving saliva.

Similar findings have been confirmed in the survey by Kudielka et al. (56), where the authors demonstrated that the total plasma cortisol concentration was higher in older women than in older men, while the opposite was observed for salivary cortisol levels.

A possible justification for the differences the cortisol concentration between genders could be that, i.e., exogenous estrogens taken by women can increase the concentration of corticosteroid-binding globulin in the blood (57, 58). Additional reports suggest that birth control pills may cause increased free cortisol through increased basal activation of the HPA axis (59, 60).

Other theories suggest a difference in the occurrence of chronotypes depending on gender. Some studies have shown that women are more of a "morning chronotype" than men, have an earlier phase of the biological clock gene expression rhythm, and

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TABLE 5 The characteristics of daily cortisol and DHEA rhythm during 64h according to age.

Parameters	Age<50years							Age>50years							
	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p	Day 1 (D1)	Day 2 (D2)	Day 3 (D3)	*p*	D1 vs. D2p	D1 vs. D3p	D2 vs. D3p	<50years vs. >50yp
Cortisol															
	n = 27							*n* = 22							
MESOR, ng/ml	5 (0.7–9.62)	5.1 (0.8–9.9)	5.22 (1.21–12.85)	ns	ns	ns	ns	5.69 (2.63–12.55)	5.3 (2.54–12.29)	5.15 (2.36–10.84)	ns	ns	ns	ns	ns
Amplitude, ng/ml	3.81 (0.14–10.23)	4.36 (0.18–10.96)	3.42 (0.28–10.17)	ns	ns	ns	ns	3.66 (1.04–9.06)	4.07 (1.05–9.8)	4.15 (0.63–8)	ns	ns	ns	ns	ns
Acrophase, clock time	8:17 (4:04–22:17)	7:35 (5:17–22:11)	8:35 (7:04–13:04)	**0.0391**	ns	ns	ns	8:17 (2:35–10:46)	7:56 (3:22–9:22)	8:11 (6:17–11:49)	ns	ns	ns	ns	ns
DHEA															
	n = 7							*n* = 8							
MESOR, pg/ml	121 (32.51–3,939)	141.8 (51.29–435.4)	101.8 (42.32–277.2)	ns	ns	ns	ns	30.88 (11.4–111.9)	34.36 (7.34–112)	26.34 (10.35–45.76)	ns	ns	ns	ns	<0.01
Amplitude, pg/ml	122.5 (23.71–235.6)	48.02 (14.82–176.5)	78.88 (29.44–128.9)	ns	ns	ns	ns	17.2 (2.96–88.39)	19.27 (4.92–48.73)	30.88 (11.4–111.9)	ns	ns	ns	ns	<0.01
Acrophase, clock time	5:11 (2:35–8:22)	4:56 (2:04–8:32)	8:35 (3:11–1:04)	ns	ns	ns	ns	6:17 (1:56–20:39)	5:39 (00:04–10:06)	5:49 (2:04–20:23)	ns	ns	ns	ns	<0.01

The data are given as medians and ranges (min-max). DHEA, dehydroepiandrosterone; ns, non-significant. The data were analyzed with a one-way ANOVA test and with two-way ANOVA for age < 50 years vs. age > 50 years. A value of $p < 0.05$ was considered significant. Bolded values are statistically significant.

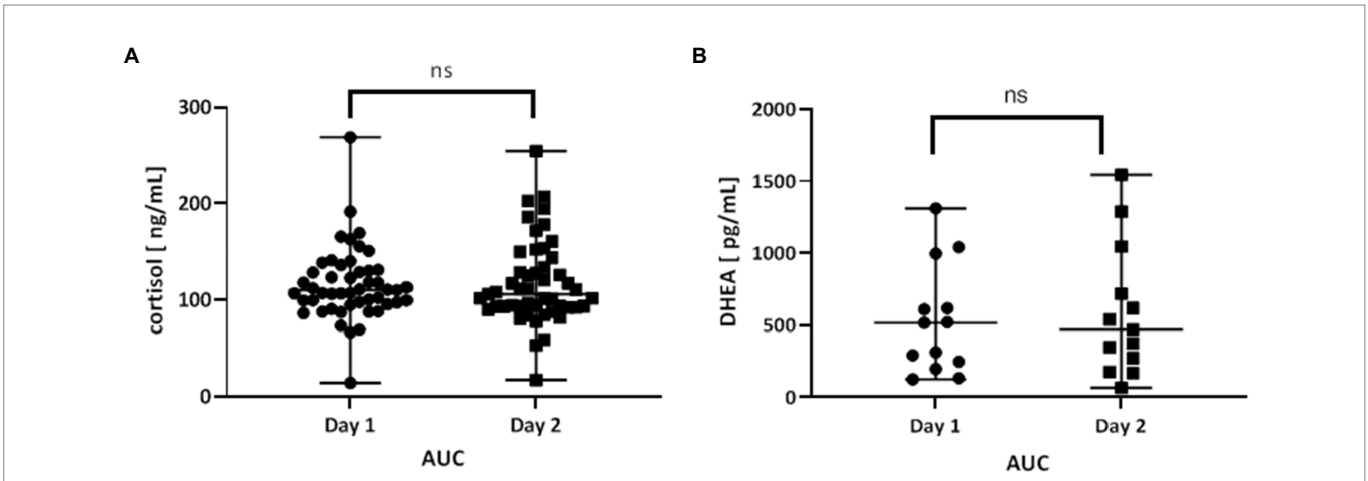

FIGURE 3
(A) Area under the curve (AUC) of the time course of cortisol levels day 1 (day before fasting) and day 2 (fasting day). The AUC was calculated for each patient separately (n=49), and the mean value of day 1 and day 2 were compared. **(B)** Area under the curve (AUC) of the time course of DHEA levels day 1 (day before fasting) and day 2 (fasting day). The AUC was calculated for each patient separately (n=15), and the mean value of day 1 and day 2 were compared. Values are expressed as median (min-max). A value of p<0.05 was considered significant. The data were analyzed with the Wilcoxon test.

have a shorter period of the circadian rhythm (61–63). Moreover, women have an earlier occurrence of acrophase in the circadian cortisol rhythm compared to the cortisol rhythm in men (58). The current study found only differences in acrophase in the morning chronotype participants. On the fasting day, acrophase occurred earlier; no differences were observed for the other rhythm parameters. Similarly, the rhythm was no different in the evening chronotypes. Finally, no differences were reported in the rhythms

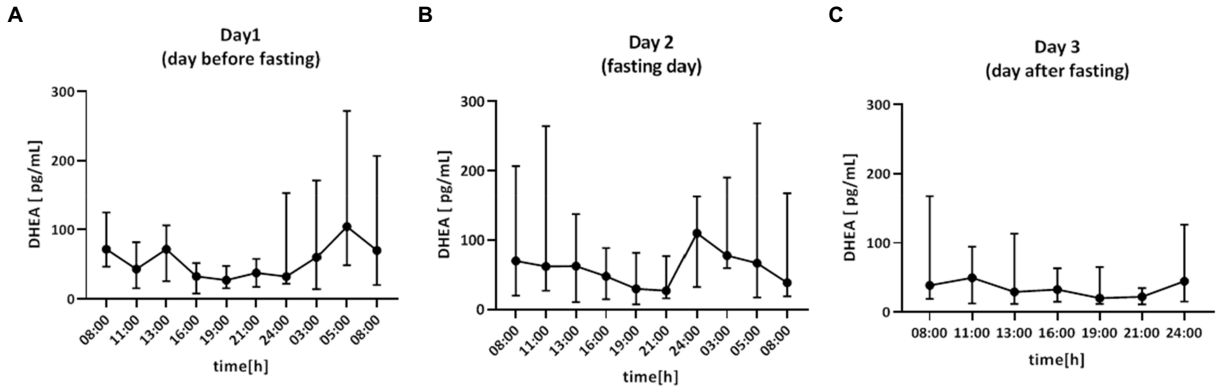

FIGURE 4
Time course of DHEA concentration (pg/ml) for 64h in: **(A)** day 1 (day before fasting), **(B)** day 2 (fasting day), **(C)** day 3 (day after fasting), *n*=15. Each value is the median (min-max).

TABLE 6 Anthropometric and metabolic characteristics of the study group (*n*=49), taking into account measurement changes before (day 0) and after caloric restriction (day 21).

Parameters	The obese before caloric restriction *n*=49	The obese after caloric restriction *n*=49	*p*-Value
Weight, kg	130.2 (89.2–189.4)	125.8 (84.6–184.2)	**<0.0001**
BMI, kg/m^2	41.5 (32.2–67.1)	40.6 (30.0–62.7)	**<0.0001**
Fat tissue, %	41.6 (32.6–53.2)	40.5 (29.8–55.1)	**0.0001**
Fat tissue, kg	51.7 (31.9–100.2)	48.8 (29.2–97.6)	**<0.0001**
Visceral fat tissue	18.5 (6.0–40.0)	18.0 (5.0–36.0)	**<0.0001**
Muscle tissue, kg	70.3 (31.7–95.6)	70.0 (47.9–94.1)	**0.001**
Body water (TBW), %	41.3 (33.4–48.6)	40.5 (29.9–55.1)	**<0.0001**
Glucose, mg/dl	103.0 (84.0–313.0)	102.0 (84.0–363.0)	0.6461
Insulin, mU/ml	18.0 (5.8–88.8)	22.1 (6.2–41.7)	0.0799
HOMA-IR	5.4 (1.5–49.3)	5.5 (1.6–10.9)	0.0934
HbA1c, %	6.4 (5.0–27.4)	5.7 (5.0–5.8)	**0.0053**
Cholesterol, mg/dl	180.5 (106.0–274.0)	166.5 (87.0–266.0)	**0.0244**
LDL, mg/dl	106.8 (37.6–204.0)	47.0 (28.0–82.0)	0.144
HDL, mg/dl	45.0 (31.0–100.0)	97.9 (39.0–198.0)	0.2576
Triglycerides, mg/dl	135.0 (50.0–454.0)	118.0 (38.0–438.0)	**0.0237**
CRP, mg/L	6.3 (0.4–16.2)	4.6 (0.3–28.9)	0.4614

BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; CRP, C-reactive protein; HbA1c, glycated haemoglobin. The data were analyzed with the Mann–Whitney test. A value of $p < 0.05$ was considered significant. Bolded values are statistically significant.

between the morning and evening chronotypes. Also, Toda et al. (64) found no differences in the circadian rhythm of cortisol concentration between the morning and evening chronotypes. On the other hand, Kudielka et al. (65) have reported that individuals with a morning chronotype may have higher cortisol levels upon awakening. These inconsistencies may be related to the differences in survey methodology. When we compared MESOR, amplitude, and acrophase of cortisol daily rhythm in the group of younger (below 50 years old) and older (above 50 years old) participants no differences were found. The results are similar to those reported in published literature (29).

The current study also assessed the rhythm of DHEA secretion. We noted that the MESOR of DHEA rhythm on fasting day increased. Furthermore, after dividing the patients by chronotype, there were differences in acrophase in the morning chronotypes and amplitude in the evening chronotypes. On fasting day, the acrophase was shifted earlier in the morning chronotypes, while in evening chronotypes, the amplitude was lower.

Lastly, we found no differences in the pattern of DHEA rhythm between the morning and evening chronotypes and between men and women. There are few reports that DHEA concentrations are usually higher in women than men (13), but others did not find differences in gender (10). When we compared MESOR, amplitude, and acrophase of DHEA rhythm in the group of women and men we could not find any differences.

However, we noted that all measured parameters of DHEA rhythm differed in younger and older patients with a trend toward a decrease in MESOR, amplitude, and delayed acrophase with age. Touitou (66), in his review described a similar reduction in DHEA rhythm, related to alterations in the biosynthesis pathways of adrenal steroids.

To date, there are only a few studies on DHEA changes in a fasting diet. A study by Harvie et al. (67) involving obese women on an IF diet for 6 months and consuming 25% energy demand for 2 days noted no difference between the concentration of DHEA before and after the dietary intervention.

In a different study, Jakubowicz et al. (68) compared the effects of consuming less than 50% of calories at dinner compared to eating more than 50% of calories at breakfast in women with polycystic ovary syndrome (PCOS) and found that DHEA was reduced by 35% in the breakfast group compared to the dinner group. Jakubowicz et al. (69) investigated the effects of a very low (1,000 kcal) and moderate (1,400 kcal) calorie restriction diet for 2 months on serum DHEA-S concentration in obese patients. The diet resulted in an increase in DHEA –S in obese men but not women.

Cortisol and DHEA are steroid hormones with opposing effects. In obese patients, there is an increased concentration of cortisol, which in turn can cause a decrease in DHEA concentrations (25). We evaluated the cortisol/DHEA ratio at multiple time points of saliva sampling. The ratio was similar on all days, with only a different concentration in the

early morning (05:00) of the fasting day. The ratio was higher, due to their lower DHEA concentration and higher cortisol level. Moreover, patients were treated for 3 weeks in a hospital setting with a moderate calorie restriction diet, which allowed reduced anthropometric parameters such as body mass, BMI, and fat mass but also muscle mass. Reduced body mass resulted in improving, HbA1c, and lipids concentration (cholesterol, triglycerides). Correlations between cortisol and DHEA rhythm and changes in anthropological and biochemical parameters after dietary restriction were calculated. No correlations were found between those parameters.

The impact that a calorie restriction diet may have on the relationship between cortisol and DHEA rhythms and changes in metabolic parameters and body weight after the restriction is limited. The published studies have focused on the correlation between obesity and those hormone levels. In the study by Abraham et al. (70) on obese patients, it was observed that the concentration of cortisol in urine samples and after the dexamethasone test did not correlate with patients' BMI. However, it was reported that cortisol in saliva tended to increase with increasing BMI. Similarly, Travison et al. (71), Jackson and Steptoe (72), and Al-Safi et al. (48) noted a relationship between BMI and cortisol concentration. Furthermore, Reynolds et al. (73) pointed out that cortisol concentration in men was inversely related with BMI. Studies by Travison et al. (71) and Jackson and Steptoe (72) were conducted with a considerable number of patients. Harithy (74) showed a negative relationship between the BMI value and DHEA-S concentration in lean and obese women. In addition, in obese patients, the level of DHEA-S showed a positive correlation with insulin concentration. There was no significant association between DHEA-S and glucose levels (74). Oltmanns et al. (75) and Reynolds et al. (73) noticed a positive correlation between cortisol concentration and glucose concentration (73, 75).

The limitations in our current study are inadequate knowledge of oral contraceptives used by patients, which may have affected cortisol concentration (76). In addition, the DHEA concentration was measured only in 15 patients due to a shortage of our funding.

Moreover, the study was performed with a relatively small sample size, and the analysis according to age range could not be achieved by sex because the number of DHEA measurements was deficient. Further studies in a larger sample of patients will help better ascertain the effect on the ratio of Cortisol/DHEA of IF. Therefore, this study would be the first approach to the impact of IF on DHEA rhythm.

In conclusion, one-day fasting affects the daily rhythms of cortisol and DHEA in obese patients. Nevertheless, very little is known about circadian rhythm-regulated secretion of DHEA and cortisol by a fasting diet. The regulation of the body clock seems to be mediated by fasting, but the mechanism is not yet fully understood. Additional studies are needed to elucidate the regulation of the body clock, daily rhythms of cortisol, and DHEA by fasting diet. Understanding the alteration of daily hormonal rhythms influenced by fasting could lead to developing new treatments for obesity and its comorbidities, which are serious public health concerns.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Poznan University of Medical Sciences (No. 249/19). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MM conceived the study, managed the patients, and collected clinical samples. MS performed statistical analyses. RR and EK performed biochemical analyses. AZ, AJ, MG, and AD managed the participants. DM, KK, AB, and JW critically reviewed the manuscript. DK designed and supervised the study and wrote draft of manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Intermittent fasting and immunomodulatory effects: A systematic review

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Introduction: strategy of periodic food restriction and fixed eating windows, could beneficially modify individuals by losing body weight, regulating glucose or lipid metabolism, reducing blood pressure, and modulating the immune system. Specific effects of IF and its mechanisms have not yet been assessed collectively. Thus, this systematic review aims to summarize and compare clinical trials that explored the immunomodulatory effects of IF.

Methods: After screening, 28 studies were included in this systematic review.

Results: In addition to weight loss, IF could benefit health subjects by strengthening their circadian rhythms, migrating immune cells, lower inflammatory factors, and enriching microbials. In addition of the anti-inflammatory effect by regulating macrophages, protection against oxidative stress with hormone secretion and oxidative-related gene expression plays a key beneficial role for the influence of IF on obese subjects.

Discussion: Physiological stress by surgery and pathophysiological disorders by endocrine diseases may be partly eased with IF. Moreover, IF might be used to treat anxiety and cognitive disorders with its cellular, metabolic and circadian mechanisms. Finally, the specific effects of IF and the mechanisms pertaining to immune system in these conditions require additional studies.

KEYWORDS

intermittent fasting, immune system, immunomodulatory effect, metabolic syndrome, obesity

1. Introduction

Fasting has recently received increasing attention for its advantages on body health (1). Dietary habits that involve fat-rich foods and snacks may lead to chronic diseases (2). Intermittent fasting (IF), as a dieting strategy, combines periodic energy restriction and fixed-duration eating windows (3). Different types of IF that incorporate varied combinations of fasting and eating windows have been proposed; examples include alternate-day fasting (36 h of fasting and 12 h of *ad libitum* eating) (4) and time-restricted fasting (16 h of fasting and 8 h of *ad libitum* eating) (5) (Figure 1).

It has been shown that IF is effective for decreasing body weight (6), and it can help to regulate glucose or lipid metabolism and reduce blood pressure (7) (Figure 1). In one study, numerous subjects with metabolic syndrome experienced improvements in lipid and glucose metabolism after IF (8). Another study had also noted that healthy and lean people may

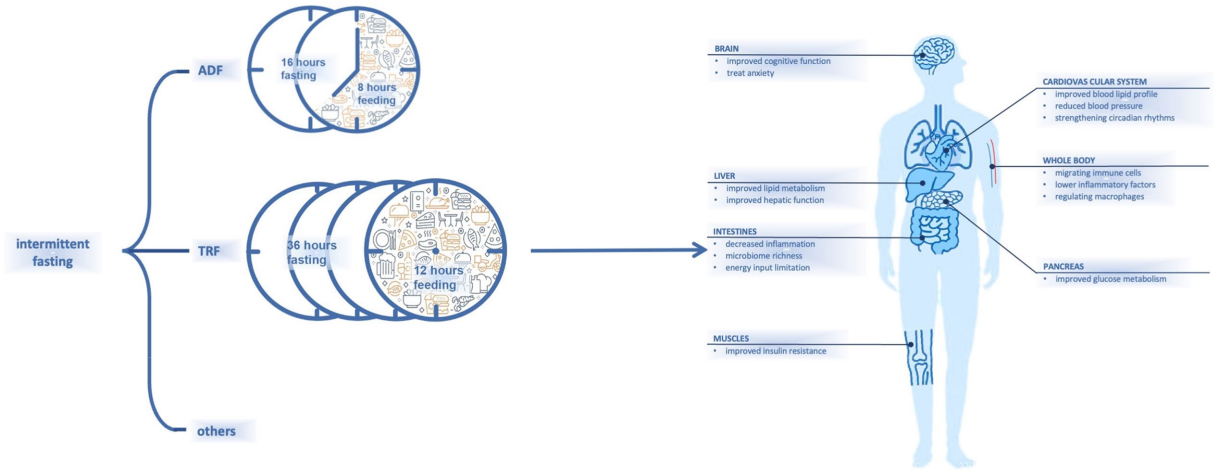

FIGURE 1
Content and presumed influences on body of intermittent fasting.

experience metabolic improvements by resetting their dietary intake with a schedule of fasting and eating (9). As studies of additional parameters including pre-inflammatory markers have been conducted, other effects of fasting have been observed.

One area of great interest is the influence of fasting on the immune system, which responds to stressful and harmful events in the body (10). The immune system can be regulated by weight reduction; changes in lipid and glucose metabolism; and other processes, such as circadian rhythm changes (10–14). Whether the influence of fasting on the immune system would benefit different populations—including healthy people, people with metabolic syndromes, and those with other physiological or pathophysiological conditions—is subject to discussion.

In this systematic review, we summarize clinical trials that studied the immunomodulatory effects of IF. All types of subjects were included and divided into different groups including healthy subjects, obese subjects and others, to clarify the cross-effect between IF and subjects under different physiological and pathophysiological situations, including pregnancy, perioperative period，endocrine disease, cancer and autoimmune diseases. The purpose of this systematic review is to analyze and compare current trials on this topic and to provide insight into the possible influence of IF on the immune system.

2. Methods

This systematic review was conducted and presented according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA) guidelines (Supplementary Table S1) and Assessment of Multiple Systematic Reviews 2(AMSTAR 2) tools (Supplementary Table S2). Various databases were searched, including Cochrane, PubMed, and Embase, from January 2005 to August 2022. The terms used for the literature research were "time-restricted feeding," "time-restricted eating," "intermittent fasting," "feeding schedule," "food timing," "meal frequency," "compressed feeding," and "restricted food intake." These terms were then united with "OR." In addition, the terms "normal human," "adult," "patient," and "human" were linked with "OR." The terms "immune," "immunity," "immunologic," "lymphocyte," "chemokine," "interleukin," "C-reactive protein," "CRP," "neutrophils," "oxidative stress," "oxidative burst," "inflammatory," "inflammation," "immunoglobulin," "autoimmune," "lipid peroxidation," "homocysteine," "malondialdehyde," "MDA," "glutathione," and "GSH" were united with "OR" and then added together with the aforementioned terms.

The inclusion criteria were as follows: randomized control trials and cohort studies; age > 18 years; one type of IF conducted; and at least one immunomodulatory marker analyzed. Exclusion criteria were as follows: intervention not strictly followed; no fasting procedure included in the intervention; IF combined with other eating interventions, such as liquid diet, protocol; and review articles.

A total of 3,558 potentially eligible articles were collected from the databases. After screening, 89 articles were selected for full-text review, of which 61 were excluded for unexpected interventions (Figure 2). Twenty-eight studies were later grouped into effects on healthy people, effects on obese subjects, and effects on other subjects according to the trial set. These grouping procedures were performed by two independent researchers before August 2022. The following parameters were extracted from the original articles for comparison: participants, trial length, intervention, control group, immunomodulatory effect, metabolic information, and body weight.

The Cochrane Collaboration tool (Supplementary Table S3) was applied to assess risk of bias in all included studies. The levels of evidence were as follows: randomized trials, nonrandomized controlled trials, historically controlled cohort studies, and single-arm noncontrolled trials. Because different trials had different levels of bias, a meta-analysis was not performed.

3. Results

3.1. Effects on non-obese healthy people

Eleven studies measured the immunomodulatory effect of IF on healthy people, and some included assessment of body weight changes or metabolic differences (Table 1).

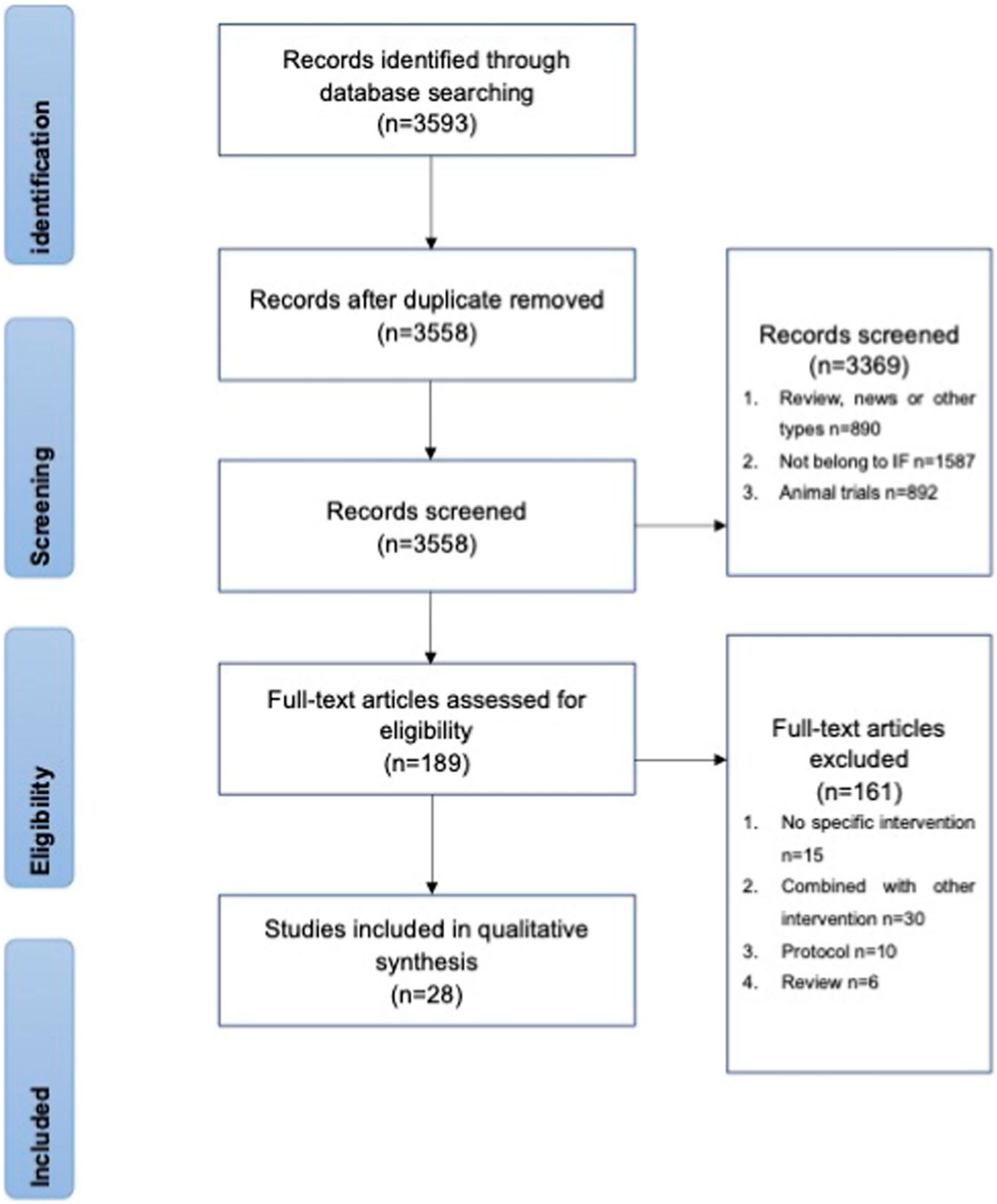

FIGURE 2
Search and study selection for systematic reviews (PRISMA) flow chart.

Various parameters were selected to investigate the immunomodulatory effects of IF in the eleven studies. Two studies measured the effects on immune cells but had different results. Madeo et al. found that almost all cell subsets remained the same (18), whereas Gasmi et al. observed that neutrophils, lymphocytes, and natural killer cells changed after a twelve-week trial of IF (17). Several studies have focused on classic inflammatory biomarkers. Lower levels of C-reactive protein (CRP), leptin, and adiponectin were observed in a study by Varady et al. (5). Similar results were reported by Paoli, both in an one-year (long-term) and an 8-week (short-term) trial (1)). However, Lauridsen et al. found that measurements of parameters such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 10 (IL-10) were not significantly changed after a course of IF (3). Mao et al. reported that lower levels of TNF-α and IL-8 could be observed after 5 weeks of IF (21). A study by Mcallister et al. (19) measured cortisol levels and found no significant change and these results were replicated in a study by Moro et al. (9). Moro et al. also reported a significant decrease in testosterone levels (9). Two studies measured microbial diversity after IF and concluded that IF generated great richness (20). Li et al. attempted to explain this change and found that sirtuin1 (*SIRT1*) expression was higher after IF compared with baseline levels (20), which was regarded as a stimulator for circadian genes and correlated with microbial diversity. A study by Wegman et al. also measured Sirt-1–related genes and reported similar results (15).

TABLE 1 Effects on non-obese healthy objects.

Reference	Intervention	Control	Participants	Trial length	Immune immunomodulatory effect	Glucose metabolism	Lipid metabolism	Others	Body weight
Varady et al. (5)	ADF	RCT	BMI26	12w	CRP: ↓($p < 0.01$) Leptin: ↓($p < 0.03$) Adiponectin: ↑($p < 0.01$)		TC: ⌀ LDL: ⌀ HDL: ⌀ TC*: ↓($p < 0.01$)	DBP: ↓ ($p < 0.05$) SBP: ⌀	↓, −6.5 ± 1.0% ($p < 0.001$), on average 5.2 kg.
Wegman et al. (15)	ADF	Crossover	BMI23	3w	Gene-upregulated*: *SIRT1, SIRT3, SOD2, TFAM*	Insulin: ↓($p = 0.0023$)			
Paoli et al. (16)	TRF	Before-after study	resistance-trained male	8w	Adiponectin: ↑($p = 0.0000$) Leptin: ↓($p = 0.0001$) IL-1b: ↓($p = 0.0235$) TT: ↓($p = 0.0476$) IGF-1: ↓($p = 0.0397$) IL-6*: ↓($p = 0.0035$) TNF-α*: ↓($p = 0.0001$)	Insulin: ↓($p = 0.0303$) Glucose: ↓($p = 0.0011$)	TG: ↓($p = 0.0201$) HDL: ↑($p = 0.0142$) LDL: ⌀		↓($p = 0.0448$)
Lauridsen et al. (3)	IF	Before-after study	lean	4w	TNF-α: ⌀ IL-6: ⌀ IL-10: ⌀ Adiponectin: ⌀ Leptin: ⌀ Cortisol: ⌀	Glucose: ⌀ Insulin: ⌀ HOMAIR: ⌀ HbA1c: ⌀	HDL: ⌀ LDL: ⌀ TG: ⌀ TC: ⌀	ALT: ⌀ SBP: ⌀ DBP: ⌀	↓($p = 0.05$), on average 1.0 kg.
Gasmi et al. (17)	TRF	RCT	Young and aged	12w	Red cells: ⌀ Monocytes: ⌀ Neutrophils: ↓ White blood cells: ↓ Lymphocytes: ↓ Natural killer cell: ↓				↓ young, ($p < 0.05$)
Madeo et al. (18)	ADF	Cross-sectional	healthy middle-aged	4w	Monocytes: ⌀ Lymphocyte: ⌀ B cell: ⌀ CD4 T cell: ⌀ β-hydroxybutyrate*: ↓, ($p = 0.003$)		TC: ↓($p = 0.004$) HDL: ⌀ LDL: ↓($p = 0.011$) VLDL: ↓($p = 0.009$) TG: ↓($p = 0.010$)	SBP: ↓($p = 0.006$) DBP: ↓($p = 0.0302$)	↓, ($p < 0.0001$), on average 3.5 kg.
McAllister et al. (19)	TRF	RCT	BMI28	4w	Cortisol*: ↓ Adiponectin*: ↑ CRP: ↑	Glucose: ⌀ Insulin*: ↑	LDL: ↑ HDL: ⌀ TG: ↓ TC: ↓	SBP: ↑ ($P = 0.04$) DBP: ⌀	
Li et al. (20)	TRF	RCT	healthy man	25d	IL-1β: ⌀ TNF-α: ⌀ Gene-upregulated: *Bmal1*($p = 0.0020$), *Clock*($p = 0.0302$), *SIRT1*($p = 00068$) Microbial richness: ↑ ($p < 0.005$)		TC: ↓ ($p < 0.0001$) TG: ↓($p = 0.0052$) LDL: ⌀ HDL: ↑($p < 0.0001$)	AKP: ↓($p < 0.009$) AST: ↓($p = 0.0268$) ALT: ↓($p = 0.0174$) Albumin: ↓, ($p < 0.0001$)	
Moro et al. (9)	TRF	RCT	cyclist	4w	TT: ↓($p = 0.0497$) CRP: ⌀ ESR: ⌀ IL-6: ⌀ Adiponectin: ⌀ TNF: ⌀ TSH: ⌀ T3: ⌀ Cortisol*: ↓($p = 0.0005$) IGF-1: ⌀	Glucose: ⌀ Insulin: ⌀	TC: ⌀ TG: ⌀	Cr: ⌀	↓,2%($P = 0.04$)
Paoli et al. (1)	TRF	RCT	healthy	2 m/12 m	TT: ↓($p < 0.001$) IGF-1: ↓($p = 0.039$) Adiponectin: ↑($p = 0.001$) Leptin: ↓($p < 0.001$) Il-6: ↓($p = 0.038$) IL-1β: ↓($p < 0.001$) TNF-α: ↓($p = 0.042$)	Glucose: ↓($p < 0.0001$) Insulin: ↓($p < 0.0001$) HOMA-IR: ↓($p < 0.0001$)	TC: ⌀($p = 0.289$) HDL: ↑($p < 0.001$) LDL: ⌀($p = 0.129$) TG: ↓($p < 0.0001$)		↓($p = 0.001$), on average 2.89 kg.
Mao et al. (21)	TRF	RCT	healthy	5w	TNF-α: ↓($p = 0.024$) IL-8: ↓($p = 0.045$) CRP: ⌀ WBC: ⌀ Microbial-diversity: ↑ ($p = 0.049$) Resistin: ⌀ Leptin: ⌀ Ghrelin: ⌀ gene-upregulated: *SIRT1, BMAL1, PER2, SIER1*	HOMA-IR: ↓, ($p < 0.001$, $p = 0.002$) Glucose: ↓($p = 0.005$)	HDL: ⌀ LDL: ⌀ TC: ⌀ TG: ⌀	SBP: ⌀ DBP: ⌀ AST: ↓($p = 0.046$) ALT: ⌀ ALP: ⌀ GGT: ⌀	↓($P = 0.009$), on average 1.6 kg.

WBC, White blood cells; NEUT, Neutrophile Granulocyte; PLT, Platelet; Hgb, hemoglobin; BCR/ABL, BCR/ABL gene; TT, testosterone; CRP, C-reactive protein; IGF-1, Insulin-Like Growth Factor 1; HOMA-IR, Homeostasis model assessment of insulin resistance; IF, intermittent fasting; RCT, randomized control study; CML, chronic myelogenous leukemia; PCOS, polycystic ovary syndrome; TC, total cholesterol; TG, triacylglycerol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, glutamic-pyruvic transaminase; NK cell, natural killer cell; IL, interleukin; TNF, tumor necrotic factor; BP, blood pressure; ADF, alternative day fasting; TRF, time restricted feeding; TNF-a, tumor necrosis factor-alpha; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDH, lactate dehydrogenase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; HbA1c, glycosylated hemoglobin; VLDL, very low-density lipoprotein cholesterol; Cr, creatinine; ESR, erythrocyte sedimentation rate; TSH, Thyroid Stimulating Hormone; T3, triiodothyronine; ⌀, no significant results; ↑, significantly increasing; ↓, significantly decreasing. Some changes in values and *p*-values are missing as they were not presented in the original manuscript.
*Indicates that the *p*-value was calculated based on the final and baseline values of participants in the TRF group because no com[illegible]ade between changes in values in a TRF group and a normal diet control group in the original manuscript.

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In eight trials, the decrease of body weight was observed after several weeks; three additional studies did not assess this factor. With regard to glucose metabolism, seven studies measured levels of fasting insulin and fasting glucose and conducted the test of homeostatic model assessment of insulin resistance (HOMA-IR) (1). Two studies found no significant changes in these parameters (3, 9), whereas improvements in these parameters were observed in five other studies (1, 15, 16, 19, 21).

Nine studies measured parameters related to lipid metabolism, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Six of them found improvements in multiple parameters; IF was associated with higher HDL, lower TC, lower TG, and lower LDL (1, 5, 18, 20). The remaining three studies found no significant changes in these parameters (3, 9, 21). Effects on different parameters, such as systolic blood pressure, diastolic blood pressure, and alanine transaminase, have been reported in other studies (18, 20).

Sleep quality and appetite were evaluated in some studies (5, 19, 21), and there was no significance after IF (21). Another study showed that during fasting, satiety and fullness of subjects were lower than controlled group, but no differences were found in nausea scores between two groups (3). Alertness, focus perceiving and mood perceiving were measured insignificantly in one study (19).

3.2. Effects on obese subjects

The effects of IF on obese subjects have received much attention. Twelve studies that assessed this topic were identified (Table 2).

Heilbronn et al. found that levels of TNF-α, IL-6, and IL-10 changed insignificantly during 8 weeks of IF, whereas macrophage counts increased significantly (25). Changes in CRP levels have been measured in several trials; however, almost no significant differences were observed (2, 10, 23, 24, 29). Conversely, Varady et al. found that 8-isoprostane decreased after 10 weeks of IF (6); these results were similar to those of a trial by Peterson et al. (23). Haus et al. reported that adiponectin and leptin levels decreased after a course of 24 weeks (29), and these results were confirmed by Varady et al. in a before–after study (22). Significant changes in IL-6 and TNF-α levels were observed in a study by Zouhal et al. (26). After a four-month trial conducted by Safavi et al., subjects had lower CRP levels (8). Mindikoglu et al. attempted to determine the immunomodulatory effects of gene expression like *AP5Z1* after finding almost no significant change on inflammatory parameters (10). Heilbronn et al. also found that gene expression like *PLIN5* may result in immune system changes (28).

Significant body weight reductions were observed in all studies except that of Horne et al., which only identified significant changes in galectin-3 levels (27). Because metabolic syndrome is often related to obesity, glucose and lipid metabolism have been extensively researched in obese subjects. Mindikoglu et al. compared fasting glucose and insulin levels before and after 4 weeks of IF and found no significant changes (10). Varady et al. also found no improvements in glucose metabolism in obese subjects who completed IF, but that study did identify higher level HDL (2). Six studies found that fasting insulin, fasting glucose, and HOMA-IR levels were improved after IF than before (6, 8, 23, 24, 26, 29). Augmentation of lipid metabolism was observed in a study by Varady et al. in obese subjects (22). However, other studies on lipid metabolism did not show such significant results. In addition to the collected metabolic findings, four studies found that IF could reduce blood pressure levels (10, 23, 24, 26).

3.3. Effects in other conditions

Five studies focused on the effects of IF on special populations, including individuals in special physiological states, such as during pregnancy or before or after an operation, and individuals with conditions such as polycystic ovary syndrome (PCOS), multiple sclerosis (MS), or chronic myelogenous leukemia (CML) (Table 3).

Ozturk et al. conducted a study of Ramadan IF in pregnant women. Total antioxidant status, total oxidant status, and related indices were measured; however, none showed significant changes after 4 weeks of the intervention. Pregnancy complications and birth weights were measured but showed no significant results between the IF-treated group and the controlled group (30). A study by Ginhoven et al. focused on IF during the perioperative period; 30 subjects who underwent kidney donation surgery were randomly assigned into a 1-day fasting group and a four-day restriction group. Many indicators were examined including CRP, white blood cells (WBCs), B cells, T cells, natural killer cells, IL-10, IL-6, TNF-α, and lipopolysaccharide. No statistically significant preoperative differences between groups were observed, with the exception of IL-8, which peaked at 6 hours after surgery in both groups but was significantly higher in the restriction group ($p = 0.018$). After surgery, the restriction group showed lower natural killer cell counts, lower WBC counts, and lower TNF-α levels (34).

Yassin et al. conducted a retrospective study of the effects of IF in subjects with CML. Forty-nine subjects were enrolled and tested before, during, and after fasting. BCR-ABL expression levels were measured and showed no significant difference among the three time points. Various hematological parameters, including WBC, hemoglobin, and platelet levels, showed no significant changes (31).

An eight-hour IF was conducted in 15 women with PCOS for 5 weeks; participants reported significant decreases in body weight (32). Metabolic parameters were also assessed, and lipid metabolism had insignificant changes, whereas fasting insulin levels and HOMA-IR decreased significantly after IF compared with their baseline levels (32). Total testosterone decreased by approximately 10%, but changes in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were not significant (32). A reduction in high-sensitivity CRP (hsCRP) and alanine transaminase (ALT) levels was observed, and insulin-like growth factor 1 (IGF-1) was upregulated (32). Fitzgerald et al. found that IF could alter T-cell subsets and metabolic markers in subjects with multiple sclerosis. The subjects in that study lost an average of 3.0 kg after the eight-week trial and had no significant changes in leptin and adiponectin levels. Individuals in the IF group showed significant reductions in memory T cells and increased naïve cell subsets (33).

4. Discussion

For non-obese healthy people, it is believed that the body could maintain a steady state in which lipid and glucose metabolism are

TABLE 2 Effects on obese subjects.

Reference	Intervention	Control	Participants	Trial length	Immune immunomodulatory effect	Glucose metabolism	Lipid metabolism	Others	Body weight
Varady et al. (22)	ADF	Before-after study	Obese	8w	CRP: ⌀ Homocysteine: ⌀ Adiponectin: ↓, −30% ($p < 0.05$) Leptin: ↓, −21 ± 6% ($p < 0.05$) Resistin: ↓, −23 ± 6%		TC 4w: ↓, −20%($p < 0.05$) LDL 4w: ↓, −31%($p < 0.05$) HDL 4w: ⌀ TG: 4w ↓, −19%		↓, −3.83%, on average 5.7 kg.
Varady et al. (2)	ADF	RCT	Obese	12 m	CRP: ⌀ Homocysteine: ⌀	Glucose: ⌀ Insulin: ⌀	HDL: ↑	BP: ⌀	*↓, −6%
Peterson et al. (23)	TRF	RCT	Prediabetes	5w	8-isoprostane: ↓, −11 pg./ml ($p = 0.05$) TNF-α: ⌀ cortisol: ⌀	Glucose: ⌀ Insulin: ↓($p = 0.13$)	HDL: ⌀ LDL: ⌀ TC: ↑($p = 0.0007$)	SBP: ↓, -11 mmHg ($p = 0.03$) DBP: ↓, -10 mmHg ($p = 0.03$)	*↓($p = 0.12$)
Bowen et al. (24)	ADF	RCT	Obese	24w (16w + 82)	CRP: ↓	Insulin: ↓ Glucose: ↓	HDL*: ↑ LDL*: ↓ TC*: ↓ TG*: ↓	SBP*: ↓ DBP*: ↓	*↓, on average 11.2 kg.
Haus et al. (5)	ADF	RCT	Obese	24w	Adiponectin: ↓ Leptin: ↓ IL-6: ↑ TNF-α: ⌀	Glucose: ↓, ($p = 0.031$) Insulin: ↓, ($p = 0.115$) HOMA-IR: ↓, ($p = 0.031$)			↓, ($p < 0.001$)
Heilbronn et al. (25)	IF	RCT	Obese	8w	TNF-α: ⌀ IL-6: ⌀ IL-10: ⌀ Macrophage: ↓	HOMA-IR: ↓			↓
Varady et al. (6)	TRF	RCT	Obese	10w	8-isoprostane: ↓($p = 0.02$) TNF-α: ⌀ IL-6: ⌀	Glucose: ⌀ Insulin: ↓, ($p = 0.02$, $p = 0.04$) Insulin resistance: ↓, ($p = 0.03$, $p = 0.04$)	LDL: ⌀ HDL: ⌀ TG: ⌀	SBP: ⌀ DBP: ⌀	↓,3.2%(4 h) ↓,3.2%(6 h)
Zouhal et al. (26)	IF	RCT	Obese	30d	IL-6*: ↓, ($p = 0.02$) TNF-α*: ↓, ($p = 0.019$)			AST: ⌀ ALT: ⌀ LDH: ⌀ Urea: ⌀	↓,2.7% ($P = 0.002$)
Mindikoglu et al. (10)	IF	Before-after study	Metabolic syndrome	4w	leptin: ⌀ Adiponectin: ⌀ CRP: ⌀ Homocysteine: ↑ ($p = 0.0004$) IL-1: ⌀ IL-6: ⌀ IL-8: ⌀ TNF-α: ⌀ Gene-upregulated: *AP5Z1, YPS8, INTS6, IGFBP5, POLRMT, KIT, CROCC, PIGR, CALU* Gene-downregulated: *POLK, CD109, SRGN, CAMP*	HOMA-IR: ⌀ Glucose: ⌀ Insulin: ⌀	TG: ⌀ HDL: ⌀ TC: ⌀ LDL: ⌀	SBP: ↓($P = 0.023$) DBP: ↓($p = 0.002$) ALT: ⌀ AST: ⌀ GGT: ⌀ ALP: ⌀ Albumin: ⌀	↓($p < 0.0001$), on average 2,5 kg.
Horne et al. (27)	IF	RCT	Metabolic syndrome	4w/13w/26w	Galectin-3: ↑($p = 0.021$)				
Heilbronn et al. (28)	IF	RCT	obese women	8w	Gene-nonregulated: *LIPE, ACACA, FASN, DGAT1* Gene-upregulated: *PLIN5* Gene-downregulated: *SOD1, SOD2* β-hydroxybutyrate:↑($p < 0.05$)				↓($p < 0.05$)

(Continued)

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TABLE 2 (Continued)

Reference	Intervention	Control	Participants	Trial length	Immune immunomodulatory effect	Glucose metabolism	Lipid metabolism	Others	Body weight
Safavi et al. (8)	ADF	RCT	Metabolic syndrome	4 m	CRP: ↓(p = 0.03) TNF-α: ↓(p = 0.60) IL-6: ↓(p = 0.49) PT: ↑(p < 0.001) APTT: ↑(p = 0.04)	Glucose: ↓(p = 0.03)			↓(p = 0.02), on average 6.43 kg.

WBC, White blood cells; NEUT, Neutrophile Granulocyte; PLT, Platelet; Hgb, hemoglobin; BCR/ABL, BCR/ABL gene; TT, testosterone; CRP, C-reactive protein; IGF-1, Insulin-Like Growth Factor 1; HOMA-IR, Homeostasis model assessment of insulin resistance; IF, intermittent fasting; RCT, randomized control study; CML, chronic myelogenous leukemia; PCOS, polycystic ovary syndrome; TC, total cholesterol; TG, triacylglycerol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; AST, aspartate aminotransferase ↑; ALT, glutamic-pyruvic transaminase ↑; NK cell, natural killer cell; IL, interleukin; TNF, tumor necrotic factor; BP, blood pressure; ADF, alternative day fasting; TRF, time restricted feeding; TNF-a, tumor necrosis factor-alpha; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDH, lactate dehydrogenase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; HbA1c, glycosylated hemoglobin; VLDL, very low-density lipoprotein cholesterol; Cr, creatinine; ESR, erythrocyte sedimentation rate; TSH, Thyroid Stimulating Hormone; T3, triiodothyronine; PT, prothrombin time; APTT, activated partial thromboplastin time; ø, no significant results; ↑, significantly increasing; ↓, significantly decreasing. Some changes in values and p-values are missing as they were not presented in the original manuscript.

*Indicates that the p-value was calculated based on the final and baseline values of participants in the TRF group because no comparison was made between changes in values in a TRF group and a normal diet control group in the original manuscript.

effective and the immune system works well (18). From some perspectives, IF could still benefit healthy people. After IF, WBC subsets in two trials changed in different ways (17, 18). The reduction in neutrophils may have resulted from the migration to extravascular lymphoid tissues (17). This process requires a long intervention, so another shorter trial conducted by Madeo et al. did not show such results (18). More studies showed that the elimination of old damaged cells would process during fasting, and more active immune cells would be generated when fasting ended (35). In this way, IF could protect various tissues against diseases with more active immune cells by hormesis mechanisms that increase cellular stress resistance (36). A decrease in natural killer cells is mainly linked to a decrease in IL-2 or IGF-1. Neither of which were measured in the trial by Madeo et al., but it could be observed in two studies by Paoli et al. (1, 16). Besides IGF-1, other measurements also show significant changes. Adiponectin may interact with adenosine 5′-monophosphate-activated protein kinase (AMPK) (19), which then helps to regulate insulin resistance (9). High level of adiponectin would stimulate fatty acid oxidation in skeletal muscle and inhibit glucose production in the liver, which benefit to energy homeostasis (37). Meanwhile, adiponectin is an anti-inflammatory agent, a reduction of inflammatory markers including CRP and TNF-α could be observed in some studies (1, 5). Changes in gene expression provide more information on immunomodulatory effects: Wegman et al. concluded that an increase in *SIRT1* and sirtuin3 (*SIRT3*) expression could be detected after a 3-week trial (15). For *SIRT1*, other studies have also shown an increase level (21). *SIRT1* is linked to circadian rhythms and cellular mechanisms, such as cell repair, division, metabolism, and growth (20). It could be concluded that IF could protect bodies from cardiovascular diseases. *SIRT3* is a member of the sirtuin family of histone deacetylases, which are primary mitochondrial protein deacetylases. Moreover, it could regulate cell metabolism, thus maintaining myocardial energy steady. *SIRT3* is also believed as a protection for cardiomyocytes from oxidative stress-mediated cell damage (38). Besides that, some animal studies showed more exciting results through *SIRT3* regulation of IF. High expressions of *SIRT3* in cerebral cortical and hippocampal cells could benefit for treating anxiety and cognitive disorders, which was found as considerable overlap mechanisms by which IF and exercise enhance brain function of Alzheimer's Disease patients (39, 40). A study by Mao et al. investigated clock genes and showed that levels of genes such as *BMAL1* and *PER2* were elevated in a five-week trial (21), indicating that IF could partly modulate the immune system by improving the circadian rhythm. The reinforcement of circadian rhythm could benefit body immune through promoting system recovery and the clearance of harmful cellular element (41). Another potential immunomodulatory effect involves microbial diversity in two studies (20, 21): Low gut microbial diversity is associated with metabolic disease (42), and high diversity may be due to the high expression of *SIRT1* and high levels of HDL (20) and improve body immune system, such as liver function mentioned in the study by Li et al. Emerging evidence showed that SIRT1 could promote gut microbial population shifts by influence inflammation and circadian rhythm (43). It has also been suggested that IF could benefit healthy people lose weight (9), even cyclists and men who practice resistance training (9, 16). After the trial, it was concluded that IF could lose almost fat and maintain muscle mass with the measurement of muscle area of the thigh and arm. Healthy individuals might already have high insulin sensitivity

TABLE 3 Effects in other conditions.

References	Intervention	Control	Participants	Trial length	Immune immunomodulatory effect	Glucose metabolism	Lipid metabolism	Others	Body weight
Ozturk et al. (30)	IF	RCT	Pregnant	4w	Oxidative stress index (OSI): ⌀ Total oxidant status (TOS): ⌀ Total anti-oxidant status (TAS): ⌀				
Nashwan et al. (31)	IF	Retrospective study	CML		WBC, NEUT, PLT, HGB*: ⌀ BCR/ABL*:⌀				
Bing he et al. (32)	Eating on 8:00–16:00	Before-after study	PCOS	5w	TT: ↓($p = 0.048$) CRP: ↓($p = 0.040$) IGF-1: ↑($p = 0.006$)	Glucose: ⌀ Insulin: ↓ ($p = 0.017$) HOMA-IR: ↓($p = 0.025$)	TG: ⌀($p = 0.715$) TC: ⌀($p = 0.328$) LDL: ⌀($p = 0.984$)	AST: ↓($p = 0.113$) ALT: ↓($p = 0.027$)	↓($p < 0.001$), on average 1.3 kg.
Fitzgerald et al. (33)	IF	RCT	Obese, multiple sclerosis	8w	Leptin: ⌀ Adiponectin: ⌀ Memory T cell subsets: ↓ Naïve subset: ↑ Th1 cell: ↓				↓
Ginhoven et al. (34)	IF	RCT	Kidney donation，BMI25		CRP: ⌀ WBC, B cell, T cell: ⌀ NK cell: ↓after surgery ($P < 0.001$) IL-10, IL-6: ⌀ TNF-α: ⌀ before surgery, ↓after surgery Cytokine: ⌀ IL-8: ↑($p = 0.018$)				

WBC, White blood cells; NEUT, Neutrophile Granulocyte; PLT, Platelet; Hgb, hemoglobin; BCR/ABL, BCR/ABL gene; TT, testosterone ； CRP, C-reactive protein; IGF-1, Insulin-Like Growth Factor 1; HOMA-IR, Homeostasis model assessment of insulin resistance; IF, intermittent fasting; RCT, randomized control study; CML, chronic myelogenous leukemia; PCOS, polycystic ovary syndrome; TC, total cholesterol; TG, triacylglycerol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; AST, aspartate aminotransferase ； ALT, glutamic-pyruvic transaminase ； NK cell, natural killer cell; IL, interleukin; TNF, tumor necrotic factor; BP, blood pressure; ADF, alternative day fasting; TRF, time restricted feeding; TNF-a, tumor necrosis factor-alpha; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDH, lactate dehydrogenase; GGT, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase; HbA1c, glycosylated hemoglobin; VLDL, very low-density lipoprotein cholesterol; Cr, creatinine; ESR, erythrocyte sedimentation rate; TSH, Thyroid Stimulating Hormone; T3, triiodothyronine; ⌀, no significant results; ↑, significantly increasing; ↓, significantly decreasing. Some changes in values and *p*-values are missing as they were not presented in the original manuscript.
*Indicates that the *p*-value was calculated based on the final and baseline values of participants in the TRF group because no comparison was made between changes in values in a TRF group and a normal diet control group in the original manuscript.

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at baseline; thus, IF seems to have less influence on glucose metabolism in these non-obese and healthy individuals (18). Things were similar when focusing on lipid metabolism. A decrease in leptin was found in many studies (1, 16), which might suggest that IF could partly strengthen lipid metabolism in healthy individuals. To sum up, IF could benefit immune system of healthy people through migration of immune cells, regulation of oxidative-related and circadian-related genes, increasing gut microbial diversity and improvement of muscle-fat ratio. Trials with longer durations and more factors including anxiety degree, cognition state, microbial diversity (44–46), key gene expression, and inflammatory markers are needed to better clarify the immunomodulatory effects of IF on healthy people.

Most obese subjects would harbor inflamed adipose tissue, which could cause a persistent, low-grade, inflammatory response. Obesity is often associated with the metabolic syndrome, because fat accumulation would cause insulin resistance (47). And evidence accumulated that persistent inflammation of adipose tissue is a central mechanism through which obesity promotes cancer risk (48). From the perspective of immune cells, A decrease in macrophages was observed in a study by Heilbronn et al. (25). Most cytokines that are produced by adipose tissue originate from nonfat cells and macrophages (29), thus the result confirmed that IF could be beneficial for inflammation associated with obesity. Recent studies have suggested that IF inhibits the nuclear factor kappa-B signaling pathway, which is an important regulator of downstream parameters including TNF-α and IL-6 (25), which is consistent with the results that IF could partly eliminate the inflammation caused by adipose tissue, with lower CRP and TNF-α (26). There were insignificant changes of some inflammatory markers in some studies, which might be related with short trial duration and inadequate weight loss (6). The concentration of galectin-3, which plays various roles in humans, was measured increasingly by Horne et al. in 2021 (27). It has been shown that galectin-3 could stimulate the expression of some antiviral genes and protect against inflammation, which may result in improvements in glucose metabolism. Although changes in inflammatory factors were less significant in obese people compared with healthy subjects, the immunomodulatory effect of IF observed in obese people might reflect a suppression of oxidative stress (26). Heilbronn et al. found that the ketone bodies, especially β-hydroxybutyrate, which protects against lipotoxicity and stimulates lipid oxidation, was significantly elevated in obese subjects (28). As it was regarded as an epigenetic regulator in terms of histone methylation, acetylation, IF could help to delay various age-related diseases. A decrease in 8-isoprostane, a marker of oxidative stress in lipids, was observed in two studies (6, 23). Oxidative stress is a definition of the imbalance between the production and elimination of reactive oxygen species (49). Some other studies have suggested that, though IF might have little effect on inflammation, it may greatly influence oxidative stress, which is linked to insulin resistance (26). Interestingly, improvements in glucose metabolism were observed in two studies that reported decreased oxidative stress markers (6, 23). Significant changes in leptin, which is regarded as a special body weight regulating hormone, were also noted (29), meaning that the resistance to leptin is partly improved in obese subjects. Besides the ability to regulate metabolic syndrome, including lowering glucose and lipid synthesis (50), leptin is one of the mediators responsible for the inflammatory state (51). In addition to the findings about immune cells and inflammatory markers, other study conduct tests of gene expression. Heilbronn et al. found that genes related to oxidative stress were down-regulated such as *SOD1* and *SOD2* (28), and Mindikoglu et al. found that the expression of other genes including the tumor activators *POLK*, *NIFK*, *SRGN*, *CAMP*, and *D109*, were downregulated (10), which are consistent with remitting oxidative effect and lowering cancer risk by IF. To sum up, besides the advantages of IF on obese subjects including losing body weight, regulate lipid metabolism and improve insulin resistance, which was almost suggested in all studies, IF could reduce oxidative stress and remit inflammatory state through macrophage adjustment and hormone secretion. Moreover, although evidence is accumulating that gut microbial is involved in the etiology of obesity (52) and altered by modified IF (4), relevant researches were still rare. Another issue waiting for more studies was the influence between IF and nervous system on obese subjects. Neuroinflammation, which has emerged as a crucial cause of cognitive dysfunction, such as Alzheimer's Disease, could be caused through inflamed adipose tissue of obesity (53). A study in obese rat showed that IF could prevent memory loss in comparison to *ad libitum* by regulating body metabolism (54), which offering a new sight for the advantages of IF to remit neuroinflammation.

Pregnancy is a state of high oxidative stress, which contributes to preeclampsia and restriction of fetal growth (30). Maternal IF resulted in detrimental influence on fetal development and maternal stress stage by changing the metabolite profiles in animal studies (55). However, IF has no significant influence on the high oxidative stress and fetal development in the human study (30). The reason could be the different circadian rhythms between rats and humans. A case related with gestational diabetes mellitus was reported that IF is a useful intervention to reduce maternal body weight, plasma glucose, and psychological distress without any adverse effects (56). Surgery is regarded as a shock or an acute stress, and IF is able to improve resistance to this stress. In a study by Ginhoyen et al. (34), a higher preoperative IL-8 level may counter the proinflammatory influence of subsequent surgery, thus TNF-α was lower in the food-restriction group after surgery. Compared with subjects in the non-fasting group, subjects in the restriction group showed a more moderate postoperative inflammatory response. For healthy people in special physiological states, such as those observed during the perioperative period, IF could reduce acute stress. More trials are needed to identify the influence on pregnant subjects, including the fetal and maternal safety, anti-stress effect and body metabolism regulation. It is worth nothing that study include in this review on pregnant subjects was a Ramadan IF trial, which might be less convincing as subjects in this study had experienced such interventions before.

PCOS is an endocrine condition closely linked to metabolic disorders. Because obesity is closely related to PCOS, it is not surprising that IF could provide benefits by reducing insulin resistance and easing hyperandrogenemia (32). Whether IF could be applied in subjects with cancer remains unclear (57), because it may also affect chemotherapy. In a study of subjects with CML, Yassin et al. reported that fasting did not result in significant immunological effects with measurements including BCR-ACL levels and hematological parameters (31). It was suggested that IF in some patients who have cancer could be capable of decreasing chemotherapy-related toxicity and tumor growth, however (58), more clinical trials were needed to clarify. MS is an autoimmune disease characterized by degeneration of the central nervous system (59). The epidemiology of this condition includes a history of

childhood obesity. Although no significant changes in leptin or adiponectin levels have been observed in studies of IF in MS, an observed difference in T-cell subsets in intestines might explain the immunological effects of IF that have been reported in studies (33, 60), which was also a kind of possible therapy for MS (59). The components of the intestinal microbiome could also raise the propensity to develop MS strongly (59). Researches about gut microbial of the influence of IF on subjects who have MS were expected as a result of migration of intestine immune cell subsets. As mentioned before, IF is beneficial for nervous system by cellular, metabolic and circadian mechanisms and a promising therapy for brain disorders, future research should disentangle whether positive effects of IF could be applied in clinical situations (61). Besides IF, other types of diet, including energy-restricted fasting and ketogenic diet (62), were also evaluated as nutrition therapy for MS (63). Some advantages were concluded that ketone bodies produced in these diets could serve as an alternative energy source for the brain (62), and during 3-day cycles of a fasting mimicking diet, it was found that the clinical symptoms of experimental autoimmune encephalomyelitis mice. More results was put forward that the improvement of this diet was related with immune system, including reducing inflammatory cytokines and immune cell migration. However, these diets might cause deficiency of various nutrients in long term (63). To sum up, a special diet could serve as a unique nutrition therapy for MS with disadvantages of nutrition deficiency, which was nowadays a popular and promising topic.

The different evidence levels should be taken into consideration when analyzing the results of these studies. Of the 28 selected trials, 19 were randomized, controlled, parallel, or crossover studies. Some trials were cohort studies, and the trial focusing on CML was a retrospective study; the lack of a control group in that trial may lead to inaccurate conclusions. Trials differed in terms of baseline characteristics, study durations, meal types, and IF types. These differences may interfere with the final results. For example, Gasmi et al. studied whether young people and old people would act differently while undertaking IF (17), Paoli et al. compared all factors in a 2-month trial and in a 1-year trial (1), and Varady et al. focused on whether the influence of IF would vary with different durations of eating windows (6). In the future, more studies on this topic should be conducted to provide new data.

This systematic review finds substantial evidence that IF can modulate the immune system in non-obese healthy people, obese people, and subjects in other physiological or pathophysiological states and these effects were clinically relevant with cognitive improvement, lipid and metabolism regulation, and inflammatory state remission. The mechanisms influenced and regulated to drive changes in each population differ. For example, non-obese healthy people can metabolize lipids and glucose efficiently, so the immunomodulatory effect is reflected in immune cell subset migration, lower inflammatory factors, upregulation of circadian rhythm–related gene expression, and greater microbial diversity. Although weight reduction has also been observed in healthy people, changes in parameters of lipid and glucose metabolism remained insignificant in most cases. In obese people, IF contributes to body health by regulating macrophages, which is related to the inflammatory stage of adipose tissue. Although many inflammatory factors did not show significant changes in obese subjects, other important factors, including 9-isoprastane, leptin, and galectin-3, had significant changes. The gene expression of cancer activators and lipid oxidative activators provides insights into the mechanisms behind these immunomodulatory effects. In pregnant women, IF seems safe to be conducted and possibly useful to treat endocrine disorders during pregnancy. Moreover, IF is able to improve resistance to the stress of surgery. IF can be beneficial for the immune system of individuals with PCOS by improving endocrine function. Limited trials studying the effects of IF on cancer have been conducted. For nervous system, IF is believed to be applicable to treat anxiety and cognitive disorders by cellular, metabolic and circadian mechanisms. However, more trials are needed to better understand the effects and mechanisms by which IF modulates the immune system.

5. Conclusion

Our systematic review, analyzing data from IF studies in different populations, suggests that IF could have immunomodulatory effects in healthy people, obese people, and people with special physiological and pathophysiological conditions. Different mechanisms may contribute to these effects. IF can benefit non-obese healthy individuals by strengthening circadian rhythms, migrating immune cells, lower inflammatory factors, and enriching microbial diversity. In addition of the anti-inflammatory effect by regulating macrophages, protection against oxidative stress with hormone secretion and oxidative-related gene expression plays a key beneficial role for the influence of IF on obese subjects. Physiological stress by surgery and pathophysiological disorders by endocrine diseases may be partly eased with IF. Moreover, IF might be used to treat anxiety and cognitive disorders with its cellular, metabolic and circadian mechanisms. Finally, the specific effects of IF and the mechanisms pertaining to immune system in these conditions require additional studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

ZH, HX, HY, and YM contributed to conception and design of the study. ZH and HX organized the methodology, investigation, and data collection. ZH and CL performed the statistical analysis. ZH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Molecular crosstalk between circadian clock and cancer and therapeutic implications

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The circadian clock governs activity of many physiological processes, thereby playing a pivotal role in human health. Circadian disruption is closely associated with cancer development; in particular, recent discoveries have provided strong evidence supporting specific functions of different molecular clock components in either promoting or inhibiting tumorigenesis. This narrative review aims to summarize the existing data on molecular connections between the clock and cancer. These results along with future efforts pave the road to targeting the circadian clock as a novel pathway for therapeutic intervention. Given the implications of chrono-nutrition interventions such as time-restricted feeding in extending lifespan, chrono-nutrition may have preventive and therapeutic applications for individuals with and at-risk of age-related diseases including cancer.

KEYWORDS

circadian rhythm, cancer, clock-targeting, chronomedicine, chronotherapy

Introduction

Evolved by most living creatures, the circadian clock allows organisms to organize their behavior and physiology to anticipate diurnal environmental changes. The mammalian circadian machinery is centrally controlled by core clock genes which form two interlocked transcriptional feedback loops. The BMAL1::CLOCK (brain and muscle ARNT-like 1 and CLOCK) heterodimer activates transcription of the clock repressors PER1/2/3 and CRY1/2, which subsequently form a heterodimer, translocate into the nucleus, and inhibit transcriptional activity of BMAL1::CLOCK. In the other loop, RORs and REV-ERBs, which are also targets of BMAL1::CLOCK, feedback to collaboratively regulate BMAL1::CLOCK by stimulating or repressing *Bmal1* transcription, respectively (1). Up to 80% of the protein-coding genes exhibit circadian transcription somewhere in the body of primates, highlighting the molecular clock as a crucial mechanism monitoring a broad range of physiological functions, including sleep, feeding, hormone secretion, metabolism, and immune responses (2, 3). Environmental or genetic disruption of circadian homeostasis exacerbates the development of clinically relevant disorders, such as sleep disorders, obesity, diabetes, and cancers, prompting pharmacological manipulation of the clock for novel treatments (4).

The normal circadian rhythms have long been acknowledged as a tumor suppressor, given that epidemiologic studies have suggested night shift work being a carcinogenic factor, particularly for breast cancer (5, 6). Systemic studies also revealed circadian clock

genes are frequently dysregulated or mutated across many human cancer types (7–9). Furthermore, the disruption of circadian rhythms is closely associated with cancer incidence, stage, and survival rate (7, 10, 11). While recent discoveries in different cancer systems have confirmed the significant roles of the circadian machinery in cancer biology, studies conducted in specific cancer systems have provided consistent evidence showing that certain central clock molecules may play oncogenic roles (10). Devising strategies for clock-based cancer therapeutics therefore calls for in-depth mechanistic studies to guide cancer-specific treatments for optimal efficacy and safety. This article will discuss the broad spectrum of molecular functions of the circadian clock in driving cancer progression.

Circadian control of cell cycle progression and DNA damage response

The circadian clock and the cell cycle are interconnected biological circuits both frequently dysregulated in cancer (12). Discovering BMAL1 and CLOCK, the forementioned master transcription factors of circadian rhythms, are required for cancer cell proliferation has encouraged experimental and clinical investigations to inform novel anticancer strategies. Downregulating *BMAL1* or *CLOCK* triggers reduced cell proliferation rate in the glioblastoma stem cells (GSCs), hepatocellular carcinoma (HCC) cells, and leukemia stem cells, featured by a symbolic cellular response of cell cycle dysregulation (13–15). *BMAL1* or *CLOCK* inhibition arrests the cell cycle at the S/G_2 phase, which is likely associated with the increased expression of p21 and reduced level of CYCLIN B1 (14, 16). Meanwhile, *BMAL1/CLOCK* knockdown induces the downregulation of the *WEE1* gene, which encodes a checkpoint kinase essential for cancer cells' survival and, consequently, leads to enhanced genome instability and apoptosis (14). Genetic studies demonstrated that transcriptional regulation of *WEE1* and *p21* cooperatively contributes to cancer cell proliferation promoted by BMAL1::CLOCK (14). Taken together, cell cycle regulation is an important cellular mechanism used by the circadian clock for tumor growth control (**Figure 1**).

Genome instability resulting from compromised DNA damage responses is a major hallmark of cancer that contributes to cancer initiation and progression (17). DNA damage response activates checkpoints that enforce cell cycle arrest to allow DNA repair before cell division (18) and therefore shares similar regulation by the clock as in the cell cycle pathways. Besides, studies have found that the core clock components may regulate earlier stages of the DNA damage response *via* interaction with the damaged DNA or molecules involved in DNA damage detection and signaling. For example, the CLOCK protein was reported to relocalize to the DNA lesion sites and participate in the checkpoint activation (19). Consequently, loss of CLOCK or BMAL1 in mice results in enhanced sensitivity to DNA cross-linking agents (20). CRYs are regulated by DNA damage through post-translational modification and subsequently protect genome integrity by modulating ATR activity (21, 22). PERs physically interact with the checkpoint proteins ATM and CHK2, promote DNA damage-induced apoptosis, and protect animals from tumor development (23–25). The clock factor TIMELESS interacts with the DNA damage response kinases CHK1 and ATR and contributes to DNA damage checkpoint response (26). Overall, most circadian clock components are generally positive regulators of the DNA damage response, thereby suppressing cancer development in healthy tissues. Notably, in prostate cancer, the CRY1 expression is elevated by androgen, endowing it with pro-tumorigenic functions to promote DNA repair and cancer cell survival after ionizing radiation treatment (27).

In addition to DNA damage response, the circadian clock machinery strictly controls the activity of DNA damage repair, particularly the nucleotide excision repair (NER) pathway which specifically repairs DNA lesions caused by UV irradiation, cigarette smoke, and the cross-linking agent cisplatin. The underlying mechanisms involve the clock-controlled circadian expression of xeroderma pigmentosum group A (XPA), an essential scaffold protein of the NER machinery, leading to its peak expression at ZT10 (28). Besides, because the NER repair is facilitated by locally activated transcription, DNA damages at rhythmically expressed genes exhibit a circadian repair on the transcribed strand in phase with the rhythmicity of gene expression (29). Another important link between the clock and DNA damage repair is that the circadian-controlled metabolite nicotinamide adenine dinucleotide (NAD+) acts as the substrate for the poly(ADP-Ribose) polymerase 1 (PARP1) and sirtuin enzymes that contribute to genome stability through participation in the base excision repair (BER), homologous recombination (HR), non-homologous end-joining (NHEJ), and NER (30, 31).

Circadian clock regulation of the classical oncogene *Myc*

The C-MYC proto-oncogene is a multifunctional transcription factor implicated in many aspects of cellular processes, including proliferation, differentiation, metabolism, and apoptosis (32). Multiple lines of evidence indicate that the cellular level of MYC protein is regulated by the circadian clock. For example, in mice, ubiquitin-mediated MYC degradation is enhanced by CRY2, which recruits the SCF^{FBXL3} E3 ligase to the phosphorylation-modified MYC (33). Consequently, the absence of CRY2 leads to MYC upregulation and enhanced incidence of lymphoma (33). On the other hand, in GSCs, BMAL1 directly binds the promoter region of MYC. Disrupting BMAL1/CLOCK or overexpressing CRY1 decreases MYC levels and impairs GSC proliferation (13). In lung cancer, conversely, *Bmal1* works as a tumor suppressor whose loss leads to increased C-MYC expression and enhanced proliferation (34). Taken together, the MYC regulation reflects cancer-specific functions of the core clock components.

Circadian clock in cancer stem cells

Cancer stem cells (CSCs) are maintained as a subpopulation of tumor cells by self-renewal. Due to this property and their inherent resistance to mainstream treatments, CSCs are key players in tumor relapses (35). As compared to normal neural stem cells, the GSCs

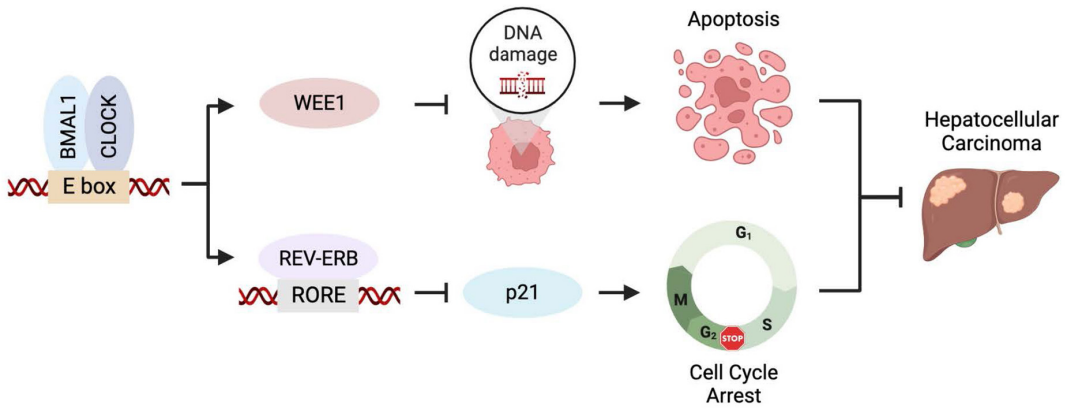

FIGURE 1
BMAL1::CLOCK promotes HCC growth by controlling cell cycle regulators. BMAL1::CLOCK directly activates the transcription of *WEE1* but inhibits p21 expression *via* regulating the REV-ERBs level. Inhibition of BMAL1::CLOCK downregulates *WEE1* leading to mitotic catastrophe and apoptosis and elevates p21 level that causes cell cycle arrest. The two mechanisms collaboratively contribute to the pro-proliferative activity of BMAL1::CLOCK in HCC.

are highly sensitive to perturbation of the circadian pathway. For example, the stemness of GSCs was lost upon *BMAL1* or *CLOCK* downregulation, as presented by reduced frequency of sphere formation and decreased expression of core GSC maintenance transcription factors, including SOX2 and OLIG2 (13). Similarly, targeting *Clock* or *Bmal1* also leads to reduced self-renew capacity and differentiation of murine leukemia stem cells (15).

Circadian clock regulation of cancer metabolism

Chronic jet lag may cause spontaneous development of HCC following global liver metabolic dysfunction and increased incidence of non-alcoholic fatty liver disease (NAFLD) (36). This suggests that metabolic disruption plays an important role in HCC development associated with circadian dysfunction. In addition, BMAL1 and CLOCK maintain GSCs by promoting glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), two major mechanisms of energy production for cancer cells, supported by reduced oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) upon *BMAL1* or *CLOCK* knockdown (13). Chromatin binding of BMAL1 is reprogrammed in GSCs to directly regulate multiple metabolic enzymes involved in glycolysis and the TCA cycle which is tightly coupled with OXPHOS (13). In addition, the molecular clock was suggested to modulate the activity of autophagy that plays a vital role in fueling the metabolic demands of cancer cells (37). Overall, the molecular mechanisms of circadian clock-regulated cancer metabolism involve both anabolism and catabolism.

Circadian regulation of tumor microenvironment

The molecular clock has been demonstrated to play a role in tumor microenvironment (TME). For example, BMAL1::CLOCK recruits immune-suppressive microglia cells to the TME of glioblastoma (GBM) *via* transcriptional activation of the *OLFML3* gene (38). Circadian expression of the co-stimulatory molecule CD80 in dendritic cells governs a circadian response of tumor antigen-specific CD8+ T cells to melanoma development (39). The CLOCK protein interacts with HIF-1 to activate VEGF expression, facilitating angiogenesis and metastasis in colorectal cancer (40).

Circadian clock roles in aging

Increasing evidence has linked disruption of the circadian clock function to aging (41). During the aging process, the circadian control declines, resulting in dampened and occasionally shifted oscillations of sleep-week cycle, body temperature, suprachiasmatic nucleus (SCN) activity, hormone release, and plasma glucose levels (41–43). Mice deficient in BMAL1, CLOCK, or PER show reduced life span and premature aging phenotypes, including sarcopenia, osteoporosis, loss of soft tissues, and cataracts (44–47).

Circadian alignment of feeding time combined with >12 h fasting interval (time-restricted feeding, TRF) enhances the lifespan benefits of calorie restriction (CR), indicating that optimizing the phase of circadian gene expression may be a powerful intervention for increasing life span and wellbeing (48). In addition to optimizing the expression of catabolic factors and disease markers, long-term CR and TRF enhance the circadian amplitude of the core clock and output genes (49, 50) and thereby entrain the clock in the SCN or peripheral organs, respectively (51). Interestingly, in both flies and mice, the lifespan extension benefits of CR require core clock genes (52–54). CR induces globally reprogrammed protein acetylation whose cycling organizes the enhancement of circadian oscillations (55). Therefore, reprogramming circadian rhythms holds significant importance for increased longevity achieved by feeding regimens.

Providing molecular mechanisms, deficiency of the *BMAL1* gene accelerates aging in both human and cynomolgus monkey mesenchymal progenitor cells (MPCs), attributable to a non-canonical transcription-independent role of BMAL1 in stabilizing

heterochromatin which prevents activation of pro-senescence retrotransposons (56). Furthermore, comparative transcriptomic analysis showed that a species' maximum lifespan negatively correlates with the expression of genes responsible for cellular energy metabolism which are more prone under circadian regulation potentially to avoid persistent high expression (57).

The NCI Annual Plan and Budget Proposal for Fiscal Year 2020 stated that the "greatest risk factor for cancer is advancing age". The fundamental roles of the clock in aging regulation indicates that anti-aging may be an important mechanism used by the circadian clock in cancer prevention. This concept encourages a healthy circadian rhythm reinforced by chrono-nutrition in everyday practice aiming to combat age-related diseases including cancer.

Circadian disruption in cancer

Transcriptome analysis of patient specimens revealed that a large body of genes cycling in non-cancerous tissues, including core clock genes, may lose oscillations in tumors. For example, serum shock stimulated clear circadian rhythms in the non-tumorigenic breast epithelial cells MCF10A, while the oscillation patterns were not observed in the breast cancer cells MCF7 (7). The CYCLOPS algorism identified quite a number of transcripts cycling in non-cancerous liver samples, including *CRY1*, *PER1*, and *ARNTL2*, lost rhythmic expression in biopsies of human HCC (9). Notably, circadian transcription and metabolism in the liver could be reprogrammed by distal tumors in the lung or breast, highlighting the effects of the tumor macroenvironment on systemic homeostasis of circadian rhythms (58, 59).

Several possible mechanisms can explain circadian disruption during tumorigenesis. C-MYC is a transcription factor constitutively expressed in over 70% of human cancers (32). It has been proposed to disrupt the circadian rhythms in tumor cells by binding an E-box motif element that drives activation of the core clock gene *NR1D1* (60). In epigenetic studies, we reported that, relative to normal neural stem cells, the genome-wide BMAL1 occupancy was considerably enhanced in GSCs coupled with reprogrammed chromatin structure (13). The ectopic recruitment of BMAL1 to thousands of additional binding sites drives essential hallmarks of cancer progression in these cells, including ramped-up metabolism and proliferative activity and maintenance of CSC state (13). Thus, cancer cells extensively reprogram the circadian rhythms to render novel oncogenic functions supporting disease physiology. Cell-specific chromatin structure guides deposition of the master circadian clock transcription factors and is thus a pivotal physiological mechanism defining heterogenous circadian rhythms in health and disease (61, 62). Notably, cancer reprogramming of the circadian machinery does not necessarily result in disturbance of the relatively robust central clock oscillation (13, 15).

Chrononutrition and circadian medicine in cancer treatment

As narrated above, maintaining a healthy circadian rhythm through consistent daily sleep patterns and diet increases resilience in fighting cancer. In parallel, efforts have been made to identify

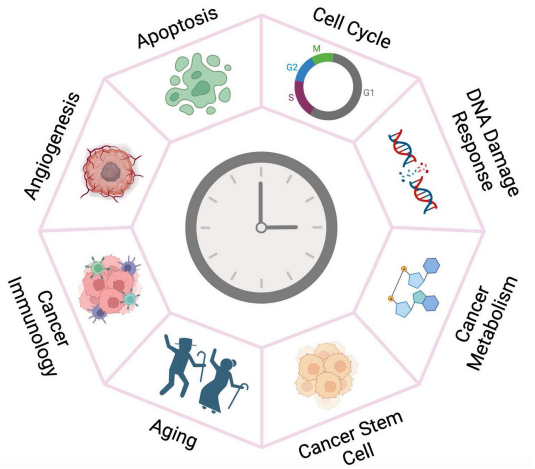

FIGURE 2
The circadian clock plays versatile roles in cancer development. The circadian clock machinery is reprogrammed by oncogenic pathways and chromatin remodeling in cancer tissues, thereby gaining fundamental functions in cancer development. Targeting the clock may therefore have more deleterious impact on the growth of tumor tissue than for the normal cells.

chemicals that can strengthen our circadian rhythms aiming to overcome circadian clock dampening associated with aging. For example, the small molecule Nobiletin, a naturally synthesized compound enriched in citrus peels, enhances the circadian clock amplitude and protects against metabolic diseases by agonizing the ROR nuclear receptors (63).

In addition to disease prevention, exciting approaches for the circadian clock management of diseases have been developed to limit eating windows, modify drug scheduling, or target clock components. Independent epidemiological studies discovered that the risk of breast cancer and prostate cancer was significantly associated with late night eating behavior (64–66). In mouse orthotopic models, TRF markedly inhibits tumor initiation, progression, and metastasis of obesity-driven postmenopausal breast cancer accompanied by restored circadian gene expression in the tumor suggesting that TRF might suppress tumorigenesis by regulating tumor clock (67). Similarly, in a transgenic MMTV-PyMT model of spontaneous breast cancer, TRF prevented the procarcinogenic effects of high-fat diet (68). These studies support the beneficial role of chrono-nutrition in cancer management and have encouraged clinical trials to prescribe TRF for the treatment of cancer in humans (ClinicalTrials.gov, identifier NCT05259410).

Clinically actionable genes strongly correlate with clock genes in transcriptional expression, suggesting that circadian drug efficacy should be necessarily investigated and considered (3, 7). Synchronizing drug delivery with a patient's biological clock, known as chronomedicine, has been clinically validated for the improvement of drug efficacy in pathological conditions such as asthma, hypertension, and cardiovascular disease (69). Extending the boundary of chronotherapy further to the surgical procedures, patients who received cardiac surgery in the afternoon and subsequently suffered from major heart damage were half the patients who underwent the same surgery in the morning (70).

In cancer clinical trials, nevertheless, the application of chronochemotherapy has produced inconsistent results within different clinical trials and patient populations. For example, chronochemotherapy benefits observed in a group of 31 women with ovarian cancer (71) were not replicated in a larger study (72). In a retrospective study reviewing 166 glioblastoma patients, Damato et al. found that patients taking morning temozolomide exhibited longer overall survival compared to evening, especially in MGMT-methylated patients (73). However, when the same group conducted a small feasibility study, they found that among 35 patients on different dosing schedules there was no significant difference in either adverse side effects or survival (74). A phase III trial of 564 colorectal cancer patients from 10 European countries receiving chronomodulated infusion of fluorouracil, leucovorin, and oxaliplatin revealed no benefit of the chronotherapy to the whole study population; however, compared with the conventional treatment group, the risk of death was decreased by 25% for men but increased by 38% for women treated with chronotherapy (75). These confusing clinical trials exemplify the heterogeneity of the circadian rhythms in cancer patients, warranting an aforehand definition of the circadian phase potentially through evaluation of appropriate blood-based biomarkers.

By contrast, the emerging fundamental roles of core clock components in cancer biology have urged developing and examining small molecules directly targeting the clock proteins. Encouragingly, existing clock-targeting small molecules have exhibited specific tumor growth inhibition in glioblastoma and other cancer types (10, 13, 37). Considering the broad roles of the core clock components in managing specific cancers, it is favorable for future endeavors to develop cancer-specific strategies with the goal of improving standard first-line regimens. A better understanding of the clock-cancer interactions will inform blood- or tissue-based biomarkers for molecular stratification of cancer patients that may show differential sensitivity to clock-targeting treatments.

Conclusion

The molecular clock is emerging as a fundamental regulator of cancer development and prognosis. Here we review recent endeavors that collectively reveal the clock's multifaceted roles in different aspects of cancer development (**Figure 2**). Further mechanistic investigation in various cancer systems and pathways assisted by clinical and omics data collected from cancer patients will expand the functional landscape of the circadian clock aiming to guide pharmacological strategies.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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High-fat intake reshapes the circadian transcriptome profile and metabolism in murine meibomian glands

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Background: Nutritional and food components reshape the peripheral clock and metabolism. However, whether food challenges affect the circadian clock and metabolism of meibomian glands (MGs) has not been fully explored. This study was designed to analyze alterations in the rhythmic transcriptome and metabolism of MGs of murine fed a balanced diet or a high-fat diet (HFD).

Methods: Male C57BL/6J mice were maintained on a 12/12 h light/dark cycle and fed *ad libitum* on normal chow (NC) or HFD for 4 weeks. MGs were collected from sacrificed animals at 3-h intervals throughout a 24-h circadian cycle. The circadian transcriptome of MGs was analyzed *via* bioinformatics approaches using high-throughput RNA sequencing (RNA-seq). In addition, circadian oscillations of lipid components in MGs were analyzed.

Results: Meibomian glands displayed robust transcriptome rhythmicity. HFD feeding significantly altered the circadian transcriptome profile of MGs—including composition and phase—and spatiotemporally affected the enriched signaling pathways. In addition, HFD feeding significantly altered the normal rhythmic oscillations of lipid components in MGs.

Conclusion: Our data show that HFD significantly affects MGs' rhythmicity, which reveals a high sensitivity of MGs' clocks to lipid composition in food.

KEYWORDS

bioinformatics, circadian rhythm, high-fat diet, meibomian gland, metabolic dysfunction, RNA-seq, transcriptome

1. Introduction

Meibomian glands (MGs) are sebaceous glands located in the palpebral plate opening at the edge of the eyelid. They provide specialized lipids to the tear film to avoid tear evaporation and overflow and maintain tears between the oily margin and the eyeball to maintain the structural and functional integrity of the ocular surface (1, 2). When the

lipids secreted by this gland are altered qualitatively and quantitatively for various reasons, it can result in increased tear evaporation, hyperosmolarity, tear film instability, and bacterial growth at the lid margin, ultimately leading to damage to the ocular surface (3). Currently, MGs dysfunction of various causes is becoming one of the most common diseases in the clinical setting of ophthalmology (3–5). It seriously affects the quality of life of patients. However, our understanding of the structure and physiological function of MGs and the factors affecting them remains extremely limited to date (6).

Given the biological evolutionary drive, the organs, tissues, and physiological processes of any mammalian species can be predicted to undergo significant rhythmic changes accompanying the daily light–dark cycle of the Earth (7–9). Similarly, ocular tissues and their physiological activities undergo synchronous rhythmic changes (10). Published studies, including our team's series of work, suggest that the cornea (11–13), lacrimal gland (14, 15), retinal pigment epithelium, and retina (16, 17) all exhibit robust rhythmic changes in the phase of the lighting cycle. However, little attention has been paid so far to the circadian rhythmical pattern of MGs and their underlying mechanisms (18, 19). Considering the importance of MGs in maintaining tear film stability through lipid secretion, understanding their circadian rhythmic activity pattern and their associated mechanisms is of clinical importance.

Circadian rhythmicity in mammals shows different patterns, depending on the organ, tissue, and physiological function (7, 20, 21). This rhythmicity is closely coordinated between various organs of the body (22). Many factors, such as high-calorie diets (22) and hypoxia (23), can significantly alter these circadian rhythms and the interconnections between the respective systems. Because of the acceleration of human economic and social activities, a Western diet characterized by high fat content has become prevalent in every corner of the world. Such diets increase the risk of developing many systemic diseases, such as metabolic syndrome, diabetes, and cardiovascular diseases (24). Similarly, the altered composition of high-calorie diets poses a challenge to the physiological function of ocular tissues and the development of disease (25). Metabolic stress from a high-calorie diet can remarkably alter the circadian activity of the cornea and lacrimal gland and the composition of the transcriptome that controls these activities (26). Furthermore, preliminary data suggest that a high-fat diet (HFD) promotes the onset and development of dry eye disease through the induction of an inflammatory response in the lacrimal gland (27, 28). However, the detailed mechanisms are unclear. Therefore, new tools are needed to revisit the HFD-induced dysfunction of MGs and their underlying mechanisms.

Here, we compared the altered transcriptomes of MGs in mice fed a balanced diet and an HFD. Then, the effect of metabolic stress generated by HFD on the circadian clock of MGs and its possible underlying mechanisms were explored by bioinformatics analysis and the detection of diurnal oscillations of lipid droplets in MGs. We found that increased lipid content in food drastically altered the characteristics of the circadian transcriptome of MGs and produced previously unobserved effects on the transcriptome of MGs. This might provide a pathophysiological basis for explaining how food components affect the physiological function of MGs and bring about certain diseases.

2. Materials and methods

2.1. Animals and dietary interventions

Six-week-old male C57BL/6J mice were obtained from Nanjing University in China and housed in light-tight circadian chambers (12/12 h light/dark daily cycle) (Longer-Biotech Co., Ltd, Guangzhou, China) (29). The Zeitgeber time (ZT) scale was used here to record the time: ZT0 referred to time of lights on (7 a.m.), and ZT12 referred to lights off (7 p.m.) (30). Mice were provided with *ad libitum* access to their respective diets throughout the study. After 2 weeks of adaption in light-tight circadian chambers, all mice (8 weeks old now) were divided randomly into two groups. The normal chow (NC) group mice were provided with standard NC with 9% kcal fat (Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) for 4 weeks. The HFD group mice were provided with HFD with 60% kcal fat (Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) for 4 weeks (**Figure 1A**), as previously described (26). RNA-Seq data for circadian analysis were collected at eight time points throughout the circadian cycle (3-h intervals) (**Figure 1B**). Circadian gene identification and circadian transcriptomic analysis (phase and amplitude) were performed by the Jonckheere–Terpstra–Kendall (JTK) cycling algorithm. The biological processes and molecular function of genes were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG), gene ontology (GO), phase set enhanced analysis (PSEA), time-series clustering analysis, and gene set enriched analysis (GSEA) (**Figure 1C**). Circadian changes in lipid droplets in MGs were studied by Oil Red O (ORO) staining. All mice were euthanized by cervical dislocation after inhalation of ether.

2.2. MG collection, total RNA extraction, and RNA-seq

After exposure to NC or HFD dietary regimens, the upper and lower MGs from the left eyelid were collected and combined from euthanized animals at 3-h intervals over the circadian cycle from NC- and HFD-fed mice, as previously described (27, 31). Total RNA was isolated from the MGs using an RNAeasy spin column kit (Qiagen). For each ZT point, RNA-Seq analysis was performed using three biological replicates (15, 30). Library preparation and sequencing for the total RNA of MGs were performed according to our previous report (26, 30, 32). In brief, total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). The cDNA was amplified by PCR, and raw reads were filtered by SOAPnuke (Version v1.5.2) (33). HISAT2 (34) and Bowtie2 were used to align the clean reads (reference: Mus_musculus, GCF_000001635.26_GRCm38.p6) (35). Differentially expressed genes (DEGs) between the NC- and HFD-fed groups were identified using the R software edgeR package.[1]

1 https://bioconductor.org/packages/release/bioc/html/edgeR.html

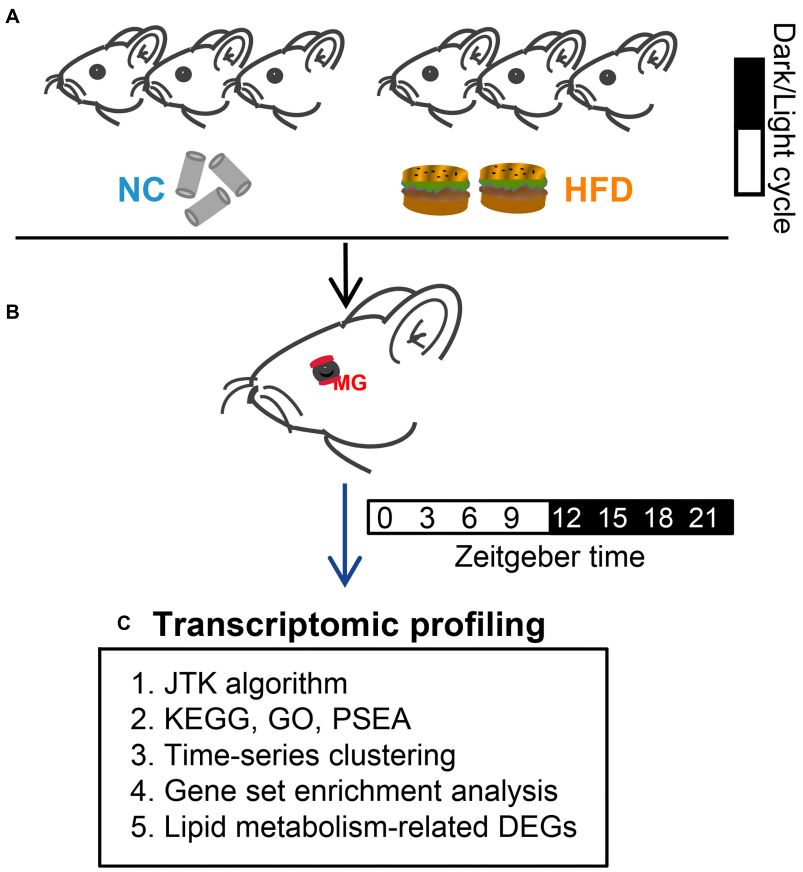

FIGURE 1
Experimental design. **(A)** After adaption, all mice were divided randomly into two groups. Mice in the NC- and HFD-fed groups were provided with standard normal chow and a high-fat diet for 4 weeks, respectively. **(B)** MGs were obtained from euthanized mice at 3-h intervals (for transcriptomic profiling analysis) or 6-h intervals (for ORO staining) over a 24-h circadian cycle. **(C)** High-throughput sequencing (RNA-Seq) was performed after MG collection. Circadian gene identification and circadian transcriptomic analysis were performed using the Jonckheere–Terpstra–Kendall (JTK) cycling algorithm. The biological processes and molecular function of genes were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), phase set enhanced analysis (PSEA), time-series clustering analysis, and gene set enriched analysis (GSEA).

2.3. Analysis of rhythmic genes

The circadian genes of MGs were identified using the JTK_CYCLE algorithm in R software, as previously described (26, 30, 32). The time-ordered fragments per kilobase of exon model per million mapped fragments (FPKM) of all MG genes were imported into the algorithm. Rhythmic genes with a period of 24 h were identified, and the phases with amplitudes of the rhythmic genes were also determined. All MG genes were composed of low expression genes (FPKM < 0.1), rhythmic genes (FPKM ≥ 0.1 and Bonferroni-adjusted $P < 0.05$), and non-rhythmic genes (FPKM ≥ 0.1 and Bonferroni-adjusted $P \geq 0.05$).

2.4. Functional annotation by KEGG, GO, PSEA, and GSEA

Biological processes and molecular function annotations of MG genes were performed using KEGG, GO, PSEA, and GSEA, as previously described (26, 30, 32). KEGG and GO enrichment analysis were performed by online bioinformatic platform Dr. Tom,[2] an online software developed by Beijing Genomic Institute (BGI) (36). PSEA software (v1.1) was used to annotate rhythmic genes at the oscillating phase's level with the reference gene set (c2.cp.kegg.v7.2.symbols.gmt) downloaded from MSigDB[3] (30). GSEA software (v3.0) was used to characterize the biological pathways of MG genes by reference gene sets c5.go.bp.v7.2.symbols.gmt and c2.cp.v7.2.symbols.gmt. The significance threshold of the *Q*- or *P*-value for the analysis was 0.05 (26, 30, 32).

2.5. Time-series clustering analysis and protein–Protein association networks

To reveal dynamic expression trends in the rhythmic genes of the MGs, the fuzzy c-means clustering algorithm in the Mfuzz package was adopted, as previously described (30). In this paper, the number of clusters in the rhythmic genes of NC- and HFD-fed

2 biosys.bgi.com/
3 https://www.gsea-msigdb.org/gsea/msigdb/

mice was set as 4 on the basis of gene expression trends, with default values for other parameters. To visualize the gene–gene interaction of lipid–metabolic genes in the NC- and HFD-fed mice, protein–protein association network (PPAN) analysis was performed *via* STRING analysis.[4] The parameters in the full STRING network were as follows: meaning of network edges, evidence; active interaction sources, experiments and databases, kmeans clustering method with 3 as the number of clusters.

2.6. Immunohistochemistry of MGs

After dietary intervention, eyelid tissues with eyeballs were collected from the right side of the NC- and HFD-fed mice at 6-h intervals throughout a 24-h circadian cycle (ZT0, 6, 12, 18), as previously described (15, 26). In brief, paraffin tissues were collected for hematoxylin and eosin staining to visualize the morphology of the MG tissues, and frozen sections were prepared for ORO staining (G1016, Servicebio Company). Eyelid tissues were cut into sagittal sections (5 μm thick). MG sections were immersed in ORO solution for 10 min in the dark. ORO staining was analyzed by mean optical density using ImageJ software (version 1.42q; National Institutes of Health, USA). Representative ORO staining images of the NC- and HFD-fed MGs were selected using CaseViewer software (3DHISTECH Ltd., Budapest, Hungary).

2.7. Statistical analysis and software

Statistical analysis and figure preparation were processed using GraphPad Prism 9.3.1. A heatmap of circadian genes was prepared using the "*pheatmap*" package in R software. Data with normal distribution were statistically analyzed using the Student's *t*-test to compare the differences between the NC- and HFD-fed mice. A value of $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. HFD alters the characteristics of circadian transcriptome in murine MGs

To visualize the transcriptomic differences between NC- and HFD-fed MGs, we performed a comparative expression analysis of RNA-seq data using a volcano plot (**Figure 2A**). We identified 1,397 and 1,722 circadian genes (**Supplementary Table 1**, JTK_adj $P < 0.05$) from all the MG genes of the NC- and HFD-fed mice, respectively (**Figures 2B, C**). In total, 338 cycling genes were shared between the two diet interventions; 1,059 were unique to the NC-fed MGs, and 1,384 were unique to the HFD-fed MGs (**Figure 2C** and **Supplementary Table 2**). HFD intervention did not significantly alter the oscillation patterns of shared rhythm genes in MGs within 24 h at 3-h intervals (**Figure 2D**). The peak expression of NC-unique cycling genes in MGs was throughout the circadian cycle, but they did not show a circadian rhythmic expression pattern in HFD-fed MGs (**Figure 2E**). In contrast, HFD-unique cycling genes were mainly expressed in the light phase, whereas they did not show a circadian rhythmic pattern in NC MGs (**Figure 2F**).

The expression phase of NC-unique rhythmic genes was mainly in ZT6 to ZT10.5 and ZT18 to ZT22.5 and throughout the circadian cycle (**Figure 2G**). Importantly, the phase of HFD-unique rhythmic genes peaked in ZT6 to ZT9 (**Figure 2H**). In contrast, the shared rhythmic genes were mainly from ZT0 to ZT1.5 in the NC-treated MGs (**Figure 2I**) and from ZT22.5 to ZT1.5 in the HFD-treated MGs (**Figure 2J**). For the shared cycling genes, 63.6% were phase shifted, whereas 36.4% were in phase (**Figure 2K**). Of the phase-shifted cycling genes, 30.2% were advanced in phase, and 69.8% were delayed (**Figures 2K–M**). There was no significant difference in the amplitude of cycling genes in shared or unique cycling genes between the MGs of NC- and HFD-fed mice (**Supplementary Figure 1**). Collectively, these data suggest that, under homeostatic conditions, HFD intervention dramatically altered the composition, number, and oscillation phase of rhythm genes in murine MGs.

3.2. HFD alters the functional characteristics of cycling genes in mouse MGs

To evaluate the effect of HFD feeding on the biological processes of cycling genes, we performed GO annotations for MG genes in NC- and HFD-fed mice. The NC- and HFD-specific cycling genes were enriched in various biological processes, especially in the immune, metabolic, and nervous systems, as shown in **Figure 3A**. PSEA analyzes were performed to characterize the effect of HFD intervention on the spatiotemporal distribution of the signaling pathways of cycling genes. The pathways enriched in the NC-fed MGs were distributed throughout the circadian cycle, whereas those in the HFD-fed group were mainly located in the light phase (**Figures 3B, C**). Importantly, more immune-related pathways were enriched in the MGs of NC- fed mice, and more important signaling pathways were enriched in the light phase of the MGs of HFD-fed mice (**Figures 3B, C**). In summary, our results indicate that HFD intervention significantly rewired the rhythmic activity in the GO and PSEA levels, which may result in changes in the potential functions of these rhythmic genes in the MGs of HFD-fed mice.

3.3. HFD alters the cluster-dependent transcriptomic map

To reveal the dynamic expression trends in the rhythmic genes of the MGs after HFD intervention, we analyzed the time series clustering analysis of cycling genes in the MGs of NC- and HFD-fed mice. Four oscillating patterns were determined on the basis of the positions of the peaks and troughs in the NC or HFD groups. The peaks of Cluster 1 were located at ZT6 and the troughs at ZT18, and the 298 and 459 cycling genes were enriched in the MGs of NC- and HFD-fed mice, respectively (**Figures 4A, B**). The peaks of Cluster

4 https://string-db.org/

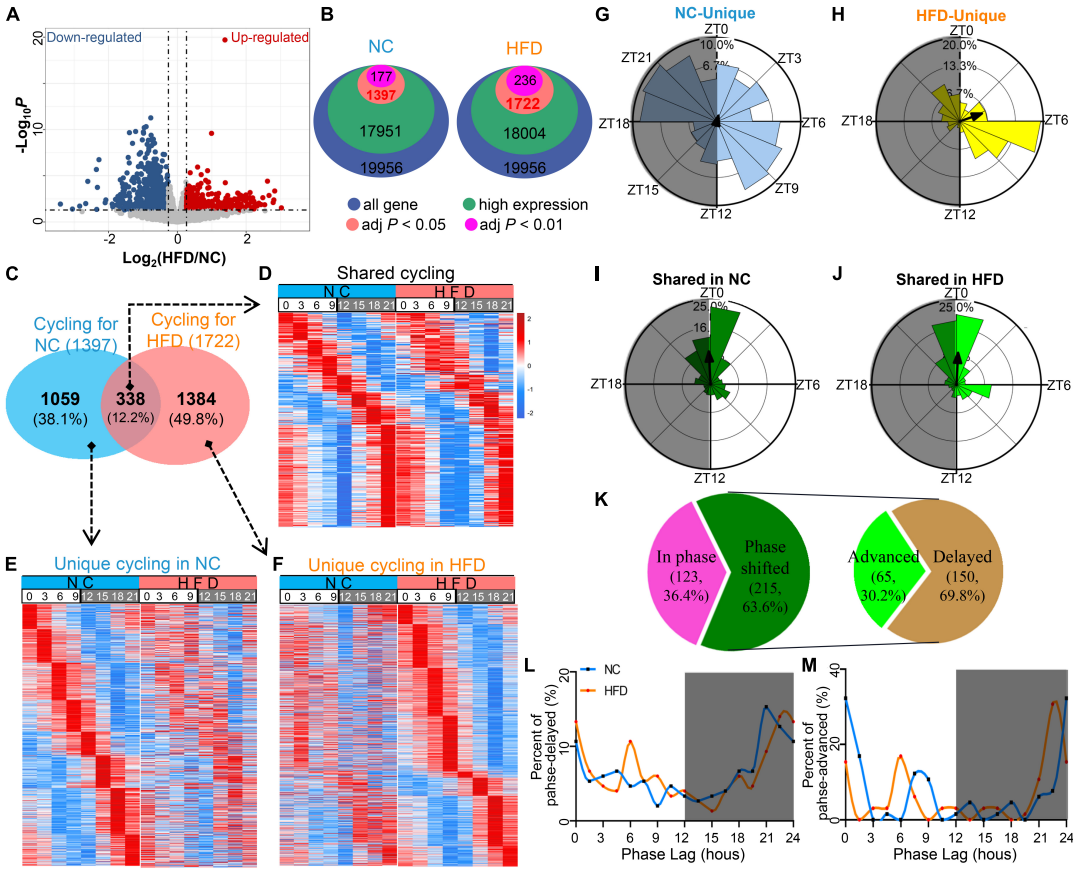

FIGURE 2
High-fat diet (HFD) reprograms the characteristics of the circadian transcriptome in murine MGs. **(A)** Volcano plot of RNA-seq data for NC- and HFD-fed MG genes. The red and blue dots denote ≥1.2-fold or ≤0.83-fold changes in expression between the NC-fed and HFD-fed MGs, respectively. **(B)** The number of rhythmic genes in NC- and HFD-fed MGs under different threshold conditions in the JTK_ algorithm. **(C)** Venn diagram showing the gene sets of the MGs of NC- and HFD-fed mice (JTK algorithm, adjusted $P < 0.05$ and expression ≥ 0.1). $n = 3$ mice per group per time point at 3-h intervals. $n = 24$ mice per group. **(D)** Heatmaps visualizing the expression levels of 338 shared rhythmic genes in the MGs of NC- (left) and HFD-fed (right) mice at different ZT points at 3-h intervals throughout the circadian cycle. The expression levels were indicated by a color bar ranging from blue to red, with the expression range normalized to ± 2. **(E)** Heatmaps visualizing the expression levels of 1,059 rhythmic genes unique in the MGs of NC-fed mice at various ZT points at 3-h intervals throughout the circadian cycle. **(F)** Heatmaps visualizing the expression levels of 1,384 rhythmic genes unique in the MGs of HFD-fed mice at various ZT points at 3-h intervals throughout the circadian cycle. **(G–J)** Phase analysis of rhythmic genes in the MGs of NC- and HFD-fed mice. Gray shading: dark phase. **(K)** Phase analysis of 338 shared rhythmic genes in the MGs of NC- and HFD-fed mice. Phase distribution plot for phase-delayed **(L)** and phase-advanced genes **(M)** in shared cycling genes. Gray shading: dark phase.

2 were located at ZT18 and the troughs at ZT6, and 337 and 325 cycling genes were enriched in the MGs of NC- and HFD-fed mice, respectively (Figures 4C, D). The peaks of Cluster 3 were located at ZT12 and the troughs at ZT0, and 161 and 198 cycling genes were enriched in the MGs of NC- and HFD-fed mice, respectively (Figures 4E, F). The peaks of Cluster 4 were located at ZT3 and the troughs at ZT15, and 263 and 402 cycling genes were clustered in the MGs of NC- and HFD-fed mice, respectively (Figures 4G, H). Cycling genes in each cluster of the MGs of NC- and HFD-fed mice are listed in Supplementary Table 3.

The KEGG annotation functions for cycling genes with similar temporal patterns between the MGs of NC- and HFD-fed mice had significantly different annotation pathways (Figures 4A–H, right of each panel). Rhythmic genes in cluster 2 of the MGs of NC-fed mice were enriched mainly in immune function (Figure 4C), whereas the cluster 4 genes of the MGs of HFD-fed mice were associated with immune pathways (Figure 4H). The cycling genes of cluster 1 in the MGs of NC-fed mice were mainly related to important signaling pathways (Figure 4A), whereas similar pathways in the MGs of HFD-fed mice were concentrated in Cluster 4 (Figure 4H). Cluster 3 genes in the MGs of HFD-fed mice were related to metabolism pathways, especially fat metabolism (Figure 4F), whereas a few pathways were associated with metabolism function in the MGs of NC-fed mice (Figure 4). Collectively, these results suggest that HFD intervention reshapes the oscillating patterns and corresponding functional pathways of rhythmic genes.

3.4. HFD does not elicit core clock desynchrony of MGs

To determine the effect of HFD intervention on the oscillatory pattern of core clock machinery genes in the mouse MG, we compared the expression levels and oscillation amplitudes of the

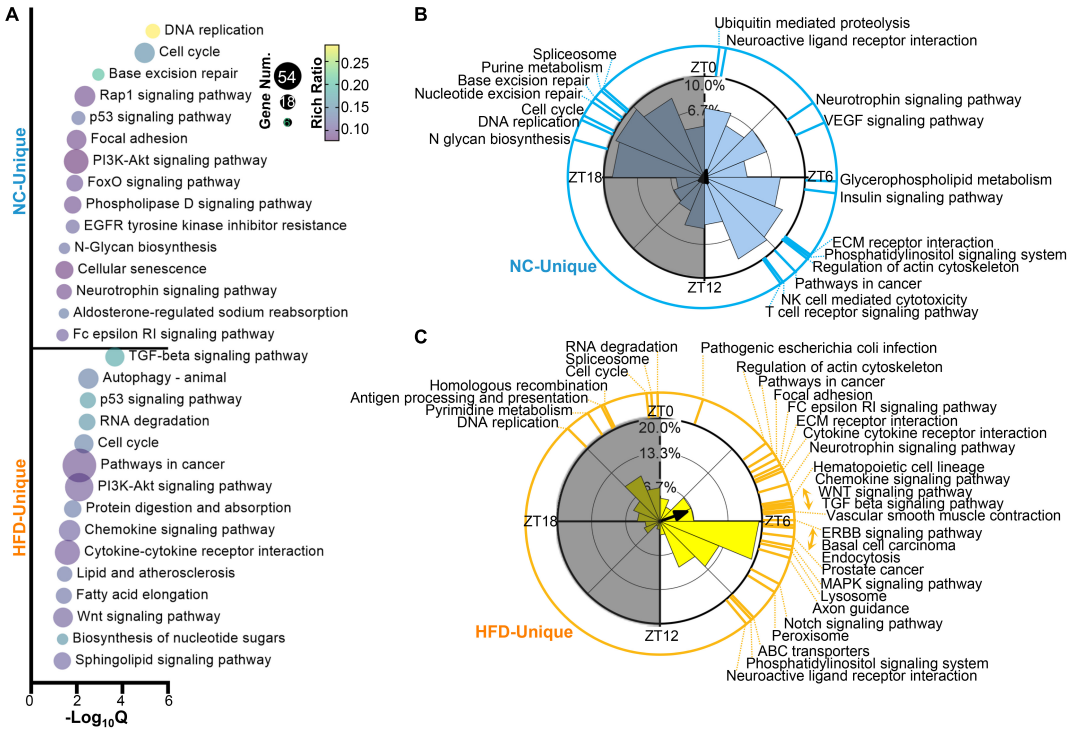

FIGURE 3
High-fat diet (HFD) alters the oscillatory characteristics of circadian transcriptomic profiling in murine MGs. **(A)** Functional annotations for NC-unique (up) and HFD-unique (down) cycling genes by GO Biological Process analysis ($Q < 0.05$). Phase distribution of significantly enriched KEGG pathways ($Q < 0.05$) in the MGs of NC-fed **(B)** and HFD-fed **(C)** mice. The blue **(B)** and yellow **(C)** lines on the outside circle indicate the enriched pathways at various phases. Gray shading: dark phase.

core clock genes, including *Arntl* (*Bmal1*), *Npas2*, *Clock*, *Per1*, *Per2*, *Per3*, *Nr1d1*, *Nr1d2*, *Cry1*, and *Cry2*, between the MGs of NC- and HFD-fed mice at 3-h intervals over a 24- h circadian cycle. The results showed that the expression of all these core clock genes exhibited significant diurnal rhythmicity in MGs from NC- and HFD-fed mice (Figure 5). However, the phase distribution and oscillation amplitude of core clock gene expression were not significantly altered in the MGs of HFD-fed mice compared to those of NC-fed mice (Figure 5). Thus, these data suggest that HFD intervention does not interfere with the synchronization of the core clock machinery in MGs.

3.5. HFD-induced lipid metabolism disorder in MGs

To verify the effects of HFD intervention on the lipid metabolism-related genes and their potential functions in murine MGs, we compared the differential expression level of genes between the MGs of NC- and HFD-fed mice (fold change $\geq$1.2 or $\leq$0.83, adjust $P < 0.05$). As shown in Figure 6A and Supplementary Table 4, 98 DEGs related to lipid metabolism were found, of which 61 were upregulated in the MGs of HFD-fed mice, and 37 genes were downregulated. The top 20 up- and down-regulated DEGs at various ZT points are shown in Figure 6B. The DEGs related to lipid metabolism were enriched in some lipid metabolism pathways ($Q < 0.05$), as shown in Figure 6C. Analyzes by PPANs (Figure 6D) and GSEA (Figures 6E–H) were performed to investigate the enrichment of genes in specific molecular functions. These data revealed that significantly enriched signaling pathways were related to specific lipid metabolism, including glycerolipid/glycerophospholipid/ether lipid metabolism, response to lipid/fatty acid, regulation of lipid storage, and lipid catabolic/metabolic process (Figure 6D). The GSEA results revealed that triglyceride metabolism/catabolism, PPAR signaling pathway, and fatty acid metabolic process were enriched specifically in the MGs of HFD-fed mice (Figures 6E–H).

To determine the effect of HFD feeding on lipid metabolism in MGs, we performed ORO staining to observe the differences in lipid droplets between the MGs of NC- and HFD-fed mice. The results showed that lipid amounts showed a strong rhythm in the MGs of NC-fed mice, with lipid droplets peaking at ZT12 and troughing at ZT0 (Figure 6I). In contrast, in the MGs of HFD-fed mice, lipid amounts peaked at ZT18 and trough at ZT12 (Figure 6I). In addition, the amount of lipids in the MGs of HFD-fed mice was significantly higher than that in the MGs of NC-fed mice (Figures 6J, K). These results suggest that HFD intervention alters lipid metabolism in murine MGs and causes lipid accumulation in MGs.

4. Discussion

To the best of our knowledge, this is the first study to show that high-fat nutritional stress uniquely affects the circadian transcriptome of murine MGs. We found that a 4 weeks high-fat

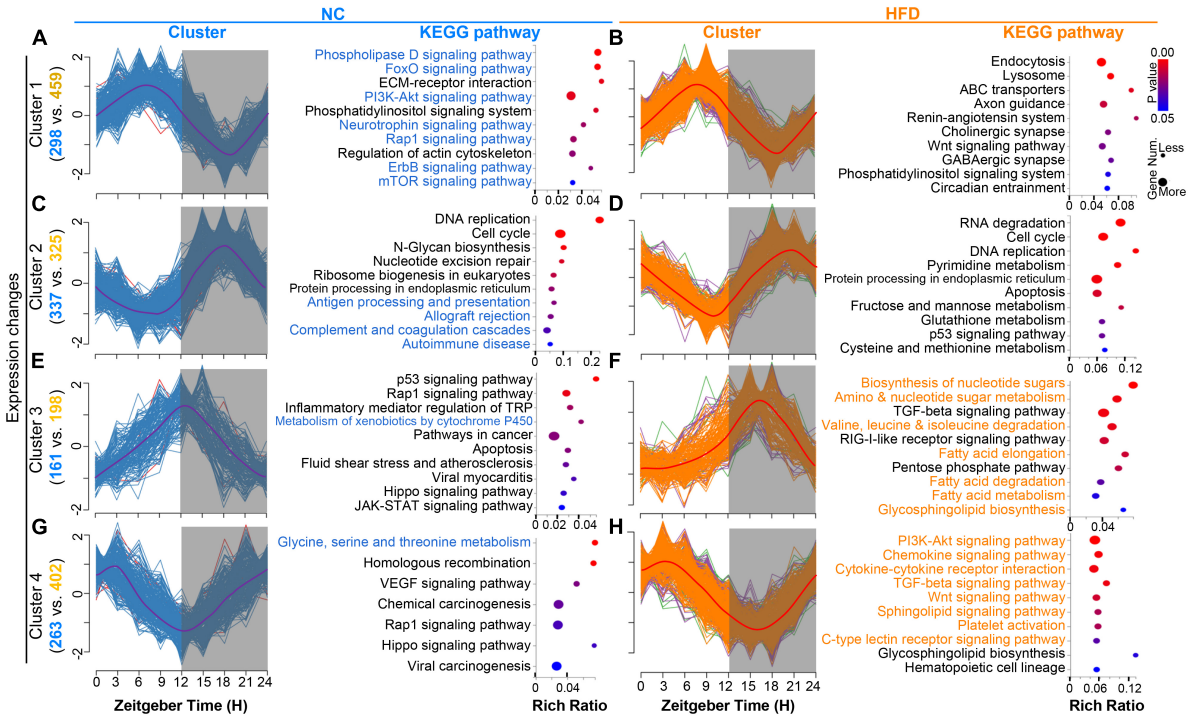

FIGURE 4
High-fat diet (HFD) alters the cluster-dependent transcriptomic map. **(A,C,E,G)** Oscillating patterns of normalized expression for rhythmic genes from four enriched clusters for the MGs of NC-fed mice (left). The enriched KEGG pathways for genes in each cluster ($P < 0.05$) are shown in the (right) panel. Gray shading: dark phase. **(B,D,F,H)** Oscillating patterns of normalized expression for rhythmic genes from four enriched clusters for the MGs of HFD-fed mice (left). The enriched KEGG pathways for genes in each cluster ($P < 0.05$) are shown in the (right) panel. Gray shading: dark phase.

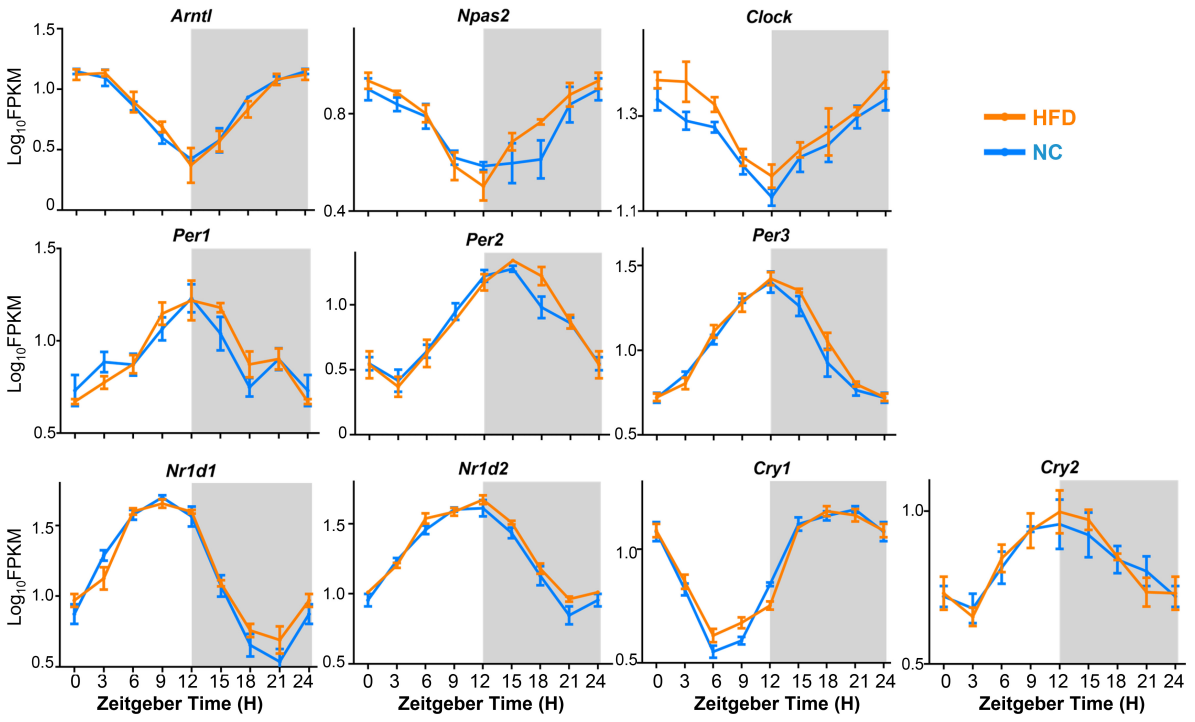

FIGURE 5
Expression and oscillation patterns of core clock machinery genes in the MGs of NC- and HFD-fed mice. $n = 3$ mice per group for each sampling time point. Student's t-test was performed for each ZT time point for the NC- and HFD-fed mice. Gray shading: dark phase.

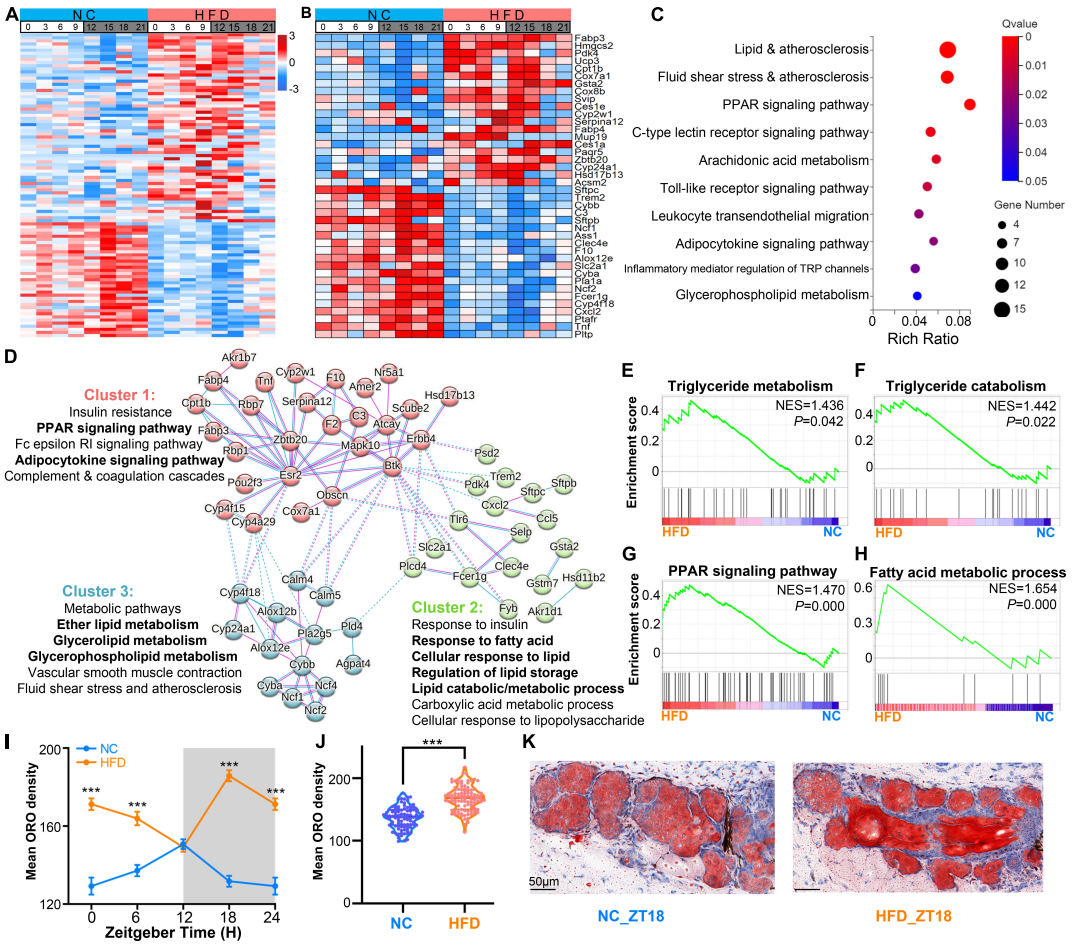

FIGURE 6

High-fat diet (HFD)-induced lipid metabolism disorder in MGs. **(A)** Heatmaps visualizing the expression levels of the differentially expressed lipid-associated genes (fold change ≥1.2 or ≤0.83, adjust $P < 0.05$) in MGs between the NC- and HFD-fed mice at various ZT points at 3-h intervals throughout the circadian cycle. The expression levels were indicated by a color bar ranging from blue to red, with the expression range normalized to ± 3. **(B)** Heatmaps visualizing the expression levels of the top 20 up- and down-regulated DEGs of lipid metabolism-related genes in the MGs between the NC- and HFD-fed mice at various ZT points. **(C)** The top 10 significant KEGG annotations of 98 DGEs associated with lipid metabolism in the MGs between the NC- and HFD-fed mice ($Q < 0.05$). **(D)** The protein–protein association networks (PPANs) and functional clusters with specific KEGG annotations of lipid metabolism-related DEGs in the MGs between the NC- and HFD-fed mice ($Q < 0.05$). **(E–H)** Enrichment plots for triglyceride metabolism/catabolism, PPAR signaling pathway, and fatty acid metabolic process were enriched specifically in the MGs of HFD-fed mice by GSEA analysis. **(I)** Temporal changes in lipid droplets in the MGs of NC- and HFD-fed mice at 6 -h intervals. Three to five right-sided MGs were randomly selected from each NC- and HFD-fed mouse. $n = 6$ mice per group per sampling time point. Student's t-test was performed for each ZT point in the NC- and HFD-fed mice. ***$P < 0.001$. The gray shading indicates the dark phase. **(J)** Average lipid droplet accumulation in the MGs of NC- and HFD-fed mice. $n = 24$ mice per group. Student's t-test between the NC- and HFD-fed mice. ***$P < 0.001$. **(K)** Representative ORO staining images of lipid deposition in the MGs of NC- (left) and HFD-fed (right) mice at ZT18. Scale bar: 50 μm.

dietary regimen significantly altered the circadian characteristics of MGs, including their cycling transcriptome profiles and content of lipid droplets. Notably, high-fat intake shifts cycling genes and their enriched functional signaling pathways that occur throughout the light–dark cycle in the MGs of balanced diet-fed mice to only the light phase of HFD-fed mice. These data suggest that the nutritional challenges posed by short-term, high-fat dietary intake reorganize the circadian rhythms of MGs.

In mammals, circadian physiology is generated or controlled by the suprachiasmatic nucleus (SCN), a central pacemaker in the hypothalamus (37, 38). The SCN generates or controls output circadian physiology through diffusible signals, including hormonal rhythms, sympathetic/parasympathetic systems, core body temperature, and feeding patterns, to control the molecular clock in peripheral tissue cells, thereby generating output circadian physiological activity (8). However, many exogenic zeitgebers (39–41), including nutritional alternations (42–44), feeding timing (29, 45), and altered sleep/wakefulness (46) can disrupt the normal circadian rhythm and promote the occurrence of some diseases, such as metabolic syndrome and type 2 diabetes (47, 48).

Previous studies by us and other teams have found that interventions, such as short-term HFD (26), high fructose intake (32), jet lag (15), and gut dysbiosis accompanying aging (14), can reformat the rhythmic profile of the murine lacrimal gland. Similarly, a high fructose intake significantly alters the rhythmic pattern of the murine corneal transcriptome and its associated physiological activities. Consistent with these studies, the present study confirms that a 4 weeks high-fat dietary regimen reshapes

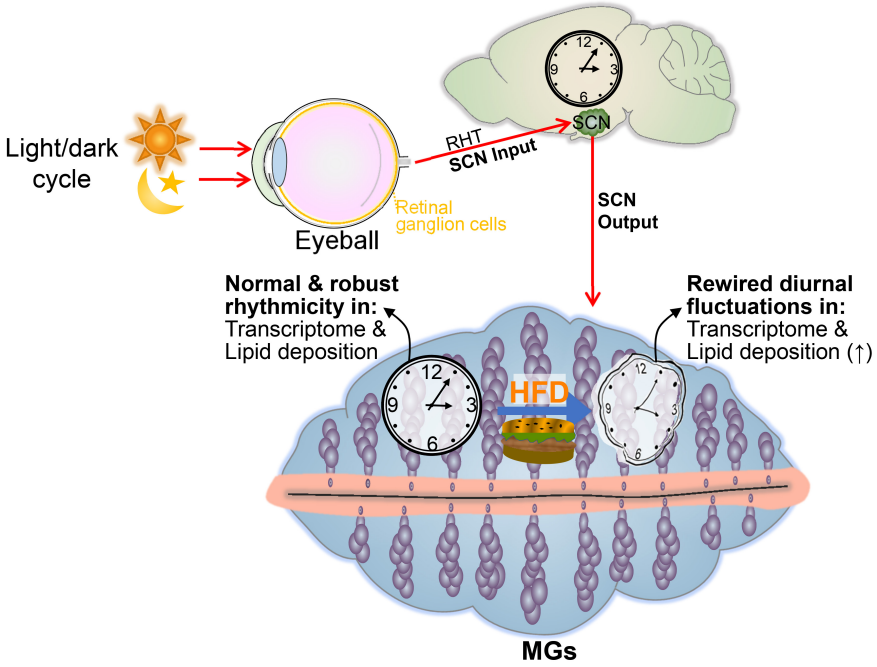

FIGURE 7
Summary displaying the effects of an HFD on the cyclical transcriptomic profile of MGs. In mice receiving a high-fat dietary regimen, the light-regulated central clock pacemaker (SCN) functions normally and expresses normal sleep/wake and fasting/feeding rhythms. However, a high-fat diet alters the normal circadian rhythmicity transcriptome profiles and lipid droplet oscillation of MGs.

the composition of rhythmic transcriptome and their functional signaling pathways enriched in mouse MGs at a spatiotemporal level. These results suggest that the nutritional challenge from an HFD alters the circadian rhythmicity of MGs in a tissue-specific manner. Therefore, further exploration of the underlying mechanisms will likely be of high significance.

Each mammalian cell contains a machinery of core clock genes that generate rhythmic oscillatory gene expression and its associated physiological activities in a 24-h cycle by binding thousands of pathways to the entire genome and driving a feedback regulatory system (49). Core clock genes are the central controllers of the biological clock system. The available data suggest that the core clock system of the cell is a relatively stable system. If the retino-hypothalamic tract (RHT) system is not disturbed, the core clock system maintains a steady state (50, 51). This stability is not only present in metabolic stress but also in aging organs and tissues (51–53). Similarly, the same stabilization phenomenon has been observed in ocular tissues subjected to nutritional challenges, such as the cornea (11) and the lacrimal gland (26) subjected to high fructose intake and the lacrimal gland subjected to a high-fat diet (26). Similarly, this asynchrony is present in the nutritionally challenged liver (54), as well as in several aging tissues (51–53). Recently, we found that the core clock of the lacrimal gland was not significantly altered, even in sleep deprivation-treated mice, without altering the light/dark cycle, although the output gene fraction was drastically altered (55). These studies further confirm the strong stability of the core clock without altering the day/night cycle. However, the cause of the altered output genes under the aforementioned nutritional stress and other factors has thus far been unclear. Recently, Deota et al. speculated that the rhythmicity of output gene expression in most tissues is not exclusively driven by the circadian clock. Systemic signals generated by other factors (e.g., feeding-fasting cycles) combined with endogenous clock modulated signals may play a dominant role in regulating the rhythmicity of gene expression in peripheral organs (56). Therefore, further exploration of the mechanisms by which HFD leads to decoupling the core clock from the downstream core clock-controlled output system is potentially valuable for addressing the pathophysiological alterations in the structure and function of MGs caused by HFD.

High-throughput RNA-seq data-based bioinformatics analysis is currently one of the main tools used to elucidate the complex molecular mechanisms behind circadian rhythm alterations. Time series clustering methods provide an effective approach for assessing the accompanying temporal features for big data analysis (57). The present study provides another dimensional analysis of the pattern of altered physiological activity of MGs due to excessive lipid intake. Consistent with previously studied ocular tissues, such as the cornea (11) and lacrimal gland (26), nutritional challenges dramatically altered the output transcriptome of circadian rhythms in both gene composition and the oscillations of their enriched signaling pathways. MGs play an important role in maintaining the stability of the tear film mainly through the lipid layer of the tear film (3, 58, 59). Therefore, we specifically analyzed the effect of HFD on the lipid metabolism-related transcriptome of MGs and the content of lipid droplets accompanying temporal oscillations. As predicted, this study confirms that HFD has a profound effect on lipid metabolism-related pathways and oscillations of lipid droplets in MGs. These data provide new insights into HFD-induced MG dysfunction. However, further exact mechanisms

would require further in-depth analysis by lipidomics, proteomics, and metabolomics.

This study has several limitations. First, the C57BL/6 mice used in this study were nocturnal animals. The sleep–wake cycle in mice is opposite to that in humans (60). Therefore, certain facts about human MGs must be interpreted with caution. Second, the present study only provided changes in the transcriptomic profile of MGs in male mice, and further observations in female mice may provide more information, especially regarding sex-specific differences (61, 62). Third, the C57BL/6 mice used in this study are melatonin-deficient mouse models (63), but melatonin plays an important role in regulating circadian rhythms in humans (64). Therefore, not all phenomena that occur in humans can be addressed. Fourth, this paper focuses on the bioinformatic interpretation of the effect of HFD on the transcriptomic rhythmicity of MGs, and we will attempt to focus more on the cellular and molecular mechanisms in future studies. Finally, this project provides only the effect of HFD on the bulk transcriptome rhythmicity of MGs. Considering the complexity of the different cell types of MGs and their existence of different oscillatory cycles, the use of single-cell RNA-seq sequencing technology in the future will offer solutions to this problem (65, 66).

5. Conclusion

In conclusion, our observations support the concept that an HFD alters the output component of the circadian rhythm of the MGs, rather than the core clock machinery (**Figure 7**). These data emphasize the importance of nutritional interventions in maintaining the health of MGs. Exploring or targeting the loss-of-coupling mechanism between the core clock and the output component has the potential to ameliorate MG dysfunction induced by HFD.

Data availability statement

The original contributions presented in this study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA924579.

Ethics statement

All animal experiments in this study were approved by the Animal Ethics Committee of Henan Provincial People's Hospital and followed the guidelines described in the ARVO Statement for the Use of Animals in Vision and Ophthalmic Research.

Author contributions

ZL and SZ designed the study and wrote the manuscript. SZ, ZL, JL, HS, DH, and DQ collected and prepared the samples. SZ performed RNA-seq and bioinformatics analysis with help from XP, DL, and SH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1146916/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Amplitudes (AMP) of shared **(up)** and unique **(down)** rhythmic genes in the MGs of NC- and HFD-fed mice. Student's *t*-test between NC- and HFD-fed mice. ns, not significant.

SUPPLEMENTARY TABLE 1
Cycling genes (JTK_adj $P < 0.05$) of all MG genes for NC- (1,397) and HFD-fed (1,722) mice in **Figure 2B**.

SUPPLEMENTARY TABLE 2
Cycling genes (JTK_adj $P < 0.05$) unique to NC-fed (1,059), unique to HFD-fed (1,384) and shared between the two groups (338) in **Figure 2C**.

SUPPLEMENTARY TABLE 3
Rhythmic genes in each time-series cluster of NC- and HFD-fed MGs in **Figure 4**.

SUPPLEMENTARY TABLE 4
Expression levels of lipid-related DEGs for NC- and HFD-fed MGs in **Figure 6A**.

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Chronic circadian desynchronization of feeding-fasting rhythm generates alterations in daily glycemia, LDL cholesterolemia and microbiota composition in mice

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Introduction: The circadian system synchronizes behavior and physiology to the 24-h light– dark (LD) cycle. Timing of food intake and fasting periods provide strong signals for peripheral circadian clocks regulating nutrient assimilation, glucose, and lipid metabolism. Mice under 12h light:12h dark (LD) cycles exhibit behavioral activity and feeding during the dark period, while fasting occurs at rest during light. Disruption of energy metabolism, leading to an increase in body mass, was reported in experimental models of circadian desynchronization. In this work, the effects of chronic advances of the LD cycles (chronic jet-lag protocol, CJL) were studied on the daily homeostasis of energy metabolism and weight gain.

Methods: Male C57 mice were subjected to a CJL or LD schedule, measuring IPGTT, insulinemia, microbiome composition and lipidemia.

Results: Mice under CJL show behavioral desynchronization and feeding activity distributed similarly at the light and dark hours and, although feeding a similar daily amount of food as compared to controls, show an increase in weight gain. In addition, ad libitum glycemia rhythm was abolished in CJL-subjected mice, showing similar blood glucose values at light and dark. CJL also generated glucose intolerance at dark in an intraperitoneal glucose tolerance test (IPGTT), with increased insulin release at both light and dark periods. Low-density lipoprotein (LDL) cholesterolemia was increased under this condition, but no changes in HDL cholesterolemia were observed. Firmicutes/Bacteroidetes ratio was analyzed as a marker of circadian disruption of microbiota composition, showing opposite phases at the light and dark when comparing LD vs. CJL.

Discussion: Chronic misalignment of feeding/fasting rhythm leads to metabolic disturbances generating nocturnal hyperglycemia, glucose intolerance and hyperinsulinemia in a IPGTT, increased LDL cholesterolemia, and increased weight gain, underscoring the importance of the timing of food consumption with respect to the circadian system for metabolic health.

KEYWORDS

chronic jetlag, hyperinsulinemia, circadian rhythm, glucose intolerance, lipidemia

1. Introduction

The circadian clock, located at the hypothalamic suprachiasmatic nucleus (SCN), consists of neuronal network oscillators based on transcription-translation feedback loops of core clock genes, whose activity is synchronized to the 24-h LD cycle through light-activated pathways (1). Briefly, the CLOCK and BMAL1 heterodimer bind to the E-boxes of their own repressors Cryptochrome (Cry1 and Cry2) and Period (Per1, 2, and 3) and of the nuclear hormone receptors Rev-erb (α and β), and Ror (α, β, and γ). In turn, this molecular clockwork is fine-tuned by ROR activation and REV-ERB repression of the Bmal1 gene expression through RRE elements (2). Outputs from the SCN relaying in several hypothalamic areas generate daily activity-rest, endocrine, physiological, and metabolic rhythms. In virtue of similar molecular machinery, peripheral clocks coordinate circadian metabolic functions downstream of the SCN (3). Feedback signals from the feeding-fasting rhythm act as strong zeitgebers (synchronizers) for oscillators in metabolic tissues controlling energy homeostasis, indeed bypassing the SCN under time-restricted feeding protocols (4), or without its participation (5). In fact, the liver clock is a central circadian integrator of feeding signals regulating rhythmic transcripts involved in daily carbohydrate homeostasis (6).

Both diurnal and nocturnal species feed when they are behaviorally active, concurrent with a high metabolic and thermogenic rate. Mice synchronized to laboratory LD cycles show nocturnal activity with about 75–85% of caloric consumption occurring at dark hours (7). This ensures the supply of carbohydrates, lipids, and amino acids needed for energy uptake, utilization, and storage, while basal energy expenditure during the rest/fasting period is sustained by the degradation and mobilization of stored fuels (i.e., glycogen and fat) (8).

Daily rhythms in basal blood glucose in mice under *ad-libitum* feeding exhibit high diurnal values which decrease at night (9, 10), depending both on SCN direct control, as well as on the feeding-fasting rhythm (11). Blood glucose during the activity/feeding phase is supplied mainly by diet, while during rest/fasting, by endogenous production from hepatic glycogen degradation (12). Pancreatic insulin and glucagon basal secretion, in turn, are mainly regulated by the feeding-fasting rhythm and/or changes in glycemia. Several circadian controls set increased glucose tolerance and insulin sensitivity at the light–dark transition to anticipate activity, such as islet clocks controlling peak insulin secretion (13), or the increase in glucose transporter type 4 (GLUT4) in muscle (14). In addition, the main functions of the liver are rhythmically regulated, generating a circadian control of nutrient metabolism and energy homeostasis (15). In consequence, hepatic lipid metabolism is controlled by the circadian clock (16). Some examples of this are the control of lipogenesis by the circadian clock *via* HDAC3 (17) and the regulation of adipogenesis *via* Bmal1 (18, 19).

Taking into account the circadian control over feeding/fasting, and nutrient and energy compounds metabolism, it is not surprising that, over the years, chronic circadian disruption has been hypothesized to be one of the main factors that generate metabolic alterations. At the gene level, mice lacking the Per2 gene exhibit dyslipidemia (20) and those lacking Clock have a predisposition to hyperlipidemia (21). In addition, Bmal1 in the central clock has been shown to be sufficient for controlling metabolic rhythms, being the central clock who drives the majority of rhythmicity in circulating metabolites (22).

Fan et al. showed that a chronically disrupted light protocol generated more glucose intolerance after glucose injection (23). In previous experiments in the laboratory, we found that desynchronized mice, by advancing CJL schedule, exhibit an increase in body weight (but similar caloric intake than controls), retroperitoneal and epididymal adipose tissue, adipocytes size, and circulating triglycerides (24). Moreover, light schedules inducing long-term circadian desynchronization (as constant light, dim light at night, or advancing CJL), uncouple feeding-fasting rhythm from activity/rest rhythms generating asynchronous signals to peripheral oscillators controlling energy homeostasis, leading to several metabolic disturbances (25). Thus, independent of daily caloric intake and nutrient quality, the daily timing of food consumption must be considered as a critical factor in maintaining metabolic health, by allowing circadian compartmentation of energy homeostasis.

Rhythms in both intestinal microbiota composition and their metabolome, strongly dependent on the feeding-fasting rhythm of the host, have been studied in regulating circadian nutrient and energy metabolism (26). Chronic changes of the microbiota composition (dysbiosis) are related to metabolic alterations, recognized due to a diminution in bacterial diversity, and an increment of facultative anaerobes followed by an increment in the Firmicutes/Bacteroidetes ratio. CJL schedules were used in several studies to generate circadian disruption and/or dysbiosis of rhythms in the microbiota, as reduced bacterial oscillations (27), changing the composition of jejunal and fecal microbiota (28), or shifting the Firmicutes rhythm (29). Taking this into account, under conditions of circadian desynchronization, the daily microbiota composition can be assessed as a prognostic marker of metabolic disturbances.

In the present work, we aim to continue exploring the effects of CJL on circadian metabolic homeostasis, by studying the desynchronization of feeding-fasting rhythm, daily variations in blood glucose, lipid homeostasis, and intestinal microbiota composition.

2. Materials and methods

2.1. Animals

C57BL/6J male mice (6 weeks old, purchased from Facultad de Veterinaria, Universidad Nacional de La Plata, Argentina) were allowed to acclimatize in groups of 5 individuals in polycarbonate cages for 1 week in stock rooms at animal facilities of the Universidad Nacional de Quilmes, with tap water and rodent chow (ACA Cooperación, Argentina) *ad libitum*, and wood bedding replaced every 3–4 days. Room temperature was set at 22–24°C, and 12 h light:12 h dark (LD) cycles were set with fluorescent lamps supplying 100–150 lux at cage microenvironment [lights ON/OFF at 7 a.m./19 p.m., setting zeitgeber time 12 (ZT12) at lights OFF].

2.2. Behavioral recordings and CJL light schedule

Mice were placed individually in cages inside ventilated closets for behavioral recording, with food and water *ad-libitum* for the entire

experimental protocol (except when indicated). General cage activity was monitored with passive infrared (PIR) motion sensors above the cage grid. Feeders were disposed inside the cage by a 5-cm diameter plastic cylinder supplied with chow and with a PIR on top for detecting food-access activity. PIR signals were processed and stored in a computer at 5 min bins, to obtain time series that were analyzed with the software El Temps (Universitat de Barcelona, Spain). From LD 12:12, the light schedule for advancing CJL (Casiraghi et al., 2012) was designed as follows: (1) advancing by 6 h the lights OFF, (2) followed by advancing 6 h the lights ON, (3) keeping this schedule for one entire LD 12:12, (4) repeating 1–3 (see Figure 1). Thus, the LD 12:12 cycle was repeated 2 times each week: one coincident with the LD cycle used as control (i.e., having ZT12 at 19 p.m., used for all experimental comparisons), and the following, with a 6-h advance.

2.3. Experimental protocol

After recording 24-h rhythms under LD 12:12 cycles for 7 days, two experimental groups were set: mice kept under LD 12:12 (control group, $n = 14$; from here, "LD"), or under CJL ($n = 14$) for 50 days, monitoring behavior, as well as measuring the weekly increase of body weight, and food intake by weighing the food remaining in feeders after 1 week. In the last 15 days of the protocol, glucose was measured in blood samples every 4 h and for 24 h, for testing daily rhythms in glycemia under *ad libitum* feeding in both CJL and LD groups. The next week, an intraperitoneal glucose tolerance test (IPGTT) was performed according to the guidelines and considerations for metabolic tolerance tests in mice (30), with previous fasting of 4 h, by delivering 2 g/kg glucose in sterile saline solution intraperitoneally, both at ZT6 and ZT18, measuring blood glucose at 0, 15, 30, 60, and 120 min, and insulin at 0, 15, 60, and 120 min post-administration.

2.4. Biochemical analysis

Mice were restrained for taking blood samples from the caudal venous sinus, in order to measure *ad libitum* glycemia rhythm (at ZT3, ZT6, ZT9, ZT12, and ZT24), and delivering an IPGTT at both ZT6 and ZT18 to measure glycemia (digital glucometer Contour TS, Bayer) and plasma insulinemia responses with an ELISA kit (EMINS, Thermofisher Scientific, United States) according to manufacturer's instructions, followed by absorbance measurement at 450 nm in Cytation 5 imaging reader (Biotek Instruments, United States).

Samples at the endpoint were taken at ZT6 and ZT18 under isoflurane (5% in oxygen, 500 ml/min) anesthesia, first by bleeding out euthanized mice by cardiac puncture, for measuring total cholesterolemia (Colestat, Wiener Lab, Argentina), HDL (HDL cholesterol, Wiener Lab, Argentina) and LDL cholesterolemia (LDL cholesterol, Wiener lab, Argentina). All measurements were performed following the manufacturer's instructions, followed by absorbance measurements (Smartspec 3,000 UV/Vis, Biorad,

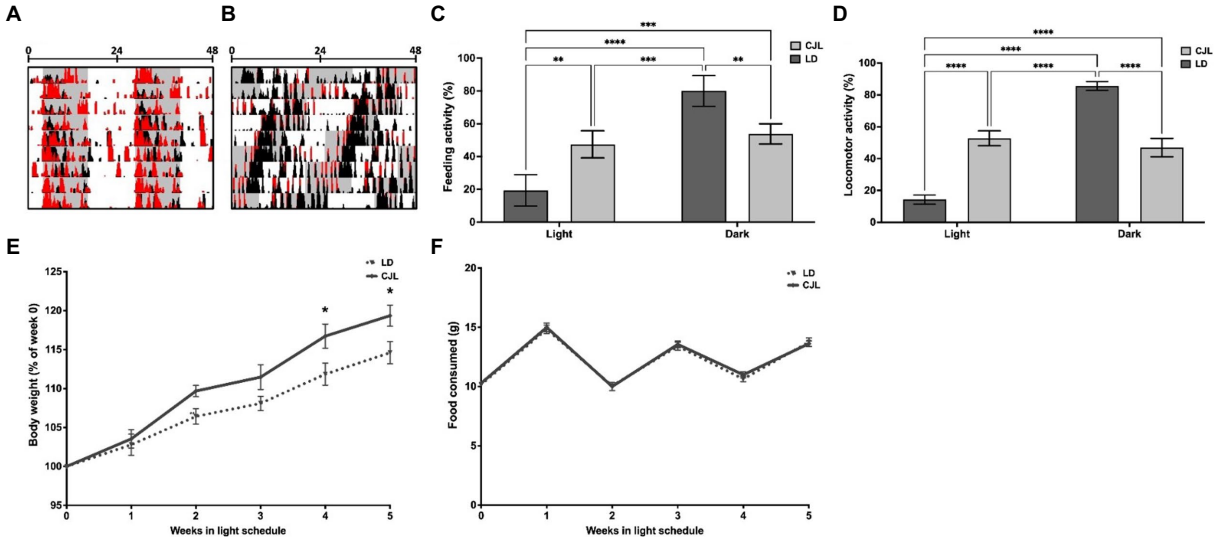

FIGURE 1
CJL induces behavioral desynchronization of general activity and feeding-fasting rhythms, together with an increase in body weight. Representative actograms of locomotor activity of mice under LD **(A)** and CJL **(B)**. Histograms in black represent general activity (black) and feeding activity (red). Mice under LD present general and feeding activity bouts at the dark phase with a period of 24h (24h ± 0.02h, $n = 4$). Mice under CJL showed behavioral desynchronization with a period close to 21h (20.97h ± 0.02h, $n = 4$). **(C)** light/dark feeding activity distribution (as a percentage of total) of mice under LD and CJL. Feeding activity under LD was significantly higher in the dark, while no significant difference was found between light and dark in mice under CJL; Two-way ANOVA, $p < 0.0001$, $F = 41.27$ for interaction; $p < 0.0001$, $F = 63.21$ for light schedule, $p = 0.826$, $F = 0.050$ for light/dark; $n = 4$, followed by Tukey's multiple comparisons test: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$; $n = 4$. **(D)** Light/dark general activity distribution (as a percentage of total) of mice under LD and CJL. General activity under LD was significantly higher in the dark, while no significant difference was found between light and dark in mice under CJL; Two-way ANOVA, $p < 0.0001$, $F = 430.2$ for interaction; $p = 0.953$, $F = 0.003$ for light schedule; $p < 0.0001$, $F = 309.1$ for light/dark; $n = 6$ per group; Tukey's multiple comparisons test: ****$p < 0.0001$. **(E)** Body weight increment (as a percentage of week 0) is significantly higher in mice under CJL conditions compared to LD. Repeated measures Two-way ANOVA, $p = 0.0036$, $F = 2.36$ for interaction; $p < 0.0001$, $F = 92.54$ for weeks; $p = 0.0040$, $F = 9{,}948$ for light schedule; $n = 4$, followed by Sidak's multiple comparisons test: **$p < 0.005$, *$p < 0.05$; $n = 4$. **(F)** Food consumed (g) remained constant over the weeks, without differences between LD and CLJ conditions. Two-way RM ANOVA, $p = 0.9682$, $F = 0.184$ for interaction; $p < 0.0001$, $F = 103$ for weeks; $p = 0.6697$, $F = 0.1858$ for light schedule; $n = 4$, followed by Sidak's multiple comparisons test: ns; $n = 4$.

United States) at 505 nm for total cholesterolemia, 600 nm for HDL, and 660 nm for LDL. A subset of mice was destined for microbiota analysis, collecting a sample of cecum content according to Tong et al. (31) at ZT0 and ZT12, in order to have one time-point at the end of the feeding phase, and the other one at the end of the fasting phase.

2.5. Microbiota analysis

About 250 mg of cecum content homogenized in buffer GA was centrifuged, obtaining DNA from supernatants with a commercial kit (PBL, Argentina). DNA integrity and concentration were determined by spectrophotometer and electrophoresis gels. To construct the libraries V3-V4, the hypervariable region of the ribosomal 16S gene was amplified from DNA. Then a second PCR cycle was performed to add P7 and P5 adapters analyzing the amplicons by spectrophotometer and electrophoresis and then purifying them by silica columns. Each library was then sequenced by means of a Miseq 300 bp, paired-end platform (Illumina Inc., San Diego, CA, United States). Operational taxonomic units (OTUs) were then identified using DADA2 software and SILVA database and analyzed by means of R packages included in the Microbiome Analyst (32) tool. The number of OTUs reads was filtered (cutoff 4 reads, 20% prevalence, cutoff 10% of variance by interquartile range) in each sample. Then, the relative abundance (% of reads in each sample) of OTUs was obtained, and those included in Firmicutes or Bacteroidetes phyla were summed to obtain the Firmicutes/Bacteroidetes ratio.

2.6. Chronobiological and statistical analyses

Behavioral time series were analyzed for detecting circadian rhythms by means of actograms and Chi-square periodograms. Both locomotor activity, as well as activity at feeders, were analyzed in 24-h waveforms as follows: in control mice, by averaging activity in representative data sections of 7 consecutive days, while in mice under the CJL schedule, waveforms were obtained by averaging 4 data sections of the LD 12:12 cycles present in the schedule. The activity counts during light or dark were summed, and calculated as percentage of total activity in 24 h waveforms for comparison. Daily rhythm in glycemia was assessed by fitting mean values obtained of each ZT to the best 24-h cosine function that minimizes the squares of the residuals. Mean values obtained by averaging ZT3, ZT6, ZT9, ZT12 for light, and ZT15, ZT18, ZT21, ZT0 for dark conditions, were also compared. Statistical analyses were done using the software GraphPad Prism 7 (GraphPad Software, San Diego, CA) by previously assessing assumptions for parametric ANOVA analyses, setting $p = 0.05$ as a level for type I error.

3. Results

3.1. CJL induces desynchronization of feeding-fasting rhythms

Advancing CJL is one of the most commonly used light schedules to desynchronize the behavioral rhythms of mice. Actograms in Figures 1A,B show general (black histograms) and feeding activity (red) patterns of representative mice subjected to CJL or LD. All mice under CJL present an activity period that approximates to 21 h (20.97 h ± 0.02 h), following the global period of the light schedules, while 8 out of 14 present a simultaneous period component under relative coordination (24.11 h ± 0.19 h), showing behavioral desynchronization. General activity (24 h ± 0.02 h) and feeding activity bouts under LD were concentrated mainly in the dark (Figures 1A,C), whereas, under CJL, feeding activity also spread into the light period (Figure 1B) generating a similar amount at light and dark (Figure 1C). In addition, weight was significantly increased from the second week of the protocol in mice under CJL with respect to controls (Figure 1D), without differences in the amount of food intake (Figure 1E).

3.2. Glucose metabolism is altered under CJL

In order to determine if chronic desynchronization by the advancing CJL schedule has an impact on glucose homeostasis, first, the 24-h daily rhythm in blood glucose *ad libitum* was measured under LD and CJL (Figure 2A). Mean values for each ZT of control mice ($n = 5$) were significantly adjusted to a cosine wave of 24 h (Ho: amplitude = 0, $p < 0.05$), having the following parameters: MESOR: 130 mg/dl, amplitude: 34.4 mg/dl, acrophase: ZT7 (higher values during light, that decreased with time at the darkness, Figure 2B). For basal glycemia measured in mice under CJL ($n = 5$), 24-h cosine fitting was not significant (amplitude = 0, $p > 0.05$), with similar values at light and dark (Figure 2B). In contrast, under the CJL condition, blood glucose values did not decrease at dark. When performing an IPGTT, blood glucose showed similar kinetics (Figure 2C) within each ZT but differs between LD and CJL groups; however, an area under the curve (AUC) test (Figure 2D) showed no significant differences at light (ZT6) for both LD and CJL, however, a significantly lower tolerance (i.e., increased AUC) was found at ZT18 under CJL. In addition, insulin release in response to IPGTT was also affected under CJL at both ZT6 and ZT18 (Figure 2E). While mice under LD increased their insulin levels from basal values to about a 3-fold change at 15 min, those under CJL showed an increased insulinemia at both ZTs. Thus, taking all these results into account, mice under CJL showed impaired daily glucose homeostasis, having increased basal nocturnal hyperglycemia *ad libitum*, both decreased glucose tolerance at night, and increased insulinemia, in response to IPGTT.

3.3. LDL levels are increased in mice subjected to CJL

Lipidemia was measured in order to analyze the impact of CJL on the daily homeostasis of lipid metabolism. While total cholesterol, LDL, HDL, HDL/LDL, and HDL/cholesterol ratio did not change with respect to controls under LD (Figures 3A–D,F), two-way ANOVA shows that LDL/cholesterol ratio significantly increased in mice under CJL, both at ZT6 and ZT18 (Figure 3E), but no significant differences were found between ZT6 and ZT18 for either LD or CJL, indicating that time of day does not have an impact on LDL/cholesterol levels, as opposed to the light schedule.

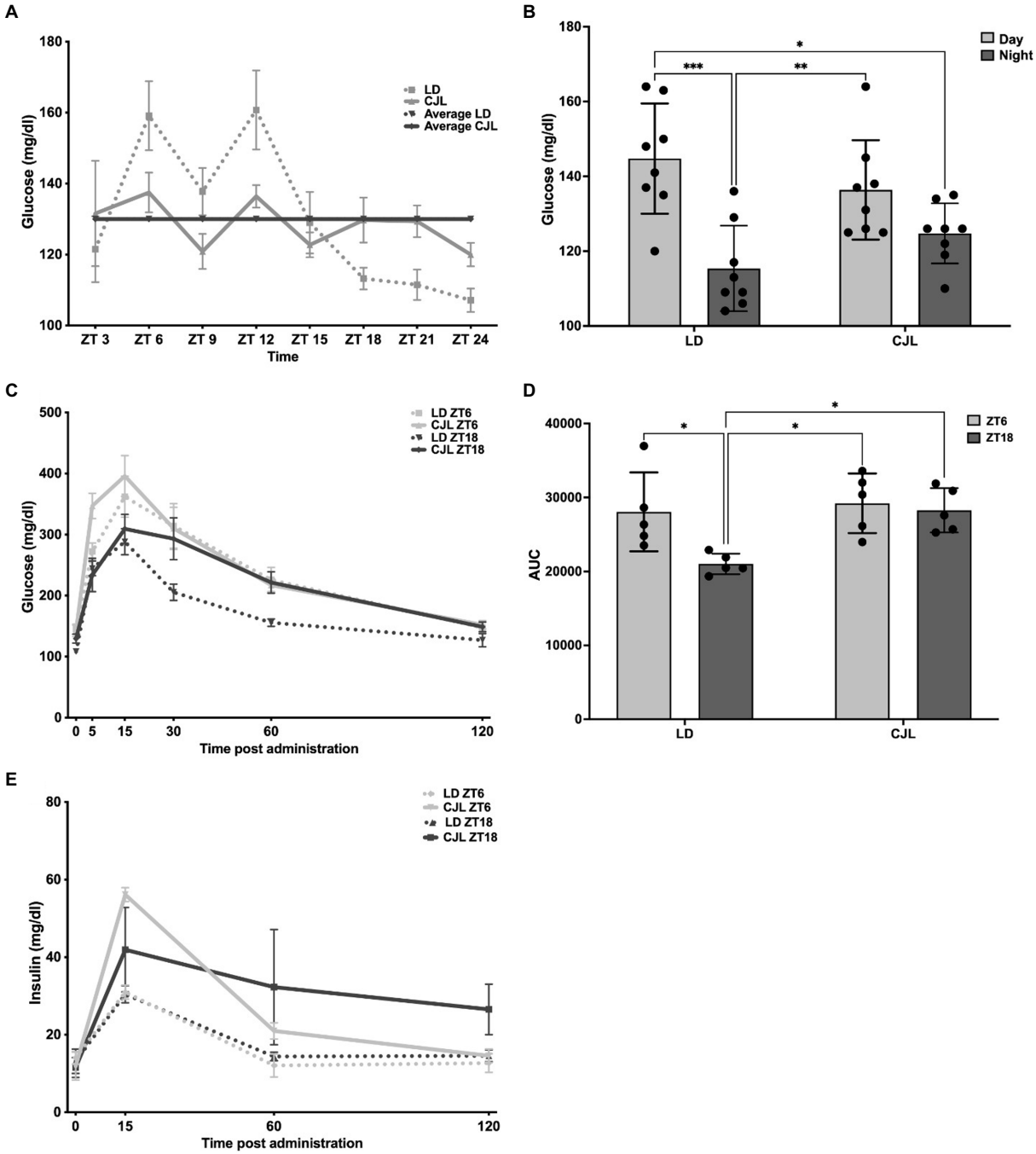

FIGURE 2
Daily glucose homeostasis is altered under CJL conditions. **(A)** Daily rhythm in blood glucose concentration (mg/dl) under *ad libitum* feeding along LD 12:12 in control mice under LD (dotted line) and under CJL (solid line). The daily mean for each group is also indicated. Two-way ANOVA, p =0.0058, F =3.05 for interaction; p <0.0001, F =6.145 for ZT; p =0.682, F =0.1685 for light schedule. Mean values for each ZT of control mice (n =5) were significantly adjusted to a cosine wave of 24h (Ho: amplitude=0, p <0.05), having the following parameters: MESOR: 130mg/dl, amplitude: 34.4mg/dl, acrophase: ZT7. **(B)** Mean values obtained by averaging ZT3, ZT6, ZT9, ZT12 for light, and ZT15, ZT18, ZT21, ZT0 for dark conditions. During the light phase, the values increase and in the dark, the values decrease for LD and CJL conditions. Two-way ANOVA, p =0.0481, F =4.271 for interaction; p <0.0001, F =22.79 for day and night; followed by Tukey's multiple comparisons test: ***p <0.001, **p <0.01, *p <0.05; n =8. **(C)** Blood glucose concentration kinetics after delivering 2g/kg (t =0) intraperitoneal glucose (IPGTT) in mice under LD (dotted line) at or under CJL (solid line), both at ZT6 (gray) or ZT18 (black). Glycemia values were measured at t =0, t =5, t =15, t =30, t =60, and t =120 (min) after glucose administration. Significant differences were found for glycemia kinetics between LD and CJL; however, no significant differences were observed for the two ZTs. Values are expressed as mean±SEM at each time. Three-ways ANOVA, p <0.0001, F =20.63 for time; p =0.006, F =26.75 for light schedule; p =0.2861, F =1.51 for ZTs; p =0.28, F =2.75 for time×time schedule; p =0.37, F =1.14 for time×ZT; p =0.56, F =0.39 for time schedule×ZT; p =0.55, F =0.72 for time×time schedule×ZT. **(D)** Value of the area under the curve (AUC) to estimate the rate of appearance/disappearance of blood glucose, as an indicator of tolerance. A lower value was observed in LD at ZT18 compared to the CJL condition. Values represented as mean±SEM. Two-way ANOVA, p =0.0871, F =3.322 for interaction; p =0.0227, F =6.357 for light schedule; p =0.0293, F =5.726 for ZT; followed by Tukey's multiple comparisons test: *p <0.05; n =5. **(E)** Blood insulin values after delivering 2g/kg (t =0) intraperitoneal glucose (IPGTT) in mice under LD (dotted line) at or under CJL (solid line), both at ZT6 (gray) or ZT18 (black). Insulin values were measured at t =0, t =5, t =15, t =30, t =60, and t =120 (min) after glucose administration. Values are expressed as mean±SEM at each time.

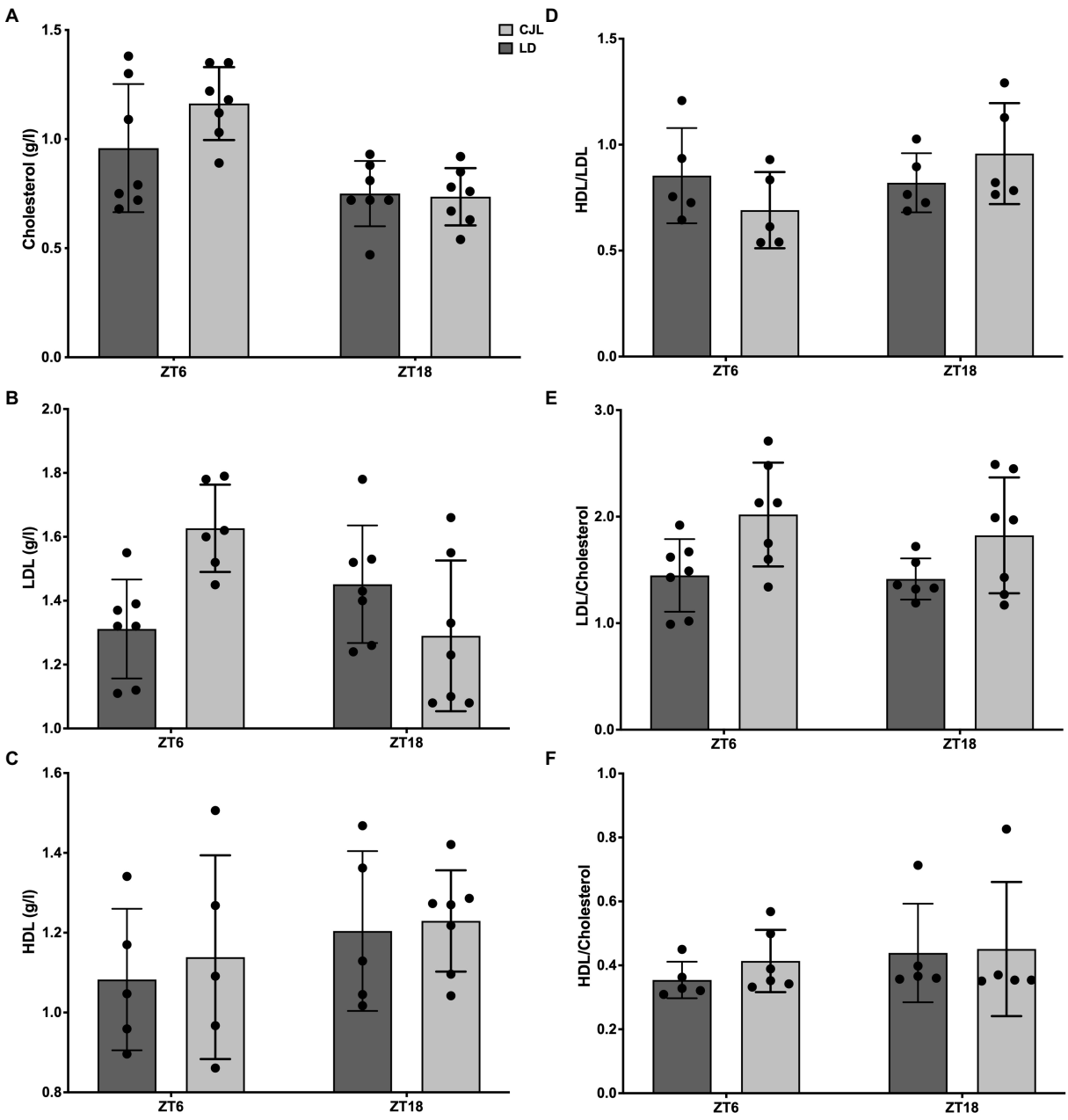

FIGURE 3
Daily changes in lipidemia (total cholesterolemia, LDL, HDL, HDL/LDL ratio, HDL/cholesterol ratio, and LDL/cholesterol ratio) in mice under LD and CJL at ZT6 and ZT18. **(A)** Total cholesterol (g/l) two-Way ANOVA, p =0.1533, F =2.174 for interaction; p =0.0003, F =18.39 for ZT; p =0.2122, F =1.643 for light schedule; followed by Sidak's multiple comparisons test: ns; n =7. **(B)** LDL (g/l) two-way ANOVA: p =0.002, F =11.33 for interaction; p =0.178, F =1.929 for ZT; p =0.288, F =1.180 for light schedule. **(C)** HDL (g/l), two-way ANOVA: ns. **(D)** HDL/LDL ratio, two-way ANOVA: ns. **(E)** LDL/Cholesterol ratio, two-way ANOVA, p =0.6226, F =0.248 for interaction; p =0.4876, F =0.4977 for ZT; p =0.0061, F =9.106 for light schedule; followed by Tukey's multiple comparisons test: ns; n =7. **(F)** HDL/Cholesterol ratio, two-way ANOVA: ns.

3.4. Microbiota composition is altered in CJL

First, samples of cecum content were collected from mice under both light schedules at ZT0 and ZT12, and microbiome composition was determined by performing sequencing of the samples, obtaining the relative abundance of the different Phylum (Supplementary Figure 1A) and Families (Supplementary Figure 1B) present on each sample. To determine differences between these compositions, the analysis was centered on determining the difference between Firmicutes and Bacteroidetes relative abundances (% of reads in each sample), obtaining the Firmicutes/Bacteroidetes ratio at ZT0 and ZT12, in order to obtain daily markers of dysbiosis. This ratio was changed at both light and dark conditions depending on light schedules (LD vs. CJL; i.e., interaction is a significant factor in the ANOVA, Figure 4). This indicates a misalignment of the microbiota populations with the time of day, due to the chronic desynchronization of the feeding/fasting rhythm.

4. Discussion

In the present work, we studied the effects of chronic CJL on the homeostasis of energy, metabolism, and weight. Mice present desynchronization of behavioral activity and feeding activity, resulting in weight gain by feeding a similar daily amount of food to controls. In addition, *ad libitum* glycemia rhythm was abolished in CJL-subjected mice and an IPGTT shows glucose intolerance in dark, with increased insulin release at both light and dark periods. When lipids were analyzed, we found that LDL was increased, but no changes in HDL were observed.

When set in opposite phases, and correctly aligned with peripheral oscillators, activity/feeding and rest/fasting cycles act as strong zeitgebers for the circadian compartmentation of the metabolism of energy compounds. In this work, chronic advances of the LD cycle induced behavioral desynchronization with several metabolic disturbances, generating increased weight gain, with a similar amount of food intake [as observed in other studies by the lab (24) or other groups (27)]. Behaviorally desynchronized mice under CJL show feeding activity still coincident with general activity (see actogram in Figure 1). Also, in mice under CJL, daily caloric intake was not different from controls but distributed similarly at light and dark.

While mice under LD decreased *ad libitum* glycemia at dark after activity onset at ZT12, desynchronization under CJL abolished this daily variation, keeping similar blood glucose at light and dark (see Figures 2A,B). Importantly, tolerance to intraperitoneal glucose was decreased at dark under CJL (Figures 2C,D), as has already been shown for other desynchronization protocols (23), despite increased insulin secretion (Figure 2E). These results indicate an impediment to managing glucose output and could explain the nocturnal hyperglycemia observed *ad libitum*. It is well described that glucose uptake by the liver, adipose, and muscle tissues increases during the night in mice, due to increased glucose tolerance and insulin sensitivity (12), to supply energy for activity. In addition, glucose transporter 4 (GLUT 4) has already been shown to be controlled by the circadian clock in muscle in mice (33) and in adipose tissue in humans (34). Thus, circadian chronic desynchronization may have an impact on GLUT4 levels, affecting consequently glucose tolerance in CJL mice.

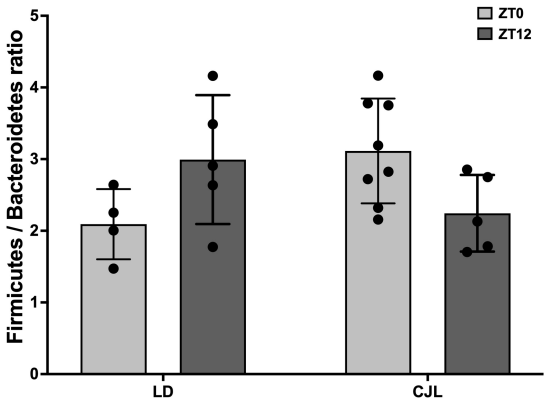

FIGURE 4
Daily changes in gut microbiota (Firmicutes/Bacteroidetes ratio) composition for mice under LD and JCL at ZT0 and ZT12. Two-Way ANOVA, p =0.0102, F =8.235 for interaction; p =0.6646, F =0.1943 for light schedule; p =0.9592, F =0.0026 for ZT; followed by Tukey's multiple comparisons test: ns; n =8.

In LD mice, intraperitoneal glucose elicited similar insulin secretion (about a 3-fold change from basal levels at 15 min) at ZT6 and ZT18, as observed in other studies (12). However, resistance to insulin actions follows a circadian rhythm, being higher at light during rest (35). Indeed, circadian disruption by advancing CJL schedule increased fasting glycemia with loss of hypothalamic insulin sensitivity (decrease of phospho Akt and insulin receptor substrate 1) (36) and delayed pancreatic genes controlling sensitivity (37). The increased glucose output at night in synchronized mice, due to increased disposal in tissues, allows daily glucose usage and/or storage according to the circadian demand for energy.

One of the limitations of the current study is that the GTT was performed with ip administration of glucose, which might result in possible artifacts derived from a less physiological route than oral administration. In addition to this, some of the effects found in this test might be limited by the small sample size. Further experiments will be conducted with oral administration and a greater number of mice. Finally, despite measuring insulin after a GTT is an accepted method, an insulin tolerance test would better measure insulin resistance. Thus, insulin tolerance tests will be conducted in the future to complete insulin resistance results.

The control of the SCN clock on daily glucose metabolism was previously assessed in SCN-ablated mice, showing a lack of rhythms in glucose tolerance and insulin sensitivity (38), oxygen consumption, and insulin resistance (39). Several genetic models (mice with Clock mutation and Bmal1 deficiency), showed disrupted circadian control of glucose, by altered gluconeogenesis (9, 40). However, these effects were assessed downstream of the SCN without a 24-h feeding-fasting rhythm as a strong feedback zeitgeber. Here, the desynchronization of mice with a functional circadian system by advancing CJL allowed the assessment of the effects of altered daily timing of feeding-fasting on the daily glucose homeostasis.

Circadian desynchronization of behavior by CJL, with increased feeding at light, generates disruption of lipid metabolism at LDL cholesterolemia, while HDL/cholesterolemia remains unaltered (Figure 3). Although a different protocol, daytime feeding was previously used to desynchronize peripheral oscillators from feeding-fasting, which increases total cholesterol due to the increased expression of cholesterol synthetic genes (41) or due to dysregulation in bile acids synthesis (42). Dissecting the central and/or peripheral control of functions at metabolic tissues depends on depict complex interactions. Oscillator in the liver is central in this network, in the circadian control at the transcriptome level of other metabolic tissues, as white adipose, upon daytime feeding (43), and its full circadian function is set by other peripheral signals dependent on photic cycle (44).

Nevertheless, LDL and HDL have not been studied under desynchronization by light schedules. This work shows that both total and HDL cholesterolemia did not change, but LDL does increase under CJL. Low-density lipoprotein receptor (LDLR) is regulated by the circadian clock *via* the complex Clock/Bmal1 (45) In addition, fatty acid binding protein 4 (FABP4,) one of the main lipid-binding proteins in adipose tissue and liver, has been shown to have a circadian expression in both tissues (46) and it is overexpressed in the liver in the contexts of morbid obesity and insulin resistance (47). It is possible that CJL is changing both levels of LDLR and FABP4, contributing to generating the differences observed in weight gain and LDL/cholesterol ratio levels. This remains to be established in further experiments. Finally,

impairments in glucose uptake/disposal leading to chronic hyperglycemia and hyperinsulinemia, as observed here due to CJL, might lead to an increased usage of fatty acids from triglycerides stores in the liver and adipose tissue, as observed in previous studies in the laboratory (24). Both elevated triglyceridemia and LDL, as well as lower HDL, are biomarkers of metabolic syndrome and type 2 diabetes (48). Although this CJL mice model generates clear dysregulation of glucose homeostasis, it was not enough with respect to lipid metabolism to characterize those alterations.

Fecal Firmicutes/Bacteroidetes ratio shows a circadian (49) and daily (50) oscillation. While the abundance of Firmicutes increases during the activity/feeding period, Bacteroidetes increase during the rest/fasting period (51), due to their ability to degrade glycans of the mucosal layer cells (52). Here, the Firmicutes/Bacteroidetes ratio observed in LD was changed under CJL. Increased postprandial glucose transport at enteroendocrine L-cells, together with short-chain fatty acids (SCFA) metabolized by Firmicutes, increased both secretions of glucagon-like peptide 1 (GLP1), which in turn stimulates insulin secretion (53) as well as insulin signaling in adipocytes and glucose uptake by the tissues (54). In addition, GLP1 sensitivity has a diurnal pattern that, in turn, depends on the intestinal clock gene expression and the presence of Rumninococcaceae and Lachnospiraceae gut bacteria (55). This so-called incretin effect supplies additional circadian controls for blood glucose and lipid homeostasis (56, 57). Decreased Firmicutes/Bacteroidetes ratio, associated with decreased SCFA-GLP1-insulin signaling and glucose changes observed here, remains to be determined.

To conclude, chronic desynchronization generates evident alterations in feeding fasting rhythm and in glucose homeostasis, with nocturnal hyperglycemia related to decreased nocturnal response to glucose, hyperinsulinemia, and increased levels of LDL. Thus, it remains to be established if this model can be assessed for the development of insulin resistance by studying GLUT4 transport at the liver or dyslipidemia by studying markers of lipid export (e.g., FABP2-4), both at liver and adipose tissues.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ebi.ac.uk/ena/browser/view/PRJEB60019.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Comitee, Universidad Nacional de Quilmes.

Author contributions

LT and ML were equal contributors and performed the experiments and wrote the manuscript. IA and RR served as technical assistance. CB and MB performed the microbiota analysis. DG served as experimental advisor and wrote the manuscript. SP and JC were both major and equal contributors in writing the manuscript, directing the study, designing experiments, and processing and interpreting data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Associations of timing of food intake with energy intake, eating behaviour traits and psychosocial factors in adults with overweight and obesity

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Introduction: Whether a late distribution of food intake impacts obesity through increased energy intake remains uncertain and the behavioural characterization of late eating needs to be further investigated. The first objective of this study was to assess the associations between late eating and body mass index (BMI) and total energy intake (TEI), and whether TEI mediates the association between late eating and BMI. The second objective was to assess the associations between late eating and eating behaviour traits or psychosocial factors and whether eating behaviour traits mediate the association between late eating and TEI.

Methods: Baseline data from 301 individuals (56% women, age=38.7 ± 8.5 years; BMI=33.2 ± 3.4 kg/m^2), who participated in four weight loss studies were used in this cross-sectional study. Total energy intake was assessed using a three-day food record from which the percentage of TEI after 17:00 and after 20:00 was calculated. Eating behaviour traits and psychosocial factors were assessed with questionnaires. Pearson correlations and mediation analyses adjusted for age, sex, underreporting of energy intake, sleep duration and bedtime were performed.

Results: Percent TEI after 17:00 and after 20:00 were associated with TEI (r=0.13, p=0.03 for both), and TEI mediated the association between percent TEI after 17:00 and BMI (β=0.01 ± 0.01, 95% CI: 0.001, 0.02). Percent TEI after 17:00 was associated with disinhibition (r=0.13, p=0.03) and percent TEI after 20:00 was associated with susceptibility to hunger (r=0.13, p=0.03), stress (r=0.24, p=0.002) and anxiety (r=0.28, p=0.0004). In women, disinhibition mediated the association between percent TEI after 17:00 and TEI (β=3.41 ± 1.43, 95% CI: 0.92, 6.47). Susceptibility to hunger mediated the association between percent TEI after 20:00 and TEI (β=0.96 ± 0.59, 95% CI: 0.02, 2.34) in men and women.

Conclusion: Late eating is associated with TEI and suboptimal eating behaviours which could contribute to explaining the association between timing of food intake and obesity.

KEYWORDS

timing of food intake, obesity, eating behaviours, late eating, psychosocial factors, energy intake

1. Introduction

Recent evidence indicates that the timing of food intake is a risk factor for obesity and associated comorbidities such as type 2 diabetes and cardiovascular diseases (1–3). Cross-sectional, prospective and interventional studies have shown that eating later during the day was associated with obesity or greater adiposity (4–9), weight gain (10) and reduced weight loss (11–16).

The distribution of food intake throughout the day is an important synchronizer of peripheral clocks, located in many organs and tissues (1). Consuming a high proportion of energy later during the day and into the night can produce chronodisruption, a state where peripheral clocks are out of synchrony with the central clock, located in the suprachiasmatic nucleus and aimed to synchronise behaviours with environmental cues (1, 2). A state of chronodisruption can impact many physiological processes (1, 2). However, the mechanisms explaining the increased susceptibility to obesity among late eaters remain to be fully understood. Some studies, but not all (17–20), have shown no associations between late eating and total energy intake (TEI) either cross-sectionally (13) or during weight loss (12, 14, 21). Based on these results and those of experimental studies exploring the effect of timing of food intake on metabolism (1, 22–24), it has been suggested that late eating impacts body weight mainly through energy expenditure (e.g., lower thermic effect of foods) (24) rather than through energy intake (1). However, late eating resulted in higher daily appetite sensations in two recent cross-over randomised controlled trials in which food intake was fully controlled (25, 26). These results seem inconsistent with the previous hypothesis and suggest that energy intake may be involved in the association between timing of food intake and obesity, but this needs to be assessed in well-designed studies conducted under free-living conditions.

In studies where food intake is not fully controlled, the lack of influence of late eating on energy intake remains uncertain as most studies relied on self-reported dietary assessment tools without consideration of misreporting of energy intake which can result in attenuated or misleading associations (27). Accounting for misreporting of energy intake may be particularly relevant in this context as underreporting is associated with obesity and is more likely to occur with foods of low nutritional value that may be perceived as socially undesirable (28) and that are associated with evening preferences (29, 30). Moreover, late eaters have been characterized as being more prone to eating when stressed, overeating at night and eating while watching television (13). Disinhibition, which refers to an overconsumption of food triggered by different cues (31), habitual and emotional susceptibility to disinhibition, and binge eating severity have also been associated with a higher proportion of TEI consumed as evening snacks (32). Eating behaviour traits such as disinhibition, emotional eating and binge eating have been associated with higher energy intake, weight gain and obesity (33–40). Although the behavioural characterization of late eaters in the literature is scarce, these results support the hypothesis that late eating may also impact body weight through energy intake.

To improve obesity treatment and prevention, there is a need to better understand how late eating impacts body weight. Importantly, more studies accounting for systematic bias associated with dietary assessment tools or using objective measurement of dietary intake are needed to shed light on the possible association between late eating and energy intake. Expanding the behavioural characterization of late eaters is also important as it could help develop targeted interventions for these individuals. To our knowledge, only two studies have assessed eating behaviour traits associated with late eating (13, 32). Based on results from these two previous studies showing that late eating is associated with eating in response to negative emotions (13, 32), it is possible that late eaters are characterized by higher levels of psychosocial factors such as stress, anxiety and depressive symptoms, but this remains to be assessed.

The first objective of this study was to assess the associations between a late distribution of food intake (i.e., late eating), body mass index (BMI) and TEI and to determine whether TEI mediates the association between late eating and BMI, while considering underreporting of energy intake. The second objective was to examine the associations between late eating, eating behaviour traits and psychosocial factors (i.e., stress, anxiety and depressive symptoms), and to investigate whether eating behaviour traits related to overeating (e.g., disinhibition and susceptibility to hunger) mediate the association between late eating and TEI. We hypothesised that late eating is associated with TEI and BMI and that TEI mediates the association between late eating and BMI. We also hypothesised that late eating is associated with overeating-related eating behaviour traits and that these traits mediate the association between late eating and TEI.

2. Materials and methods

2.1. Participants

This cross-sectional study included baseline data from 301 individuals with overweight or obesity from the Weight Loss Intervention Studies (WeLIS) Cohort, which includes four previous weight loss studies with similar designs conducted at Université Laval (41–44). These studies aimed to assess the effect of various supplements (i.e., probiotic, calcium and vitamin D, or a multivitamin and mineral) compared to placebo as part of a 12–15-week energy-restricted intervention (42–44), or the effect of a non-restrictive satiating diet compared to standard nutritional guidelines (i.e., Canada's Food Guide 2007) for 16 weeks (41), on weight loss. Inclusion

Abbreviations: TEI, total energy intake; Percent TEI, percentage of total energy intake; TFEQ, Three-Factor Eating Questionnaire; rEI, self-reported energy intake; TEE, total energy expenditure; RMR, resting metabolic rate; PAL, physical activity level.

criteria were to be aged 20 to 55 years, living with overweight or obesity, having a body weight variation of less than 4 kg for at least 2 months before the study, being inactive to low active (i.e., maximum of three periods of 30 min of low to vigorous intensity physical activity per week), being in apparent good health (i.e., absence of any disease or condition that may pose a risk to the participant or alter the results of the study) based on a standard medical examination performed by a physician during the screening visit of each study, having no comorbidities such as type 2 diabetes or cardiovascular diseases, not taking medications or supplements that could impact study outcomes, consumption of less than 10 alcoholic beverages per week and a maximum of 2 drinks per day, consumption of less than 5 cups of coffee per day, not being pregnant or lactating and absence of menopause for women. Individuals working at night during the completion of the three-day food record ($n = 1$) and those who completed less than 2 days of food record ($n = 3$) were excluded from the present study (Supplementary Figure S1). Each study was approved by the Research Ethics Board of Université Laval and written informed consent was obtained from each participant before the study.

2.2. Anthropometric measurements

Anthropometric measurements were performed according to standardised procedures recommended at the Airlie Conference (45). Body weight was measured to the nearest 0.1 kg using a digital scale and height was measured to the nearest 0.1 cm using a standard statiometer. Body mass index was calculated as kg/m^2.

2.3. Dietary assessment and distribution of food intake

Dietary intakes were assessed with a three-day food record completed on two weekdays and one weekend day at baseline (46). Participants received instructions on how to complete the food record and measure quantity of food consumed. The research dietitian reviewed the completed food record with the participant to ensure that all information was clear and complete. Food records were analyzed using the Nutrific software (47) linked to the Canadian Nutrient File version 1997 or 2005, depending on the studies (48, 49).

The distribution of food intake was assessed by calculating the percentage of TEI from six intervals throughout the day. Period 1 corresponded to the first moment of food consumption recorded until 8:59, period 2 was from 9:00 to 11:29, period 3 was from 11:30 to 14:29, period 4 was from 14:30 to 16:59, period 5 was from 17:00 to 19:59 and period 6 was from 20:00 until the time of the last food consumption recorded. The percentage of TEI from each period was calculated as the sum of the three-day energy intake from each period divided by the sum of the three-day TEI, multiplied by 100. These periods were based on hours delimiting periods in previous American and Canadian studies (6, 17, 18, 32) and adapted to the usual Canadian meal pattern (i.e., frequency and time of intakes) (50–52). They were also designed to capture late eating and main peaks of intake (i.e., meals) and snacks in different periods. Further details about the rationale behind the definition of time periods established in this study to assess the distribution of food intake have been previously published (53). To obtain the percentage of TEI from morning, afternoon and evening, the periods were combined as follows: morning (periods 1 and 2), afternoon (periods 3 and 4) and evening (periods 5 and 6). In the present study, we used the percentage of TEI consumed after 17:00 (i.e., periods 5 and 6 combined) and after 20:00 (period 6). A higher percentage of TEI consumed during the evening reflects a later distribution of food intake.

2.4. Eating behaviour traits and psychosocial factors

Eating behaviour traits were assessed with the Three-Factor Eating Questionnaire (TFEQ) (31, 54) and the Binge Eating Scale (BES) (55). The TFEQ is a 51-item questionnaire that measures three main dimensions of eating behaviour traits, namely cognitive restraint, disinhibition and susceptibility to hunger, with higher scores reflecting higher levels of these eating behaviours. Cognitive restraint reflects the tendency to restrain food intake to control or lose body weight. It is assessed with 21 items and can be separated into specific types, namely rigid and flexible restraint, each assessed with 7 items (56). Disinhibition is based on 16 items and reflects an overconsumption of food triggered by different cues representing its three subscales, namely habitual (5 items), emotional (3 items) and situational (5 items) susceptibility to disinhibition (57). Susceptibility to hunger (14 items) represents a susceptibility to experience feelings of hunger triggered by internal (internal locus of hunger, 6 items) or external (external locus of hunger, 6 items) cues (57). Thirty-six items are in a true or false format coded as 0 or 1 and 15 items are based on a 4 or 6-point scale coded as 0 or 1. The Binge Eating Scale assesses binge eating severity with 16 items describing the behavioural manifestations of binge eating and the feelings and cognitions surrounding binge eating episodes (55). Items are on a scale of 0 to 2 or 0 to 3, providing a total score of 0 to 46, with higher scores denoting higher binge eating severity.

Psychosocial factors, namely stress, anxiety and depressive symptoms were assessed with questionnaires. The Perceived Stress Scale measures the degree to which situations in one's life are considered stressful during the last month (58). This questionnaire comprises 10 items assessed on a 5-point scale coded as 0 to 4, providing a total score ranging from 0 to 40 (59). The anxiety trait was assessed with 20 items of the State and Trait Anxiety Inventory (STAI) (60). These items are measured on a 4-point scale, ranging from 1 to 4, providing a total score for the trait section ranging between 20 and 80. Depressive symptoms were assessed with the Beck Depression Inventory (BDI) (61). The questionnaire comprises 21 items assessed on a 4 to 6-point scale, scored from 0 to 3, which provide a total score for the questionnaire ranging between 0 and 63. Higher scores on these questionnaires reflect higher levels of stress, anxiety or depressive symptoms (55, 58, 59, 61).

2.5. Assessment of covariates

Information on sex and age were collected at screening by the research staff. Sleep duration and habitual bedtime over the last month were self-reported with two questions based on the Pittsburgh Sleep Quality Index (62). Misreporting of energy intake was assessed by the method of Huang et al. (27) according to which under- and over reporting of energy intake are identified based on confidence limits around a ratio of self-reported energy intake (rEI) to total energy

expenditure (TEE) calculated from a formula accounting for measurement error in rEI and TEE. Since an objective measure of resting metabolic rate (RMR) was available for 99% ($n = 298$) of participants, TEE was based on a factorial method using RMR and physical activity level (PAL) (63) rather than on equations such as those developed by the National Academy of Medicine as done in the original method of Huang et al. (27, 64). Resting metabolic rate was measured in a fasted state using indirect calorimetry in each study, as detailed elsewhere (65). For participants with missing data on RMR ($n = 3$), the latter was estimated using the Mifflin St-Jeor equation (66), which was found to be the most reliable equation to predict RMR in adults with normal weight and obesity, with the narrowest error range (67). Since participants had to be inactive to low active to be included in each study, RMR was multiplied by a PAL coefficient of 1.4 to determine TEE. A PAL coefficient of 1.4 represents the cut-off value between inactive and low active (64). Assuming a standard PAL for each participant in the assessment of misreporting of energy intake has been previously done (68). The ±1 standard deviation (SD) for confidence limits was established by using a within-individual coefficient of variation (CV) of 23.0% for rEI, n = 3 days for dietary assessment, a CV of 16.8% for TEE and a CV of 4.0% or 8.5% to account for within-individual day-to-day variation and error associated with objective measurement of TEE for measured or estimated RMR, respectively (69). To account for skewness of TEI, the ±1 SD confidence intervals were exponentiated using a multiplicative factor of 1 (70). For participants with an objective measure of RMR, the resulting confidence interval was 0.80 to 1.24 whereas the confidence interval was 0.79 to 1.26 for participants with estimated RMR. Individuals with values corresponding to or within the confidence interval were considered plausible reporters. Underreporting of energy intake was defined as rEI/TEE <0.80 and < 0.79 for participants with measured and estimated RMR, respectively. Overreporting of energy intake corresponded to rEI/TEE >1.24 and > 1.26 for participants with measured and estimated RMR, respectively. Based on these values, two indicator variables were created to represent underreporting (yes, 1; no, 0) and overreporting (yes, 1; no, 0). Because misreporting is based on a deviation from an exact correspondence between rEI and TEE (27), overreporting may also represent overconsumption in some individuals. Consequently, the main analyses were only adjusted for underreporting, but overreporting was further considered in supplemental analyses. Adjustment, rather than exclusion of under- and overreporters was used as it has been suggested as a more appropriate way to address misreporting since it avoids selection bias and reduction in statistical power (71). Ethnicity was also assessed at screening, but was not used as a covariate since there was low diversity in this sample.

2.6. Statistical analyses

Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). Sex differences in baseline characteristics, distribution of food intake, eating behaviour traits and psychosocial factors were assessed with Student's T tests and Chi-square tests. Differences in these variables between studies were assessed with general linear models and Chi-square tests.

The associations between the percentage of TEI consumed in the evening (after 17:00 and after 20:00) and BMI, TEI, eating behaviour traits or psychosocial factors were assessed with Pearson correlations. The main analyses were adjusted for age, sex (men, 0; women, 1), underreporting of energy intake, sleep duration and bedtime. Due to sex differences in eating behaviour traits (34, 35), linear regressions were used to investigate sex interactions in these associations. In case of significant sex interaction, Pearson's correlations were performed separately among men and women. The strength of associations for Pearson's correlations was interpreted based on Funder and Ozer guidelines, where coefficients of 0.10, 0.20, and 0.30 represent small, moderate and large effect sizes, respectively (72).

Mediation analyses were conducted to assess if TEI mediates the potential associations between the percentage of TEI after 17:00 or after 20:00 and BMI. Mediation analyses were also performed to determine if the association between late eating and TEI is mediated by some eating behaviour traits that confer a susceptibility to overeating and showed a significant and positive association with late eating in correlation analyses. These analyses were performed separately among men and women when a sex interaction was previously observed in regression analyses. Mediation analyses were performed with model 4 of the Process macro for SAS, version 3.4.1 (73). Process is an ordinary least square regression path analysis modelling tool that assesses the mediating (or indirect) effect through which an independent variable influences a dependent variable using percentile bootstrap confidence intervals (73). The current study used 5,000 bootstrap samples. In the mediation model, the association between the independent variable and the mediator is represented by path *a*, and the association between the mediator and the dependent variable, adjusted for the independent variable, is represented by path *b*. The total effect (*c*) represents the association between the independent (late eating) and dependent variables (BMI or TEI) and the direct effect (*c'*) represents this same association but adjusted for the mediator (TEI or eating behaviour traits). Mediation analyses were adjusted for the same covariates as correlations. According to Hayes, a mediation effect could occur despite no evidence that the association between the independent and dependent variables is different from zero since the indirect effect is not determined nor constrained by the size of the total effect (73).

In secondary analyses, correlations and mediation models were adjusted for (1) age, sex and underreporting (i.e., model 2), (2) age, sex, misreporting (i.e., both under-and overreporting), sleep duration and bedtime (i.e., model 3) and (3) age, sex, underreporting, sleep duration, bedtime and studies (i.e., model 4). Adjustment for the different studies was performed by creating three indicator variables for Major et al. (42, 43) and Arguin et al. (41) studies. The Sanchez et al. (44) study was used as reference since this study had all of the questionnaires available and was performed in both men and women. For the associations between late eating and binge eating severity, perceived stress and anxiety, only one indicator variable for Sanchez et al. (44) was used as these questionnaires were only available in Arguin et al. (41) and Sanchez et al. (44).

3. Results

3.1. Participant characteristics

This study included 168 women and 133 men, with a mean age of 38.7 ± 8.5 years (range 19.7 to 55.2 years) and a mean BMI of

TABLE 1 Participant characteristics.

	Total (n=301)	Women (n=168)	Men (n=133)	p
Women, n (%)	168 (55.8)			0.04
Age, y	38.7 ± 8.5	38.0 ± 8.7	39.5 ± 8.1	0.14
Ethnicity, n (%)[a]				
White	284 (96.0)	161 (96.4)	123 (95.4)	0.65
Other	12 (4.1)	6 (3.6)	6 (4.7)	
BMI, kg/m^2	33.2 ± 3.4	33.0 ± 3.5	33.5 ± 3.2	0.18
Total energy intake, kcal/day	2,524 ± 622	2,263 ± 541	2,853 ± 561	<0.0001
% Energy intake before 11:30	23.3 ± 7.2	21.1 ± 7.3	23.6 ± 7.1	0.50
% Energy intake between 11:30 and 16:59	33.0 ± 8.8	33.2 ± 8.6	32.7 ± 9.1	0.57
% Energy intake after 17:00	43.7 ± 8.7	43.7 ± 8.4	43.7 ± 9.0	0.98
% Energy intake after 20:00	9.2 ± 10.0	9.4 ± 9.7	9.0 ± 10.3	0.75
Reporting status, n (%)				
Underreporters	42 (14.0)	26 (15.5)	16 (12.0)	0.68
Plausible reporters	209 (69.4)	114 (67.9)	95 (71.4)	
Overreporters	50 (16.6)	28 (16.7)	22 (16.5)	
Eating behaviour traits[b]				
Cognitive restraint (0–21)	7.2 ± 3.8	8.4 ± 4.0	5.8 ± 3.0	<0.0001
Rigid restraint (0–7)	2.3 ± 1.6	2.7 ± 1.6	1.8 ± 1.4	<0.0001
Flexible restraint (0–7)	2.1 ± 1.6	2.5 ± 1.7	1.6 ± 1.3	<0.0001
Disinhibition (0–16)	8.5 ± 3.1	8.6 ± 3.0	8.4 ± 3.2	0.51
Habitual susceptibility (0–5)	1.6 ± 1.4	1.7 ± 1.4	1.4 ± 1.4	0.12
Emotional susceptibility (0–3)	1.7 ± 1.3	1.9 ± 1.2	1.4 ± 1.3	0.002
Situational susceptibility (0–5)	3.5 ± 1.3	3.3 ± 1.4	3.7 ± 1.2	0.01
Susceptibility to hunger (0–14)	6.3 ± 3.5	5.7 ± 3.3	7.1 ± 3.6	0.001
Internal locus of hunger (0–6)	2.4 ± 1.9	2.1 ± 1.8	2.7 ± 1.9	0.008
External locus of hunger (0–6)	2.8 ± 1.6	2.5 ± 1.6	3.1 ± 1.7	0.003
Binge eating severity (0–46)	12.9 ± 6.9	12.5 ± 6.4	13.2 ± 7.3	0.53
Psychosocial factors[c]				
Perceived stress (0–40)	13.6 ± 5.9	15.3 ± 6.0	12.5 ± 5.7	0.002
Anxiety -Trait (20–80)	36.6 ± 7.3	37.4 ± 7.0	36.1 ± 7.4	0.28
Depressive symptoms (0–63)	4.7 ± 4.9	5.1 ± 5.3	4.2 ± 4.2	0.11

[a]n = 296, Other: Black, n = 5, North African n = 3, Middle Eastern, n = 1, Hispanic, n = 2, mixed-race individuals, n = 1.
[b]Cognitive restraint, disinhibition and susceptibility to hunger, n = 261–289; Binge eating severity, n = 153.
[c]Perceived stress, n = 180, Anxiety trait, n = 172, depressive symptoms, n = 267. BMI, body mass index.

33.2 ± 3.4 kg/m^2 (range 26.2 to 45.4 kg/m^2) (Table 1). Participants consumed 43.7 ± 8.7% of TEI during the evening (after 17:00). Of this value, 9.2 ± 10.0% of TEI was consumed after 20:00. Except for TEI, there were no differences in these baseline characteristics nor distribution of food intake between men and women. However, as expected, there were several sex differences in eating behaviour traits, with women presenting higher levels of cognitive restraint (8.4 ± 4.0 vs. 5.8 ± 3.0, p < 0.0001) and emotional susceptibility to disinhibition (1.9 ± 1.2 vs. 1.4 ± 1.3, p = 0.002), and lower levels of situational susceptibility to disinhibition (3.3 ± 1.4 vs. 3.7 ± 1.2, p = 0.01) and susceptibility to hunger (5.7 ± 3.3 vs. 7.1 ± 3.6, p = 0.001). Women also reported higher levels of perceived stress (15.3 ± 6.0 vs. 12.5 ± 5.7, p = 0.002). Differences in participant characteristics, distribution of food intake, eating behaviour traits and psychosocial factors between studies included in this cohort are presented in Supplementary Table S1. Note that differences between studies are mainly attributed to sex.

3.2. Associations between the percentage of TEI in the evening, TEI and BMI

There was a non-significant trend for the association between the percentage of TEI after 17:00 and BMI (r = 0.11, p = 0.07), but no association between the percentage of TEI after 20:00 and BMI (r = 0.05, p = 0.44). However, the percentage of TEI consumed after 17:00 or after 20:00 were positively associated with TEI (r = 0.13,

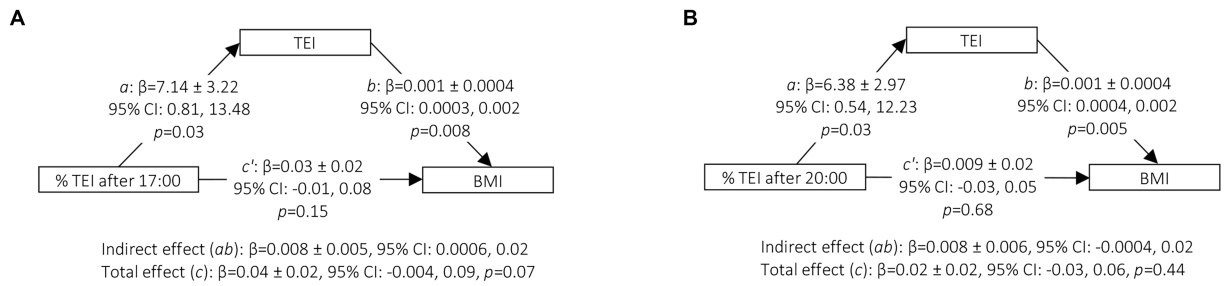

FIGURE 1
Mediation of TEI in the association between the percentage of TEI after 17:00 **(A)** or after 20:00 **(B)** and BMI. Mediation analyses were conducted using the Process Macro v. 3.4.1 for SAS that uses percentile bootstrap confidence intervals to assess the mediating or indirect effect. 95% CI for indirect effect are estimated through 5, 000 bootstrap samples. Models were adjusted for age (continuous), sex (men, 0; women, 1), underreporting of energy intake (yes, 1; no, 0), sleep duration (continuous) and bedtime (continuous). *a*, Association between the percentage of TEI after 17:00 or 20:00 and the TEI; *b*, Association between TEI and BMI adjusted for the percentage of TEI after 17:00 or after 20:00; total effect (*c*), association between the percentage of TEI after 17:00 or 20:00 and BMI without adjustment for the mediator (TEI); direct effect (*c'*), association between the percentage of TEI after 17:00 or 20:00 and BMI adjusted for the mediator (TEI); indirect effect (*ab*), mediation effect; Boot, Bootstrap; CI, confidence interval; TEI, total energy intake; BMI, body mass index. *n* =278.

$p = 0.03$ for both). No sex interaction was observed for these correlations (data not shown). Similar results were observed for correlations that did not consider sleep duration and bedtime or that were further adjusted for overreporting of energy intake or the different studies (Supplementary Table S2).

Mediation analyses showed a significant mediating effect of TEI on the association between the percentage of TEI after 17:00 and BMI ($\beta = 0.008 \pm 0.005$, 95% CI: 0.0006, 0.02) (Figure 1A). Although of similar magnitude, the mediating effect for the model related to the percentage of TEI after 20:00 did not reach significance ($\beta = 0.008 \pm 0.006$, 95% CI: −0.0004, 0.02) (Figure 1B). Similar results were observed after adjustment for the different sets of covariates (Supplementary Table S3). The model adjusted for age, sex and underreporting of energy intake showed a significant mediating effect of TEI in the association between the percentage of TEI after 20:00 and BMI, but the effect was similar to the original model.

3.3. Associations between the percentage of TEI in the evening, eating behaviour traits and psychosocial factors

The percentage of TEI after 17:00 was positively associated with disinhibition ($r = 0.13$, $p = 0.03$) (Table 2). The percentage of TEI after 20:00 was positively associated with habitual susceptibility to disinhibition ($r = 0.13$, $p = 0.04$), susceptibility to hunger (r = 0.13, $p = 0.03$), perceived stress ($r = 0.24$, $p = 0.002$) and anxiety trait ($r = 0.28$, $p = 0.0004$). A sex by distribution of food intake interaction indicated different patterns of association in men and women for some eating behaviour traits and depressive symptoms. The percentage of TEI after 17:00 was positively associated with disinhibition ($r = 0.27$, $p = 0.0009$) and its subscale habitual susceptibility to disinhibition ($r = 0.21$, $p = 0.01$) in women (Table 3). In men, the percentage of TEI after 17:00 was negatively associated with susceptibility to hunger ($r = -0.20$, $p = 0.04$) and its subscale external locus of hunger ($r = -0.19$, $p = 0.04$). Depressive symptoms were not associated with the percentage of TEI after 20:00 neither in men nor in women. Again, these results remained similar after adjustment for the different sets of covariates (Supplementary Tables S2, S4).

In women, mediation analyses showed that disinhibition and its subscale habitual susceptibility to disinhibition mediated the association between the percentage of TEI after 17:00 and TEI ($\beta = 3.41 \pm 1.43$, 95% CI: 0.92, 6.47 and $\beta = 3.53 \pm 1.80$, 95% CI: 0.66, 7.68, respectively) (Figures 2A,B). In men and women combined, susceptibility to hunger mediated the association between the percentage of TEI after 20:00 and TEI ($\beta = 0.96 \pm 0.59$, 95% CI: 0.02, 2.34) (Figure 2D) but no mediating effect of habitual susceptibility to disinhibition in the association between the percentage of TEI after 20:00 and TEI was observed ($\beta = 0.83 \pm 0.63$, 95% CI: −0.11, 2.27) (Figure 2C). These results remained similar after adjustment for the different sets of covariates (Supplementary Table S5).

4. Discussion

The timing of food intake has been identified as a risk factor for obesity. However, whether a late distribution of food intake impacts body weight through increased energy intake remains controversial and the behavioural characterization of late eaters needs to be further investigated. This study first examined the associations between a late distribution of food intake and TEI, BMI and eating and psychosocial traits, while considering underreporting of energy intake. This study also aimed to assess whether TEI and eating behaviour traits mediate the association between late eating and BMI or TEI, respectively. The results showed that a higher percentage of TEI during the evening was positively associated with TEI and that TEI mediated the association between the percentage of TEI after 17:00 and BMI. The results also showed that late eating was associated with higher levels of disinhibition, susceptibility to hunger, stress and anxiety, and that disinhibition in women and susceptibility to hunger in men and women combined mediated the association between late eating and TEI. Although cross-sectional, this study suggests that late eating could influence obesity through increased energy intake and that suboptimal eating behaviour traits could contribute to explaining the susceptibility to overeating among late eaters.

The association between the percentage of TEI in the evening and TEI was small, but consistent with previous observational studies

TABLE 2 Associations between the percentage of TEI after 17:00 or after 20:00, eating behaviour traits and psychosocial factors[a].

	% TEI after 17:00			% TEI after 20:00		
	r	*p*	*p* sex int[b]	*r*	*p*	*p* sex int[b]
Eating behaviour traits[c]						
Cognitive restraint	0.05	0.47	0.48	0.03	0.67	0.44
Rigid restraint	0.09	0.14	0.90	−0.01	0.81	0.40
Flexible restraint	0.03	0.69	0.43	0.04	0.53	0.50
Disinhibition	**0.13**	**0.03**	**0.02**	0.09	0.13	0.61
Habitual susceptibility	0.10	0.12	**0.047**	**0.13**	**0.04**	0.84
Emotional susceptibility	0.09	0.15	0.28	0.10	0.11	0.23
Situational susceptibility	0.07	0.23	0.07	−0.01	0.87	0.89
Susceptibility to hunger	−0.04	0.47	**0.02**	**0.13**	**0.03**	0.54
Internal locus of hunger	−0.03	0.63	0.08	0.10	0.10	0.48
External locus of hunger	−0.06	0.35	**0.04**	0.07	0.23	0.82
Binge eating severity	0.05	0.56	0.64	0.12	0.19	0.65
Psychosocial factors[d]						
Perceived stress	0.12	0.13	0.51	**0.24**	**0.002**	0.65
Anxiety -Trait	0.10	0.22	0.85	**0.28**	**0.0004**	0.21
Depressive symptoms	0.05	0.40	0.50	0.02	0.75	**0.04**

[a]Values are Pearson correlation coefficients adjusted for age (continuous), sex (men, 0; women, 1), underreporting of energy intake (yes, 1; no, 0), sleep duration (continuous) and bedtime (continuous).
[b]*p* values for sex interaction obtained from linear regression. Int, interaction.
[c]Cognitive restraint, disinhibition and susceptibility to hunger, $n = 249$ to 276, Binge eating severity, $n = 143$.
[d]Psychosocial factors, $n = 162$ to 252. TEI, Total energy intake.

(17–20). Moreover, although late eating (i.e., percentage of TEI after 17:00) showed a non-significant trend towards higher BMI, mediation analyses showed that late eating could be associated with BMI through higher energy intake. As indicated previously, an indirect effect can be different from zero even if the total effect is not (73). This is explained by the fact that the size of the indirect effect is not constrained nor determined by the size of the total effect (73). As shown in path *a* of Figures 1A,B each percent increase in TEI after 17:00 or after 20:00 resulted in an increase of approximately 7 kcal/day. When considering women only, the total effect (path *c*) of Figures 2A,B indicates that each percent increase in TEI after 17:00 results in an increase in energy intake of approximately 12 kcal/day. Through energy intake, the mediation models showed that each percent increase in TEI after 17:00 resulted in 0.008 unit of BMI. For an individual 1.75 m tall, an increase of 5% in TEI after 17:00 would result in an extra 0.12 kg of body weight. Given that the average increase in body weight among Canadian adults is 0.5 to 1 kg per 2-year period (74), the effect of the timing of food intake through energy intake may be a meaningful factor to consider in the aetiology of obesity.

Several factors may explain why a late distribution of food intake may lead to higher energy intake. Ghrelin, hunger, appetite for specific foods (i.e., sweets, salty and starchy foods, fruits and meat), desire to eat, prospective food consumption and fullness showed endogenous circadian rhythms with higher levels in the evening and lower levels in the morning and the opposite for fullness (75, 76). This potentially facilitates a positive energy balance towards the end of the day. These results were corroborated in an experimental study comparing appetite and food reward in response to a test meal during morning or late afternoon (77). Appetite, liking and wanting for high-fat foods were higher and post-meal fullness was lower in the late meal condition (77). The distribution of food intake may also influence appetite, as late eating resulted in higher daily levels of ghrelin and hunger and lower levels of fullness during weight loss (12). Results of this latter study were recently corroborated in two cross-over randomised controlled trials in which all foods were provided to participants during weight loss or weight stable conditions (25, 26). These studies reported higher daily levels of hunger, prospective food consumption and desire to eat in the late eating condition (25, 26). Homeostatic and hedonic mechanisms may thus be implicated in the effect of late eating on TEI.

The possible effect of late eating on energy intake is also supported by positive, small to moderate associations between late eating and eating behaviour traits associated with overeating, namely disinhibition and susceptibility to hunger. Moreover, these eating behaviours mediated the associations between late eating and TEI in women or in the whole group, respectively. These results are in line with previous studies showing that late eating was associated with overeating at night and emotional eating (13) and that evening snacking was positively associated with disinhibition (32). More broadly, these results are also in line with studies on chronotype showing that evening type individuals, who also present a delayed food pattern (78, 79), are more likely to present higher levels of disinhibition, susceptibility to hunger and binge eating and lower levels of cognitive restraint (80, 81). While cross-sectional, these results collectively suggest that late eating could lead to overconsumption through suboptimal eating behaviour traits.

Disinhibition behaviours and overconsumption triggered by susceptibility to hunger may be more likely to occur during the evening as a result of homeostatic and hedonic mechanisms potentially facilitating overeating during the evening presented above. Overeating associated with these eating behaviours may also be more likely to occur during the evening as a result of lower self-regulation capacity and increased tiredness (82), eating because others are eating (83, 84), eating in front of television (83), expecting eating to be more rewarding (83), alcohol consumption (83, 85), and more opportunities to eat alone (83) or when at home compared to at work or at school. It may also be a consequence of reduced energy intake during the day. Tani et al. showed that a lower proportion of TEI in the morning and in the afternoon were associated with higher energy intake during the evening, and a higher proportion of TEI during the evening was associated with higher TEI (19). A reduced energy intake earlier in the day may also be a consequence of overeating during the evening. Indeed, late eaters showed reduced morning appetite (13) and a Mendelian randomisation study showed that evening chronotype was causally associated with breakfast skipping, the latter also being causally associated with obesity (86).

Sex differences were observed in the associations between late eating and eating behaviour traits. Women with disinhibition seem more susceptible to overeating after 17:00, while both men and women presenting higher levels of susceptibility to hunger, showed a higher percentage of TEI after 20:00. This may be related to gender

TABLE 3 Associations between the percentage of TEI after 17:00 or after 20:00 and eating behaviour traits or depressive symptoms, respectively, in men and women[a].

	Women		Men	
	r	*p*	*r*	*p*
% of TEI after 17:00				
Disinhibition	**0.27**	**0.0009**	−0.03	0.76
Habitual susceptibility	**0.21**	**0.01**	−0.05	0.63
Susceptibility to hunger	0.09	0.25	**−0.20**	**0.04**
External locus of hunger	0.08	0.33	**−0.19**	**0.04**
% of TEI after 20:00				
Depressive symptoms	−0.10	0.23	0.17	0.08

[a]Values are Pearson correlation coefficients adjusted for age (continuous), underreporting (yes, 1; no, 0), sleep duration (continuous) and bedtime (continuous). Women, $n = 146$ to 154, men, $n = 106$ to 115. TEI, Total energy intake.

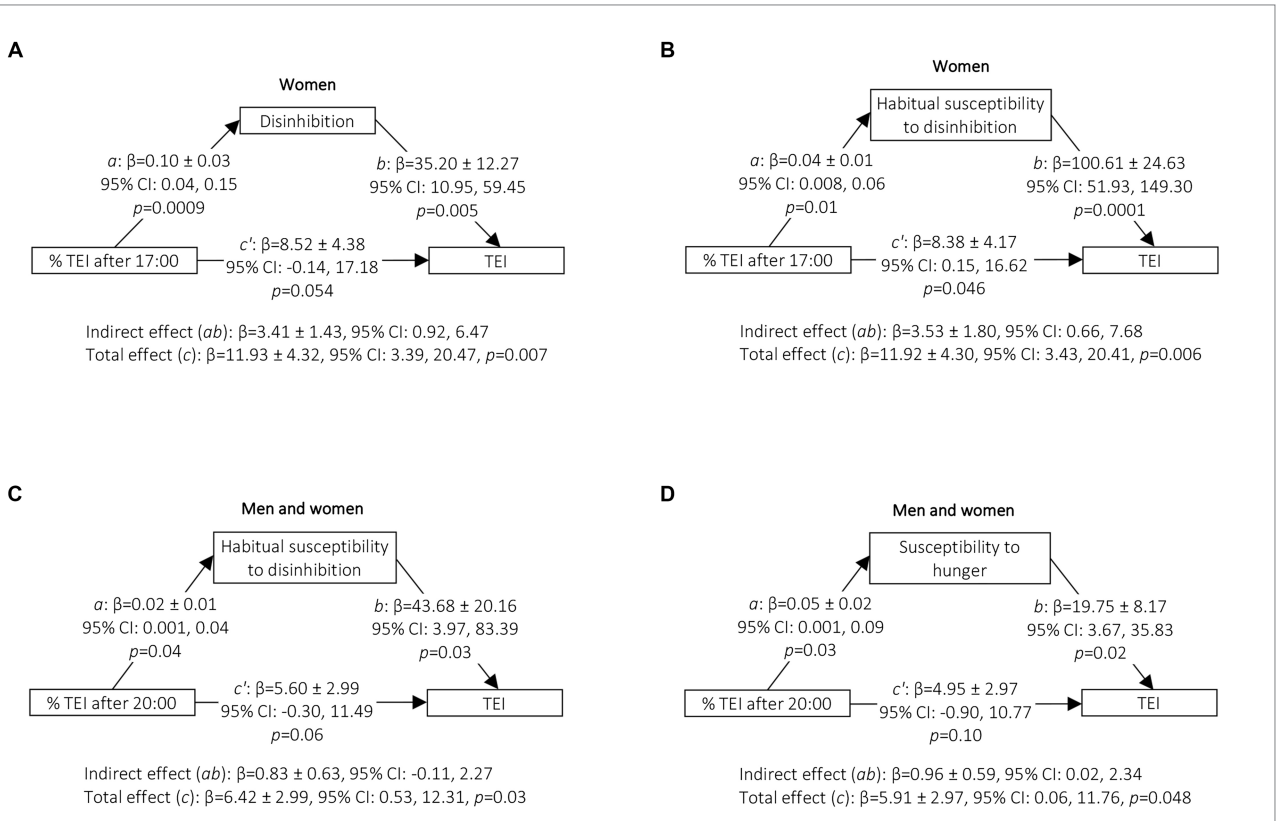

FIGURE 2
Mediation of disinhibition, habitual susceptibility to disinhibition and susceptibility to hunger in the association between the percentage of TEI after 17:00 or 20:00 and TEI in women (% TEI after 17:00) or in men and women (% TEI after 20:00). Mediation analyses were conducted using the Process Macro v. 3.4.1 for SAS that uses percentile bootstrap confidence intervals to assess the mediating or indirect effect. 95% CI for indirect effect are estimated through 5,000 bootstrap samples. Models were adjusted for age (continuous), underreporting of energy intake (yes, 1; no, 0), sleep duration (continuous) and bedtime (continuous). **(C,D)** are further adjusted for sex (men, 0; women, 1). *a*, association between percentage of TEI after 17:00 or after 20:00 and eating behaviour trait; *b*, association between eating behaviour trait and TEI adjusted for the percentage of TEI after 17:00 or after 20:00; total effect (*c*), association between the percentage of TEI after 17:00 or after 20:00 and TEI without adjustment for the mediator (eating behaviour trait); direct effect (*c'*), association between the percentage of TEI after 17:00 or after 20:00 and TEI adjusted for the mediator (eating behaviour trait); indirect effect (*ab*), mediation effect; Boot, Bootstrap; CI, confidence interval; TEI, total energy intake. **(A)** n =152, **(B)** n =147, **(C)** n =259, **(D)** n =267.

differences in social norms regarding food intake and body weight, as eating light meals are perceived as more feminine and heavy meals as more masculine (87). Eating smaller meals during the day may promote overeating in women with disinhibition during the evening, when at home, as opposed to men who may not perceive such social pressure to restrain eating during the day. Sex differences in chronotype may also be involved, as men usually present a later chronotype (78).

The positive associations between the proportion of TEI consumed after 20:00 and psychosocial factors such as stress and anxiety are in line with a previous study showing that late eaters were more likely to eat when stressed (13). More broadly, this is also in line with studies indicating that individuals with evening chronotype were more likely to experience negative psychological symptoms including anxiety (88), emotional overeating and stress-related eating (79, 89, 90). Because affective states and cognitive functioning worsen later in the day, resulting in a decrease in self-regulation capacity (82), it may be hypothesised that individuals with higher levels of stress and anxiety are more susceptible to overeating during the evening or at night. This hypothesis is supported by a study showing a positive association between psychological stress and self-reported overeating at dinner in workers (91). More broadly, this is also in line with the literature showing a positive association between emotional overeating and evening snacking (32) and that evening and night eating are used to regulate negative emotions among individuals with night eating syndrome (92). Participants of this latter study also reported that evening and night eating resulted in calmness (92). Lastly, a positive association between stress and hunger was found to be predominant during late afternoon and evening (93), which may also explain the increased susceptibility to overeating during the evening in individuals with stress and anxiety.

This study has several strengths and limitations. One of the main strengths is the use of a three-day food record with the consideration of underreporting of energy intake, and overreporting in supplemental analyses, to mitigate the effect of systematic bias in self-reported dietary assessment (27). As day-to-day variation was observed in the timing of food intake, combining several days of food intake is more likely to reflect a more habitual daily distribution of food intake than a single day (78, 94). The distribution of food intake was based on meal and snack times reported on the food record, which is more precise than using meals and snacks without considering hours. However, this classification is limited by not being defined relative to endogenous circadian timing or sleep–wake cycle (3). Yet, the consideration of mean sleep duration and bedtime over the last month as covariates suggests that the associations observed are not simply reflecting a shifted sleep–wake cycle providing more opportunities to eat in the evening in individuals with later bedtime and shorter sleep duration. It should be noted that these sleep parameters could have been more accurately assessed using objective measurements (e.g., accelerometer) or if sleep duration had been derived from self-reported data on usual bedtime, wake time, and length of time to fall asleep (95). One main limitation is the cross-sectional design that precludes causation to be inferred. Moreover, the relatively low sample size resulted in lower capacity to detect statistically significant results for some analyses. In addition, since the WeLIS cohort includes individuals living with overweight and obesity who are interested in losing weight, the results of this study may not be generalizable to other populations. Replication of these results within larger longitudinal cohorts and with objective measurements of food intake are needed.

In conclusion, this study suggests that late eating is positively associated with TEI, which may be related to higher BMI, in the long term. This association is supported by positive associations between late eating and eating behaviour traits, which contributed to explaining the susceptibility to overeating among late eaters. These results suggest that the timing of food intake and eating behaviour traits are important determinants to consider in obesity treatment and prevention.

Data availability statement

The datasets presented in this article are not readily available because it will be made available upon request pending approval from the authors as well as the funding agency. Requests to access the datasets should be directed to vicky.drapeau@fse.ulaval.ca.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Board of Université Laval. The participants provided their written informed consent to participate in their respective study.

Author contributions

VD and RJ designed research. RJ collected data related to the timing of food intake, analyzed data, and wrote the first draught of the manuscript. VD had primary responsibility for the final content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1155971/full#supplementary-material

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Association of breakfast styles such as Japanese, Western, and cereals with sleeping habits, eating habits, and lifestyle in preschool and elementary school children

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Introduction: In Japan, breakfast styles are categorized into five groups; Japanese breakfast (JB; rice and miso soup), Western breakfast (WB; bread and milk), Japanese-Western breakfast (J-WB; alternative daily serving), cereal breakfast (CB), and breakfast skipping. In our recent studies, breakfast style was highly associated with the daily sleep–wake phase (chronotype), and healthy eating habits. In contrast with other breakfast style consumers, JB-consumers were positively associated with the morning chronotype and healthy eating habits such as a high consumption of a variety of protein sources, vegetables, and dietary fibers, and low consumption of sweetened juices. These previous studies included only adult participants; hence, in the current study, we investigated whether similar observations can be made in children.

Methods: Preschool (aged 3–5years) and elementary school children (6–8years) (N=6,104, 49.87% boys, 50.13% girls, mean body mass index 15.39±0.03kg/m^2 for preschoolers and percentage of overweight −2.73±0.22 for elementary school children) participated in this cross-sectional online survey on lifestyle, including eating and sleep habits, through their mother's responses.

Results: The results showed that the morning-evening type index values (chronotype indicator, smaller indicates morning type) were negatively correlated with JB intake (−0.05, p <0.01) and positively correlated with WB (0.03, p <0.05) and CB intake (0.06, p <0.01), suggesting that the JB group exhibited the morning chronotype and the WB and CB groups exhibited the evening chronotype. The JB group consumed a variety of protein sources (mean±SE; days/week) with more frequency (fish 2.95±0.038 p <0.001, soy 3.55±0.043 p <0.001, egg 3.82±0.044 p <0.001) compared with the WB group (fish 2.58±0.033, soy 3.00±0.038, egg 3.49±0.039). On the other hand, the JB group consumed snacks (5.48±0.042 p <0.001) and sweetened juice (2.50±0.050 p <0.001) less frequently than the WB group (snacks; 5.80±0.037 and sweetened juice; 2.74±0.049).

Discussion: JB-eating children with a morning chronotype exhibited better sleep and eating habits than WB-eating children with an evening type pattern. The results suggest that JB eating habits may be associated with good eating and sleeping lifestyles, even among preschool and elementary school children.

KEYWORDS

Japanese breakfast, Western breakfast, cereal, sleep, eating habits, protein sources, morning type, evening type

1. Introduction

Sleep has a significant impact on a healthy life. Particularly, longitudinal studies in Japan have shown that sleeping hours have decreased progressively because of the late time to fall asleep, and sleep quality has declined over the past few decades (1). However, the actual sleep state and its relationship with sleeping habits and behavior in developing children are not fully understood (2). Children's sleeping habits are directly influenced by those of their parents, and irregular or late sleeping habits in parents are associated with children's sleep disturbances, daytime sleepiness, and irregular eating habits (3). Furthermore, comparing mothers and fathers, the former's sleeping habits had a stronger impact on children's sleep than did the latter's in adolescents (3, 4). A few studies have examined the association of preschool/elementary school children's sleeping habits and behavior with those of their parents. Adolescents' sleep patterns are correlated with those of their parents, with factors such as the adolescents' sex, father's level of education, and parents' alcohol consumption frequency, particularly influencing the adolescents' sleep duration (4, 5). These studies focused on sleep duration and quality but not on circadian rhythm-related factors such as chronotype. Sleep rhythms, such as chronotypes and social jet lags, are directly linked to the rhythms of life.

Dietary factors can influence sleep quality and rhythm (6). Individuals with morning chronotypes eat healthier than those with evening types, with a more frequent intake of fish, fruits, whole grains, and rye (7). Cross-sectional and experimental studies have demonstrated the benefits of eating earlier in the day regarding postprandial blood glucose levels. People with the evening type are driven to consume food later in the day (8). The evening type is associated with largely unhealthy eating habits associated with obesity (9, 10). Individuals with the evening type consume snacks more frequently and later at night; this chronotype shows significantly higher fasting total cholesterol and low-density lipoprotein cholesterol levels and significantly lower postprandial insulin sensitivity (11).

Breakfast is the most important meal of the day (12), and studies have shown that consuming protein sources for breakfast effectively elevates exercise habits. Examining the relationship between sleeping and eating habits can help reduce the incidence of disease and lower blood pressure and blood glucose levels. Breakfast has an immediate positive effect on children's brain development and cognitive function (13–15).

Japanese people have a low incidence of coronary artery disease and are known for their longevity (16). This has long attracted the interest of other countries because of the possible contribution of the Japanese diet. However, certain problems are associated with the Japanese diet (17); excess salt intake through the consumption of miso soup, salted vegetables, soy sauce, and commercial seafood is an example. Despite the high sodium intake, the Japanese have an overall low incidence of cardiovascular diseases. This is probably due to the high potassium intake through vegetables. Japanese meals are characterized by high fish and soy products and low animal fats and meat consumption. Therefore, this characteristic could be a valuable tool for supporting healthy eating habits (18). In addition, foodstuffs in Japanese meals prevent the onset of diseases; frequent consumption of seaweed, vegetables, mushrooms, legumes, potatoes, and starches lowers the risk of non-alcoholic fatty liver disease in Japanese men (19) and reduces the risk of sarcopenia in Japanese men and women (20). It has also been introduced as a healthy diet for preventing COVID-19, and its effectiveness has been evaluated in scientific studies (21, 22). Furthermore, soy reduces blood pressure and glucose levels (23, 24). A recent study suggested that Japanese breakfast (JB) consumption is associated with the intake of protein sources such as fish, eggs, and soy and is beneficial for good eating habits, including a balanced diet (1). In addition, a study reported the benefits of JB on nutrition intake and physical activity (25, 26). Adults who consumed JB preferred mornings, while those who consumed Western breakfast (WB) or cereal breakfast (CB) preferred evenings (27). Many of these studies included only adults and older adults, and there are no reports examining the relationship between chronotype and breakfast style among preschool and early elementary school children. Healthy eating habits at an early age are important for supporting good eating habits throughout life. This study aimed to elucidate the relationship between breakfast style and chronotype in children.

Good eating habits are defined as having a balanced diet rich in protein sources, vegetables, and fruits, with a low consumption of snacks and sweets. Good eating habits and breakfast intake styles are related and directly connected to health (25); in particular, they correlate with physical activity levels, cardiovascular indices, and the cognitive and academic performance of young people (28). Children are also influenced by their parents' eating habits. The body mass index (BMI) values were influenced more by the mother (29–31). Poor eating habits established in childhood can persist into adulthood and increase the risk of developing obesity and obesity-related complications such as type 2 diabetes. Modification of eating habits in childhood has been shown to improve health and reduce the risk of developing diseases later in life (30).

The primary aim of this study was to examine the effect of differences in breakfast styles (JB, J–WB [Japanese–Western breakfast], WB, and CB) on sleep parameters such as wakeup/sleep onset time, sleep duration, and chronotype of children aged 3–8 years and their parents. We also examined the intake frequency of various protein sources, vegetables, and fruits as healthy eating habits, and that of snacks and sweetened juice as unhealthy eating habits across breakfast styles. A high frequency of intake of various protein sources, vegetables, and fruits indicated good eating habits, whereas a high frequency of intake of snacks and soft drinks, such as juice, indicated unhealthy ones (25). We also aimed to identify the differences in the food intake patterns between JB with the morning chronotype as a healthy group and WB with the evening chronotype as an unhealthy group.

2. Materials and methods

2.1. Ethical approval

The Ethics Review Committee on Research Involving Human Subjects at Waseda University approved this study (No. 2021-101). In addition, the guiding principles of the Declaration of Helsinki were followed.

2.2. Target population and data collection

The respondents to the questionnaire items were mothers of children aged 3–8 years; the children and their parents were residents of Japan (from Hokkaido to Okinawa). An online survey company (Macromill Inc., Tokyo, Japan) was commissioned to conduct this survey. Data were collected from 6,180 people. The aforementioned company has a large pool of applicants who could respond to the survey; thus, it was relatively easy to recruit 6,180 participants. Moreover, participants could respond to the web-based survey quickly and efficiently; hence, the survey was completed within 4 days (July 1–4, 2022). Among the 6,180 respondents, three reported living outside the country and were excluded. Respondents with missing values for sleep-related variables were excluded, and 6,104 people were ultimately analyzed. Using our previous cross-sectional web-based survey on elementary and high school students, we performed a power analysis for multiple regression analysis with confounding factors to determine the sample size (32).

2.3. Questionnaire

The questionnaire aimed to determine the sleeping habits of children aged 3–8 years and the eating habits of their parents and siblings. The questionnaire could be completed within 20 min. All questionnaires were completed by the mothers instead of the children or their fathers. Participants who did not live with their children were excluded from this survey; we focused on mothers who lived with their children to ensure accurate responses. Four questions on basic information were asked, including the sex, age, height, and body weight of the respondent's children, and these values were self-reported by the mothers.

Four questions on sleep parameters were asked: wake-up time on weekdays with or without an alarm, that on weekends and holidays without wake-up alarm, and sleep onset time on weekdays and weekends/holidays. Following recent papers (25–27), the breakfast style of the respondent's children was categorized into five groups: JB, a pattern in which Japanese food items such as rice are included; J–WB, a pattern in which Japanese and Western food items are eaten alternately; WB, a pattern in which bread is included; CB, a pattern in which cereals are included; and missing breakfast (SB) (Supplementary Table 1). In Japan, the aforementioned breakfast styles were easy to understand as JB: including rice and soy sauce; WB: including bread and salad or milk products; and CB: including cereal and milk or soy milk. J–WB included alternately served JB and WB. We did not ask about the breakfast styles of the parents; however, we believe that the entire family would have taken the same breakfast.

For analyzing eating habits, there were seven questions regarding the respondents' children's intake frequency of food items in their usual diet (eight levels, 0–7 days/week). In the current study, the food items were categorized into three groups. The first group consisted of several questions to examine the variation in protein sources: meat, fish, eggs, soy, and dairy products (Supplementary Table 2). The second group included two questions to investigate the intake frequency of vegetables and fruits (Supplementary Table 3) to estimate healthy nutrients such as potassium and dietary fibers (33). The third group consisted of two questions examining the intake of unhealthy foods, such as snacks and soft drinks (Supplementary Table 3). As this study aimed to investigate the impact of different breakfast styles on sleep, eating habits, and lifestyle, data from participants who did not respond about their breakfast style was excluded.

MSFsc (Chronotype Index; Midpoint time of sleep time on weekdays and holidays), SJL (Social Jet Lag), and SLOSS (Sleep Deprivation Index) were used as the sleep indices. Smaller MSFsc values indicate more morning-type characteristics, and a large MSFsc value indicates more evening-type characteristics. Smaller SJL values indicate less social jet lag. SLOSS indicates the degree of sleep deprivation per week, with higher numbers indicating greater sleep deprivation. The calculations of these sleep parameters have been previously published (34).

The MSFsc can be expressed by the following equation:

$$\mathrm{MSFsc} = \mathrm{MSF}^{*1} - \left(\mathrm{SDh}^{*2} - \mathrm{SDweek}^{*3}\right)/2.$$

*1 Midpoint time of sleep time on weekdays and holidays.
*2 Sleep duration on holidays.
*3 Sleep duration on weekdays and holidays.

SJL can be expressed by the following equation:

$$\mathrm{SJL} = \text{Time of waking on holidays} - \text{Time of waking on weekdays}.$$

Absolute values were calculated and analyzed as data.

SLOSS score can be expressed by the following equation:

$$\text{When SDweek} > \mathrm{SDw}^{*4} : \mathrm{SLOSSweek} = \left(\mathrm{SDweek} - \mathrm{SDw}\right) \times \mathrm{WD}^{*5}.$$
$$\text{when Sdweek} \leq \mathrm{SDw} : \mathrm{SLOSSweek} = \left(\mathrm{Sdweek} - \mathrm{SDh}\right) \times \left(7 - \mathrm{WD}\right).$$

*4 Sleep duration on weekdays.
*5 Number of days per week in preschool or elementary school.

2.4. Chronotype division

The two groups, divided into morning and evening types, were divided by two near-equal numbers of children calculating MSFsc for each grade in boys and girls because MSFsc values were higher in older girls (32). The actual MSFsc limits were 2:00 AM in boys aged 3 years, 2:04 in girls aged 3 years, 2:00 in boys aged 4 years, 2:04 in girls aged 4 years, 2:04 in boys aged 5 years, 2:15 in girls aged 5 years, 2:00 in boys aged 6 years, 2:04 in girls aged 6 years, 2:08 in boys aged 7 years, 2:08 in girls aged 7 years, 2:15 in boys aged 8 years, and 2:15 in girls aged 8 years.

2.5. Body mass index and percentage of overweight

We calculated BMI (Kg/m^2) for children aged 3–5 years because there are similar BMI values among 3–5 years old Japanese boys and girls (35). Conversely, BMI is not a good marker for 6–8 year-old children. Therefore, we calculated POW by adjusting age and sex using the growth curve for Japanese children (35). Thin and fat children were defined as having <13 and > 18 kg/m^2 BMI, and < −20% and > +20% POW, respectively.

2.6. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 28 (IBM Ltd., Armonk, NY, United States). Student's *t*-test was used for sex-based comparisons of data on children's basic information, sleeping habits, and eating habits. Mann–Whitney U-test was used for POW Chi-square test was used for the sex-based comparison of children's breakfast styles. Spearman's rank correlation coefficient was used to examine the relationship of children's breakfast style with their sleeping and eating habits. Kruskal–Wallis and Dann tests were used to compare the breakfast style and chronotype groups. Multiple regression analysis was used to examine the relationship between chronotype and usual food intake. In this case, the objective variable was the MSFsc value. The explanatory variables were meat, fish, eggs, soy, dairy products, vegetables, fruits, snacks, and juice. Adjustment factors were age, sex, and BMI. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characteristics of the target group and results of the questionnaire

Participants ($n = 6{,}104$) comprised 49.87% boys and 50.13% girls. The mean age was 5.50 ± 0.02 years. Comparisons between boys and girls were made using Student's *t*-test. The JB, J–WB, WB, CB, and SB styles were followed by 2,188, 1,389, 2,300, 172, and 55 children, respectively. Comparisons between boys and girls were performed using Fisher's exact test (Table 1). The proportion of JB boys was significantly higher than that of JB girls (Table 1). Children's mean wake-up times were 6:37 on weekdays and 7:14 on weekends, and their mean bedtime was 21:09 on weekdays and 21:25 on weekends (Supplementary Table 4). The MSFsc, SJL, and SLOSSweek for children were 2:08, 0:29, and 0:56 h, respectively (Supplementary Table 4). Comparisons between boys and girls were made using Student's *t*-test (Supplementary Table 4). Holiday wake-up time, holiday sleep length, MSFsc, SJL, and SLOSS values were significantly higher in girls than in boys (Supplementary Table 4). The frequency of weekly intake of meat, fish, eggs, soy, dairy products, vegetables, fruit, sweets, and juices is presented in Supplementary Table 5. The intake frequencies of meat ($p < 0.05$), dairy products ($p < 0.001$), and snacks ($p < 0.05$) were significantly higher in boys than in girls. In contrast, the intake frequency of vegetables ($p < 0.05$) was significantly higher in girls than in boys.

3.2. Relationship between breakfast style and sleep habits

To examine the relationship between breakfast style and sleep habits, Spearman's rank correlation coefficients were calculated using data for children, mothers, and fathers (Tables 2–4). Correlation analysis demonstrated that JB was negatively associated with sleep markers, including MSFsc, in children, mothers, and fathers, while WB and CB were positively associated. The correlation values in the JB groups were larger for mothers than for children and fathers. Comparisons were also made between breakfast-style groups regarding actual wakeup time, sleep onset time, sleep duration, and MSFsc, SJL, and SLOSS for children, mothers, and fathers (Figures 1–3). On both weekdays and holidays, families that consumed JB woke up earlier than those that consumed WB (Figures 1A, 2A, 3A). On both weekdays and holidays, the different styles arranged in increasing order of wake-up times was JB, J–WB, WB, and CB; this order was clearer and more significant on holidays (Figures 1A,B, 2A,B). The respective holiday wake-up time in the JB, WB, and CB groups for children were 7:12, 7:16, and 7:34; for mothers 6:52, 7:02, and 7:12; and for fathers 7:28, 7:38 and 7:45. Thus, the time difference between JB and WB was approximately 10 min and that between JB and CB was 20 min. Wake-up time was earlier for mothers, followed by children and fathers on both weekdays and holidays. Similar to the wake-up times, the increasing order of sleep onset time was JB, J–WB, WB, and CB (Figures 1C,D, 2C,D). On

TABLE 1 Basic information of participants.

Category	ALL (n =6,104)	Boys (n =3,044)	Girls (n =3,060)	p value Boys vs. Girls
Age [years]	5.50 ± 0.02	5.50 ± 0.03	5.50 ± 0.03	0.95*[1]
BMI [kg/m^2]	15.18 ± 0.03	15.27 ± 0.05	15.09 ± 0.05	$p < 0.01$*[1]
POW [%]	−2.73 ± 0.22	−2.74 ± 0.35	−2.61 ± 0.37	0.65*[2]
JB [people]	2,188	1,139	1,049	$p < 0.05$*[3]
J-W B [people]	1,389	664	725	0.08*[3]
WB [people]	2,300	1,123	1,177	0.21*[3]
CB [people]	172	85	87	0.91*[3]
SB [people]	55	33	22	0.13*[3]

Data are expressed as mean ± SE. *[1]Student's *t*-test was used. *[2]Man-Whitney U test was used. *[3]Chi-square test was used.

TABLE 2 Correlation between breakfast style and sleep in children.

	Wake-up time		Time of sleep onset		Sleep duration		MSFsc	SJL	SLOSS
	weekday	holiday	weekday	holiday	weekday	holiday			
JB	−0.04**	−0.04**	−0.03*	−0.04**	−0.00	0.00	−0.05**	−0.02	−0.01
J-WB	−0.02	−0.01	−0.02	−0.00	0.01	−0.01	−0.01	0.00	0.01
WB	0.04**	0.03*	0.02	0.02	0.01	0.02	0.03*	0.00	−0.00
CB	0.03**	0.06**	0.05**	0.06**	−0.03*	0.00	0.06**	0.04**	0.02

*$p < 0.05$, **$p < 0.001$. Spearman's rank correlation coefficient was used.

TABLE 3 Correlation between breakfast style and sleep in mothers.

	Wake-up time		Time of sleep onset		Sleep duration		MSFsc	SJL	SLOSS
	weekday	holiday	weekday	holiday	weekday	holiday			
JB	−0.06**	−0.06**	−0.04**	−0.06**	0.01	0.01	−0.073**	−0.03*	0.01
J-WB	−0.03*	−0.02	−0.01	0.00	−0.01	−0.02	−0.00	0.02	0.00
WB	0.09**	0.06**	0.04**	0.05**	0.01	0.00	0.07**	−0.00	−0.02
CB	0.01	0.04**	0.04**	0.04**	−0.05**	−0.01	0.04**	0.04**	0.03*

*$p < 0.05$, **$p < 0.001$. Spearman's rank correlation coefficient was used.

weekdays and holidays, children and their mothers who consumed JB went to bed earlier, while those who consumed WB or CB went to bed later. There were no significant differences in sleep duration between breakfast styles in children (Figures 1E,F) and fathers (Figures 3E,F). MSFsc values in the JB group were smaller among children (Figure 1G) and mothers (Figure 2G) than in the WB and CB groups, but not among fathers (Figure 3G). SJL values in the JB group were smaller among children (Figure 1H) and mothers (Figure 2H) than in the CB group, but not among fathers (Figure 3H). There were no significant differences in SLOSS among breakfast styles (Figures 1I, 2I, 3I). This suggests that consuming JB leads to earlier waking and sleeping times, more preference for morning hours, and less social jetlag. On the other hand, consuming WB or CB results in later waking and sleeping times, longer evening hours, more social jet lag, and worse sleep deprivation.

3.3. Relationship between breakfast style and frequency of food item intake

Spearman's rank correlation coefficient was calculated for children's data to examine the relationship between breakfast style and the intake frequency of various food items (Table 5). A comparison between breakfast-style groups was also made regarding children's frequency of food item intake (Figure 4). The JB group was positively associated with fish, eggs, and soy and negatively associated with dairy products such as protein sources. In contrast, the WB and CB groups were negatively associated with fish, eggs, and soy and positively associated with dairy products. J–WB was positively associated with vegetables and fruits. JB was negatively correlated with WB snacks and juice intake frequency. Comparisons were also made between breakfast-style groups regarding the actual frequency (days/week) of food intake for children (Figure 4). There were no significant differences in meat intake frequency among breakfast styles (Figure 4B). Children who usually consumed JB had more protein sources such as fish, eggs, and soy than the WB or CB groups (Figures 4C–E). In contrast, the JB group had a significantly lower frequency of dairy products than the WB and CB groups (Figure 4F). There were almost no differences in vegetable and fruit intake frequency between the JB, WB, and CB groups (Figures 4G,H). The JB group had a lower intake of snacks and juices than the WB group (Figures 4I,J). There is a link between the consumption of JB and the high intake of various protein sources, excluding dairy protein sources, and the low intake of snacks and juices.

3.4. JB or WB characteristics with morning or evening type

We recently reported that those who consume JB are more likely to be morning-type individuals, while those who consume WB are more likely to be evening type (27). Based on these results, we examined whether the intake of each food group was influenced by chronotype or by the difference between JB and WB. In the current experiments, the percentage of JB (36%) and WB (38%) was similar, and previous adult data (20–60 years old) showed a similar percentage of JB (30%) and WB (31%) (27). However, the percentages of J–WB (23%) and CB (3%) were lower in the present study; hence, we divided the JB and WB groups into morning and evening types. First, we divided the children into morning and evening types and further grouped them according to whether they consumed JB or WB. The characteristics and results of food intake are presented in Supplementary Table 4. The morning and evening type groups were each subdivided into JB and WB groups, and the food intake in each group was compared (Figure 5). As expected, the JB and WB groups among boys and girls were associated with the morning and evening types, respectively. The morning types had lower MSFsc values, while the evening type had higher ones. The mean intake frequencies of fish, eggs, and soy, were highest in the JB group with the morning types and lowest in the WB group with the evening type. The differences in mean values between the JB morning and EB evening types were 0.51, 0.50, and 0.84 for fish, egg, and soy intake,

TABLE 4 Correlation between breakfast style and sleep in fathers.

	Wake-up time		Time of sleep onset		Sleep duration		MSFsc	SJL	SLOSS
	weekday	holiday	weekday	holiday	weekday	holiday			
JB	−0.04*	−0.05**	−0.03	−0.03	−0.00	−0.02	−0.03*	−0.02	−0.01
J-WB	−0.02	−0.01	−0.02	−0.01	0.01	−0.00	−0.01	0.01	−0.00
WB	0.04**	0.05**	0.03*	0.03	−0.00	0.02	0.04*	0.01	0.01
CB	0.01	0.02	0.02	0.03	−0.02	−0.01	0.03	0.00	0.01

*$p < 0.05$, **$p < 0.001$. Spearman's rank correlation coefficient was used.

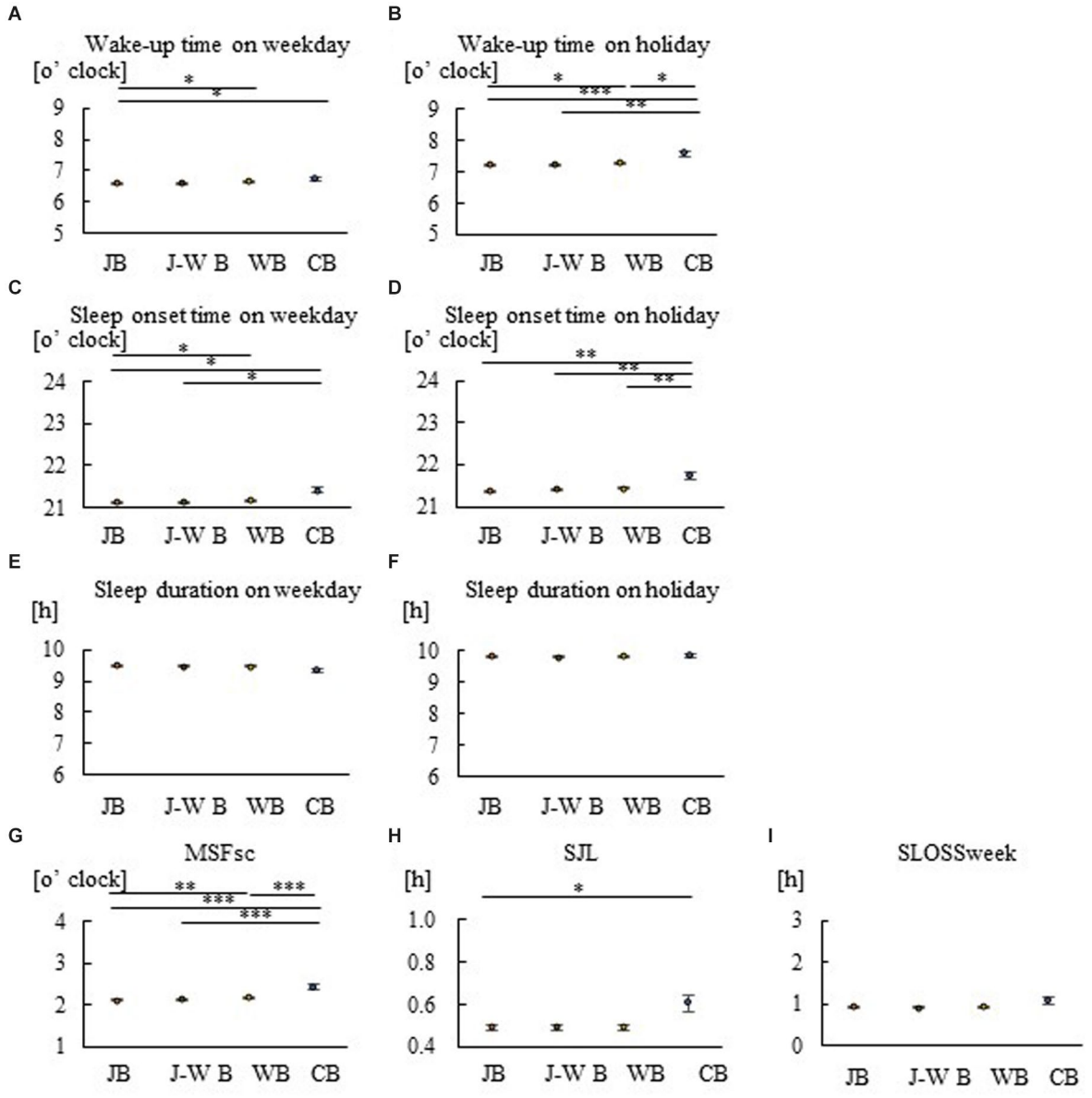

FIGURE 1
Group comparison of breakfast styles for sleep in children. Wake-up time on weekdays **(A)**, wake-up time on holidays **(B)**, sleep onset time on weekdays **(C)**, sleep onset time on holidays **(D)**, sleep duration on weekdays **(E)**, sleep duration on holidays **(F)**, MSFsc **(G)**, SJL **(H)**, and SLOSS **(I)**. Consumption of JB leads to earlier waking and sleeping times on weekdays and holidays, more preference for morning hours, and less social jetlag. Consumption of WB or CB, on the other hand, results in later waking and sleeping times, longer evening hours, and more social jet lag. *p<0.05, **p<0.005, ***p<0.001. A Kruskal–Wallis test with Bonferroni correction was used.

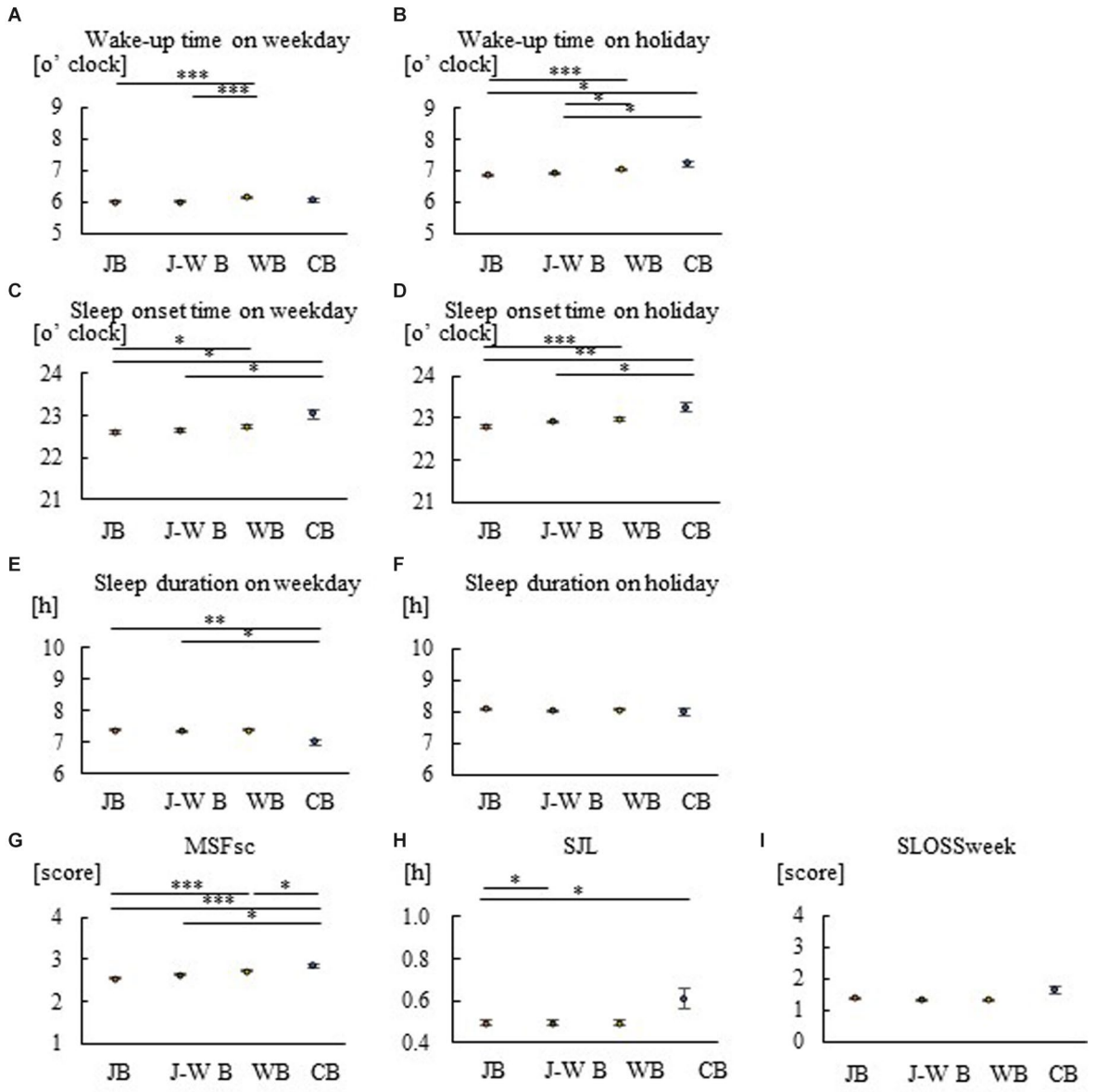

FIGURE 2
Group comparison of breakfast styles for sleep in mothers. Wake-up time on weekdays **(A)**, wake-up time on holidays **(B)**, sleep onset time on weekdays **(C)**, sleep onset time on holidays **(D)**, sleep duration on weekdays **(E)**, sleep duration on holidays **(F)**, MSFsc **(G)**, SJL **(H)**, and SLOSS **(I)**. Consumption of JB leads to earlier waking and sleeping times on weekdays and holidays, more preference for morning hours, and less social jetlag. Consumption of WB or CB, on the other hand, results in later waking and sleeping times, longer evening hours, and more social jet lag. *p<0.05, **p<0.005, ***p<0.001. A Kruskal–Wallis test with Bonferroni correction was used.

respectively. The differences in mean values between the JB morning and EB evening types were 0.57 and 0.80 for snacks and juice intake, respectively. There were no significant differences in meat intake among the four groups (Figure 5B). The frequency of food intake by fish (Figure 5C), total protein sources (Figure 5A), eggs (Figure 5D), soy (Figure 5E), vegetables (Figure 5F), and fruits (Figure 5G) were significantly higher in the JB group with the morning types than in the WB group with the evening type. The intake frequencies of snacks (Figure 5G) and juice (Figure 5H) were significantly lower in the JB group with the morning types than in the WB group with the evening types. In general, those who consumed JB with morning types exhibited healthy eating habits. On the other hand, those who consumed WB with evening types exhibited less consumption of healthy food.

3.5. Association of food consumption with MSFsc

The results of the multiple regression analysis showed that eating breakfast was associated with lower MSF. Multiple regression analysis also showed that the intake of soy, dairy products, vegetables, and fruits lowered MSF. On the other hand, the intake of meat, snacks, and juice increased MSF (Table 6).

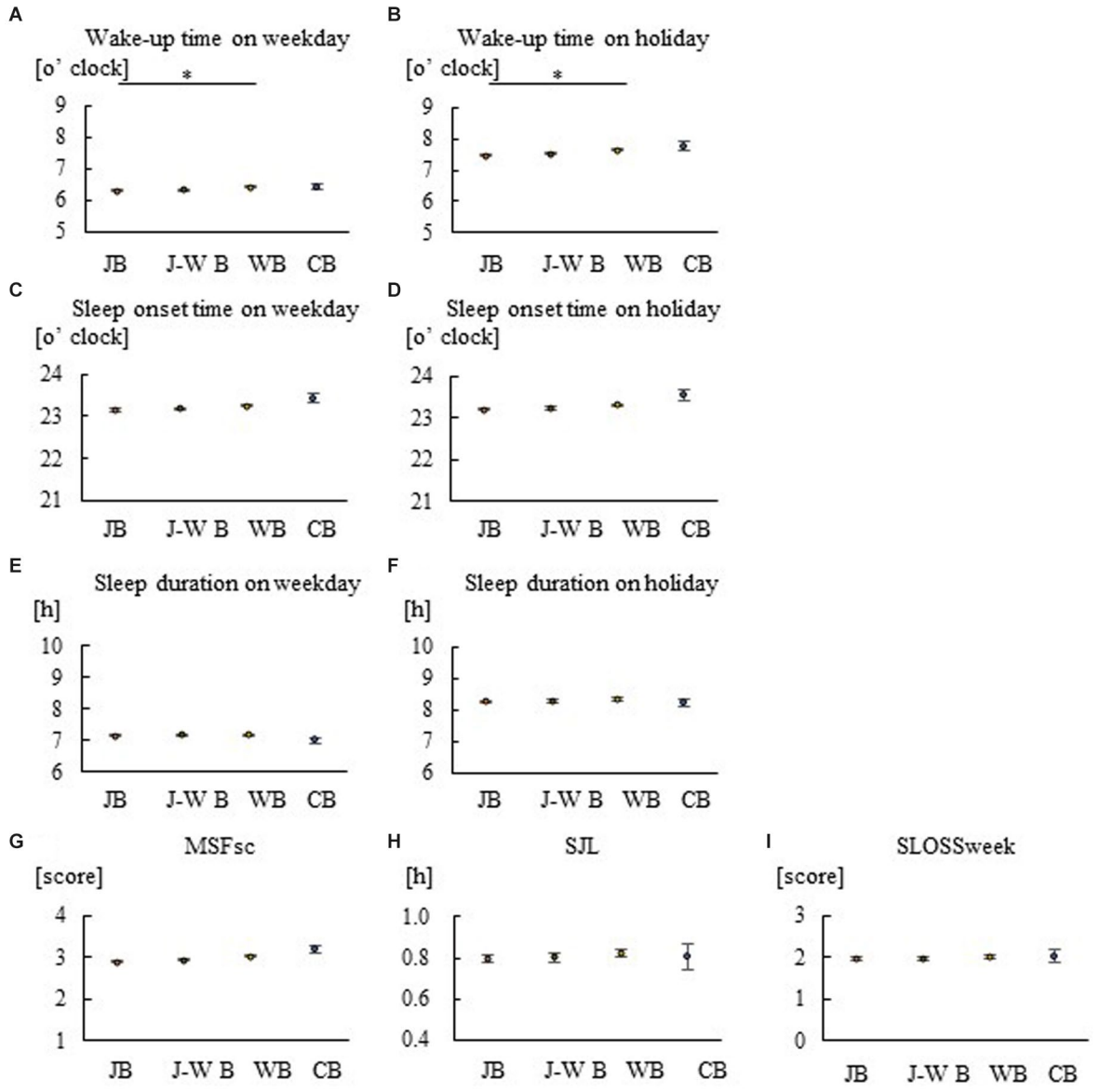

FIGURE 3
Group comparison of breakfast styles for sleep in fathers. Wake-up time on weekdays **(A)**, wake-up time on holidays **(B)**, sleep onset time on weekdays **(C)**, sleep onset time on holidays **(D)**, sleep duration on weekdays **(E)**, sleep duration on holidays **(F)**, MSFsc **(G)**, SJL **(H)**, and SLOSS **(I)**. Consumption of JB leads to earlier waking times compared with WB. *$p<0.05$. A Kruskal–Wallis test with Bonferroni correction was used.

4. Discussion

To the best of our knowledge, this is the first cross-sectional study in Japan to examine the association between breakfast type, sleep, and eating habits among children aged 3–8 years. Our results showed that JB consumption was associated with good sleeping and eating habits.

4.1. Sleep and breakfast style

Adequate sleep from infancy onwards is important for development. However, many children have difficulties sleeping for the recommended duration (36). In this study, we found a close association between sleep and breakfast, which is consumed after long periods of starvation.

The National Sleep Foundation recommends 11–13 h of sleep for toddlers aged 3–5 years and 10–11 h for school-aged children aged 6–11 years (37, 38). The average duration of sleep is reported to be 10.4 h for toddlers aged 3–5 years and 8.9 h for school-aged children aged 6–11 years. Therefore, the average time of 9.47 h of sleep on weekdays and 9.83 h of sleep on weekends of participants in the current study was insufficient to meet the required sleep time. There were almost no differences in sleep duration on weekdays and holidays among breakfast styles (JB, J–WB, WB, and CB) in children, mothers, and fathers, suggesting that sleep duration is independent of breakfast style.

When compared with the WB and CB groups, the JB group showed an earlier pattern of wakeup and sleep onset times on weekdays and holidays, suggesting that children, mothers, and fathers who consume JB are more likely to be of the morning types rather

TABLE 5 Correlation between breakfast style and frequency of food items in children.

	Total protein source	Meat	Fish	Eggs	Soy	Dairy products	Vegetables	Fruits	Snack	Juice
JB	0.05**	0.01	0.08**	0.06**	0.10**	−0.07**	0.02	−0.02	−0.07**	−0.06**
J-W B	0.02	−0.00	0.01	0.04**	0.01	0.01	0.03*	0.04**	0.03*	0.02
WB	−0.05**	0.00	−0.08**	−0.08**	−0.11**	0.07**	−0.03	−0.00	0.06**	0.03**
CB	−0.02	−0.01	−0.03**	−0.03*	−0.02	0.03*	−0.02	−0.02	0.01	−0.00

*$p < 0.05$, **$p < 0.001$. Spearman's rank correlation coefficient was used.

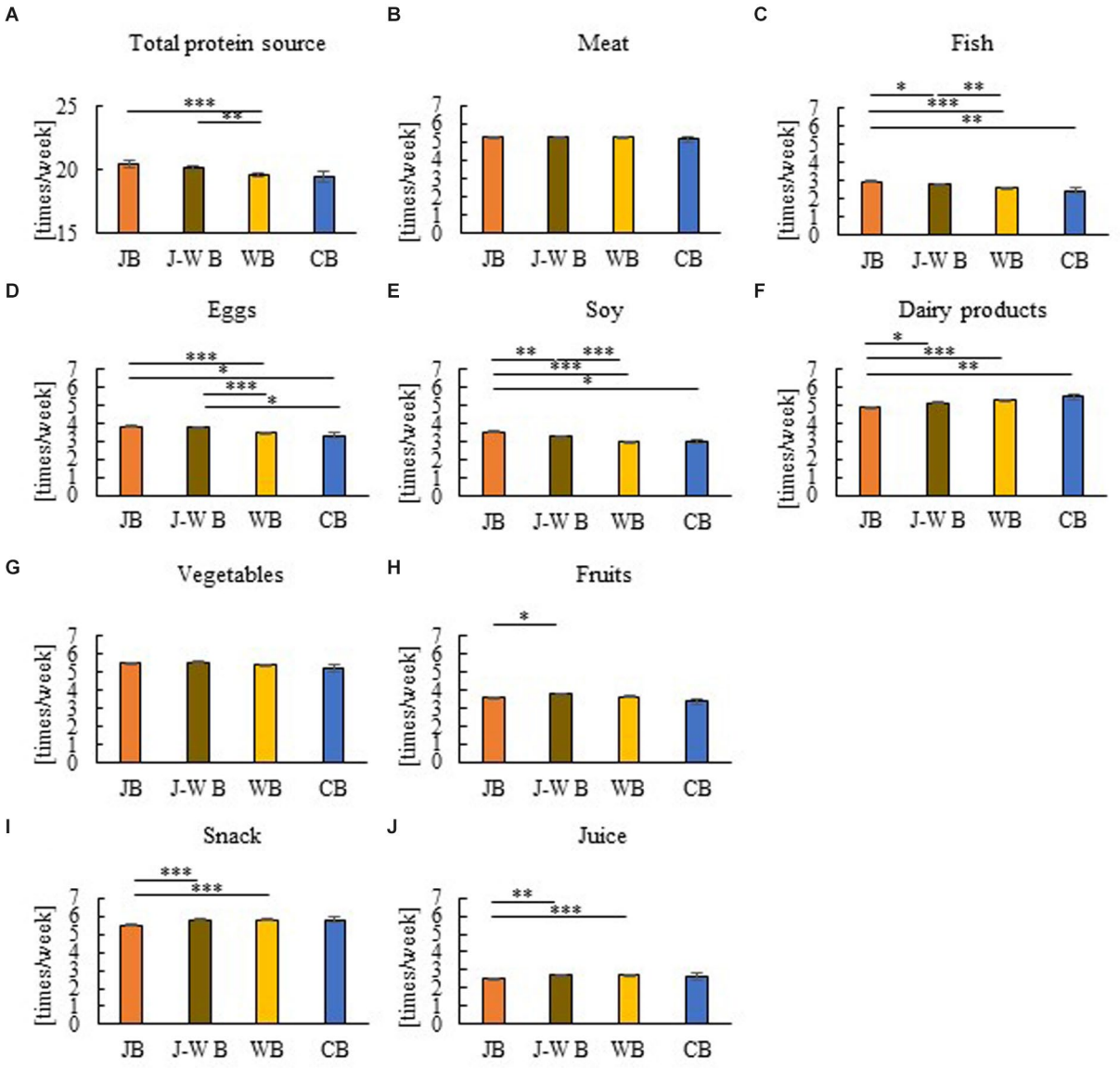

FIGURE 4
Group comparison of breakfast styles for frequency of food items in children. Total protein source **(A)**, Meat **(B)**, Fish **(C)**, Eggs **(D)**, Soy **(E)**, Dairy products **(F)**, Vegetables **(G)** Fruits **(H)**, Snack **(I)**, and Juice **(J)**. There is a link between the consumption of JB and the high intake of various protein sources (Fish, Eggs, and Soy), excluding dairy protein sources, and the low intake of snacks and juices. *p<0.05, **p<0.005, ***p<0.001. A Kruskal–Wallis test with Bonferroni correction was used.

than the evening. These findings are consistent with those of our recent studies on JB groups aged 20–60 years participating in body weight reduction programs (70% women) and JB groups aged 20–50 years participating in daytime work (70% men); individuals who often consume JB tend to be morning types when compared with the WB or CB groups (26, 27). The respective holiday wake-up times

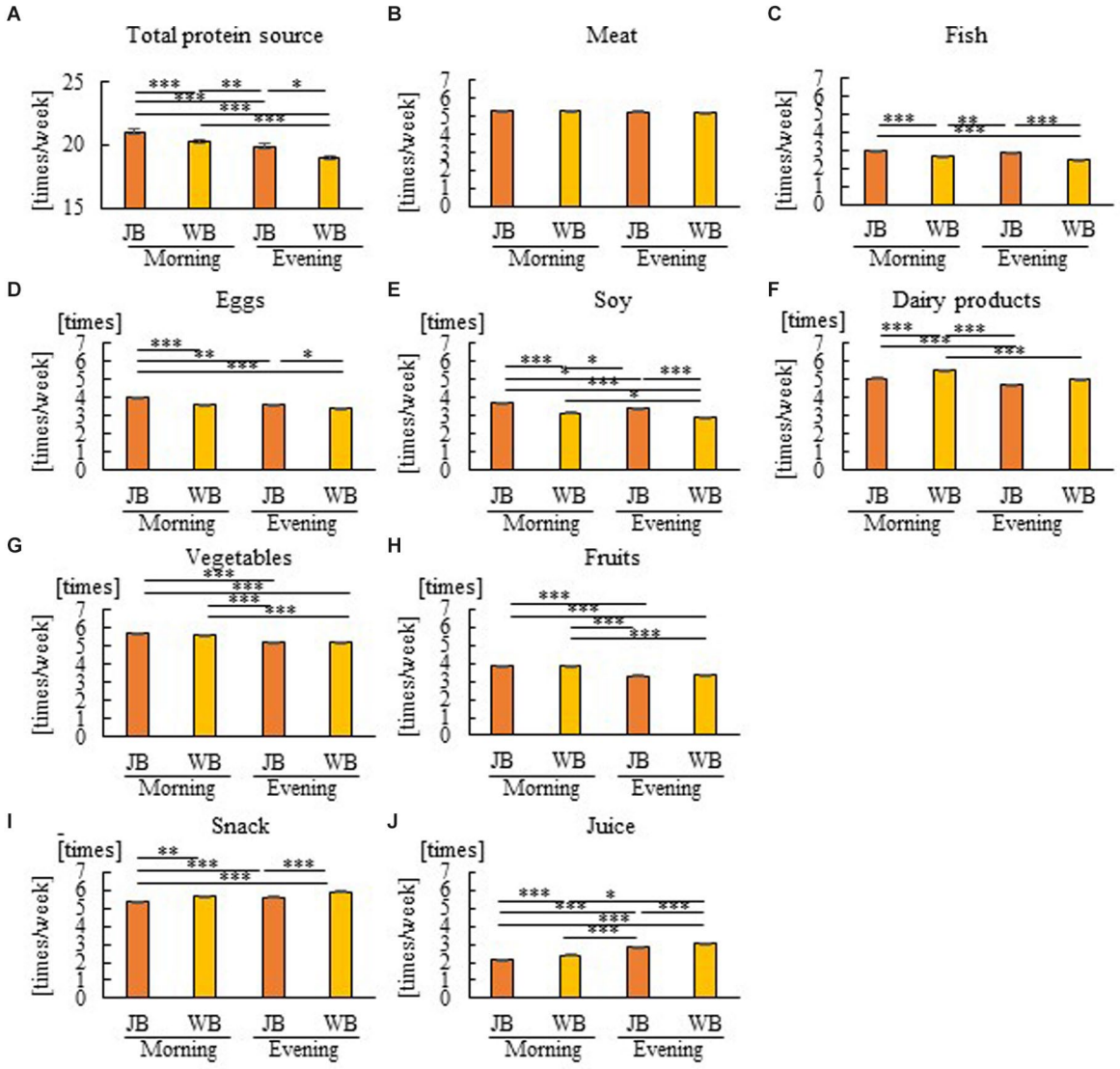

FIGURE 5
Group comparison of breakfast styles for frequency of food item in children between groups when divided into morning and evening types for JB and WB, respectively. Total protein sources **(A)**, Meat **(B)**, Fish **(C)**, Eggs **(D)**, Soy **(E)**, Dairy products **(F)**, Vegetables **(G)**, Fruits **(H)**, Snack **(I)**, and Juices **(J)**. JB groups with morning types exhibit healthy food consumption (Fish, Eggs, Soy, Vegetables, and Fruits). On the other hand, WB groups with evening types showed less consumption of healthy food and more consumption of unhealthy food (Snacks and Juice). * $p<0.05$, ** $p<0.005$, *** $p<0.001$. A Kruskal–Wallis test with Bonferroni correction was used.

in the JB, WB, and CB groups for children were 7:12, 7:16, and 7:34; for mothers 6:52, 7:02, and 7:12; and for fathers 7:28, 7:38 and 7:45. Thus, the time difference between the JB and WB groups was approximately 10 min, and that between the JB and CB groups was 20 min. Every morning, 15 min of phase advance are required to adjust to a 24 h rhythm from circadian free running with 24.2–24.5 h (39). Thus, habitual changes in breakfast style from WB or CB to JB may move 10–20 min earlier than the circadian clock. The present study revealed that people with a pattern of consuming WB or CB tend to exhibit evening-type preference behaviors. The longer screen-time exposure by later sleep onset (40) may contribute to delaying the circadian clock time the next morning. CB and WB may further promote the evening type. Comparing children, mothers, and fathers, the results for mothers showed more differences in group comparisons and correlations with breakfast style. The order of wake-up time on weekdays and holidays was as follows: mothers, children, and fathers. This may be because mothers are often responsible for feeding their children breakfast in Japan, while many fathers are not much concerned about their children and lead their own lifestyles. It may take more time to prepare JB compared to WB or CB; therefore, JB may require earlier wake-up times on weekdays. However, the JB groups showed earlier wake-up times not only on weekdays, but also on holidays. The JB group had smaller SJL and better eating habits. There is a significant relationship between the number of times a child wakes up at night and the amount of sleep a mother gets; however, fathers are somewhat less affected by their children's sleep disturbances (41), and it can be argued that a close relationship exists between children and mothers' sleep. This suggests that mothers and children may influence each other regarding sleep. Moreover, insufficient sleep can lead to chronic diseases related to hypertension, diabetes,

TABLE 6 Multiple regression analysis of MSFsc with breakfast style and food consumption.

Target variable: MSFsc, adjustment factor: Age, Sex, BMI or POW								
	3–5years				6–8years			
	β	p	R^2	F	β	p	R^2	F
JB	−0.03	0.16	0.01	5.13	−0.04	0.03	0.02	11.84
J-W B	−0.02	0.27	0.01	4.95	−0.00	0.98	0.02	10.56
WB	0.02	0.37	0.01	4.84	0.02	0.21	0.01	10.96
CB	0.06	0.00	0.01	7.53	0.06	0.00	0.02	13.00
Total protein source	−0.15	0.00	0.03	21.7	−0.13	0.03	0.03	22.92
Meat	−0.03	0.10	0.01	5.32	0.00	0.82	0.02	10.57
Fish	−0.80	0.00	0.01	9.15	−0.05	0.01	0.02	12.40
Eggs	−0.10	0.00	0.02	12.13	−0.08	0.00	0.02	15.00
Soy	−0.14	0.00	0.03	19.20	−0.10	0.00	0.03	17.60
Dairy products	−0.11	0.00	0.02	12.83	−0.14	0.00	0.04	25.65
Vegetables	−0.12	0.00	0.02	15.87	−0.15	0.00	0.04	26.07
Fruits	−0.14	0.00	0.03	19.84	−0.12	0.00	0.03	20.43
Snack	0.10	0.00	0.02	11.24	0.05	0.00	0.02	12.18
Juice	0.18	0.00	0.04	27.50	0.15	0.00	0.04	27.70

R^2 indicates the contribution ratio and β the standardized partial regression coefficient. BMI (Body Mass Index) is for 3–5 years and POW (Percentage of overweight) is for 6–8 years.

depression, and obesity, and reduced quality of life (42). An examination of the association between sleep and breakfast style revealed that consuming JB contributes to higher sleep scores. This is because people who can afford a JB tend to be early risers in the morning and tend to be morning types.

4.2. Effects of consuming Japanese food for breakfast on eating habits

Japanese food can provide an ideal nutritional balance (43, 44), and consuming Japanese food in the morning can result in a higher intake of protein sources such as fish, eggs, and soy. Since there is a relationship between consuming varying types of protein sources, excluding dairy protein sources, and JB, and since there is a relationship between increasing sleep scores and JB, there may also be a relationship between improving sleep scores and consuming protein sources.

Soy can prevent lipid-related diseases, including stroke and coronary heart disease (45). Furthermore, seafood prevents lifestyle-related diseases such as hypertension, diabetes, and lipid disorders. Furthermore, consuming JB was associated with a lower frequency of sweets and juice consumption. Consumption of high-calorie food and unhealthy snacks leads to obesity (46); therefore, consumption of Japanese food, which can reduce the frequency of sweets and juice intake, is directly linked to a healthy lifestyle. Soy and fruits are low-glycemic index (GI) foods (47). Consuming JB can lower total blood glucose levels during the day. In addition, Japanese food is rich in pulses, such as soy, which has a low GI. Eating low-GI foods in the morning improves the glycemic response and provides greater benefits than eating low-GI foods in the evening (48). Consuming Japanese food in the morning provides various protein sources and health benefits and controls blood glucose levels. In the future, analysis of studies over the years may help us understand the importance of breakfast style habits among preschool and elementary school children to protect against lifestyle-related diseases.

4.3. Relationship between morning-type tendencies, food intake, and health

In the present study, we evaluated the amount of food intake according to the time of day per week. Food frequency questionnaires are a popular way to estimate food intake; therefore, the present questionnaires may compare the amount of food consumed among different breakfast styles. The differences in mean values between JB with morning type and WB with evening type were 0.51, 0.50, and 0.84 for fish, egg, and soy intake, respectively. The differences in mean values between these two groups were 0.57 and 0.80 for snack and juice intake, respectively. These differences may lead to a 10% change in food intake. In general, a 10% change in food intake may be a factor for body weight, blood pressure, and metabolic changes. Similar results were obtained from the multiple regression analyses in the present study. Intake of soya, dairy products, vegetables, and fruit may contribute to changes in the morning types. Snacks and juice intake may contribute to the changes in the evening type.

In the present study, chronotypes, such as the morning or evening types, were found to influence food intake. The number of protein sources was significantly influenced by whether a person ate JB or WB, but was also influenced to a lesser extent by morning or evening types; individuals with morning type consumed more food. The results showed that the group that was morning-oriented and consumed JB usually consumed more protein sources, excluding dairy products. People who sleep late and eat WB, on the other hand, have fewer protein sources. The intake of vegetables, fruits, sweets, and soft drinks was also significantly influenced by whether they were morning or evening types. Compared to evening types, being a morning-type individual with JB is associated with a more active intake of vegetables and fruits. Furthermore, being an evening type individual with WB is associated with a more active intake of sweets and soft drinks.

Circadian rhythms influence diet (48). However, it is a new finding that morning-type individuals consume more vegetables and fruits, whereas evening-type ones consume more sweets and soft drinks. Furthermore, the circadian clock is involved in weight gain and obesity; being awake at night promotes obesity (48). In addition, vegetable and protein sources intake suppresses blood glucose levels and prevents obesity, while the consumption of sweets and soft drinks promotes obesity. In other words, being a morning-type individual prevents obesity and leads to good health through the consumption of vegetables and protein sources. Being a night owl increases the consumption of sweets and soft drinks, leading to obesity. Fruits also have the potential for cardiovascular protection, and reduced risk of colon cancer, depression, and pancreatic disease. Furthermore, vegetable intake reduces the likelihood of colon and rectal cancer, hip fracture, stroke, depression, and pancreatic disease (49). Reducing children's consumption of energy-dense snacks and

promoting healthier foods such as fruits and vegetables are effective and necessary means of improving their dietary intake and reducing their risk of chronic diseases later in life. Thus, our results strongly suggest that JB rather than WB or CB can be beneficial for evening-type children.

5. Limitations

Our study had certain limitations. First, food items and eating habits were collected through self-reports, and this might have resulted in self-efficacy. Second, although there are 50 characteristics of respondence, we used only three variables in this study (sex, age, and BMI). Therefore, more variables should be used to analyze the associations in future studies. Third, we did not include the age, BMI, and breakfast styles of mothers and fathers. Fourth, the association between sleep and eating habits should be assessed including not only the breakfast, but also the lunch and dinner styles to better understand the role of traditional Japanese food in healthy eating behavior. As a strength of the current study, the findings may be generalized to children in Japan, because we included participants without bias, such as people from cities and rural areas, and those from northern and southern areas of Japan.

6. Conclusion

Our results showed that JB may help develop morning-type habits regarding circadian rhythm and healthy eating habits. Earlier wake-up/sleep-onset times and eating habits established in infants may help prevent chronic diseases later in life.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The Ethics Review Committee on Research Involving Human Subjects at Waseda University approved this experiment (No. 2021-101). The guidelines of the Declaration of Helsinki were followed. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YT and SS: conceptualization and research ideas. LN, AF, YN, SM, and NM: methodology and data collection. MK and SS: data analysis, writing the original draft, and editing. SS: funding acquisition, writing the revised draft, and review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

YN, SM, and NM were employed by Benesse Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1131887/full#supplementary-material

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The interplay between macronutrients and sleep: focus on circadian and homeostatic processes

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Sleep disturbances are an emerging risk factor for metabolic diseases, for which the burden is particularly worrying worldwide. The importance of sleep for metabolic health is being increasingly recognized, and not only the amount of sleep plays an important role, but also its quality. In this review, we studied the evidence in the literature on macronutrients and their influence on sleep, focusing on the mechanisms that may lay behind this interaction. In particular, we focused on the effects of macronutrients on circadian and homeostatic processes of sleep in preclinical models, and reviewed the evidence of clinical studies in humans. Given the importance of sleep for health, and the role of circadian biology in healthy sleep, it is important to understand how macronutrients regulate circadian clocks and sleep homeostasis.

KEYWORDS

sleep, macronutrients, circadian process, homeostatic process, process C, process S, clock, protein

1. Introduction

1.1. Sleep disturbances and metabolic disturbances: an inseparable duo

The association between obesity and sleep has been increasingly studied over the last years. The duration of sleep has been put in relation to metabolic disturbances in many clinical studies. Metabolism and sleep mutually influence each other, in a complex relationship. Lack of sleep is associated to a higher body mass index (1–5) and to central obesity (4, 6–10). The recommended amount of sleep, according to the consensus statement of the American Academy of Sleep Medicine and Sleep Research Society, is at least 7 h per night on a regular basis (11).

Central obesity, which is characterized by visceral fat deposition, is a metabolic feature associated with a higher cardiovascular risk. Lack of sleep is associated to glucose metabolism impairment and type 2 diabetes (12, 13). A sleep duration longer of just 1 h per night is associated with a lower prevalence of obesity and type 2 diabetes (14, 15). Similarly, a reduction of just 1 h of sleep per night is associated with weight gain (1).

It is known that sleep restriction can stimulate appetite (16–18) and seeking for high calorie food (16, 19, 20). This is particularly evident in the conditions in which the sleep–wake cycle is

disturbed, as in shift work (21). In fact, it may lead to an increased preference for calorie-dense foods (22), reduction in energy expenditure (23) and alterations in appetite-controlling hormones (23). On the other hand, food acts as an external regulator of the circadian rhythm of the individual, of which sleep is a major manifestation (24).

There is some evidence that the relationship between BMI and sleep duration is U-shaped, so that long or short sleep durations are both associated to increased adiposity (15, 25). Similarly, the relationship between sleep duration and impaired glucose tolerance or diabetes is U-shaped (12, 13), so that we may suppose that metabolic disturbances, whatever they are, are similarly interconnected with sleep duration, but that sleep duration cannot be considered as a linear variable. The variability in sleep duration can be itself considered a risk factor for metabolic alterations (26), diabetes (14) and adiposity parameters (4, 27, 28) at any age. Also sleep quality has its own importance. Given the fact that sleep is composed by quantitative features that are objectively measurable, as sleep latency and sleep duration, and some aspects that are mainly subjective and impossible to measure objectively, as depth and restfulness (29), sleep quality is a parameter that is difficult to define in its entirety. In fact, it is determined by the combination of many factors, as sleep duration, latency, fragmentation, and the perception of restorative sleep, which are strictly connected to the presence of daytime dysfunction. A higher degree of sleep fragmentation, calculated considering movement and immobile epochs with the actigraphy, is related to a shorter total sleep time and associated with obesity (25), and a reduced sleep efficiency is associated to central obesity in women (30).

On the other hand, obesity is a risk factor for developing sleep disturbances. Obesity can increase the risk of developing obstructive sleep apnoea (31), and the psychological and environmental implications of obesity can affect sleep quality, so that sleep disturbances and obesity sit in a vicious circle in which one worsen the other (32).

The mechanisms of the association between lack of sleep and metabolic disturbances are not clarified yet. Metabolic disturbances, included diabetes and non-alcoholic liver disease, are strictly connected among each other, and they similarly interact with lack of sleep. Metabolic disturbances are often associated to hormonal imbalances, which may play a role in their development, in a complex relationship of mutual influence (33–36). Some hormones may be involved in the association between metabolic disturbances and reduced sleep. The higher levels of cortisol due to the condition of chronic stress related to sleep deprivation may explain this relationship, through the increased levels of insulin resistance and inflammation (37). Moreover, the appetite regulation hormones are altered, and lead to an increased energy intake. Lack of sleep is associated to higher levels of pro-inflammatory cytokines and altered sympathetic activity, which lead not only to weight gain itself, but also to the development of a higher cardiovascular risk through the induction of endothelial dysfunction (32, 38).

1.2. Sleep structure and functioning

1.2.1. Sleep architecture

Sleep is a physiological element which is particularly important for health, and specifically, for metabolic and cardiovascular health. It is composed by a rapid-eye movement phase (REM) and non-REM phases. The non-REM phases include the phases N1 and N2, which are the "light sleep" phases, and N3 that is the "slow wave sleep" phase (SWS), that is a deep sleep phase. Both REM and slow-waves sleep contribute to the restorative function of sleep. Sleep stages succeed one after the other in cycles that last from 90 to 110 min. Each stage lasts differently: N1 last from 1 to 5 min, N2 from 10 to 60 min and SWS from 20 to 40 min, while REM phase lasts from 10 to 60 min. These lengths vary during the sleep, and REM length increases along the night, and the non-REM phase gets shorter. The majority of sleep is spent in N2 phase, and non-REM phase lasts about 75% of sleep. During a night, normally there are 4 o 5 sleep cycles, with the progression across sleep stages as N1, N2, N3, N2, REM (39).

1.2.2. Sleep components: the homeostatic and circadian processes

Four decades ago, Borbély proposed the two-process model of sleep (40): a conceptual model that has shaped the field of sleep research and is still the primary model used to describe sleep processes. This model describes the continuous interaction of two processes, termed process S and C, that are responsible for driving sleep and wake timing (see Figure 1). Process S, or the homeostatic process, represents sleep debt, which builds up during the wake phase and decreases during sleep. When it reaches its lowest value at the end of sleep, it triggers the body to awaken, and when it reaches its highest value after a long period of being awake, it triggers sleep. Meanwhile, process C represents the circadian process. This is controlled by the master circadian regulator, which resides within the Suprachiasmatic Nuclei (SCN). Process C oscillates across a day-night cycle, as entrained by external light cues, and controls the timing of sleep and arousal. The two components are interconnected and mutually influence each other to determine sleep propensity (40) in a complex relationship, not yet completely elucidated, that influences some characteristics of

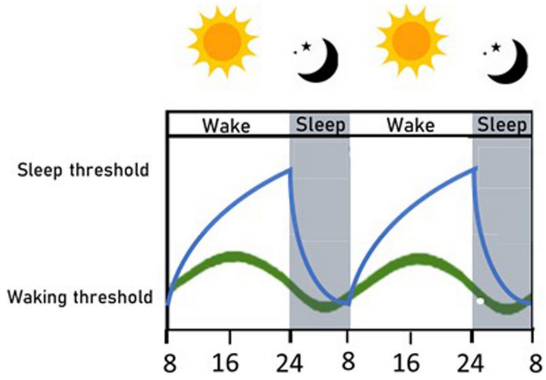

FIGURE 1
Circadian and homeostatic sleep processes. A simplified graphical representation of the two sleep processes, within a 2 days-time, in physiological conditions. The circadian process (green line) helps set the timing of sleep, and its fluctuations are regulated by the alternance of light and dark. The homeostatic process (blue line) is related to sleep debt, which accumulates while awake, and once the sleep threshold is reached, promotes sleep; then sleep debt decreases and awakening occurs when the waking threshold is reached. The two processes are interconnected and mutually interact, so that sleep is harmoniously regulated (40).

sleep, as length and depth; in fact, circadian amplitude may be reduced when sleep pressure increases (41). In physiological conditions, the two processes are harmoniously concordant, so that sleep onset is favored by both processes during the night, when it is dark, and sleep debt has accumulated during the day. Clock genes, the fundamental bricks of the circadian process, are also implicated in sleep homeostasis; the expression of some clock genes changes with wake and sleep phases and with sleep deprivation, and conversely sleep homeostasis is altered in many clock gene mutants (42).

For the purpose of this review, we will focus on how macronutrients influence the sleep components.

2. Circadian process and macronutrients in pre-clinical models

2.1. The circadian clock and the role of dietary nutrients on its entrainment and function

The two-process model of sleep regulation describes the interaction of a homeostatic process S dependent on time spent asleep/awake, and process C which is controlled by the central circadian pacemaker (43). The 'master' circadian clock resides within the Suprachiasmatic Nuclei (SCN) which is located in the hypothalamus. The SCN receives direct light input from the retinal ganglia in order to 'tell time'. In practice, this means that the master clock of the SCN is entrained by exposure to external light cues, and can shift by approximately 1 h per day in humans and mice. The period of the circadian clock in the SCN (the amount of time it takes to complete one cycle) is approximately 24 h in order to match the rotation of the Earth about its axis. The exact amount of time for one oscillation in the absence of external stimulation is known as the 'free-running period' and varies on average between species and individuals, and may be slightly more or less than 24 h. The core circadian clock controls 24-h cycles not only of sleep/wake, but also other physiological parameters, such as core body temperature and blood pressure, as well as secretion of hormones such as melatonin, cortisol, and prolactin.

At a molecular level, the circadian clock is composed of a transcription-translation feedback loop. The positive arm of this loop includes the proteins BMAL1 and CLOCK, which activate the transcription of the inhibitory PERIOD and CRYPTOCHROME (PER1-3 and CRY1&2) proteins. The oscillations are stabilized by an auxiliary loop including REVERB and ROR proteins. This molecular clock operates not only in the SCN but also in peripheral tissues and cell types, with signals from the central SCN pacemaker synchronizing the peripheral clocks (44). Peripheral clocks are influenced by feeding and fasting states and are kept synchronized with the central clock. In this physiological situation, light and dark cycles succeed harmoniously, in accordance with feeding and fasting status. However, peripheral clocks can become decoupled from the central clock, due to stimuli including, for instance, mis-timed feeding, i.e., eating out of synchrony with the biological clock, during normal physiological rest phase (45). This decoupling has been linked to the pathogenesis of a wide variety of diseases, including metabolic disorders. Both central and peripheral circadian clocks are extremely important for regulating the metabolism of macronutrients and maintaining energy homeostasis (46).

Metabolites from feeding play a role in regulating cellular rhythmicity, therefore diet composition play a role in influencing circadian clock activity and reprogram the clock in mice (47). The mechanisms through which nutrients impact circadian metabolome, and consequently biological rhythm, have not yet been fully elucidated and results are not completely concordant (see Figure 2). Research in animal models showed that different diets have a different impact on circadian phase.

Nutrients act mainly on the peripheral clocks, but some preliminary evidence suggest that food is able to directly interact with the central clock (48) (see Figure 3). During fasting conditions, the production of ghrelin from the stomach activated AgRP/NPY neurons, that project to the arcuate nucleus of the hypothalamus, and then connect to the CNS. Also the intergeniculate leaflet receives information form the periphery and is able to project to the CNS. Another mechanism could involve the pancreas, that in response to food intake produces pancreatic polypeptide that activate NPY6-receptor (Npy6r) that is coexpressed with vasoactive intestinal polypeptide (VIP) in the CNS of mice, and synchronizes CNS neurons. Leptin may be involved as well, since it has been observed that *in vitro* is able to forward shift the peak time of CNS and has a role in modulating the CNS response to light. Mendoza et al. (49) administered HF diet to mice and observed that the induction of the early gene c-fos by light in CNS was reduced, maybe through VIP signaling, so that it is possible hypothesize that a HF diet modifies circadian synchronization to light. *c-fos* is a regulatory protein found within the SCN, considered as a molecular marker of SCN resetting. The production of ghrelin, VIP and leptin is differently influenced by different macronutrients, as for example glucose markedly suppresses ghrelin concentration, medium-chain triglycerides reduce it less strongly, while amino acids determine a rise in ghrelin levels (50), therefore we may hypothesize that different macronutrients, through gastrointestinal hormones levels, differentially impact central clocks.

2.2. Impact of macronutrients on circadian process in pre-clinical models

2.2.1. Dietary fat effects on central and peripheral clocks

Experiments in mice have served as the main model for examination of the mechanisms of circadian clock function, and it is important to note that laboratory mice are naturally nocturnally active whereas humans are naturally diurnally active, i.e., the circadian active phase of mice is at night and the active phase of humans is during the day. Importantly, studies in mice have shown that the circadian phase of the SCN is not affected by restricting food intake to the rest phase, whereas the phase of peripheral organs, especially the liver, can be (51, 52). This observation indicates that circadian clocks in different tissues can become uncoupled, and this uncoupling, or internal desynchrony, has been associated with multiple human diseases (53). Examination of this phenomenon in mice using jet lag studies and genetic knockouts has shown a direct link between the circadian clock genes and metabolic disease phenotypes (54). Furthermore, disruption of the circadian clock by high-fat diet (HFD) and constant light have independent and additive effects on weight gain in mice, indicating

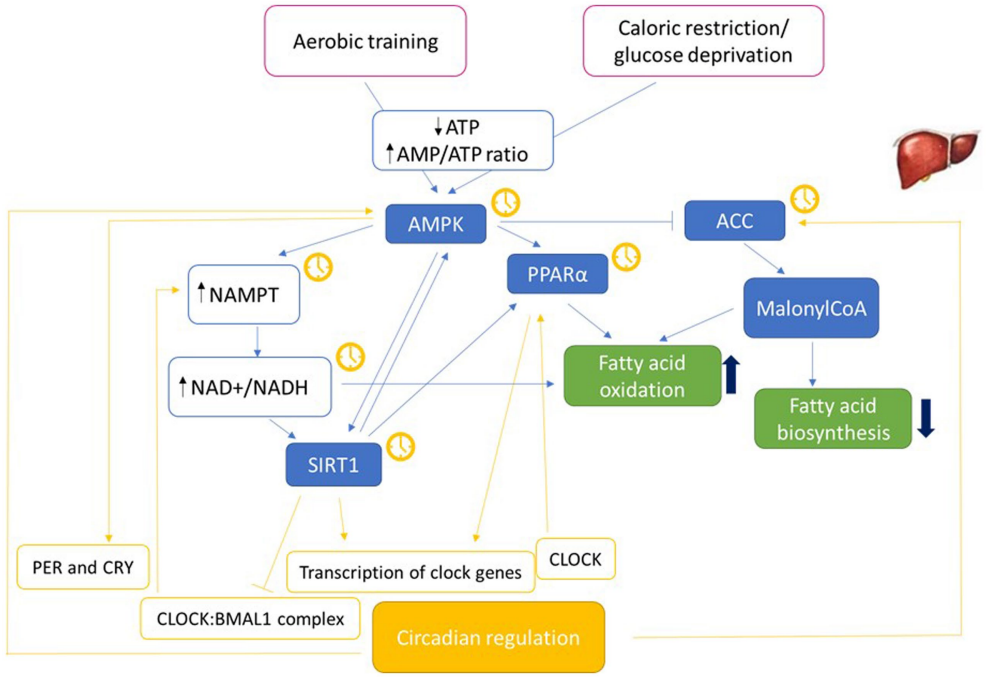

FIGURE 2
Molecular pathways possibly involving metabolic functions and circadian rhythm. Molecular pathways that may represent the connection between metabolism and circadian machinery (46, 71, 175–184). NAD+ acts as a cofactor for fatty acid oxidation and SIRT1 activity. The yellow clock indicates the molecules whose expression is circadian. Adenosine monophosphate-activated protein kinase (AMPK); Nicotinamide phosphoribosyltransferase (NAMPT); nicotinamide adenine dinucleotide (NAD+/NADH); Sirtuin 1 (SIRT1); Peroxisome proliferator-activated receptors (PPAR); Acetyl-CoA carboxylase (ACC).

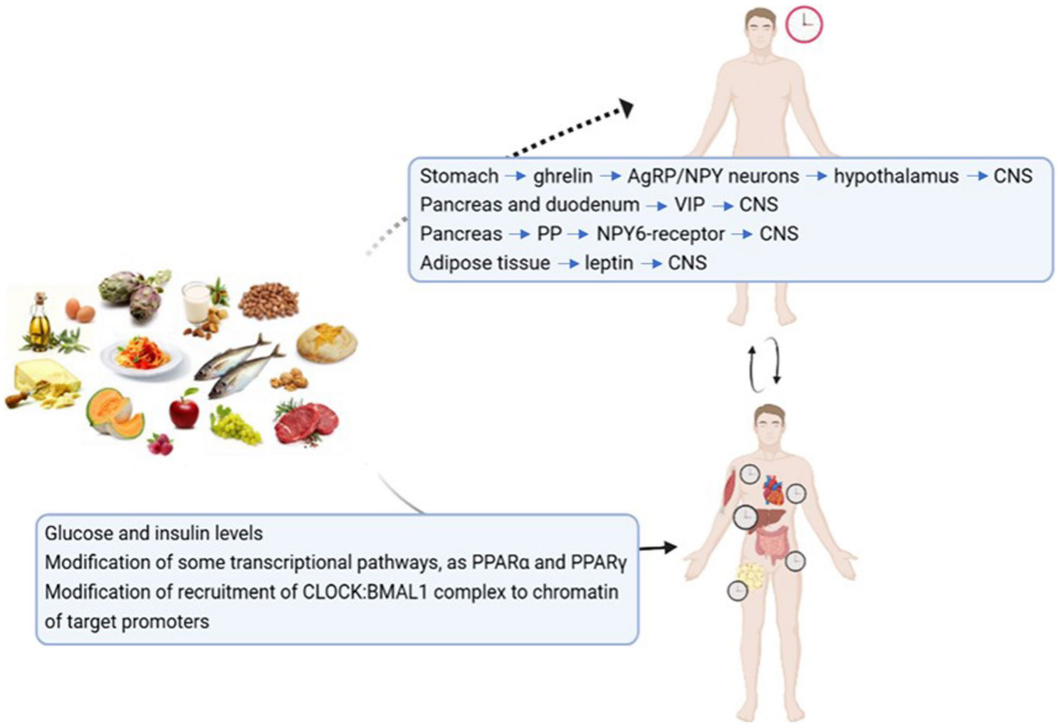

FIGURE 3
How food may affect the central and the peripheral clocks. Possible mechanisms through which nutrients may act on the central and the peripheral clocks (47–49, 185–187). Central Nervous System (CNS); Vasoactive intestinal Peptide (VIP); Pancreatic Polypeptide (PP); Peroxisome-Proliferator Activated Receptor (PPAR).

that disrupted circadian rhythms potentiate the detrimental effects of high-fat diet (55).

The SCN receives metabolic feedback from peripheral organs, as evidenced by the observation that the free-running period of mice is altered by exposure to high fat diet (49). The induction of the gene c-fos by light was reduced in the SCN of mice fed high-fat diet, and this effect may be mediated by vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) (49). Metabolic entrainment of the SCN may also occur through the adipokine leptin, which can cause dose-dependent phase-shift of SCN tissue *in vitro* (56). Administration of exogenous leptin did not shift the behavioral circadian rhythm of mice *in vivo*, but did potentiate the effect of light-pulse induced phase-shift through induction of the clock genes Per1 and Per2 (57). Other studies have found that neurons from the arcuate nucleus and intergeniculate leaflet, which are sensitive to the appetite related hormone ghrelin, transmit signals to the SCN, and thus NPY and GABA may also provide metabolic feedback to the central circadian clock (58).

While dietary fat has been shown to exert some feedback on the central pacemaker, a much greater effect is observed on clocks in peripheral tissues. Clocks in peripheral metabolic tissues, such as adipose tissue, are hypothesized to regulate energy partition in response to regular daily feeding patterns to help maintain energy homeostasis. Therefore, timing of macronutrient intake may be of high importance, and indeed mice fed HFD at the end of the active phase exhibit greater adiposity than those fed HFD at the beginning of the active period. This phenomenon may be an effect of disturbed temporal regulation of β-oxidation (59). Other studies have shown that restriction of HFD to the rest phase leads to significantly higher weight gain than restriction of HFD to the active phase (60). Furthermore, restriction of HFD to active phase when compared to *ad libitum* HFD reduces body weight gain, increases glucose tolerance, and prevents the development of metabolic liver disease. This may be related to a restored efficiency in the pathways function of CREB, mTOR and AMPK, and improved oscillation of the clocks and their target genes (61). HFD significantly alters the rhythmic expression of clock genes in the liver, as well as clock output genes (62, 63) (see Figure 4). This includes, Pparα and Ampk which promote β-oxidation as part of the adiponectin signaling pathway, and disruption of their normal rhythmic expression may lead to impaired liver lipid metabolism. HFD determines the loss of rhythmic metabolite expression, such as NAD+, that parallels the dampened cyclic transcription of Nampt. These modifications in the rhythm of gene expression induced by the HFD may alter the circadian clock by disrupting, through a phase-shift or a reduction, of the recruitment of BMAL1: CLOCK to its target promoters on chromatin (47). At the same time, HFD determines oscillatory gain of normally non-rhythmic transcripts with known or predicted methyltransferase activity, such as *Ehmt*, *Trmt2b*, *Whsc1*, and *Dph5*. The transcriptional reprogramming by a HFD is related to the oscillation and chromatin recruitment of PPARγ, whose expression is induced by a HFD (47).

2.2.2. Glucose effects on central and peripheral clocks

Glucose homeostasis is subject to circadian control, as surgical lesion of the SCN in rats abolishes the 24-h rhythm of basal blood glucose and insulin (64). But glucose may also exert a reciprocal effect on the circadian clock, and there is evidence of regulation in the SCN. Parenteral administration of glucose caused a phase-shift in SCN Per2 mRNA expression in rats (65). Furthermore, *in vivo* administration of insulin to induce hypoglycaemia in mice decreased the magnitude of light-pulse induced phase shifts, indicating that brain glucose levels are important for circadian entrainment (66).

In vitro, glucose has been shown to downregulate Per1 and Per2 gene expression in rat fibroblasts, possibly mediated by transforming growth factor β-inducible early gene 1 (Tieg1 (67, 68);. Conversely, insulin upregulates Per1 and Per2 gene expression in rat fibroblasts (69). Mice treated *in vivo* with streptozotocin to induce non-obese insulin resistance, showed alterations in hepatic expression of Bmal1, Per2, and Cry1, which were reversible by PPARα agonist pioglitazone (70). This might suggest that PPARα mediates the feedback of glucose homeostasis on to the core circadian clock. Indeed, multiple studies have shown that PPARα agonists change the rhythmic expression of core clock genes in multiple mouse tissues (71–74). Interestingly, PPARα agonist bezafibrate caused behavioral phase-advance in SCN-lesioned mice, indicating that the peripheral PPARα may be able to exert circadian control independently of the SCN master clock (75). Other potential mediators include the kinases Mapk and Gsk3, both of which are regulated by insulin signaling. Mapk can downregulate Bmal1, whereas GSK3 phosphorylates and thus degrades CRY2 (76, 77). The exact role of GSK3 in circadian entrainment is poorly understood, with opposing observations in different systems. Pharmacological inhibition and siRNA knockdown of GSK3 caused period lengthening in cultured mouse SCN and fibroblasts (78, 79). Whereas in cultured human U2OS cells, inhibitors and knockdown of GSK3 lead to period shortening (80). Another glucose sensing kinase, Ampk, can also phosphorylate and destabilize CRY1 which may add another layer of complexity to the feedback mechanisms between glucose and the circadian clock (81).

2.2.3. Dietary protein effects on central and peripheral clocks

Relatively less research has been conducted on the role of dietary protein in circadian clock entrainment. Most of the few research articles examining this have used "Essence of Chicken" (EC), which is a mixture of water-soluble substances derived from cooking whole chicken. Whilst EC contains many proteins and peptides, it will also contain many other water-soluble factors, and it is important to note that standardization is likely to be difficult to achieve. However, supplementary dietary protein has been shown to enhance the circadian oscillation of glucocorticoid secretion in mice (82), indicating a potential role for certain amino acids. In mice exposed to 12 weeks of light-induced circadian disruption, dietary EC supplementation was reported to decrease the time to reset clock genes in the pineal gland (83). Further examination of this model, which induces decoupling of hepatic clock genes (Per1, Cry1, Bmal1) and key metabolic regulators (mTOR, AMPK), and leads to liver injury, showed that dietary EC reduced markers of liver inflammation and improved synchronicity of clock genes in liver (84). It should be stressed that the role of specific proteins or amino acids cannot be delineated from other non-protein constituents of the dietary supplement in these studies. However, another study, showed that protein-only diet and/or amino acid (cysteine) administration, can elicit entrainment of liver clock via glucagon secretion and production of IGF-1 (85). Furthermore, an *in vitro* study demonstrated that exogenous methionine and arginine can alter the phosphorylation of mTOR and the expression of circadian clock proteins, offering a

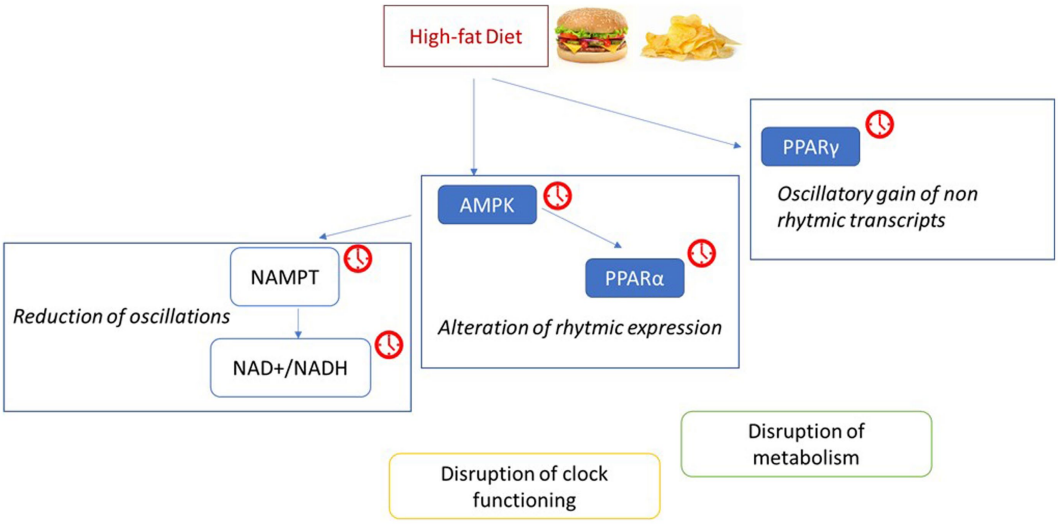

FIGURE 4
The high-fat diet induces a reprogramming of metabolic pathways and circadian rhythm. Possible mechanisms through which a high fat diet disrupts clock function and metabolism (47, 62, 188), inducing an altered or a phase shifted recruitment to chromatin of target genes by CLOCK:BMAL1. The red clocks indicate the molecules whose expression during the day is altered by the consumption of a high-fat diet. Adenosine monophosphate-activated protein kinase (AMPK); Nicotinamide phosphoribosyltransferase (NAMPT); nicotinamide adenine dinucleotide (NAD+/NADH); Peroxisome proliferator-activated receptors (PPAR).

plausible mechanism for the *in vivo* observations mentioned above (86). Lastly, it is noteworthy that protein ingestion has differential effects on intestinal microbiota in different circadian phases, and intestinal microbiota may play a role in regulating peripheral circadian clocks (87).

3. Homeostatic process and macronutrients in pre-clinical models

3.1. Molecular mechanisms of process S

Unlike the rhythmic circadian process C, homeostatic process S is cumulative – with sleep debt accumulating to its maximal value while awake, and decreasing exponentially during sleep. Higher levels of sleep debt result in an increased intensity in sleep, affecting the amount of stimuli required to awaken (88–91). While the exact molecular mechanisms involved in process S have yet to be elucidated, current thinking in the field centers around the hypothesis that process S is driven by the build-up of neuro-modulating substances in the brain, as originally proposed by Feinberg et al. and Borbély et al. (92, 93).

The primary marker used to measure process S is the presence and amplitude of slow wave activity (SWA) as recorded by electroencephalogram (EEG). This is typically considered to be in the range of 0.5–4.0 Hz (43). SWA correlates almost perfectly with sleep debt: slowly increasing over the course of the waking period, peaking during early sleep, and declining over the course of sleep. SWA is caused by the rhythmic firing of cortical neurons, particularly during non-rapid eye movement (NREM) sleep, in which neurons synchronously switch between a bursting and resting phase. As sleep need decreases, synchrony is diminished and the SWA pattern is lost (94).

One of the proposed neuro-modulating substances responsible for driving homeostatic sleep debt is adenosine (95). This was first proposed by Benington and Heller, where they postulated that the role of sleep was to replenish depleted glycogen stores, and that SWA was caused by the release of extracellular adenosine by glycogen-depleted astrocytes (96). While this model has proved to be a very simplistic view of sleep (97), the proposed links to adenosine secretion have been verified by further studies (98). Adenosine is bound via the adenosine A1/A2 receptor in various parts of the brain and, interestingly, can trigger certain pathways typically involved in light-sensing via cAMP-CREB and ERK signaling (99). This may indicate a potential cross-over point for process C and process S. However, knockdown of adenosine A1 receptor has been described to disrupt SWA, a readout of homeostatic sleep drive, but not total sleep duration, which would typically be controlled by process C (100).

More recently, a phosphorylation-centric model of sleep debt has been proposed in which synaptic protein phosphorylation may be the neuro-modulator responsible for dictating sleep pressure (101). This is evidenced by the discovery of sleep-inducing kinases, including calmodulin-dependent protein kinase (CaMK) II (102), salt-inducible kinases (SIK)3 (103), and extracellular signal-regulated kinases (ERKs) (104), which increase in activity over the wake period and show elevated function during sleep deprivation. In addition, the synaptic phospho-proteome shows a peak cluster of phosphorylation events at sleep–wake transition periods that is independent from circadian control (105), as demonstrated by sleep deprivation studies. It is therefore plausible that phosphorylation events are a key molecular mechanism underpinning process S. Moreover, these sleep-inducing kinases are implicated in metabolic pathways as well. CaMKII is activated by exercise and influence lipid metabolism (106), SIK3 belongs to the AMP-activated protein kinase (AMPK) family, regulates cholesterol and bile acid metabolism and influence lipid metabolism (107, 108), while the MAPK/ERK pathway recently

emerged as a key regulator of the Warburg effect, an aerobic glycolysis metabolic reprogramming occurring in cancer cell proliferation and some physiological processes (109).

There is a growing body of evidence that different macronutrients can affect various sleep parameters, including sleep intensity and sleep pressure. The next section of this review will focus on the impact of different macronutrient groups on the homeostatic component of sleep in pre-clinical models.

Circadian rhythm is tightly controlled and fundamental for the health of the living organism. In fact, the regulation of circadian rhythm is redundant, given the role of neuronal PAS domain protein 2 (NPAS2), which can functionally substitute CLOCK in the central clock in mice (110). NPAS2 is a conserved gene whose transcript can dimerize with BMAL1 and activate the transcription of genes related to circadian rhythm, as PER1/2/3 and CRY1/2 (111). Sleep deprivation reduces the binding of CLOCK, NPAS2 and BMAL1 to the promoters of target circadian genes. In particular, NPAS2 is able to induce sleep when the sleep need increase, even when it is not the time to sleep, therefore playing a major role in sleep homeostasis, probably acting at the thalamus and cortex, where it is more expressed (112). In addition, NPAS2 has also a metabolic role, since it boosts the Warburg effect in hepatocellular carcinoma cells through increasing the expression of glycolytic genes (111).

3.2. Impact of macronutrients on homeostatic component of sleep in pre-clinical models

3.2.1. Carbohydrates and homeostatic sleep

Carbohydrates are one of the best described macronutrients that affect sleep. In particular, it has been proposed that high levels of glucose result in the uptake of tryptophan (Trp) in the brain (113). Following ingestion of a high carbohydrate meal, insulin influences tryptophan supply to the brain by both promoting the binding of tryptophan to albumin, and at the same time reducing the levels of several other amino acid species that would usually compete with tryptophan for transport proteins into the brain (114). Tryptophan is a precursor for serotonin and ultimately melatonin, which is the primary sleep-promoting hormone, suggesting that carbohydrate intake could promote sleep pressure.

Unfortunately, few studies have been performed looking at the role of carbohydrates during sleep in rodent models. However, one paper found that glucose activates sleep-promoting neurons in the ventrolateral preoptic nucleus in mice (115), which are essential for the initiation and maintenance of SWA. The authors found that physiological levels of glucose led to the selective excitation of sleep-promoting neurons, which is normally gated by K_{ATP} channels. Induction of SWA is a marker of increased sleep pressure, and therefore suggests that glucose intake promotes sleep in mice. SWA has also been shown to regulate glycolytic metabolism directly (116). Measurement of lactate levels in real time reveal that lactate builds up during wakefulness and depletes during SWA sleep proportionally to the amplitude of SWA oscillations. Therefore, this suggests that SWA reduces the glycolytic rate of the cerebral cortex, and thus glucose metabolism is an essential part of homeostatic sleep.

The sleep modulating role of carbohydrates has been better studied in Drosophila, where response to sugar intake appears to be dose dependent. Sleep pathways between Drosophila and mammals are generally considered to be very highly conserved, with both exhibiting the two-process model described by Borbély et al. (43), therefore conclusions from Drosophila are likely to be relevant to human biology. Starvation in flies results in a dampening of sleep, leading to hyperactivity as they redirect their priorities to locating a new food source (117). This is conserved in mice, with food restriction promoting wakefulness and delayed onset of sleep during daytime (118). This can be rescued by detection of a sweet gustatory stimulus, promoting an induction of sleep and reduction in locomotor activity, such as feeding of low sucrose concentrations (119–121). This gustatory rescue of the starvation effect may be of interest in humans, for maintaining a good sleep in cases of food shortages and unstable environmental conditions (119). Interestingly, meals containing high sucrose concentrations (between 5 and 33%) have a polar effect, instead resulting in increased movement and delayed sleep (122). When maintained on a high sucrose diet over a long time period, flies show a reduction in number of sleep bouts but increased average sleep length compared to low-sucrose fed flies (123). As flies age, their sleep patterns fragment and sleep quality drops. Chronic feeding of high sucrose in aged flies has no effect on reduction of age-induced sleep fragmentation, however a controlled short exposure to a high sucrose feed can rescue this phenotype (124), suppressing sleep fragmentation in aged flies for a short time period, highlighting that the feeding of different macronutrients may be used as a treatment for certain types of sleep disruption. The effect of dietary sugar on sleep behavior is independent of circadian rhythm (123), as it is conserved even in models where circadian rhythm is disrupted, indicating that it primarily acts on the homeostatic sleep process.

Similarly to taste, also smell has a connection with sleep, as the perception of odors is reduced in some sleep stages and, conversely, odors can affect sleep, through the modulations of arousal and sleep latency, duration and quality. Moreover, sleep improves the odor memory (125). Some odors are related to ancestral mechanisms, so that the sleep of newborn infants is ameliorated by the smell of the mother. In rodents, the odor of food stimulates the awakening, which is stronger when the animal is food-deprived (125). The odor itself is able to determine a metabolic response in mice, so that increased or reduced smell ability is associated to modifications in body weight, probably due to modifications of energy expenditure, and not only to the modifications in food intake that may occur (126). Metabolism is strictly connected with aging and life span, therefore the connection between smell, metabolism and sleep may be of particular interest. If these findings are applicable to humans, which express much less olfactory receptor genes, and have a smaller olfactory bulb, is still to be elucidated.

3.2.2. Protein and homeostatic sleep

Consumption of protein has also been described to affect sleep architecture. In particular, addition of 2% yeast protein to the diet of drosophila resulted in increased locomotor behavior, reduced sleep duration, and lowered the arousal threshold required to awaken, indicating shallower sleep (122). However, this effect may depend on the specific ratio of carbohydrate: protein, as the addition of protein to a high sucrose diet instead promotes the consolidation of sleep in flies (123), reverting the sleep disruption normally observed in high sucrose diet fed flies. Therefore, it may be more useful to look at the relative composition of different macronutrients in a meal for therapeutic purposes, rather than study each macronutrient in isolation.

In mice, maternal diet composition plays a significant role on offspring health (127). Offspring born to murine dams fed a restricted protein diet display altered energy expenditure and disrupted sleep architecture compared to normal protein fed controls, despite being fed identical diets after weaning. Interestingly, these changes were independent from circadian machinery, and may indicate that protein consumption during gestation is particularly important for development of homeostatic sleep circuitry.

Tryptophan-rich proteins have a particular role in sleep promotion, as mentioned in the previous section. Consumption of the essential amino acid tryptophan directly facilitates sleep via the generation of melatonin (114). This is dependent upon tryptophan availability in the brain, which is controlled by the ratio of tryptophan to branched chain amino acids in circulation. This is aided by insulin signaling, which actively reduces the availability of branched chain amino acids and therefore promotes the uptake of tryptophan by transport proteins in the brain. However, melatonin appears to control sleep primarily via circadian factors, as it does not seem to alter sleep need as measured by sleep duration, and therefore this is unlikely to affect the homeostatic process (128). Some other authors observed that tryptophan has a direct effect on the homeostatic regulation of sleep. The re-feeding of rats after 4 days of fasting with a diet rich in α-lactalbumin was able to restore the time spent in SWS and wakefulness in just 1 day of refeeding, contrarily to the rats fed with a control-chow with lower amount of lactalbumin, who needed 4 to 6 days of refeeding to restore the alteration in the sleep architecture induced by the fasting (129). Probably, this happened through an increased transport of tryptophan through the brain–blood barrier, as lactalbumin has a higher Trp: large neutral amino acids (LNAA) ratio, and therefore the availability of brain serotonin increased. In fact, tryptophan is the serotonin precursor, through a short metabolic pathway of two enzymes, the tryptophan hydroxylase and the amino acid decarboxylase (130). Serotonin is a neurotransmitter implicated in the regulation of several behavioral and physiological function, as mood, cognition, but also sleep and wakefulness (130), and serves as a precursor for the synthesis of melatonin in the pineal gland during the night.

3.2.3. High fat diet and sleep need

The effects of lipid intake on sleep have primarily been studied in high fat diet obesity models, rather than isocaloric meals containing altered lipid proportions, so it is difficult to separate the effects of dietary fats from that of obesity-related behavioral changes. In mice, high fat diets have been shown to increase sleep pressure, reduce wakefulness, and fragment sleep, as supported by a significant increase in SWA during both wake and rest phases (131, 132). Alterations to fatty acid metabolism also alter sleep parameters. For example, ACADS, a component of the β-oxidation pathway, has been shown to affect rapid eye movement sleep, as its deficiency affects theta oscillations during sleep (133).

Fatty acid metabolism is also disrupted by high fat diets in drosophila (134), which may therefore have a downstream consequence on sleep. Interestingly, it has been found that medium- and long-chain dietary fatty acids, which range from approximately 6-10 carbons in length, promote sleep in flies (135), although their varying effects on sleep duration, number of sleep bouts, and activity during wakefulness vary massively. This may be as a result of differential oxidation of fatty acid species (136), as free fatty acids can be either used to produce Acetyl-CoA or stored as triglycerides, which may affect their relative effects on sleep architecture.

4. Clinical evidence in humans

The composition of the diet can influence both sleep quality and structure (137–146). Anyway, in humans, establishing the effects of a specific nutrient on sleep parameters is very complex, because they are not consumed isolated but within dietary patterns. A recent interesting review summarized many findings regarding the effects of macronutrients on sleep in humans (147).

Some studies observed that a healthy dietary pattern, with a large consumption of fruit, vegetables, fish and fibers, and regular meals, is associated to a better sleep quality (139, 148–150). The consumption of specific variety of cherries or their juice, which are rich in melatonin and serotonin, is often reported to be associated to a better sleep (151, 152). Vegetarians seem to have less sleep disorder, quantified with a lower Athens Insomnia Scale score (given by the sum of the scores for difficulty in sleep induction, awakenings during the night, early-morning awakening, total sleep time, overall quality of sleep and day functioning) and lower diurnal sleepiness, than non-vegetarians, and this is particularly evident in women (153). Anyway, the unprocessed red meat is not clearly related to a better or a poorer sleep (148). The Mediterranean diet, which is based on vegetables, fruit, whole grain and fish consumption, is associated with a better sleep quality, in particular with adequate sleep duration, reduced sleep latency, better sleep efficiency and reduced day dysfunction (154, 155).

Some studies reported that protein intake is associated to a better sleep quality and duration (142, 144–146, 156) but not all studies are completely concordant (138, 144, 145).

As already discussed, Trp is an essential amino acid that, after passing the brain blood barrier, is converted in serotonin and ultimately to melatonin, which promotes sleep. Sleep duration is positively associated with the dietary Trp: LNAA ratio (37). The reason for which this ratio among dietary intake of Trp and LNAA is related to sleep duration may be due to the fact that LNAA compete with Trp to bind to a carrier protein to pass the blood–brain barrier (157), influencing brain Trp bioavailability.

A study in more than 1,900 Japanese healthy adults observed an association between self-reported quality of sleep and self-assessed dietary habits. In terms of food, the intake of pulses, bread, fish and shellfish was correlated with sleep duration. Interestingly, these associations were present only in men, and not in women. The authors observed that sleep duration was directly related to the protein intake (140). A study in 104 healthy adults and older individuals from Singapore investigated the relationship among protein intake and sleep, focusing on tryptophan intake (37). Sleep duration was positively associated to dietary Trp: LNAA ratio but also to plant Trp and plant Trp: LNAA, and this interestingly suggest that the source of protein may play a role. Plant sources had a higher Trp:LNNA ratio, in comparison with animal sources, and moreover, they contain carbohydrates, which can increase plasma Trp:LNAA up to 50%. Dairy protein intake was negatively associated with sleep duration, despite its high content of tryptophan. Also sleep efficiency seemed to be negatively influenced by protein from diary sources, since people with a sleep efficiency of more than 85% ate less protein from diary sources in comparison with people with lower sleep efficiency

($p = 0.033$). This result may be related to the fact the dairy products contain not just alpha-lactalbumin, but also other proteins, which may increase the LNAA amount and therefore reduce the ratio. The different effect of the protein quality on sleep may also be related to the interaction of the gut microbiota with the peptide produced with the protein digestion, with the eventual development of dysbiosis and inflammation (150), but not all studies observed this different effect on sleep by proteins of different sources (146). Similarly, the ingestion of an evening whey protein supplementation, rich in tryptophan, did not improve sleep parameters in 15 elite male athletes. However, as authors point out, the high protein/energy intake snack did not negatively affect sleep, which is interesting for the importance of this kind of snacks for athlete recovery (158). Another study in 36 elite male football players observed that evening protein intake was associated with shortened sleep onset latency (159).

Tryptophan supplementation from non-diary sources is not invariably associated to an amelioration of sleep quality. In a recent study, 22 women with fibromyalgia were administered an eucaloric Mediterranean diet enriched with walnuts as source of tryptophan and magnesium for 16 weeks, but sleep quality did not ameliorate (160).

Data on carbohydrate intake and sleep are controversial. High-CHO (carbohydrate) diets may reduce the sleep onset latency (161). The glycaemic index may influence this parameter, as a study observed that the consumption of a high glycaemic index meal 4 h before bedtime shortened the sleep latency (113). Other sleep parameters were not affected. High-glycaemic index carbohydrates can increase the ratio tryptophan: LNAA through insulin action, which promotes the uptake of LNAA from muscles (162). Furthermore, the timing of carbohydrate intake plays a role, as the same high glycaemic index meal given 1 h before bedtime did not have a significant effect on sleep latency (113). On the other hand, another study reported no changes in sleep onset nor sleep efficiency, but a reduction in the restorative sleep in the first sleep cycle, after consumption of a high CHO meal 4 h before bedtime (163), and another one reported a reduced sleep quality associated to a high CHO intake (139). In fact, the consumption of higher percentage of energy from carbohydrates has been associated with arousals (137). A study in 32 female athletes reported an increase in wake after sleep onset and decrease of sleep efficiency along with increase in CHO intake, and on the contrary a decrease of sleep onset latency along with saturated fat intake (164).

In healthy adults administered a very low carbohydrate diet, the slow wave phase increased, and the rapid eye movement (REM) sleep decreased, in comparison to an isocaloric control mixed diet (165). Another study observed a reduction of sleepiness during a very low-calorie ketogenic diet during the reduced ketosis phase, but no evidence of modifications of other sleep parameters (166).

Regarding fat intake, less data is available. A short sleep is often associated to a higher fat intake (143), but this is not invariably observed (167). Conversely, saturated fats, which are commonly present in the Western diet, have a negative effect on sleep quality according to some studies. *Ad libitum* eating over 3 days, with a great intake of saturated fat and sugars, is associated to a less restorative sleep and increased latency (137). In particular, the percent of energy from saturated fat predicted a reduced SWS. On the other hand, other studies reported a better self-reported sleep with a high-fat diet (168), or a neutral effect (161, 163).

The effects on sleep of omega-3, of which fatty fishes are rich, and omega-6 long-chain polyunsaturated fatty acids, are not completely elucidated yet (169, 170). Fish consumption has been often associated to a better sleep quality evaluated with the Pittsburgh Sleep Quality Index Questionnaire and a longer sleep duration in men (139, 140).

As some interesting reviews and metanalysis recently pointed out, at the moment there are no striking evidence about the effects of a specific nutrient on sleep, and further research is warranted (150, 156, 171).

Conversely, deprivation of sleep can induce a modification in the pattern of the preferred foods. In fact, short sleepers, very short sleepers and individuals after a nightshift tend to eat less healthy food, increase their intake of saturated fats and carbohydrates, and consume less vegetables (20, 22). The mechanisms through which this happens may be related to a longer time of food availability, or research for gratification through calorie-dense food, or to higher energy needs to maintain the extended wakefulness, or to imbalances in appetite hormones (172).

The time of the administration of food may influence sleep and circadian rhythm. Time-restricted eating (TRE) consists in eating within a restricted time window during the day, and fast for the rest of the day. TRE is used mainly in obesity, since it favors weight loss and an amelioration of metabolic disturbances (173). Since time-restricted feeding (TRF) has been linked with an alignment of circadian rhythms in animal models, and the prolonged fasting stimulates SIRT-1 activity and ketone bodies production, which can influence circadian rhythm, it has been hypothesized that TRE may influence sleep. A recent review of human trials studied the effects of TRE on sleep in overweight and obese humans, trough questionnaires, self-reported diaries, and accelerometers (174). The authors reported that overall sleep quality did not change, sleep duration did not increase, nor insomnia was modified. Of note, the effects on sleep latency and efficiency did not align across studies. The insignificant impact on sleep quality may be attributed to the minimal weight loss occurred; the duration of sleep as well as the presence of insomnia remained unchanged probably due to the fact that participants in the trials were already sleeping for more than 7 h per night and did not suffer from insomnia. Therefore, TRE deserves more studies on larger samples of patients.

Some minerals and vitamins have been related to sleep duration and efficiency, in particular sodium, iron, zinc and vitamins D, B9, and B12 (37, 140, 164), but this topic goes beyond the aim of our review.

5. Conclusion

Dietary composition and sleep are bidirectionally related. Macronutrients influences sleep, and on the other hand, lack of sleep is related to the preferential choice of certain food. The mechanisms through which this mutual influence takes place, remain poorly defined. Both sleep processes C and S are involved. The role of circadian clocks in metabolic homoeostasis and the implications for health and disease are gradually being elucidated. However, besides regulating the metabolism of macronutrients, conversely the circadian clock is also regulated by macronutrients, both centrally and peripherally. Furthermore, regulation of peripheral circadian clocks can feed back on to the central pacemaker in the SCN.

Clinical studies have been inconclusive regarding the effects of a single macronutrient on sleep parameters in humans, probably also

because the inevitable association of the macronutrients in dietary patterns.

The effects of food on sleep are not connected just to the food itself, but comprehend the response that its taste and its smell elicit in the organism. In fact, sweet taste is able to modify sleep and reduces sleep deprivation due to starvation, and the odors are able to influence sleep latency, duration and quality, and to induce a metabolic effect in animal models. These data may be of interest for ameliorating the management of patients suffering from sleep deprivation. If these results are translatable in humans, it has still to be determined.

Further research should be conducted to explore TRE as a tool for managing sleep disruption in humans: in obese patients, where the effect may be related to the weight loss achieved, but also in normal weight individuals suffering from sleep disturbances.

Further studies aimed to better understand the relationship between nutrients and sleep are needed, given the importance of sleep for metabolic health in humans.

Author contributions

EG: conceptualization. EG, MB, and MV: writing and original draft preparation. AL, LG, and DR: review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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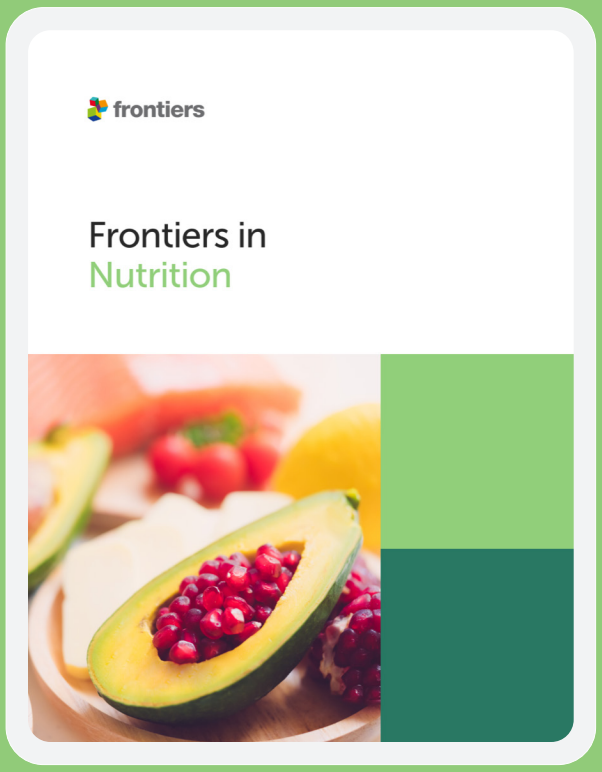

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